Factors influencing arbovirus transmission: vector competence and the effects of virus infection on repellent response, oxidative stress, and glutathione-*S*-transferase activity.

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Abstract (Academic)

Zika (ZIKV), La Crosse (LACV), and Cache Valley (CVV) viruses are mosquito-vectored diseases that cause significant morbidity and mortality in humans and animals. Transmission of these viruses are dependent on numerous factors including vector competence and the effects of mosquito-virus interactions. We conducted vector competence studies of local *Aedes* and *Culex* mosquitoes for ZIKV and CVV, and found that all *Aedes* mosquitoes were competent for CVV and only *Aedes albopictus* and *Aedes japonicus* were competent for ZIKV. Vector competence for CVV was dose-dependent, where mosquitoes orally infected with high titers developed higher transmission rates. We also found that vector competence for ZIKV was limited by midgut and salivary gland barriers. Second, we looked at the effects of LACV and ZIKV infection on repellent response in *Aedes* mosquitoes and found that infected mosquitoes were refractory to low concentrations of DEET, picaridin, and PMD. Increasing concentrations of the repellents to ≥10% was able to increase percent protection (%*p*) against infected and uninfected mosquitoes. Lastly, we determined the effects of ZIKV and LACV infection on oxidative stress and glutathione-*S*-transferase (GST) activity in *Aedes albopictus.* Virus infection had no effect on oxidative stress, but GST activity was significantly different for mosquitoes 3-days post-exposure. We found that oxidative stress levels and GST activity had an inverse relationship for infected and uninfected mosquitoes, where oxidative stress decreased and GST activity increased over the 10-day test period. This indicates that GSTs may aid in controlling byproducts of oxidative stress. The results from this entire study identified competent vectors for emerging arboviruses and demonstrated the behavioral and physiological effects of virus infection in the mosquito vector.

Abstract (Public)

Zika (ZIKV), La Crosse (LACV), and Cache Valley (CVV) viruses are transmitted by mosquitoes and can make humans and animals very sick. There are many biological factors that determine if a mosquito can transmit a virus and these viruses can change the biology of a mosquito. We conducted laboratory studies to see if *Aedes* and *Culex* mosquitoes can transmit ZIKV and CVV. We found that all *Aedes* mosquitoes were able to transmit CVV and only the Asian tiger mosquito and Asian rock pool mosquito were able to transmit ZIKV. Mosquitoes infected with high amounts of CVV developed higher transmission rates. We also found that transmission of ZIKV was limited by barriers in the mosquito midgut and salivary glands. Second, we looked at the effects of LACV and ZIKV infection on how *Aedes* mosquitoes respond to repellents and found that infected mosquitoes were less sensitive to low concentrations of DEET, picaridin, and PMD. Increasing concentrations of the repellents to 10% or higher was able to provide adequate protection against infected and uninfected mosquitoes. Lastly, we determined the effects of ZIKV and LACV infection on oxidative stress and glutathione-*S*-transferase (GST) activity in the Asian tiger mosquito. Virus infection did not change oxidative stress, but GST activity was higher in infected mosquitoes tested after 3 days after infection. We found that oxidative stress decreased and GST activity increased over the 10-day test period. This indicates that GSTs may help control damaging products from oxidative stress. The results from this entire study identified what mosquitoes were able to transmit emerging mosquito-borne viruses and demonstrated the biological effects of virus infection in the mosquitoes.

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Chapter 1

LITERATURE REVIEW

Arbovirus: La Crosse virus:

La Crosse virus (LACV, Peribunyaviridae, Orthobunyavirus) is a negative-sense, single-stranded RNA virus with a tripartite genome. The virus was first isolated from the brain tissue of a child who died after developing encephalitis [Thompson, 1965]. Clinical manifestations of LACV infection include aseptic meningitis or encephalitis, but most cases are asymptomatic or show minor febrile symptoms [McJunkin, 1998]. LACV is the leading cause of pediatric encephalitis in the United States, with severe cases in children resulting in neurological sequelae [Balkhy, 2000]. The principal vector for LACV is *Aedes triseriatus* (Say), and the virus is naturally maintained in small mammals such as chipmunks, gray foxes, raccoons, and red foxes [Pantuwatana et al. 1974; Beaty and Thompson 1975; Amundson, 1981].

Arbovirus: Cache Valley virus

Cache valley virus (CVV, Peribunyaviridae, Orthobunyavirus) is a negative-sense, single-stranded RNA virus with a tripartite genome from the Bunyamwera serogroup [Blitvich, 2012, Holden, 1959]. Only a handful of human CVV cases have been reported, but infection may result in fever, meningitis, encephalitis, and meningoencephalitis [Campbell, 2006; Nguyen, 2013, Wilson, 2017]. CVV is a concern with domesticated ruminants, such as cattle, sheep, and horses [McConnell, 1981; McLean, 1980; Neitzel, 1991; Sahu, 2002], where infection may result in stillbirths, congenital malformations, spontaneous abortions, and death [Chung, 1990; Edwards, 1989]. Although CVV has caused illness in humans and has been isolated from mosquitoes, not much is known about the virus's primary vectors and host. CVV has a widespread distribution in North America and has been isolated from several mosquitoes,

including *Ae. albopictus* and *Ae. japonicus* [Armstrong, 2015; Calisher, 1986; Yang, 2018; Ngo, 2006]. Although the principal vector is unknown, vector competence studies and field isolations have shown that *Anopheles quadrimaculatus* and *An. punctipennis* may be involved in the transmission cycle with white-tailed deer serving as potential amplifying host [Blackmore, 1998; Andreadis, 2014; Neitzel, 1991].

Arbovirus: Zika virus

Zika virus (ZIKV, Flaviviridae, Flavivirus) was first isolated in 1947 from a sentinel Rhesus monkey during a research study supported by the Rockefeller Foundation in the Zika Forest of Uganda [Dicks, 1952]. The primary intent of that study was to understand the enzootic cycle of the yellow fever virus and identify emerging arboviruses. The first case of human disease caused by ZIKV infection occurred in Nigeria during 1954 [Macnamara, 1954]. The first detection of ZIKV in Asia and the first isolation from *Aedes aegypti* occurred in Malaysia in 1966 [Marchette et al., 1969]. In 2007, the first large outbreak of ZIKV occurred on Yap Island of the Federated States of Micronesia [Duffy, 2009] followed by another outbreak in 2013 in French Polynesia [Musso, 2014]. The virus eventually made its way to the Americas, where it was responsible for the 2015 outbreaks in Brazil that resulted in numerous microcephaly cases in newborn infants [Schuler-Faccini, 2016; Campos; 2015**]**. Microcephaly is a condition in which the brain of a fetus does not develop properly, resulting in a smaller than normal head size [Klase, 2016; Calvet, 2016]. ZIKV infection has also been linked to Guillain-Barre syndrome in adults, where the immune system attacks the peripheral nerves, causing paralysis and muscle weakness [Goodfellow, 2016]. *Ae. aegypti* is implicated as the primary vector with *Ae. albopictus* serving as an accessory vector for human transmission [Ayres, 2016; Ayllón, 2017; Wong, 2016]. Non-human primates are likely to serve as reservoirs for ZIKV in sylvatic

transmission cycles [Musso, 2016]. The virus can also be spread by non-vector routes including sexual transmission from an infected person [Atkinson, 2016; Musso, 2015] and from mother to child during breastfeeding [Dupont, 2016].

Mosquito vector: *Aedes albopictus* (*Stegomyia albopicta*)

The Asian tiger mosquito, *Aedes albopictus* (Skuse) was introduced into the United States in 1985 from a used tire shipment that originated from Asia. Since its introduction into Houston, Texas, the mosquito has spread rapidly throughout the U.S. and is found extensively throughout the east coast, southeast, Midwest, and parts of the west coast [CDC, 2017; Richards, 2006]. *Ae. albopictus* is a peridomestic mosquito that can be found in a variety of rural, urban, and suburban habitats where they breed in artificial containers such as tires, flower vases, cement tanks, plastic bottles, and bird baths [Barker, 2003; Chareonviriyaphap, 2003]. *Ae. albopictus* is a very aggressive daytime biter of humans, domestic, and wild animals and is a competent vector of at least 22 arboviruses including ZIKV, LACV, Dengue virus (DENV), yellow fever virus (YFV), and Chikungunya virus (CHIKV) [Cully, 1992; Gratz, 2004; Liu, 2017; Miller, 1989; Whitehorn, 2015; Mangiafico, 1971; Hugo, 2016; Trexler, 1997].

Mosquito vector: *Aedes aegypti* (*Stegomyia aegypti*)

Aedes aegypti (Linnaeus), the Yellow Fever mosquito, is a highly anthropophilic mosquito that is a major vector of DENV, CHIKV, and YFV. There are two subspecies of the mosquito: *Ae. aegypti aegypti* and *Ae. aegypti formosus*. The former is the domesticated subspecies that is responsible for arbovirus outbreaks between humans, and the latter form is presumably the ancestral subspecies found in sub-Saharan Africa that feeds on non-human mammals [Mattingly, 1957; Gouck, 1972]. Introduction of *Ae. aegypti* into the New World most likely occurred when European ships transported native Africans from West Africa during the

slave trade [Eltis, 2010; Powell, 2013]. *Ae. aegypti* is competent for DENV, YFV, CHIK, and ZIKV and serves as an important vector in urban transmission between humans [Reed, 1900; Schule, 1928; Cornet, 1979; Vega-Rua, 2014; Ayres, 2016]. *Ae. aegypti* is capable of breeding indoors and outdoors, utilizing natural and artificial containers that retain stagnant water [Chareonviriyaphap et al., 2003].

Mosquito vector: *Aedes triseriatus* (*Ochlerotatus triseriatus*)

The eastern tree-hole mosquito, *Ae. triseriatus* (Say), is the principal vector of LACV [Pantuwatana et al. 1974, Beaty and Thompson 1975]. *Ae. triseriatus* primarily live in isolated woodlots or forest edges, and the larvae are capable of inhabiting tree holes and artificial containers [Nasci et al., 2000]. *Ae. triseriatus* tolerates a wide range of temperatures and conditions and is found extensively throughout the eastern United States and parts of Central America [Sánchez-Trinidad, 2014; Walker, 1992]. The adults are aggressive daytime biters that feed primarily on small wood-land rodents that also serve as the amplifying hosts of LACV [Yuill, 1983; Wright, 1970]. The primary mechanism of LACV maintenance by *Ae. triseriatus* is transovarial transmission to overwintering eggs [Watts, 1973]. Laboratory transmissions studies suggest that *Ae. triseriatus* may play a role in the enzootic transmission of West Nile virus (WNV) [Erickson, 2006].

Mosquito vector: *Aedes japonicus japonicus* (*Ochlerotatus japonicus japonicus* and *Hulecoeteomyia japonica japonica*)

The Asian bush or Asian rock pool mosquito, *Ae. japonicus* (Theobald), is a new invasive species. Originally from Korea and Japan, this mosquito species has spread to parts of the United States and Europe (Kaufman, 2014). The first discovery of *Ae. japonicus* in the U.S. was in New

Jersey and New York in the late 1990s (Peyton, 1999). *Ae. japonicus* larvae can utilize diverse habitats, including artificial containers, rock pools, tree holes, and bamboo stumps [Sota, 1994]. Although the adults are not major vectors of human pathogens, they are capable of transmitting Japanese encephalitis virus (JEV), eastern equine encephalitis (EEE), West Nile virus (WNV), St. Louis encephalitis (SLEV), and La Crosse virus (LACV) [Sardelis, 2001; Sardelis, 2002; Sardelis, 2002; Sardelis, 2003; Sardelis, 2011; Takashimi, 1989; Turrell, 2001]. Furthermore, blood-meal analysis of field-caught *Ae. japonicus* have shown high incidences of human blood in addition to birds, horses, opossums, chipmunks, and deer [Apperson, 2004; Molaei, 2009]. Due to cold tolerance by the larval and adult stages and overwintering capabilities, *Ae. japonicus* can now be found extensively throughout temperate regions of the United States and Canada [Kaufman, 2014].

Repellents

During the West Nile virus epidemic in the United States, there was an immense public concern over the spread of diseases by mosquitoes [Barnard, 2004]. The most common approach against mosquito disease transmission is personal protection. Personal protection allows a person to select from several options such as avoidance techniques, physical or chemical barriers, treatment of clothing with toxicants, and topical repellents [Barnard, 2000]. Topical repellents for skin is the most common personal protection practice due to accessibility, cost, and ease of use. Effectiveness can vary depending on environmental factors and mosquito species [Barnard, 1998]. The Center for Disease Control and Prevention (CDC) recommends EPA-registered repellents for protection against arthropod-transmitted diseases. These repellents include DEET (N,N-deithyl-m-toluamide or *N,N*-diethyl-3-methyl-benzamide), picaridin (2-(2-hydroxyethyl)-

1-piperidinecarboxylic acid 1-methylpropyl ester), oil of lemon eucalyptus (or PMD) and IR3535 (Ethyl 3-[acetyl(butyl)amino]propanoate) [CDC, 2018].

Repellent: DEET

DEET (N,N-Diethyl-3-methylbenzamide) is a broad spectrum repellent that effectively protects against hematophagous insects [Moore, 2007]. DEET was formulated by the United States Department of Agriculture (USDA) in 1946 and was registered as a commercial repellent in 1957 [Frances, 2007; USEPA, 1998]. DEET was initially believed to work by inhibiting L-lactic acid detection or blocking insect olfactory receptors (OR) responsible for sensing 1-octen-3-ol, a substance that can be found in human sweat and breath, blinding them to the chemical stimulus [Dogan, 1999; Ditzen, 2008]. However, recent studies have shown that exposure to DEET results in an excitatory response in the odorant receptors, indicating a true repellency effect [Syed et al. 2008; Xu, 2014]. In addition, a field study utilizing carbon dioxide as the main attractant observed repulsion of mosquitoes in the presence of DEET, which provides further evidence that DEET elicits a repellent response [Hoffmann, 2003].

Repellent: Oil of Lemon Eucalyptus

Oil of lemon eucalyptus (OLE) is a botanical oil that is refined from the lemon-scented gum, *Corymbia citriodora*. The active ingredient in OLE, *p*-Menthane-3,8-diol (PMD), was first isolated in China in 1960 during a mass screening of plants for repellent properties [Curtis, 1991]. PDM, also known as quwenling in China, is a byproduct generated during the distillation of *C. citriodora* leaves [Maia, 2011]. Compared to most plant oils, PMD has a lower vapor pressure, which reduces the evaporation rate and provides long-lasting protection [Barasa, 2002; Carroll, 2006]. When tested against *Aedes* and *Anopheles* mosquitoes, 30% PMD performed

equal to 10-20% DEET [Shreck, 1991]. In some cases, 30% PMD outperformed 15% DEET in 4-hour tests against *Anopheles* mosquitoes [Moore, 2002].

Repellent: Picaridin

Picaridin (KBR 3023), also known as 2-(2-hydroxyethyl)-1-piperidinecarboxylic acid 1-methylpropyl ester, is a piperidine derivative developed by Bayer. Picaridin is a relatively new repellent that was not registered in the U.S. until 2001 by the EPA [EPA, 2005]. Unlike DEET, picaridin is odorless, less likely to irritate the skin, does not damage plastics, and does not feel greasy. The mechanism of picaridin is thought to be similar to DEET, where it forms a vapor barrier that deters biting arthropods [Katz, 2008]. Picaridin has been shown to be effective against mosquitoes, ticks, biting flies, and fleas [Barnard, 2002; Carroll, 2010]. Studies have shown that equal concentrations of picaridin and DEET provide the same level of protection against mosquitoes and routine use of topical formulations does not lead to significant toxicological risks [Yap, 1998; Frances, 2002; Antwi, 2008].

Glutathione-*S*-transferase

Glutathione *S*-transferases (GSTs) are a diverse family of phase II detoxification enzymes that are found in almost all eukaryotic cells [Hayes, 2005; Wu et al., 2012]. GSTs are involved in the detoxification of toxic substances, such as xenobiotics, products of oxidative stress, environmental pollutants, and carcinogens (Li, 2007; Prapanthadara et al. 1996; Wu et al., 2012). GSTs aid in detoxification by conjugating reduced glutathione (GSH) to the metabolites or xenobiotics to increase water solubility for excretion (Hemingway 2002; Grant and Matsumura 1988). The majority of GSTs exist as cystolic dimeric proteins, microsomal enzymes, or mitochondrial Kappa GSTs [Pearson, 2005; Pemble, 1996]. Currently, there are at least six classes of mosquito GSTs that have been identified in insects: delta, epsilon, omega, sigma, theta, and zeta

[Ranson 2002]. Elevated levels of GSTs are implicated in insecticide resistance against orgaonphosphates, organocholorines, and pyrethroids [Clark and Shaman, 1984; Vontas, 2001]. There are also antenna-specific GST genes that have been identified for their potential involvement in signaling or odorant degradation in the olfactory system [Durand, 2018; Rogers, 1999; Wang, 2004; Leal, 2009; Tan, 2014]. GSTs also play an important role in oxidative stress by metabolizing or inactivating reactive oxygen species. ROS are toxic by-products of cellular metabolism in plants and animals that can be a result of environmental stress or infection by pathogens. When an organism experiences oxidative stress, the cells will attempt to activate or silence genes to maintain the redox balance [Scandalios, 2004]. Additionally, GST and oxidative stress can play a role in pathogen development within the mosquito midgut. Kumar et al. demonstrated that *Plasmodium* development was inhibited by oxidative stress in *Anopheles gambiae* [Kumar, 2003]. For the most part, the role of GST detoxification and pathogen survival is unclear, but there may be implications that mosquitoes with higher GST activity can serve as more efficient vectors of pathogens. This is due to the reduction of ROS in the midgut, which favors pathogen development. If left uncontrolled, the harmful effects of ROS result in degradation of DNA or RNA, damage to cell membranes and amino acids, and inactivation of enzymes [Ranson, 2005; Birben, 2012]. According to Tripathy et al. 2016, GSTs may affect vector competence and vectorial capacity due to the possibility of GST causing direct damage to the pathogen, accelerating the activation of the immune response, or aiding in pathogen development inside the mosquito [Tripathy, 2016].

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Chapter 2

Vector competence of Virginia mosquitoes for Zika and Cache valley viruses

ABSTRACT

Vector-borne diseases are a major public health concern and cause significant morbidity and mortality. Zika virus (ZIKV) is the etiologic agent of a massive outbreak in the Americas that originated in Brazil in 2015, and shows a strong association with congenital ZIKV syndrome in newborns. Cache Valley virus (CVV) is a bunyavirus that causes mild to severe illness in humans and ruminants. In this study, we investigated the vector competence of Virginia mosquitoes common in urban and suburban environments for ZIKV and CVV to explore their abilities to contribute to potential outbreaks and help inform local mosquito control strategies. To determine vector competence after oral infection, mosquitoes were fed an infectious artificial blood meal comprised of defibrinated sheep blood and virus. The presence of midgut or salivary gland barriers to ZIKV infection were determined by intrathoracic inoculation vs. oral infection. After 14-days post-exposure (DPE), individual mosquitoes were separated into bodies, legs and wings, and saliva expectorant. Virus presence was detected by plaque assay on Vero cell cultures to determine midgut infection, dissemination, and transmission rates. *Ae. albopictus* and *Ae. japonicus* were competent for ZIKV following oral exposure to the virus. *Ae. albopictus* orally infected (24%) and intrathoracically inoculated (63%) with ZIKV had similar transmission rates to *Ae. aegypti* (48% and 71%, respectively). Transmission rates of ZIKV in *Ae. japonicus* were low, and showed evidence of a midgut infection barrier demonstrated by low midgut infection and dissemination rates from oral infection (3%), but increased transmission rates after intrathoracic inoculation (19%). *Ae. triseriatus* was unable to transmit ZIKV following oral

infection or intrathoracic inoculation. CVV transmission was dose-dependent where mosquitoes fed high titer (ht) virus blood-meals developed higher rates of midgut infection, dissemination, and transmission compared to low titer (lt) virus blood-meals. CVV was detected in the saliva of *Ae. albopictus* (ht: 68%, lt: 24%), *Ae. triseriatus* (ht: 52%, lt: 7%), *Ae. japonicus* (ht: 22%, lt: 0%), and *Ae. aegypti* (ht: 10%; lt: 7%). *Culex pipiens* and *Culex restuans* were not competent for ZIKV or CVV.

INTRODUCTION

Vector-borne pathogens are a major public health concern, and cause significant morbidity and mortality globally. In recent years, vector-borne pathogens have appeared in new regions, even as endemic diseases have increased in incidence. Human travel and trade are often responsible for the introduction of invasive pathogens but ecological factors such as climate and presence of competent vectors will determine whether the pathogen becomes established. For example, since its introduction in 1999, West Nile virus (WNV) (family *Flaviviridae,* genus *Flavivirus)* is now the leading cause of vector-borne encephalitis in the United States [1]. Also impacting vector-borne disease emergence are invasive mosquitoes that may alter the transmission cycles of pathogens, whether native or introduced [2]. *Aedes albopictus* and *Ae. japonicus* are two of the most invasive mosquito species worldwide [3] and both have been known to function as competent vectors for several enzootic mosquito-borne viruses in the U.S. [4, 5].

Zika virus (ZIKV) (family *Flaviviridae,* genus *Flavivirus)* is an arthropod-borne virus (arbovirus) of humans and has been linked to congenital malformations and microcephaly in developing fetuses, and Guillain-Barre syndrome in adults [6]. Since its introduction to Brazil in

2015, ZIKV has spread into many new areas within the Americas [7]. ZIKV is transmitted primarily by urban and sylvatic *Aedes* mosquitoes, with *Ae. aegypti* serving as the main vector for human infection outside of Africa [8, 9, 10]. This emerging mosquito-borne virus has caused epidemics throughout Africa, Asia, the Pacific Islands, and the Americas [11, 12]. Due to the lack of knowledge of ZIKV replication in North American mosquitoes, experimental vector competence studies are necessary to better understand the potential transmission of ZIKV by additional species. Recent studies have shown that some *Aedes, Culex,* and *Coquillettidia* mosquitoes from temperate regions of North America were not competent for ZIKV [13, 14] but this is a small representation of the species and strain diversity of mosquitoes that are found in the United States.

Cache Valley virus (CVV) (family *Peribunyaviridae,* genus *Orthobunyavirus)* is a neuroinvasive arbovirus that is also spread by mosquitoes. Although CVV infection typically causes mild symptoms in humans, fever, meningitis, and encephalitis have been reported [15]. The symptoms of CVV infection are more severe in ruminants, such as sheep or cattle, and include stillbirths, congenital malformations, spontaneous abortions, and death [16]. CVV has a widespread distribution in North America, and has been isolated from many species of mosquitoes including *Ae. albopictus* and *Ae. japonicus* [17, 18, 19, 20]. The principal vector is unknown, but vector competence studies and field isolations have shown that *Anopheles quadrimaculatus* and *An. punctipennis* may play a significant role in the natural transmission cycle [21, 22]. Laboratory transmission studies have also shown that *Cx. tarsalis, Ae. taeniorhynchus, Ae. sollicitans,* and *Cq. perturbans* are competent vectors of CVV [22, 23, 24].

Aedes aegypti and *Ae. albopictus* are the most important mosquito species responsible for virus transmission to humans in urban environments. Both species are competent vectors for ZIKV, dengue virus (DENV), and yellow fever virus (YFV) [25, 26, 27]. The Asian rock pool mosquito, *Ae. japonicus*, is a relatively new invasive species that can be found in subtropical and temperate regions of the United States. Although *Ae. japonicus* is not an aggressive human biter, blood meal analysis from field collected mosquitoes have shown high incidences of human blood consumption [28]. Laboratory transmission studies show that *Ae. japonicus* is a competent vector of WNV, La Crosse virus (LACV), Eastern equine encephalitis virus (EEE), and St. Louis encephalitis virus (SLEV) [29, 30, 31, 32]. *Aedes triseriatus*, the principal vector of LACV, is found extensively throughout eastern United States and parts of central America [33]. Under laboratory conditions, *Ae. triseriatus* is a competent vector for WNV, DENV, YFV, EEE, and SLE [34]. WNV has been isolated from *Culex pipiens* and *Cx. restuans* and both species have been shown to be competent vectors of the virus [35, 36]. Laboratory transmission studies have found that *Cx. pipiens* is refractory to CVV and ZIKV infections [13, 23, 25, 37, 38].

Between 2015 and 2018, there were more than 5,000 imported ZIKV cases in the United States, with over 100 cases in Virginia [39]. Within the continental United States, reports of local transmission by mosquito vectors have occurred in Florida and Texas [40, 41, 42]. There have been no human CVV cases reported in Virginia, but the virus has been isolated from field-collected mosquitoes [20]. Although CVV has been detected in field mosquitoes, only a few transmission studies have been conducted to determine potential vectors for the virus. With the wide distribution of *Ae. albopictus, Ae. japonicus, Ae. triseriatus, Cx. pipiens* and *Cx. restuans* throughout Virginia [43], it is crucial to determine the vector competence of these local mosquito strains. In this study, we investigated the vector competence of Virginia mosquitoes common in

urban and suburban environments for ZIKV and CVV to explore their abilities to contribute to potential outbreaks and help inform local mosquito control strategies

MATERIALS AND METHODS

Mosquito collection and rearing

All eggs were derived from female mosquitoes collected using gravid traps in forested areas around Blacksburg, VA. After laying eggs, adult mosquitoes were tested for arboviruses by Vero cell plaque assay to ensure the absence of virus in the F1 progeny. A laboratory strain of *Ae. aegypti* from Vero Beach, FL was used as our reference vector species and was subsequently tested for CVV vector competence. Mosquitoes were reared in environmental chamber conditions set at 24°C with 75% RH and 16L: 8D photoperiod using methods by Jackson et al. [44] to ensure consistent adult size.

Cells and Virus

African green monkey kidney (Vero) cells (American Type Culture Collection, Manassas, VA, USA) were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Corning, Corning, NY) with 10% fetal bovine serum (FBS), 100 U/mL of penicillin and 100 µg/mL of streptomycin, and maintained at 37 °C with 5% CO_2.

The Asian lineage of ZIKV, PRVABC59 (GenBank accession no. KU501215), and CVV strain 4B (GenBank accession no. KX583998) was used in this study. PRVABC59 was isolated from the serum sample of a patient traveling from Puerto Rico in 2015. CVV4B was isolated from field-caught *Ae. japonicus* during a 2015 field study in Blacksburg, VA. Both viruses were maintained through passage on Vero cells and stored at -80^{o}C. Infected blood-meals consisted of 1ml of virus mixed with

9ml of defribinated sheep blood (Colorado Serum Company, Denver, CO).

Mosquito infection

For oral infection, 1-week-old female mosquitoes were starved 24 hours before blood-feeding and provided cotton balls soaked with deionized water. Approximately 40-50 female mosquitoes were placed into 1-gallon cages covered with a mesh screen top. The mosquitoes were offered infected blood meals contained in a glass water-jacketed membrane feeder attached to a circulating 37°C water bath. Pig intestine sausage casing was used as the membrane. After a 2-hour feeding period, fully engorged females were anesthetized on ice and transferred to a new 1-liter cage. A 0.5ml sample of the infected blood was removed after the feeding period and stored at -80°C for later virus titer. Parenteral infection was done by intrathoracic inoculation of week-old females that had never taken a blood meal with 0.2ul of virus [45]. Table 2.1 shows the titers of virus infected blood-meals and virus inoculum. Infected mosquitoes were maintained at 24°C with 75% RH and 16L: 8D photoperiod and provided 10% sucrose solution for sustenance.

Table 2.1. ZIKV and CVV blood-meal titers

Species	CVV high blood-meal titer (pfu/mL)	CVV low blood-meal titer (pfu/mL)	ZIKV blood-meal titer (pfu/mL)	ZIKV intrathoracic inoculation titer (pfu/mL)
Aedes albopictus	$5.25x10^6$	$2.9x10^3$	$3x10^6$	5.25×10^4
Aedes aegypti	$1.98x10^7$	$6.28x10^3$	$6.5x10^6$	5.25×10^4
Aedes japonicus	$1.99x10^6$	4.6×10^3	$3.72x10^7$	7.5×10^4
Aedes triseriatus	$2.99x10^6$	1.4×10^3	4.5×10^7	1.8×10^5

Saliva Extraction

After 14-DPE, female mosquitoes were removed from cages and immobilized by chilling on ice. Saliva was extracted by inserting the proboscis into a capillary tube filled with a 1:1 mixture of 10% sucrose and fetal bovine serum (FBS) [46]. The mosquitoes were given 30 minutes to feed and salivate. The saliva, legs and wings, and body were placed into separate microcentrifuge tubes with DMEM and stored at -80°C until virus testing.

Virus detection

Mosquito bodies, leg and wing, and saliva samples were homogenized with metal pellets in 2ml of Vero media on a vortex mixer and then clarified by centrifugation at 1500 x g for two minutes. Supernatants were tested for infection using Vero cell plaque assay following the methods of Barker et al. [47]. If virus was recovered from the body but not the legs and wings, the mosquito was classified as having a non-disseminated infection; if virus was detected in the wings and legs, the mosquito was classified as having a disseminated infection; mosquitoes with virus in saliva were classified as transmitting. Infectious blood-meals were thawed at room temperature, diluted with a series of 10-fold serial dilutions, and tested for virus concentration using plaque assay.

Statistical analysis

A Chi-squared test was used to compare mean infection, dissemination, and transmission rates among mosquito species followed by Fisher's exact tests for pair-wise comparisons [48]. GraphPad Prism 6.0 (La Jolla, CA) was used for all statistical analysis. All statistical analysis were carried out at a significance level of α=0.05.

RESULTS

Vector competence for ZIKV following oral infection

There was a significant difference among infection ($P < 0.05$, $X^2 = 58.73$, df = 5), dissemination ($P < 0.05$, $X^2 = 71.21$, df = 5), and transmission ($P < 0.05$ $X^2 = 60.17$, df = 5) rates for *Ae. aegypti, Ae. albopictus, Ae. japonicus,* and *Ae. triseriatus* after oral infection with ZIKV (Figure 1). Rates for infection, dissemination and transmission were highest for *Ae. aegypti* (68%, 60%, and 48%, respectively) and *Ae. albopictus* (49%, 41%, and 24%, respectively). *Aedes japonicus* rates of infection, dissemination and transmission (20%, 9%, and 3%) were significantly lower than *Ae. aegypti* (Fisher's exact, $P < 0.0001$, OR: 0.9257, 95% CI: 0.03259-0.2629; $P < 0.0001$, OR: 0.05970, 95% CI: 0.01877–0.1899; $P < 0.0001$, OR: 0.03052, 95% CI: 0.006101–0.1527) and *Ae. albopictus* (Fisher's exact, $P < 0.0006$, OR: 0.2077, 95% CI: 0.08494–0.5076; $P = 0.0002$, OR: 0.1313, 95% CI: 0.04539–0.3800; $P= 0.0008$, OR: 0.08764, 95% CI: 0.01780–0.4314) (Figure 1). Although 25% of *Ae. triseriatus* became infected after imbibing an infectious blood meal, there was no dissemination or transmission of the virus. Neither *Cx. pipiens* nor *Cx. restuans* were infected after oral exposure to ZIKV (Table 2.2).

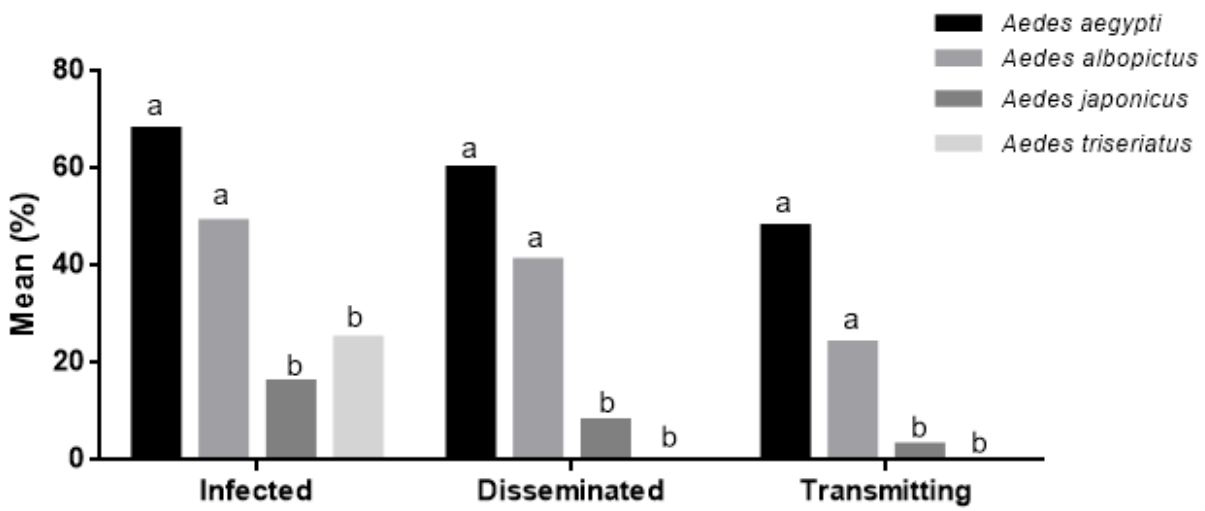

Figure 2.1. Vector competence for ZIKV PRVABC59. *Ae. albopictus* (n= 37), *Ae. triseriatus* (n= 28), *Ae. japonicus* (n= 73), and *Ae. aegypti* (n= 25) were provided infectious blood-meals with an average titer of 2.57 x 10^7 PFU/mL (range= 5.75 x 10^6 to 7.5 x 10^7 PFU/mL). After 14 days, mosquitoes were dissected and the number infected (% mosquitoes with virus in the body), disseminated (% mosquitoes with virus in legs and wings, independent of infection status), and transmitting (% mosquitoes with virus in saliva expectorant, independent of infection status) were determined by Vero cell plaque assay. Different letters denote significance by two-tailed Fischer's exact test and presented as mean % infected, disseminated, and transmitting, α=0.05.

Table 2.2. Vector competence of ZIKV PRVABC59 in *Cx. pipiens* and *Cx. restuans.*

Mosquito Species	Sample size (n)	Mean Titer (PFU/mL)	Mean non-disseminated infection	Mean disseminated infection	Mean transmitting
Culex pipiens	30	3 x 10^7	0%	0%	0%
Culex restuans	28	5.25 x 10^6	0%	0%	0%

Transmission of ZIKV following parenteral infection

Parenteral infection by intrathoracic inoculation resulted in significantly higher rates of transmission compared to oral infection in *Ae. albopictus* (63% parenteral vs. 24% oral) (Fisher's exact, $P = 0.0080$, OR: 0.1875, 95% CI: 0.05662–0.6209) and *Ae. japonicus* (19% parenteral vs. 3% oral) (Fisher's exact, $P = 0.0212$, OR: 0.1197, 95% CI: 0.02022–0.7088) (Table 3). Mode of infection had no effect on transmission by *Ae. aegypti* (71% parenteral vs. 48% oral) (Fisher's exact, $P = 0.1395$, OR: 0.3693, 95% CI: 0.1079–1.263). No virus was detected in the saliva of *Ae. triseriatus* from either orally or parenterally infected groups (Table 2.3).

Table 2.3. Transmission rate of *Ae. japonicus* and *Ae. triseriatus* intrathoracically inoculated with ZIKV.

Mosquito species	Infection method	Sample size (*n*)	Virus titer (pfu/mL)	Transmission
Aedes albopictus	Intrathoracic	19	5.25×10^4	63% [a]
Aedes albopictus	Oral	37	3×10^6	24% [b]
Aedes aegypti	Intrathoracic	21	5.25×10^4	71% [a]
Aedes aegypti	Oral	25	6.5×10^6	48% [a]
Aedes japonicus	Intrathoracic	21	7.5×10^4	19% [a]
Aedes japonicus	Oral	73	3.72×10^7	3% [b]
Aedes triseriatus	Intrathoracic	23	1.8×10^5	0% [a]
Aedes triseriatus	Oral	28	4.5×10^7	0% [a]

Differing letters denote significance of transmission rates of the same species after oral or intrathoracic infection by two-tailed Fischer's exact test, α=0.05.

Vector competence to CVV

When fed a high titer (ht) virus blood-meal, *Ae. albopictus* and *Ae. triseriatus* showed significantly higher rates of infection ($P < 0.0001$, $X^2 = 127.5$, df = 5), dissemination ($P <$

0.0001, X^2= 107.8, df = 5), and transmission ($P < 0.0001$, X^2 = 88.08, df = 5) than *Ae. japonicus* or *Ae. aegypti* (Table 4). However, when fed low titer (lt) blood-meals, there were no differences among rates for any of the species (X^2, P > 0.05) (Table 4). *Ae. albopictus* was the most susceptible to CVV oral infection (ht: 100%, lt: 24%) and had the highest rate of dissemination (ht: 85%, lt: 24%) and transmission (ht: 68%, lt: 24%). *Ae. triseriatus* was also susceptible to CVV infection (ht: 72%, lt: 15%), dissemination (ht: 69%, lt: 11%), and transmission (ht: 52%, lt: 7%). None of the *Ae. japonicus* fed a low titer blood-meal developed midgut infections. For *Ae. aegypti*¸ CVV was able to establish midgut infections (ht: 48%, lt: 11%), cause a disseminated infection (ht: 25%, lt: 11%) and transmit virus (ht: 10%; lt:7%). Figure 2 shows significant differences in infection, dissemination, and transmission between high and low titer blood-meals for *Ae. albopictus*, *Ae. triseriatus*, and *Ae. japonicus*. Virus titer resulted in significant differences in infection for *Ae. aegypti* but not dissemination or transmission rates. Neither of the *Culex* species was able to transmit CVV. No *Cx. restuans* became infected and only one *Cx. pipiens* was positive for infection and dissemination (Table 2.5).

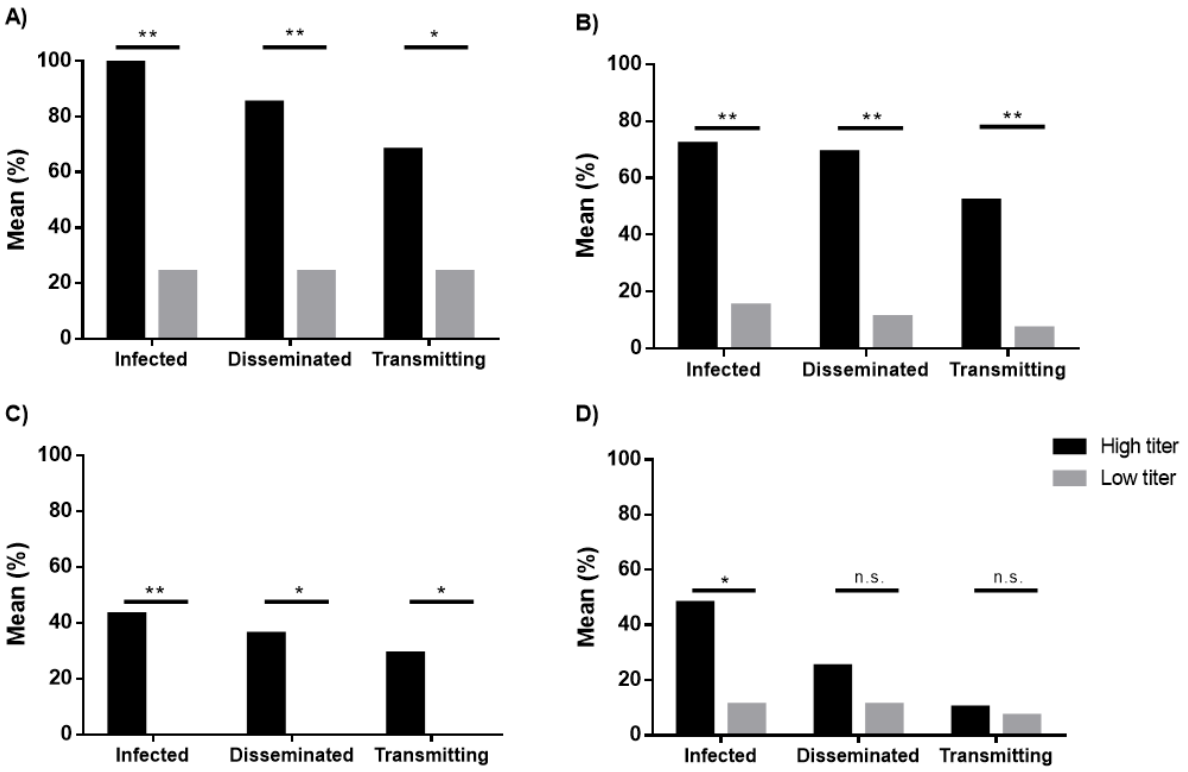

Figure 2.2. Vector competence for CVV with high versus low titer blood-meals. Mosquitoes were provided low titer (lt) (1.2 x 10^3 to 4.6 x 10^3 PFU/mL) or high titer (ht) (1.6 x 10^5 to 5.5 x 10^7 PFU/mL) infectious blood-meals. After 14 days, the mosquitoes were dissected and the number infected (% mosquitoes with virus in the body), disseminated (% mosquitoes with virus in legs and wings, independent of infections status), and transmitting (% mosquitoes with virus in saliva expectorant, independent of infection statues) were determined using Vero cell plaque assay. A) *Ae. albopictus* (lt: *n*= 21; ht: *n*= 34), B) *Ae. triseriatus* (lt: *n*=55; ht *n*=29), C*) Ae. japonicus* (lt: *n*=21; ht: *n*=74), and D*) Ae. aegypti* (lt: *n*=44; ht: *n*=52). Date is presented as mean % infected, disseminated, and transmitting. *= $p<0.01$, **= $p<0.001$, and n.s.= not significant by two-tailed Fischer's exact test.

Table 2.4. Vector competence for CVV 4B by *Ae. albopictus, Ae. triseriatus, Ae. japonicus,* and *Ae. aegypti* presented as mean % infected, disseminated, and transmitting.

	Species	Sample size (n)	Mean blood-meal titer (pfu/ml)	% infected	% disseminated	% transmitting
High titer	*Aedes albopictus*	34	5.25×10^6	100%[a]	85%[a]	68%[a]
	Aedes triseriatus	29	2.99×10^6	72%[a]	69%[a]	52%[a]
	Aedes japonicus	74	1.99×10^6	41%[b]	38%[b]	28%[b]
	Aedes aegypti	52	1.98×10^7	48%[ab]	25%[b]	10%[b]
Low titer	*Aedes albopictus*	21	2.9×10^3	24%[a]	24%[a]	24%[a]
	Aedes triseriatus	55	1.4×10^3	15%[a]	11%[a]	7%[a]
	Aedes japonicus	21	4.6×10^3	0%[a]	0%[a]	0%[a]
	Aedes aegypti	44	6.28×10^3	11%[a]	11%[a]	7%[a]

Different letters denote significance between different species within respective categories of high or low titer blood-meals by two-tailed Fischer's exact test, α=0.05.

Table 2.5. Vector competence of CVV 4B in *Cx. pipiens* and *Cx. restuans.*

Mosquito Species	Sample size (n)	Mean Titer (PFU/mL)	Mean non-disseminated infection	Mean disseminated infection	Mean transmitting
Culex pipiens	67	1.12×10^8	1%	1%	0%
Culex restuans	30	7.7×10^7	0%	0%	0%

DISCUSSION

Assessing the vector competence of local mosquitoes for imported and emerging viruses is critical for public health officials to anticipate patterns of arbovirus transmission, determine the relative roles of the different species for virus amplification and spread, and to select appropriate control responses. This study aimed to determine the risk of local ZIKV transmission and the emergence potential of CVV in Virginia by evaluating the vector competence of the most common mosquito species found in urban and suburban habitats.

A meta-analysis by McKenzie et al. [49] suggested that the vector competence of *Ae. albopictus* for Zika virus varied among geographically disparate populations. We found that the vector competence of a Virginia strain of *Ae. albopictus* was equivalent to that of a Florida strain of *Ae. aegypti*. *Culex* mosquitoes were found to be refractory to ZIKV infection. Other studies have also observed similar results, suggesting that it is unlikely this group plays a role in ZIKV transmission [13, 37, 50, 51]. We also found that *Ae. japonicus* from Virginia was capable of transmitting ZIKV, but at a much lower rate compared to *Ae. aegypti* and *Ae. albopictus*. A study by Aliota et al. [13] showed that lab strains of *Ae. triseriatus* were able to become infected with ZIKV PRVABC59, the same strain that we used, but no dissemination or transmission resulted. Our study showed similar results working with an F1 generation of field-caught *Ae. triseriatus* where only midgut infections resulted from oral exposure.

Upon ingesting an infectious blood-meal, the virus must surmount several tissue barriers associated with the midgut and salivary glands [52]. Both *Ae. japonicus* and *Ae. triseriatus* had low infection rates, which may indicate the presence of a midgut barrier that is preventing the virus from infecting midgut epithelial cells. We assessed the presence of tissue barriers by

intrathoracic inoculation of ZIKV. Injecting virus directly into the hemolymph bypasses the midgut and permits the virus to reach and infect the salivary glands. We detected infectious virus in salivary expectorant of *Ae. japonicus*, but not *Ae. triseriatus,* which indicated the presence of salivary gland barriers. Although transmission for intrathoracically inoculated *Ae. japonicus* was significantly higher than orally infected mosquitoes, the rates were low. The low midgut infection and transmission rates leads us to believe that there are potential midgut and salivary gland barriers that limit *Ae. japonicus* and prevent *Ae. triseriatus* from ZIKV transmission. Although *Ae. albopictus* was capable of ZIKV transmission after oral infection, intrathoracic inoculation significantly increased its transmission rates. This shows that midgut barriers may limit ZIKV transmission by reducing virus dissemination from the midgut epithelial cells. Virus particles disseminated in the hemolymph will likely reach and infect the salivary glands, potentially resulting in virus transmission. Studies of virus and vector systems have shown that these barriers play an important role during the extrinsic incubation period and may limit the ability of the virus to infect the mosquito for successful transmission [53, 54]. In addition, gut microbiota and immune pathways may also be involved when the virus enters the midgut [55, 56, 57]. It has been hypothesized that midgut and salivary gland barriers are responsible for the geographic variation in vector competence seen in *Ae. aegypti* and *Ae. albopictus* for ZIKV [58, 59, 60].

Although *Ae. japonicus* was capable of ZIKV transmission, it is not an aggressive human biter and predominantly inhabits forested areas, which limits its role in ZIKV transmission. Surprisingly, *Ae. triseriatus*, *Cx, pipiens*¸ and *Cx. restuans* were not competent for ZIKV even though they are competent vectors of other *Flaviviruses,* such as WNV or SLEV [61, 62, 63]. *Aedes albopictus,* on the other hand, was highly competent for ZIKV and exhibits aggressive,

anthropophilic behavior. The likelihood for this species to contribute to ZIKV transmission in Virginia is much higher compared to other *Aedes* mosquitoes in this region.

This study also found that *Ae. albopictus*, *Ae. triseriatus*, *Ae. japonicus*, and *Ae. aegypti* were susceptible to CVV infection and capable of virus transmission. The combination of high vector competence, previous isolations from the field, and anthropophilic behavior suggests that *Ae. albopictus* could play a major role in CVV transmission in endemic areas [64, 65]. *Aedes triseriatus* and *Ae. japonicus* were also competent for CVV and blood-meal analysis have shown that all three species feed on deer, the primary amplifying vertebrate host for CVV [28, 66, 67, 68, 69]. We used high and low CVV blood-meal titers that bracketed the range of titers found in experimentally infected deer [22] and showed that *Ae. albopictus*, *Ae. triseriatus*, and *Ae. aegypti* were susceptible to CVV infection and subsequently transmitted virus even when exposed to low titer blood-meals. There is currently no evidence of field isolation of the virus from *Ae. aegypti*, but the distribution of CVV includes the southern United States where *Ae. aegypti* is commonly found [18]. Even though CVV has been isolated from wild *Ae. japonicus* [20], and this species has been shown to feed on deer [28], it is unclear if it serves as a major vector in enzootic or local transmission of the virus. We also tested vector competence of field-caught *Cx. pipiens* and *Cx. restuans* for CVV, and found no evidence of transmission by either species. The existence of a dose-dependent infection or escape barrier can determine how certain mosquito species and strains are refractory to infection. Studies looking at dose-dependent interactions between mosquito vectors and the virus typically find that high titers result in greater midgut infection and transmission potential while low titers result in low midgut infection and transmission rates [70, 71, 72, 73]. The dose-dependent tissue barriers are often associated with midgut escape barriers or RNA interference (RNAi) pathways [56, 72], while incompatibility between the virus

and cells of the midgut or salivary glands are dose-independent barriers [52, 53]. With laboratory evidence of low titer vector competency and abundant distribution throughout North America, *Ae. aegypti*, *Ae. albopictus*, *Ae. japonicus*, and *Ae. triseriatus* could play major roles in CVV transmission.

Outbreaks of mosquito-borne diseases can have large economic and devastating impacts on human and animal health. Experimental vector competence studies allow us to understand the potential for a mosquito species to contribute to an outbreak and facilitate more targeted surveillance and control. Due to the wide variability of mosquito and virus infectivity, it is not possible to make the assumption that studies involving vectors from different geographic locations will have similar competencies. Therefore, it is not appropriate to extrapolate results from other studies for a single conclusion. Several studies clearly show considerable variability in the susceptibility of the same vector species for viral infection for DENV [74, 75], CHIKV [76], and ZIKV [59, 77]. In addition, when arboviruses are detected in field-caught mosquitoes, we cannot assume that it is competent and able to transmit the virus. The mosquito may have an undigested blood-meal that was recently taken from an infected host, which can yield a false positive. Laboratory vector competence studies allow us to determine if a mosquito species is capable of transmitting the virus. When conducting laboratory vector competence studies, it is important to consider laboratory-reared versus field-caught mosquitoes. For example, some vector competence studies have shown that lab-reared *Cx. quinquefasciatus* is able to become infected and transmit ZIKV [78, 79], while studies using field populations were not able to transmit the virus [38].

There are many knowledge gaps in CVV dynamics, especially our understanding of its natural cycle of competent vectors and susceptible amplifying hosts. In addition, CVV infections are often misdiagnosed for other flu-like illness, which presents itself as a challenge for accurate reporting to local or state health departments. In contrast, the ZIKV outbreak in 2015 sparked high demand for all areas of research to understand and control the virus. Although cases have dropped significantly in the United States, ZIKV is still present in parts of Africa, Asia, and South America, and may remain indefinitely [11, 80]. Our studies show that a species that has not been tested for ZIKV vector competency, *Ae. japonicus*, was able to transmit the virus, but at a low rate. *Aedes japonicus*, however, was competent for CVV transmission. *Aedes albopictus*, the most widespread anthropophilic mosquito in Virginia, was competent for both ZIKV and CVV. *Aedes aegypti* was competent for both viruses, but its inability to overwinter in colder climates reduces this species' likelihood of ZIKV transmission in Virginia.

In summary, we found that multiple *Aedes* species from Virginia are competent vectors for ZIKV and CVV. With the abundance of highly competent mosquito species, there may be greater concern for CVV transmission in temperate regions of the United States.

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Chapter 3

The effects of La Crosse and Zika virus infection on repellent response in *Aedes* mosquitoes

ABSTRACT

Because vaccines are not available for most arboviruses, insect repellents are recommended to prevent disease transmission from arthropod vectors. Little research, however, has been conducted on repellent response by virus-infected mosquitoes. This study looked at the effects of virus infection on repellent response in LACV and ZIKV-infected mosquitoes using a short-range artificial membrane feeder assay and a human arm assay. Here we tested LACV-infected *Aedes albopictus* and *Ae. triseriatus* and ZIKV-infected *Ae. albopictus* and *Ae. aegypti* against DEET, picaridin, and p-Menthane-3,8-diol (PMD). Infected mosquitoes were more likely to land, probe, and blood-feed from surfaces treated with 5% repellent for both assays. Increasing repellent concentrations significantly increased percent protection (%*p*) and reduced the number of infected and uninfected mosquitoes landing on the artificial membrane and human arm, thus reducing potential for probing or blood-feeding. This study demonstrated that pathogen infection has the potential to modify vector behavior to enhance transmission and the importance of maintaining effective repellent concentrations in areas with high mosquito biting pressure.

INTRODUCTION

La Crosse virus (LACV, family *Peribunyaviridae*, genus *Orthobunyavirus*) is the most common and important endemic arthropod-borne virus (arbovirus) of children in the United States [Calisher, 1994; Rust, 1999]. This virus was first isolated in 1960 from a child who died in Wisconsin after suffering from encephalitis [Thompson, 1965]. Infection with LACV typically results in flu-like symptoms, but the elderly, young children, and immunocompromised are at higher risk of developing neuroinvasive disease [McJunkin, 1998; Haddow, 2011]. An average

of 65 LACV cases are reported each year, with majority of cases occurring east of the Mississippi river [CDC, 2018a]. The virus is maintained in nature by *Ae. triseriatus* through horizontal transmission to small woodland mammals and transovarial transmission to overwintering eggs [Beaty, 1991; Miller, 1977; Pantuwatana, 1974].

Zika virus (ZIKV, family *Flaviviridae*, genus *Flavivirus*), is another mosquito-borne virus that is linked to Guillain-Barre syndrome in adults and microcephaly in developing fetuses [Krauer, 2017]. Since its discovery in Uganda, ZIKV has spread into many new areas, including Asia, Pacific Islands, and the Americas [Zanluca, 2015; CDC, 2018b]. Unlike most arboviruses that rely on mosquito vectors for transmission, ZIKV can also spread human-to-human via unprotected sex [Foy, 2011; Freour, 2016; Hills, 2016; Musso, 2015], infected blood [Aubry, 2015, Musso, 2014] and mother-to-child during pregnancy or breast-feeding [Brasil, 2016; Calvet, 2016; Dupont-Rouzeyrol, 2016]. Within the United States, there have been approximately 5,746 reported cases resulting from the combination of travelers returning from affected areas, local mosquito transmission, or sexual transmission [CDC, 2019]. The virus is primarily spread by urban and sylvatic *Aedes* mosquitoes, with *Ae. aegypti* serving as the main urban vector [Ayres, 2016].

Aeedes aegypti and *Ae. albopictus* are anthropophilic mosquitoes responsible for disease transmission in urban environments and are competent vectors for ZIKV, dengue virus (DENV), yellow fever virus (YFV), and chikungunya virus (CHIKV) [Liu, 2017; Miller, 1989; Whitehorn, 2015; Mangiafico, 1971; Hugo, 2016]. *Aedes triseriatus*, the principle vector for LACV, is found throughout eastern United States and parts of central America [Sánchez-Trinidad, 2014], and is also competent for West Nile virus and DENV [Erickson, 2006; Freier, 1983]. *Aedes albopictus* also serves as a potential bridge vector of LACV [Bewick, 2016].

Due to the unavailability of vaccines or treatment for ZIKV or LACV, the Center for Disease Control and Prevention (CDC) advises the best way to prevent infection against all arthropod-borne diseases is to protect yourself from mosquito bites (CDC, 2018c). Personal protection methods such as avoidance techniques, physical or chemical barriers, treatment of clothing with toxicants, and topical repellents can be used to prevent bites from mosquitoes [Barnard, 2000]. Topical repellents for skin application is the most common practice due to accessibility, cost, and ease of use. The CDC recommends the use of DEET (*N*,*N*-diethyl-3-methyl-benzamide), picaridin (Icaridin, Bayrepel, butan-2-yl 2-(2-hydroxyethyl) piperidine-1-carboxylate), and p-methane-3,8-diol(PMD) as few of the Environmental Protection Agency (EPA)- registered repellents effective against arthropod-transmitted diseases [CDC, 2018c]. Studies comparing the efficacy of common repellent ingredients and formulations have shown comparable efficacy when used properly. Although mosquito species, test parameters, and results vary between studies, DEET, picaridin, and PMD are able to provide >90% repellency [Barnard, 2002; Carroll, 2006; Frances, 2004; Uzzan, 2009]. Both ZIKV and LACV are neurotropic in the mosquito and infection has been shown to cause behavioral changes in the vector (Jackson et al, 2012; Yang et al. 2019; Gaburro et al. 2018). Since most repellency studies are conducted with uninfected mosquitoes, it is important to determine if virus infection affects the efficacy of repellents. This study used laboratory bioassays to determine if ZIKV and LACV-infected mosquitoes respond differently to three commercially available repellents: DEET, picaridin and PMD.

MATERIALS AND METHODS

Mosquitoes

Ae. albopictus and *Ae. triseriatus* eggs were collected from female mosquitoes caught with

gravid traps in Blacksburg, VA. After oviposition, all adult mosquitoes were screened for virus using Vero cell plaque assay to ensure that the strains were not infected. *Ae. aegypti* used for this study was a lab strain from Vero Beach, FL. Mosquitoes were reared in an environmental chamber with conditions set at 24°C with 75% RH and 16L: 8D photoperiod following the methods of Jackson et al. 2012. Larvae were reared in 33 x 17.5 x 11cm containers with 1,600ml of deionized water and fed on bovine liver powder (7.5g/ 500ml). After eclosion, adults were placed into 50 x 50 x 50 cm cages and provided with cotton balls soaked in 10% sucrose solution.

Cells and Virus

Vero cells (American Type Culture Collection, Manassas, VA, USA) were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Corning, Corning, NY) with 10% fetal bovine serum (FBS), 100 U/mL of penicillin and 100 µg/mL of streptomycin. Cell cultures were maintained at 37 °C with 5% CO_2. The Asian lineage of ZIKV, PRVABC59 (GenBank accession no. KU501215), and LACV strain VA0921075 was used for this study. PRVABC59 was isolated from the serum of a patient traveling from Puerto Rico in 2015. VA0921075 came from *Ae. triseriatus* mosquitoes collected from Wise County, VA (Barker et al. 2003). Both viruses were passaged through Vero cells and maintained at -80C for storage.

Mosquito infection

Three to five-day old female mosquitoes were intrathoracically injected with 0.2 µl of virus using methods from Rosen and Gubler (1974). Mosquitoes injected with DMEM were used as uninfected controls. After injection, mosquitoes were placed into 0.5-liter plastic buckets and reared in environmental chamber conditions for seven days and provided with 10% sucrose solution. Sucrose was removed 24 hours before testing and replaced with a cotton ball soaked with deionized water.

To ensure all mosquitoes intrathoracically inoculated with virus were infected, mosquitoes were tested for virus after bioassays were performed. Individual mosquitoes were placed into 2ml microcentrifuge tubes with 1ml of DMEM and metal BB pellets. The samples were homogenized for approximately 30 seconds and centrifuged at 1500 x g for two minutes. The supernatant was plated onto 24-well plates and tested for virus using Vero cell plaque assay following the methods of Barker et al. 2003. All mosquitoes intrathoracically inoculated with LACV or ZIKV were positive for virus infection.

Repellents and test parameters

Table 3.1 shows the repellents used for this study. Repellents were diluted using 200 proof ethanol to reach the desired concentrations of 5%, 10%, and 15%. In order to maintain consistency and reduce the effects of external variables, all tests were performed by the same researcher within the same BSL-2 facility at Virginia Tech between 10am to 2pm. Tests were conducted using one active ingredient at a time to reduce the effects of air-space contamination. Mosquitoes at the time of experimentation were 10 to 12-days old.

Table 3.1. List of repellents used for this study.

Product name	Active ingredient	Product type	Estimated protection time*
Repel 100 Insect Repellent	98.11% DEET (WPC Brands Inc., Bridgeton, MO)	Spray	10 hours
Repel Plant-Based Lemon Eucalyptus Insect Repellent	30% Oil of lemon eucalyptus (65% p-menthane-3,8-diol) (WPC Brands Inc., Bridgeton, MO)	Spray	6 hours
Sawyer	20% Picaridin (Sawyer Products Inc., Safety Harbor, FL)	Spray	12 hours

*manufacturer estimated protection time against mosquitoes

Short-range artificial membrane feeder (SRAMF) assay

Seven-days post-infection, groups of 30 infected or uninfected mosquitoes were placed in a plastic, cylindrical cage (11cm high X 12cm diameter) with a metal screen (Fig. 3.1a). A glass water-jacketed mosquito feeder containing defibrinated sheep blood (Lampire Biological Products, Pipersville, PA) maintained at 37°C was placed on the screen (Fig. 3.1b). Natural pork sausage casing was used as the membrane. The membrane was treated with repellent or ethanol control. Landing, probing, and blood-feeding events were recorded for 15 minutes during which time the observer was emanating human odor. A landing event was recorded when a mosquito contacted the surface for at least five seconds using all forelegs and mid legs. This ruled out disoriented flight contact with the membrane surface. A probing event was when a mosquito inserted the proboscis into the membrane. Multiple probing actions from the same mosquito were not considered additional probing events. Visible blood in the abdomen was considered positive blood-feeding event.

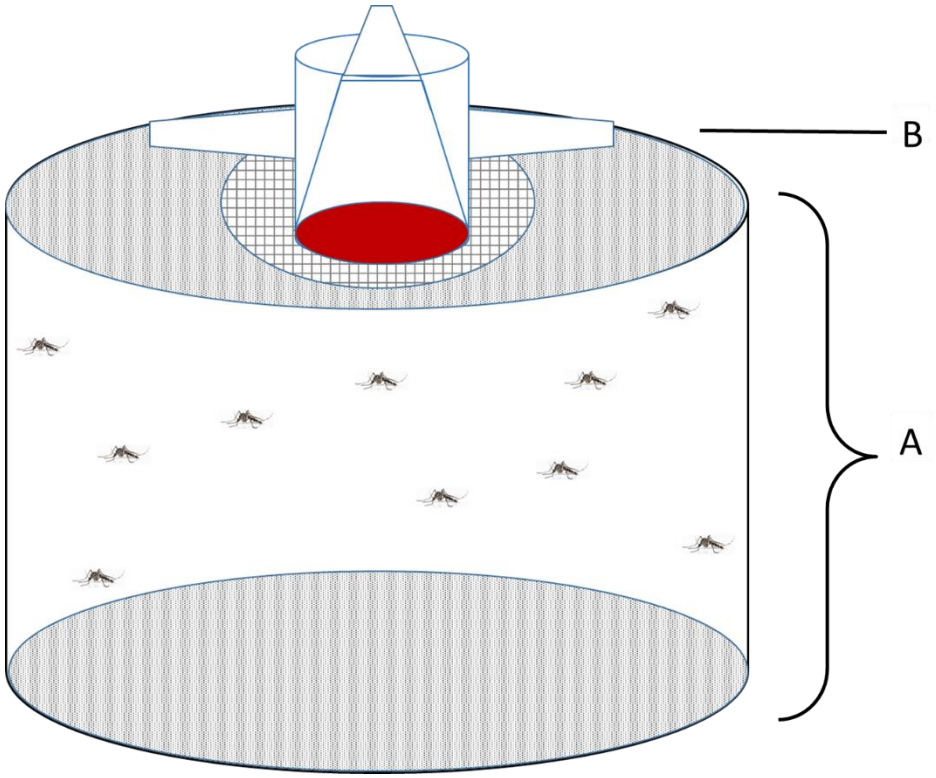

Figure 3.1. Short range artifical membrane feeder (SRAMF). Thirty female mosquitoes were placed into the assay arena (A) with an artificial membrane feeder (B) containing defibrinated sheep blood. The membrane was treated either with repellent or ethanal as a control. The number of mosquitoes that landed, probed, and blood-fed were recorded for 15 minutes.

Human arm assay

Seven-days post-infection, female mosquitoes were placed in a petri dish on ice for anesthetization. Approximately half of the proboscis was removed using dissection scissors (Fig. 3.2b). Mosquitoes were placed into 5,000 ml plastic buckets for at least 1 hour and provided with cotton balls soaked with DI water. Ten mosquitoes were placed into a 12cm x 6.5cm glass tube sealed with nylon mesh netting on both ends (Fig. 3.2c). The researcher's forearm was treated with 1ml of repellent solution or ethanol control using cotton swabs. The opening of the glass

tube was placed directly onto the forearm for two minutes and the number of mosquitoes landing on the arm was recorded (Fig. 3.2d).

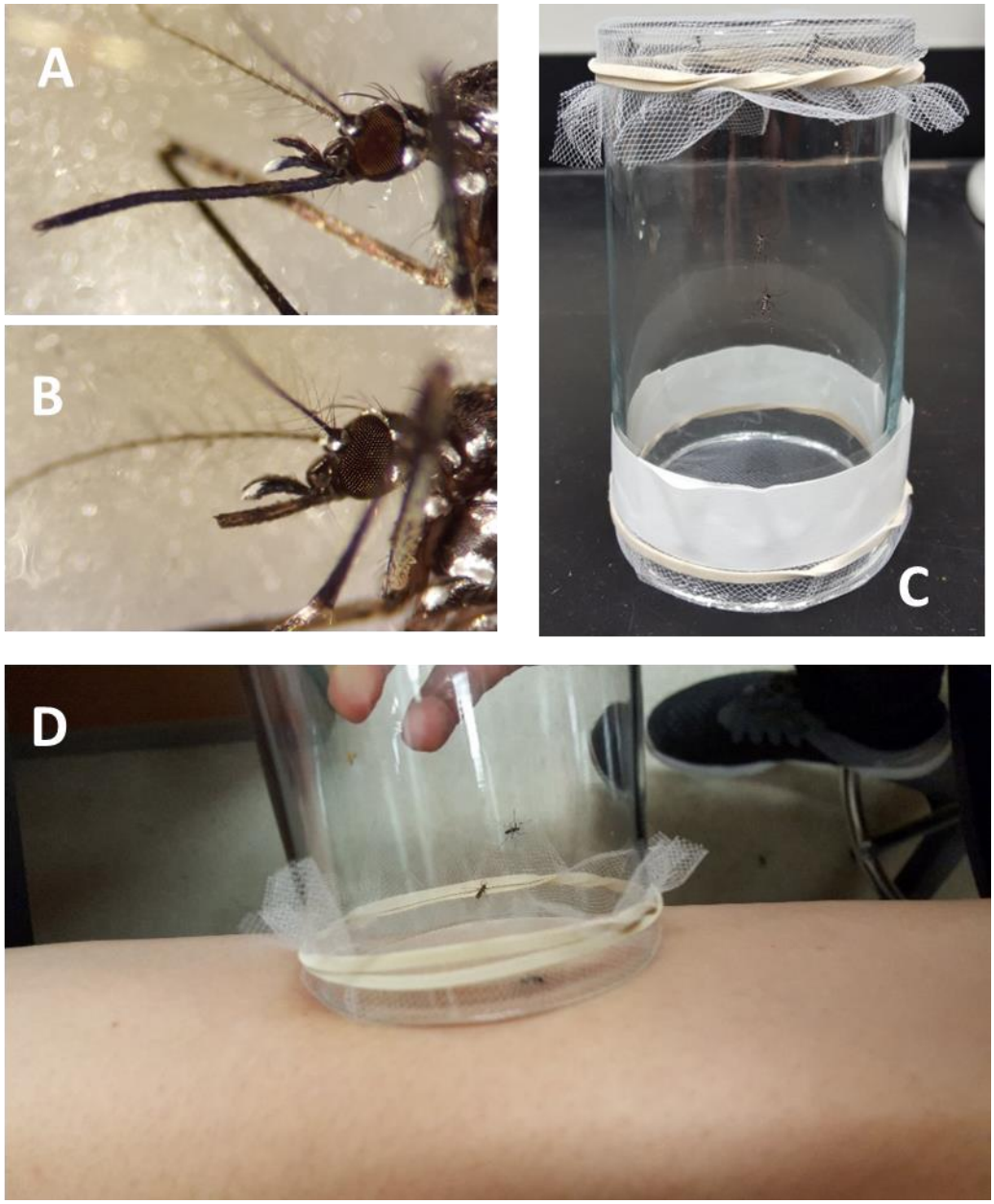

Figure 3.2. Human arm assay. After cold anesthitization, approximately half of the proboscis was removed using dissection scissors (A:. normal; B: transected). The mosquitoes were then placed into 5,000ml buckets with water-soaked cotton balls for at least one hour prior to testing.

Ten female mosquitoes with transected proboscises were placed into the assay arena (C). The number of landing events was recorded for untreated and repellent-treated arms for two minutes (D).

Statistical analysis

Comparison of mean landing, probing, and blood-feeding by infected and uninfected mosquitoes and concentrations of different repellents was analyzed by two-way analysis of variance (ANOVA) with a post hoc Tukey's multiple comparison test. Percent protection over time (*%p*) was determined following WHO guidelines for efficacy testing of mosquito repellents on human skin [WHO, 2009]. *%p* was calculated using the equation:

$$\%p = \frac{C - T}{C} \text{ x } 100$$

where *T* is the average number of mosquitoes landing on the repellent-treated membrane or skin within a given test period and *C* is the average number of mosquitoes landing on the untreated membrane or skin in the same test period. %*p* between infected and uninfected mosquitoes was analyzed using two-way ANOVA. All data were analyzed using GraphPad Prism 6.0 (La Jolla, CA). All statistical analysis were carried out at a significance level of α=0.05.

RESULTS

Effect of LACV infection on repellent efficacy

Aedes triseriatus and *Ae. albopictus* were chosen for this portion of the study because *Ae. triseriatus* is the primary LACV vector and *Ae. albopictus* serves as a potential bridge vector. Both species are competent to carry and transmit LACV.

Aedes triseriatus

Table 3.2 shows %*p* and the mean landing, probing, and blood-feeding for the SRAMF assay and %*p* and landing for the human arm assay by LACV (+) and LACV (-) *Ae. triseriatus*. At the 5% concentration for DEET and picaridin, significantly more infected *Ae. triseriatus* landed, probed and blood fed than did uninfected siblings in the SRAMF assay (Figure 3.3) and the %p was more than 25% lower (Table 2). However, no significant effect of virus infection was seen for PMD at any concentration. In the human arm assay, a significant difference in landing was observed only when mosquitoes were exposed to 5% DEET (Table 2, Figure 3.4) with a lower %p for the infected mosquitoes (55% v 82%).

Table 3.2. Mean landing, probing, blood-feeding, and percent protection (%*p*) ± SEM and P-values of LACV (+) and LACV (-) *Ae. triseriatus* exposed to DEET, picaridin, or PMD for the SRAMF and human arm assays.

SRAMF assay

Concentration		DEET			Picaridin			PMD		
		LACV +	LACV -	*P* - value	LACV +	LACV -	*P* - value	LACV +	LACV -	*P* - value
5%	Land	6.3 ± 0.61	1.7 ± 0.33	**0.0001**	6.8 ± 1.40	1.3 ± 0.42	**< 0.0001**	8.7 ± 0.71	5.7 ± 1.28	**0.0799**
	Probe	4.8 ± 0.56	0.8 ± 0.48	**< 0.0001**	5.3 ± 1.23	0.5 ± 0.34	**< 0.0001**	6.5 ± 0.48	4.3 ± 1.20	0.1264
	Blood-feed	3.5 ± 0.76	0 ± 0	**< 0.0001**	4.2 ± 1.45	0 ± 0	**< 0.0001**	5.8 ± 0.60	3.8 ± 0.87	**< 0.0001**
	%*p*	63% ± 0.09	89% ± 0.03	**< 0.05**	62% ± 0.09	92% ± 0.03	**< 0.05**	54% ± 0.04	63% ± 0.11	**< 0.05**
10%	Land	1.5 ± 0.56	1.2 ± 0.31	> 0.9999	1.5 ± 0.43	1 ± 0.26	> 0.9999	2.2 ± 0.7	2 ± 0.54	> 0.9999
	Probe	0.2 ± 0.17	0.2 ± 0.17	> 0.9999	0 ± 0	0.2 ± 0.17	> 0.9999	0.5 ± 0.22	0.2 ± 0.17	> 0.9999
	Blood-feed	0 ± 0	0 ± 0	> 0.9999	0 ± 0	0 ± 0	> 0.9999	0 ±0	0 ± 0	> 0.9999
	%*p*	92% ± 0.02	93% ± 0.03	> 0.05	91% ± 0.03	93% ± 0.03	> 0.05	88% ± 0.04	85% ± 0.06	> 0.05
15%	Land	0.8 ± 0.31	1 ± 0.26	> 0.9999	1 ± 0.26	1 ± 0.26	> 0.9999	1.5 ± 0.5	0.8 ± 0.31	> 0.9999
	Probe	0 ± 0	0.2 ± 0.17	> 0.9999	0 ± 0	0 ± 0	> 0.9999	0.7 ± 0.33	0 ± 0	> 0.9999
	Blood-feed	0 ± 0	0 ± 0	> 0.9999	0 ±0	0 ± 0	> 0.9999	0 ±0	0 ± 0	> 0.9999
	%*p*	95% ± 0.02	94% ± 0.01	> 0.05	93% ± 0.03	93% ± 0.03	> 0.05	91% ± 0.03	95% ± 0.02	> 0.05

Human arm

Concentration		DEET			Picaridin			PMD		
		LACV +	LACV -	*P* - value	LACV +	LACV -	*P* - value	LACV +	LACV -	*P* - value
5%	Land	3.6 ± 0.51	1.4 ± 0.51	**0.0127**	3.6 ± 0.4	2 ± 0.32	0.2448	5 ± 0.55	4 ± 0.55	0.4708
	%*p*	55% ± 0.06	83% ± 0.06	> 0.05	54% ± 0.07	72% ± 0.05	> 0.05	37% ± 0.08	47% ± 0.02	> 0.05
10%	Land	0.8 ± 0.37	1 ± 0.32	> 0.9999	1 ± 0.32	0.8 ± 0.2	> 0.9999	1.6 ± 0.24	1 ± 0.32	0.9996
	%*p*	90% ± 0.05	88% ± 0.04	> 0.05	87% ± 0.04	90% ± 0.03	> 0.05	80% ± 0.03	86% ± 0.05	> 0.05
15%	Land	0.6 ± 0.4	0.2 ± 0.2	> 0.9999	0.4 ± 0.24	0.2 ± 0.2	> 0.9999	1 ± 0.4	0.8 ± 0.32	> 0.9999
	%*p*	92% ± 0.05	98% ± 0.02	> 0.05	95% ± 0.03	98% ± 0.03	> 0.05	90% ± 0.05	83% ± 0.06	> 0.05

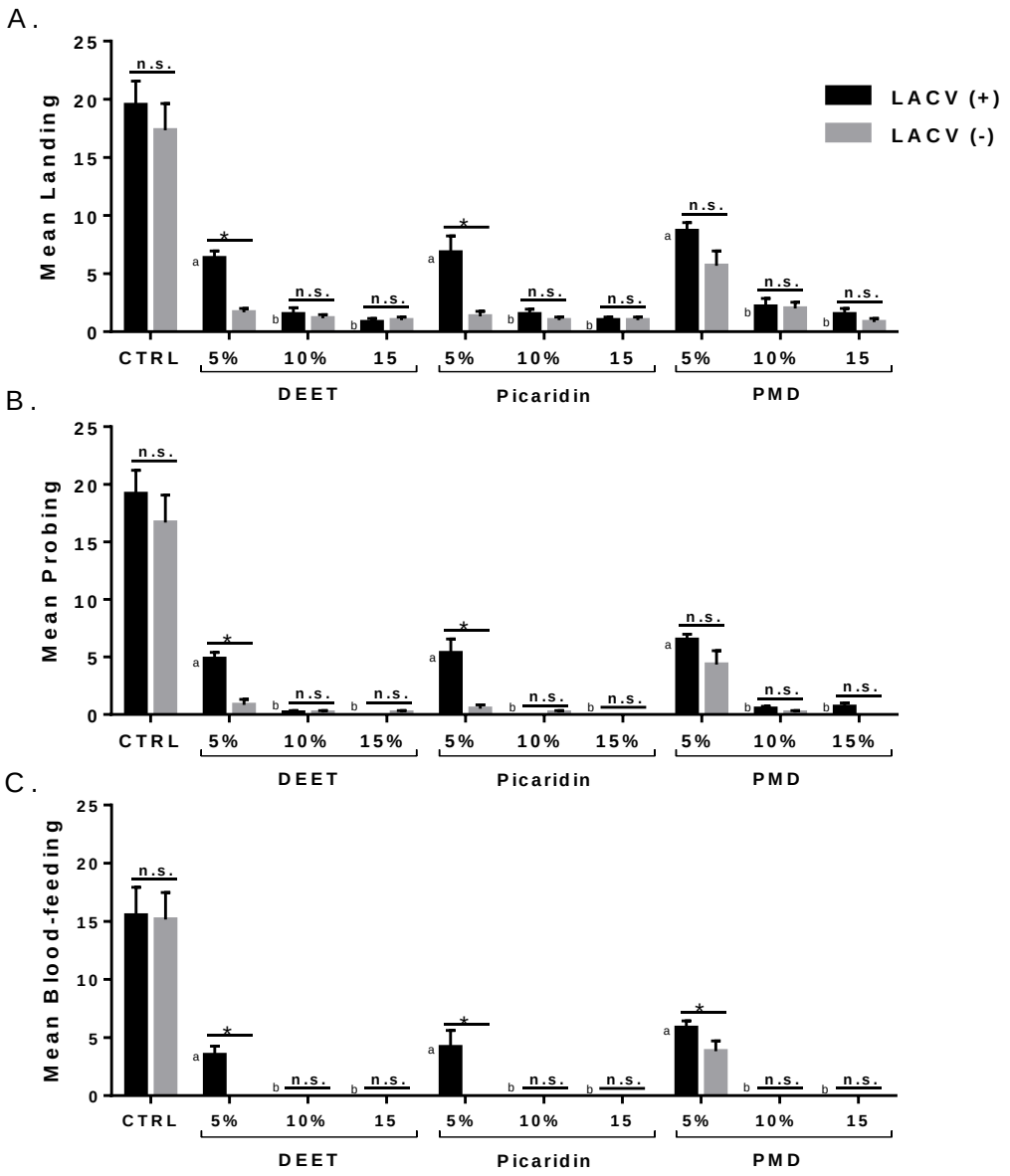

Figure 3.3. Mean landing (A), probing (B), and blood-feeding (C) on the SRAMF assay with LACV (+) and LACV (-) *Aedes triseriatus* (*N* = 6) treated with DEET, picaridin, or PMD. Asterisks denote statistical significance between infected and uninfected mosquitoes and letters denote statistical significance of increasing repellent concentrations with LACV (+) mosquitoes at α=0.05 using 2-way ANOVA followed by a post-hoc Tukey's multiple comparison test. *N* = number of replicates.

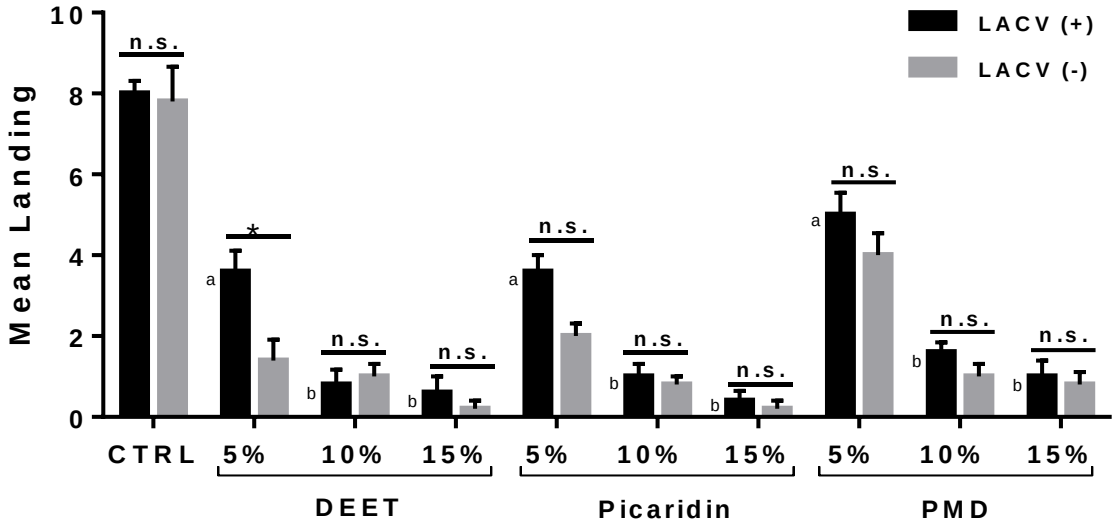

Figure 3.4. Mean landing of LACV (+) and LACV (-) *Aedes triseriatus* (*N* = 5) on arms treated with DEET, picaridin, or PMD in the human arm assay. Asterisks denote statistical significance between infected and uninfected mosquitoes and letters denote statistical significance of increasing repellent concentrations with LACV (+) mosquitoes at α=0.05 using 2-way ANOVA followed by a post-hoc Tukey's multiple comparison test. *N* = number of replicates.

Aedes albopictus

Table 3.3 shows %*p* and the mean landing, probing, and blood-feeding for the SRAMF assay and landing for the human arm assay by LACV (+) and LACV (-) *Ae. albopictus* females. The %*p* was higher for uninfected mosquitoes exposed to low repellent concentrations. Increasing repellent concentrations led to increased %*p* for both infected and uninfected *Ae. albopictus* (Table 3). LACV (+) *Ae. albopictu*s were significantly more likely to land, probe, and blood-feed on membranes treated with 5% DEET, picaridin and PMD compared to LACV (-) mosquitoes in the SRAMF assay (Figure 3.5). There was also significantly more landing by LACV (+) mosquitoes for the 10% PMD-treated SRAMF assay. A similar pattern of higher landing rates for infected mosquitoes when exposed to the 5% repellent was seen in the human arm assay (Figure 3.6). When the repellent concentration was increased to 10% and 15%, there

were no significant differences in landing, probing, and blood-feeding between infected and uninfected groups in the SRAMF assay (Figure 3.5) or in landing in the human arm assay (Figure 3.6).

Table 3.3. Mean landing, probing, blood-feeding, and percent protection (%*p*) ± SEM and P-values of LACV (+) and LACV (-) *Ae. albopictus* exposed to DEET, picaridin, or PMD for the SRAMF and human arm assays.

SRAMF assay

Concentration		DEET			Picaridin			PMD		
		LACV +	LACV -	*P* - value	LACV +	LACV -	*P* - value	LACV +	LACV -	*P* - value
5%	Land	11.8 ± 0.70	2.3 ± 0.42	< **0.0001**	12.3 ± 1.41	5.8 ± 0.48	**0.0002**	17.5 ± 1.98	7.2 ± 1.92	< **0.0001**
	Probe	11.2 ± 0.70	0.8 ± 0.40	< **0.0001**	10.3 ± 0.84	2.5 ± 0.43	< **0.0001**	15.5 ± 1.75	4.8 ± 1.92	< **0.0001**
	Blood-feed	9.2 ± 0.48	0 ± 0	< **0.0001**	5.2 ± 0.79	0.2 ± 0.17	< **0.0001**	6.7 ± 0.71	0.5 ± 0.34	< **0.0001**
	%*p*	34% ± 0.04	87% ± 0.03	< **0.05**	31% ± 0.08	67% ± 0.04	< **0.05**	3% ± 0.12	62% ± 0.11	< **0.05**
10%	Land	3.8 ± 0.83	0.3 ± 0.21	0.3405	4.3 ± 0.71	1.2 ± 0.98	0.5217	5.7 ± 0.76	0.7 ± 0.33	**0.0153**
	Probe	2.3 ± 0.92	0 ± 0	0.7994	2.5 ± 0.89	0.7 ± 0.67	0.9686	1.7 ± 0.61	0.3 ± 0.21	0.999
	Blood-feed	0.2 ± 0.17	0 ± 0	> 0.9999	0.2 ± 0.17	0 ± 0	> 0.9999	0.3 ± 0.33	0 ± 0	> 0.9999
	%*p*	79% ± 0.05	98% ± 0.01	> 0.05	74% ± 0.07	93% ± 0.06	> 0.05	69% ± 0.05	97% ± 0.02	< **0.05**
15%	Land	1 ± 0.26	0.5 ± 0.22	> 0.9999	0.8 ± 0.31	0.7 ± 0.33	> 0.9999	2.3 ± 0.56	1.5 ± 0.43	> 0.9999
	Probe	0.2 ± 0.17	0 ± 0	> 0.9999	0 ± 0	0 ± 0	> 0.9999	0.8 ± 0.31	0.2 ± 017	> 0.9999
	Blood-feed	0 ± 0	0 ± 0	> 0.9999	0 ±0	0 ± 0	> 0.9999	0 ±0	0 ± 0	> 0.9999
	%*p*	94% ± 0.02	97% ± 0.01	> 0.05	95% ± 0.02	96% ± 0.02	> 0.05	87% ± 0.03	92% ± 0.02	> 0.05

Human arm

Concentration		DEET			Picaridin			PMD		
		LACV +	LACV -	*P* - value	LACV +	LACV -	*P* - value	LACV +	LACV -	*P* - value
5%	Land	6 ± 0.45	1.4 ± 0.51	< **0.0001**	5.2 ± 0.58	1.2 ± 0.37	**0.0052**	9.8 ± 0.86	3.2 ± 0.37	< **0.0001**
	%*p*	58% ± 0.07	93% ± 0.02	< **0.05**	63% ± 0.09	89% ± 0.03	< **0.05**	30% ± 0.15	81% ± 0.03	< **0.05**
10%	Land	3.2 ± 0.37	1.2 ± 0.37	0.4964	4.2 ± 0.37	2 ± 0.55	0.3275	4.8 ± 0.97	3.4 ± 1.03	0.9384
	%*p*	78% ± 0.05	91% ± 0.03	> 0.05	72% ± 0.03	89% ± 0.03	> 0.05	64% ± 0.12	80% ± 0.06	> 0.05
15%	Land	0.6 ± 0.24	0.8 ± 0.37	> 0.9999	1.4 ± 0.4	1 ± 0.45	> 0.9999	4.2 ± 0.37	3.6 ± 0.51	> 0.9999
	%*p*	96% ± 0.02	96% ± 0.02	> 0.05	90% ± 0.04	95% ± 0.03	> 0.05	72% ± 0.04	78% ± 0.05	> 0.05

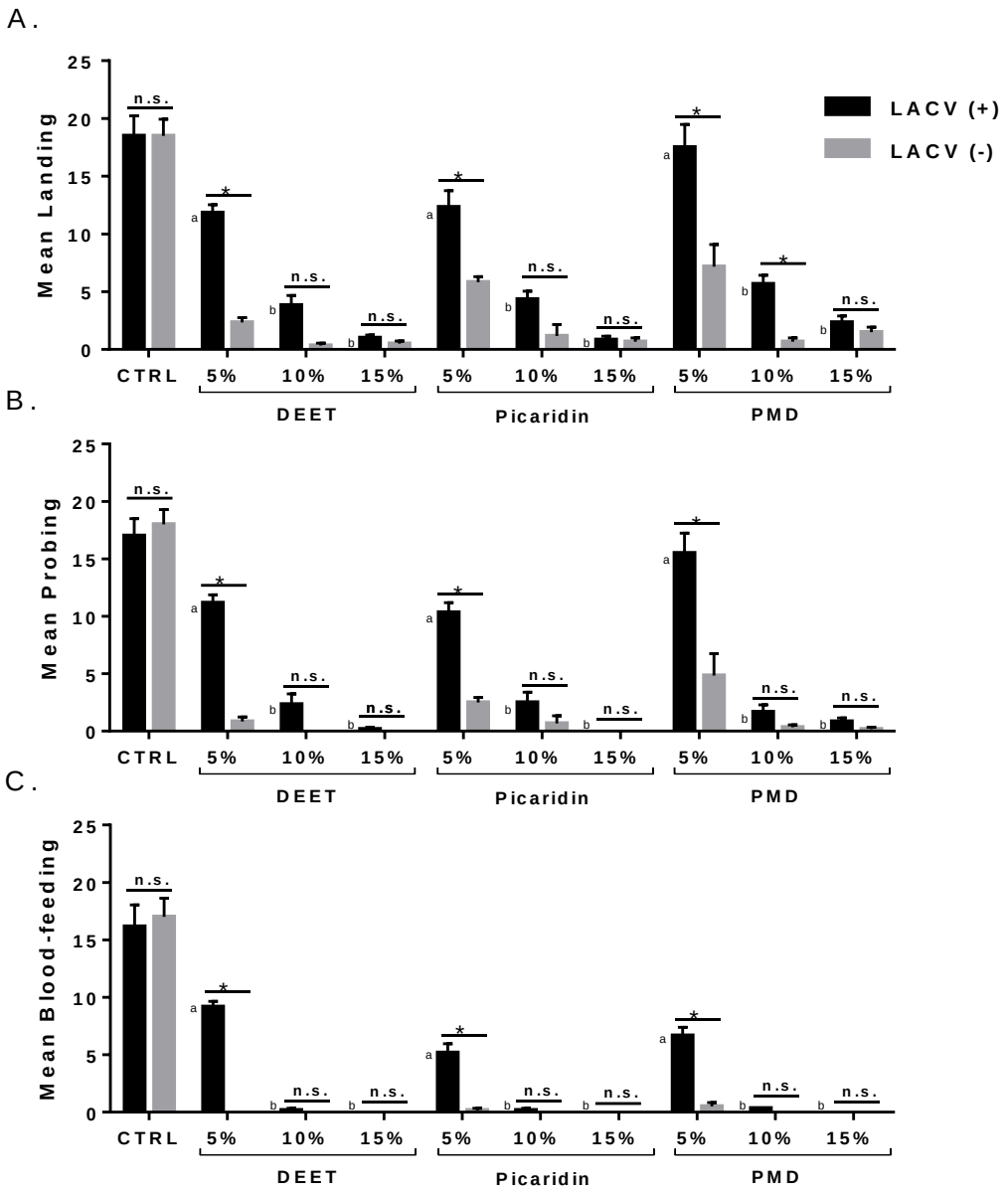

Figure 3.5. Mean landing (A), probing (B), and blood-feeding (C) on the SRAMF assay with LACV (+) and LACV (-) *Aedes albopictus* (*N* = 6) treated with DEET, picaridin, or PMD. Asterisks denote statistical significance between infected and uninfected mosquitoes and letters denote statistical significance of increasing repellent concentrations with LACV (+) mosquitoes at α=0.05 using 2-way ANOVA followed by a post-hoc Tukey's multiple comparison test. *N* = number of replicates.

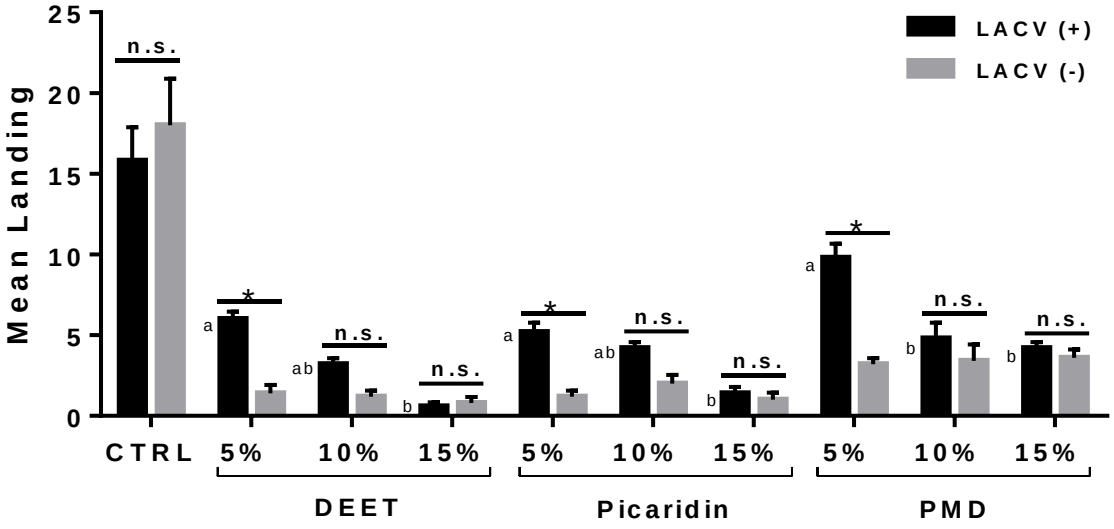

Figure 3.6. Mean landing of LACV (+) and LACV (-) *Aedes albopictus* (*N* = 5) on arms treated with DEET, picaridin, or PMD in the human arm assay. Asterisks denote statistical significance between infected and uninfected mosquitoes and letters denote statistical significance of increasing repellent concentrations with LACV (+) mosquitoes at α=0.05 using 2-way ANOVA followed by a post-hoc Tukey's multiple comparison test. *N* = number of replicates.

Effect of ZIKV infection on repellent efficacy

Aedes aegypti, the primary ZIKV vector, and *Ae. albopictus* were chosen for this portion of the study. Both species are competent vectors to carry and transmit ZIKV [McKenzie, 2019]. For most cases, *Ae. albopictus* serves as a secondary vector for ZIKV, but in areas with high *Ae. albopictus* distribution and limited *Ae. aegypti* distribution, *Ae. albopictus* are potentially the primary vector for ZIKV transmission [Liu, 2017].

Aedes aegypti

Table 3.4 shows %*p* and the mean landing, probing, and blood-feeding for the SRAMF assay and landing for the human arm assay by ZIKV (+) and ZIKV (-) *Ae. aegypti.* The %*p* was lower for 5% concentration for all repellents and both ZIKV-infected and uninfected mosquitoes. In the SRAMF assay, the only significant differences detected between ZIKV (+) and ZIKV (-)

mosquitoes was for landing at 5% DEET and for landing and probing at 5% PMD (Figure 3.7). There were no significant differences in landing, probing, or blood-feeding between infected and uninfected mosquitoes for any picaridin concentration in this assay. For the human arm assay, significant differences between ZIKV (+) and ZIKV (-) mosquitoes were observed only for the 5% PMD treatment (Figure 3.8).

Table 3.4. Mean landing, probing, blood-feeding, and percent protection (%*p*) ± SEM and P-values of ZIKV (+) and ZIKV (-) ZIKV *Ae. aegypti* exposed to DEET, picaridin, or PMD for the SRAMF and human arm assays.

SRAMF assay

Concentration		DEET			Picaridin			PMD		
		ZIKV +	ZIKV -	*P* - value	ZIKV +	ZIKV -	*P* - value	ZIKV +	ZIKV -	*P* - value
5%	Land	3.8 ± 0.37	1.6 ± 0.51	**0.0465**	4 ± 0.45	2.2 ± 0.37	0.2368	5.6 ± 0.75	2.8 ± 0.74	**0.0019**
	Probe	1.2 ± 0.37	0.6 ± 0.4	0.9995	1.6 ± 0.93	0.8 ± 0.37	0.987	4.4 ± 0.75	2 ± 0.32	**0.0027**
	Blood-feed	0.8 ± 0.37	0.2 ± 0.2	0.9685	1.4 ± 0.4	0.6 ± 0.24	0.7409	2 ± 0.45	1 ± 0.71	0.3596
	%*p*	52% ± 0.13	83% ± 0.05	> 0.05	51% ± 0.10	76% ± 0.05		34% ± 0.10	70% ± 0.08	**< 0.05**
10%	Land	1 ± 0.45	1 ± 0.32	> 0.9999	1.2 ± 0.37	0.8 ± 0.37	> 0.9999	1.6 ± 0.51	1 ± 0.45	> 0.9999
	Probe	0.4 ± 0.24	0.6 ± 0.24	> 0.9999	0.4 ± 0.24	0.6 ± 0.24	> 0.9999	0.8 ± 0.37	0.6 ± 0.24	> 0.9999
	Blood-feed	0 ± 0	0 ± 0	> 0.9999	0 ± 0	0 ± 0	> 0.9999	0.2 ± 0.2	0 ± 0	> 0.9999
	%*p*	89% ± 0.05	89% ± 0.04	> 0.05	86% ± 0.04	93% ± 0.03	> 0.05	83% ± 0.06	89% ± 0.05	> 0.05
15%	Land	0.4 ± 0.24	0.2 ± 0.2	> 0.9999	0.6 ± 0.24	0.8 ± 0.2	> 0.9999	0.8 ± 0.37	0.4 ± 0.24	> 0.9999
	Probe	0 ± 0	0 ± 0	> 0.9999	0.2 ± 0.2	0 ± 0	> 0.9999	0.2 ± 0.2	0.2 ± 0.2	> 0.9999
	Blood-feed	0 ± 0	0 ± 0	> 0.9999	0 ± 0	0 ± 0	> 0.9999	0.2 ± 0.2	0 ± 0	> 0.9999
	%*p*	96% ± 0.02	98% ± 0.02	> 0.05	92% ± 0.04	92% ± 0.02	> 0.05	90% ± 0.05	96% ± 0.03	> 0.05

Human arm assay

Concentration		DEET			Picaridin			PMD		
		ZIKV +	ZIKV -	*P* - value	ZIKV +	ZIKV -	*P* - value	ZIKV +	ZIKV -	*P* - value
5%	Land	3.2 ± 0.37	1.4 ± 0.4	0.202	4 ± 0.32	2 ± 0.32	0.0905	6.8 ± 0.58	4 ± 0.84	**0.0013**
	%*p*	62% ± 0.07	85% ± 0.05	> 0.05	53% ± 0.08	79% ± 0.03	> 0.05	22% ± 0.10	58% ± 0.10	**< 0.05**
10%	Land	0.6 ± 0.4	0.8 ± 0.37	> 0.9999	1 ± 0.55	0.4 ± 0.25	0.9999	1.2 ± 0.37	1.6 ± 0.25	> 0.9999
	%*p*	94% ± 0.04	91% ± 0.04	> 0.05	91% ± 0.05	95% ± 0.03	> 0.05	86% ± 0.04	83% ± 0.03	> 0.05
15%	Land	0.4 ± 0.24	0.4 ± 0.24	> 0.9999	0.4 ± 0.4	0.8 ± 0.49	> 0.9999	1.4 ± 0.4	0.8 ± 0.49	> 0.9999
	%*p*	96% ± 0.02	96% ± 0.03	> 0.05	96% ± 0.04	98% ± 0.03	> 0.05	85% ± 0.04	92% ± 0.05	> 0.05

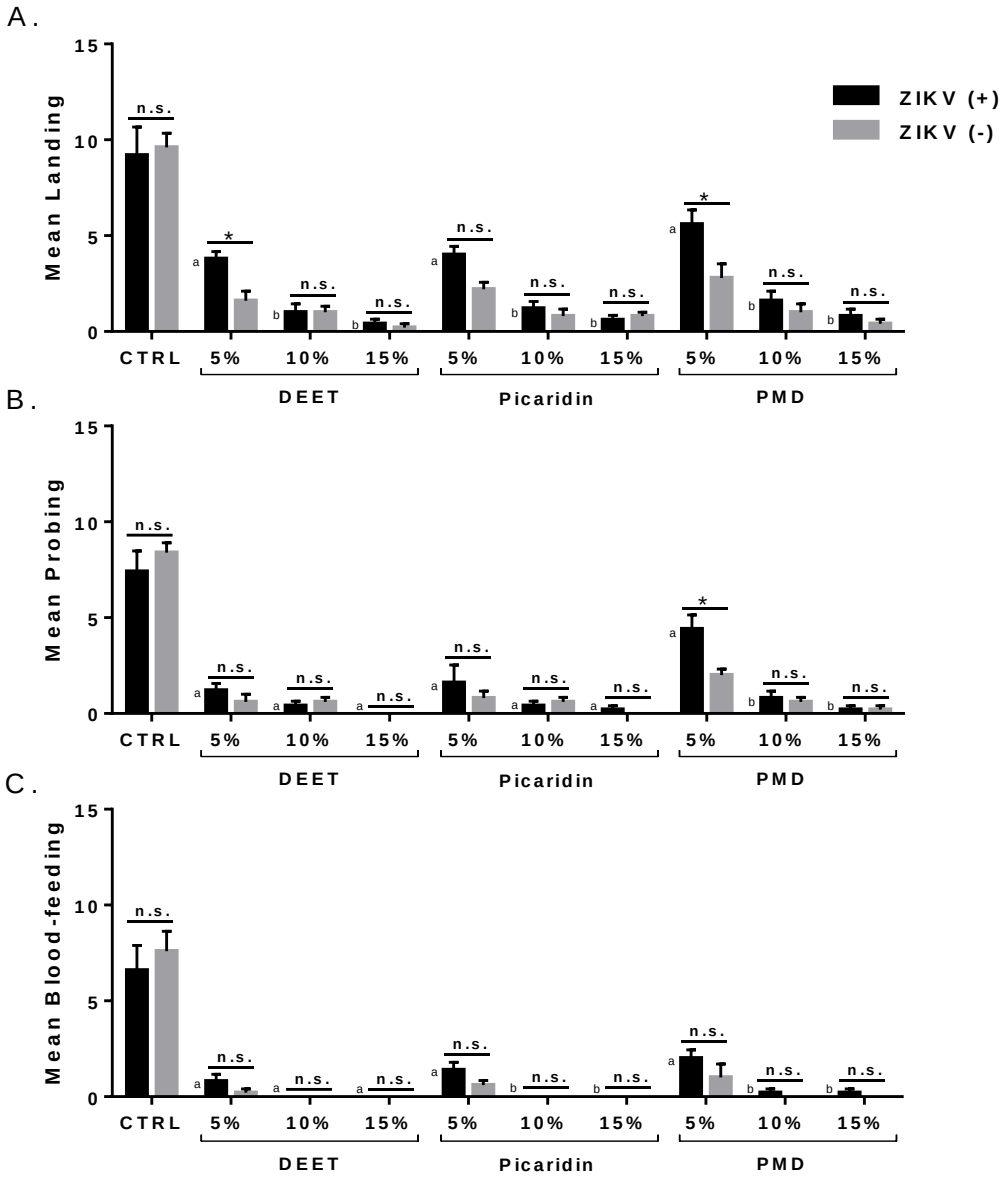

Figure 3.7. Mean landing (A), probing (B), and blood-feeding (C) on the SRAMF assay with ZIKV (+) and ZIKV (-) *Aedes aegypti* (*N* = 6) treated with DEET, picaridin, or PMD. Asterisks denote statistical significance between infected and uninfected mosquitoes and letters denote statistical significance of increasing repellent concentrations with ZIKV (+) mosquitoes at α=0.05 using 2-way ANOVA followed by a post-hoc Tukey's multiple comparison test. *N* = number of replicates.

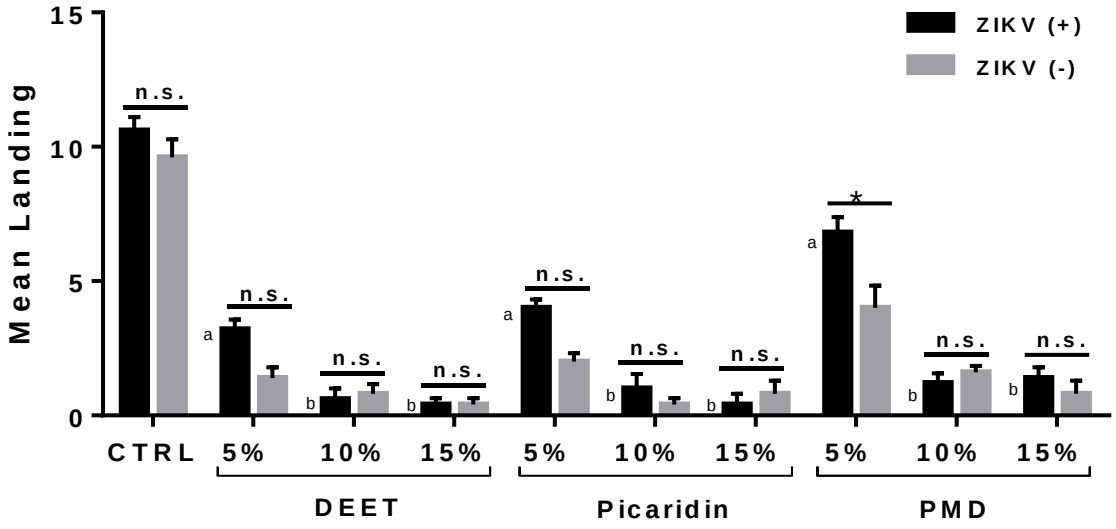

Figure 3.8. Mean landing of ZIKV (+) and ZIKV (-) *Aedes aegypti* (*N* = 5) on arms treated with DEET, picaridin, or PMD in the human arm assay. Asterisks denote statistical significance between infected and uninfected mosquitoes and letters denote statistical significance of increasing repellent concentrations with ZIKV (+) mosquitoes at α=0.05 using 2-way ANOVA followed by a post-hoc Tukey's multiple comparison test. *N* = number of replicates.

Aedes albopictus

Table 3.5 shows %*p* and the mean landing, probing, and blood-feeding for the SRAMF assay and landing for the human arm assay by ZIKV (+) and ZIKV (-) *Ae. albopictus*. Increasing repellent concentrations led to increased %*p* for both ZIKV-infected and uninfected mosquitoes. The ZIKV (+) mosquitoes showed significantly higher mean landing, probing, and blood-feeding rates in the SRAMF assay when the membrane was treated with 5% DEET, picaridin, or PMD (Figure 3.9). For the human arm assay, significant differences in landing between ZIKV (+) and ZIKV (-) *Ae. albopictus* was only observed in 5% DEET and 5% picaridin (Figure 3.10). No difference between groups was seen at any concentration when the repellent was PMD.

Table 3.5. Mean landing, probing, blood-feeding, and percent protection (%*p*) ± SEM and P-values of ZIKV (+) and ZIKV (-) *Ae. albopictus* exposed to DEET, picaridin, or PMD for the SRAMF and human arm assays.

SRAMF assay

Concentration		DEET			Picaridin			PMD		
		ZIKV +	ZIKV -	*P* - value	ZIKV +	ZIKV -	*P* - value	ZIKV +	ZIKV -	*P* - value
5%	Land	10.6 ± 0.81	2.4 ± 0.47	**< 0.0001**	15.8 ± 0.97	6.2 ± 0.34	**< 0.0001**	12 ± 0.95	7 ± 0.76	**< 0.0001**
	Probe	9.2 ± 0.49	1.2 ± 0.34	**< 0.0001**	12.2 ± 1.66	2.2 ± 0.34	**< 0.0001**	9.2 ± 0.58	4.6 ± 1.06	**< 0.0001**
	Blood-feed	3.2 ± 0.73	0 ± 0	**0.0003**	4.6 ± 0.87	0.4 ± 0.37	**< 0.0001**	4 ± 1.71	1.2 ± 0.53	**0.0027**
	%*p*	33% ± 0.06	86% ± 0.03	**< 0.05**	<1% ± 0.11	65% ± 0.04	**< 0.05**	23% ± 0.09	62% ± 0.03	**< 0.05**
10%	Land	2 ± 0.32	0.3 ± 0.21	0.7997	1.8 ± 0.37	0.4 ± 0.24	0.9793	2.8 ± 0.66	0.7 ± 0.33	0.1573
	Probe	0.4 ± 0.24	0 ± 0	> 0.9999	0.8 ± 0.37	0.7 ± 0.67	> 0.9999	1 ± 0.32	0.3 ± 0.21	> 0.9999
	Blood-feed	0 ± 0	0 ± 0	> 0.9999	0 ± 0	0 ± 0	> 0.9999	0 ± 0	0 ± 0	> 0.9999
	%*p*	87% ± 0.02	98% ± 0.01	> 0.05	88% ± 0.03	97% ± 0.02	> 0.05	83% ± 0.03	98% ± 0.01	> 0.05
15%	Land	0.2 ± 0.2	0 ± 0	> 0.9999	0.6 ±0.4	0 ± 0	> 0.9999	1.2 ± 0.58	0.4 ± 0.24	0.9998
	Probe	0 ± 0	0 ± 0	> 0.9999	0 ± 0	0 ± 0	> 0.9999	0.2 ± 0	0 ± 0	> 0.9999
	Blood-feed	0 ± 0	0 ± 0	> 0.9999	0 ± 0	0 ± 0	> 0.9999	0.2 ± 0	0 ± 0	> 0.9999
	%*p*	99% ± 0.01	100% ± 0	> 0.05	96% ± 0.02	100% ± 0	> 0.05	92% ± 0.04	97% ± 0.02	> 0.05

Human arm assay

Concentration		DEET			Picaridin			PMD		
		ZIKV +	ZIKV -	*P* - value	ZIKV +	ZIKV -	*P* - value	ZIKV +	ZIKV -	*P* - value
5%	Land	4.2 ± 0.49	1.4 ± 0.51	**0.0214**	6.6 ± 0.6	1.2 ± 0.37	**< 0.0001**	5.2 ± 0.58	3.2 ± 0.37	0.3413
	%*p*	66% ± 0.01	93% ± 0.02	**< 0.05**	44% ± 0.07	89% ± 0.03	**< 0.05**	55% ± 0.06	81% ± 0.03	**< 0.05**
10%	Land	0.8 ± 0.37	1.8 ± 0.37	0.9944	4.2 ± 0.37	3.2 ± 0.37	> 0.9999	5.2 ± 0.37	4 ± 1.14	0.9653
	%*p*	93% ± 0.03	89% ± 0.03	> 0.05	73% ± 0.08	81% ± 0.02	> 0.05	56% ± 0.04	75% ± 0.10	> 0.05
15%	Land	0.4 ± 0.24	0 ± 0	> 0.9999	0.6 ± 0.24	0.4 ± 0.4	> 0.9999	1 ± 0.32	0.8 ± 0.2	> 0.9999
	%*p*	96% ± 0.02	100% ± 0	> 0.05	94% ± 0.02	98% ± 0.02	> 0.05	91% ± 0.04	96% ± 0.01	> 0.05

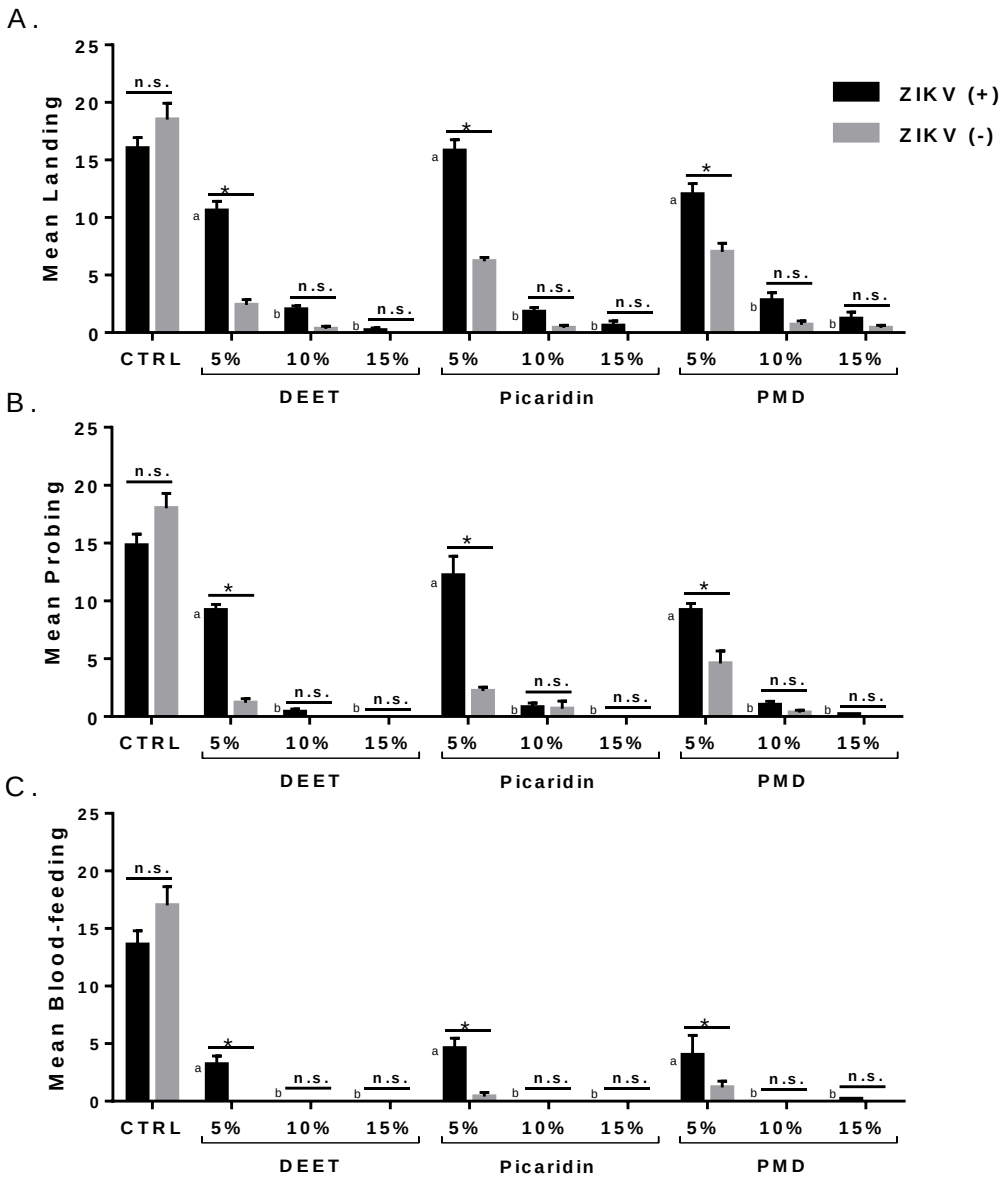

Figure 3.9. Mean landing (A), probing (B), and blood-feeding (C) on the SRAMF assay with ZIKV (+) and ZIKV (-) *Aedes albopictus* (*N* = 6) treated with DEET, picaridin, or PMD. Asterisks denote statistical significance between infected and uninfected mosquitoes and letters denote statistical significance of increasing repellent concentrations with ZIKV (+) mosquitoes at α=0.05 using 2-way ANOVA followed by a post-hoc Tukey's multiple comparison test. *N* = number of replicates.

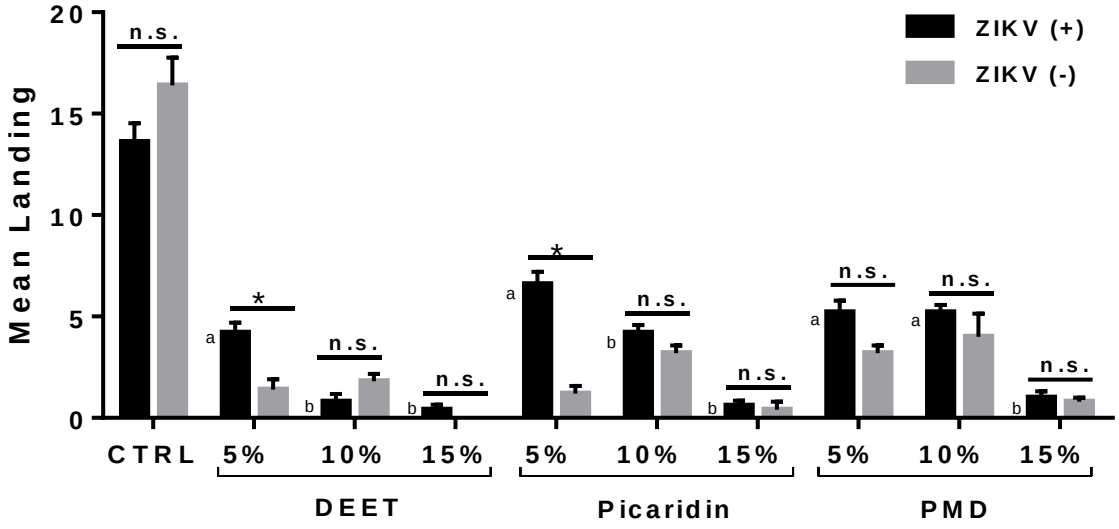

Figure 3.10. Mean landing of ZIKV (+) and ZIKV (-) *Aedes albopictus* (*N* = 5) on arms treated with DEET, picaridin, or PMD in the human arm assay. Asterisks denote statistical significance between infected and uninfected mosquitoes and letters denote statistical significance of increasing repellent concentrations with ZIKV (+) mosquitoes at α=0.05 using 2-way ANOVA followed by a post-hoc Tukey's multiple comparison test. *N* = number of replicates.

DISCUSSION

Several studies have documented behavioral changes in insect vectors from pathogen infection. A few examples include: LACV infection increased probing and refeeding rates and decreased blood-meal size in *Ae. triseriatus* [Jackson, 2012; Grimstad, 1980]; DENV 3 infected *Ae. aegypti* required more time to complete feeding compared to uninfected controls [Platt, 1997]; *Ae. aegypti* infected with DENV 2 virus developed an increase in locomotory activity compared to uninfected controls [Lima-Camara, 2011]; *An. gambiae* infected with *Plasmodium falciparum* exhibited increased attraction to human odors, blood-meal size, and biting rate [Smallegange, 2013; Koella, 1998; Wekesa, 1992]; and Tomato spotted wilt virus infection in *Frankliniall occidentalis* increased probing rate [Stafford, 2011]. Previous studies looking at the effects of virus infection on repellent response showed that *Ae. albopictus* and *Ae. aegypti*

infected with DENV 1-4 showed no significant difference when exposed to 5% DEET [Francis, 2011], while *Ae. aegypti* infected with Sindbis virus (SINV) exhibited a desensitized response to DEET and altered blood-feeding response [Qualls, 2011; Qualls, 2012]. A study looking at ZIKV-infected *Ae. aegypti* showed that there was reduced protection against infected mosquitoes exposed to lower concentrations of DEET [Leal, 2017].

Persistent virus infection within the mosquito may damage olfactory neurons, resulting in reduced sensitivity to odorants. The impairments of the olfactory or gustatory system can prevent repellent odorants from being received and processed by receptor neurons [Bohbot, 2011]. Multiple studies have shown neurotropism of arboviruses and their effects on mosquito behavior and physiology. ZIKV infection was neurotropic in *Ae. aegypti* and caused a sustained excitatory state [Gaburro, 2018]. Infection of *Ae. triseriatus* with LACV affected host-seeking and serotonin levels [Yang et al., 2019] and altered blood-feeding behaviors [Jackson et al. 2012]. DENV infection altered the feeding behavior of *Ae. aegypti* along with infection of the brain, Johnston's organ, compound eyes, and abdominal ganglion [Platt, 1997].

Another mechanism that may be responsible for the desensitized repellent response is the use of metabolic enzymes. When odorants enter the sensillum of the antennae, odorant binding proteins (OBP) in the sensillar lymph bind to odorant molecules and transport them to odorant receptors [Pelosi, 2014]. While odorant detection and processing is an important part of olfaction, the degradation of an odorant is equally important when insects rely on odors for orientation towards or away a particular source. Odorant degrading enzymes (ODE) serve an important function in processing odorant molecules and are present in the sensillar lymph. The most studied ODEs are antennal esterases, but other studies have shown that glutathione-*S*-transferases (GST), cytochrome P4540s, and aldehyde dehydrogenases also aid in odorant

degradation [Leal, 2013; Thiebaud, 2013]. Studies have demonstrated that GSTs are important for detoxification of harmful chemicals and have been identified in the antenna of *Bombyx mori* and *Manduca sexta* [Rogers, 1999; Tan, 2014]. Increased production of GSTs may result from oxidative damage of virus infection. If the result is the increase of GST activity, xenobiotics, such as repellents, may be degraded more rapidly in virus-infected mosquitoes.

Overall, LACV and ZIKV-infected mosquitoes were more likely to contact repellent-treated surfaces compared to uninfected mosquitoes. This can be prevented by utilizing concentrations of repellents that are 10% active ingredient or more. Most commercial repellents are offered at a wide range of percentages, with DEET from less than 10% to over 99%, picaridin from less than 9% to 25%, and PMD from 20% to 75% [Lo, 2018]. However, the combination of the virus and mosquito species may play a larger role than just the virus itself. The effectiveness of repellents can vary depending on environmental factors and mosquito species [Barnard, 1998; Van Roey et al., 2014]. *Ae. albopictus* is an aggressive daytime biting mosquito that is emerging as a worldwide public health concern due to its rapid spread from globalization and ability to vector numerous diseases [Bonizzoni, 2013]. The Blacksburg strain of *Ae. albopictus* used for this study showed more aggressive host-seeking and feeding behaviors compared to *Ae. triseriatus* and *Ae. aegypti* strains. For example, *Ae albopictus* exhibited a higher mean landing, probing, and blood-feeding for the SRAMF compared to *Ae. aegypti* and higher mean landing for the human arm assay compared to *Ae. aegypti* and *Ae. triseriatus.* This difference was seen in the controls which was independent of virus infection.

Due to the lack of vaccines or antivirals for most arboviral diseases, the CDC recommends the use of repellents as a way to prevent illness, especially in areas with ongoing vector-borne disease transmission. Repellents are a low-cost and effective way to minimize

mosquito bites and can be implemented with other personal protection methods for successful disease prevention programs [Debboun, 2012]. To achieve optimal repellent performance, it is important to follow the label guidelines and take into consideration of appropriate repellent concentrations and the need for reapplication. DEET and picaridin act as low volatile repellents and work when mosquitoes come into close proximity to treated skin. Over time, the effectiveness of repellents wears off due to volatilization or absorption into the skin. Even high concentrations of DEET need to be reapplied every several hours [Norris, 2017]. Reapplication, without overuse, is needed to maintain durability, especially in conditions of high air temperature, high physical activity, exposure to water, or if the user has a history of attractiveness to biting arthropods [USEPA, 2017]. Although insect repellents work to reduce and prevent mosquito bites, human behavior plays a significant role in the overall effectiveness of those products [Gryseels, 2015].

In summary, arbovirus infection in mosquitoes alters their response to repellents by increasing the likelihood of an infected mosquito to land, probe, and blood-feed from repellent-treated surfaces. However, increasing the repellent concentration was able to significantly increase %*p* and reduce the number of infected and uninfected mosquitoes from contacting repellent-treated surfaces, indicating the importance of active ingredient concentration for mitigating mosquito bites. This study shows the importance of repellents in disease prevention and public health programs and demonstrates behavioral effects of virus-vector interactions. The results suggest that DEET, picaridin, and PMD are effective against uninfected and infected mosquitoes when adequate concentrations are used and underscore the need to ensure that repellents are correctly applied and reapplied as needed to remain protective.

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Chapter 4

The effects of Zika and La Crosse virus infection on oxidative stress and glutathione-*S*-transferase activity in *Aedes albopictus*

ABSTRACT

Aedes albopictus, the Asian tiger mosquito, is a major public health concern due to its potential ability to vector numerous arthropod-borne pathogens. With the increasing use of chemicals for vector control, insecticide resistance poses a serious threat for pest management professionals. Little is known of the physiological impacts of virus infection on the regulation of oxidative stress and detoxification enzyme activity, which are both affected by insecticide exposure. In this study, adult *Ae. albopictus* females were infected with Zika or La Crosse virus, and the amount of oxidative stress and glutathione-*S*-transferase (GST) activity was measured on 0-, 3-, 7-, and 10-days post-exposure (DPE). Virus infection did not affect oxidative stress, which was measured by thiobarbituric acid reactive substances (TBARS). There was a general trend for increasing GST activity over time regardless of infection status but significant differences in enzyme activity was only observed on 3-DPE. Further studies are needed to determine the effects of virus infection on other detoxification enzymes and biomarkers for oxidative stress.

INTRODUCTION

Reactive oxygen species (ROS) are toxic byproducts from aerobic metabolism that can cause oxidative stress by damaging DNA, lipids, and proteins [Cross, 1987]. An organism is in a state of oxidative stress when detoxification is outpaced by the production of ROS [Betteridge, 2000]. Glutathione-*S*-transferase (GST), an enzyme responsible for the detoxification of

environmental pollutants, pharmaceuticals, and endogenous compounds, can also provide protection against byproducts of oxidative stress [Maity, 2008]. GSTs are found in almost all living organisms and function by catalyzing the reaction between reduced glutathione (GSH), an antioxidant, to hydrophobic electrophiles for excretion [Deponte, 2013]. In order for the mosquito to survive, a delicate balance of antioxidative enzymes is required to prevent self-damage.

Aedes albopictus (Skuse), the Asian tiger mosquito, is a vector of Dengue (DENV), Zika (ZIKV), La Crosse (LACV), and Chikungunya (CHIKV) viruses [Cully, 1992; Liu, 2017; Whitehorn, 2015; Hugo, 2016]. *Ae. albopictus* are active during the daytime and live around humans where they breed in artificial containers and tree holes (Barker, 2003; Trexler, 1997). Due to its public health concern as an accessory vector for major pathogens, control practices are critical in controlling mosquito populations [Gratz, 2004]. Recent evidence has shown that populations of *Ae. albopictus* were either developing resistance or are resistant to certain pyrethroids, organophosphates, carbamates, or organochlorines [Li, 2018; Richards, 2019; Pichler, 2018;].

ZIKV (Flaviviridae, Flavivirus) is an arbovirus responsible for the 2015 outbreaks in South and Central America and has been a major public health concern. The virus was identified as the causative agent of microcephaly in newborn infants and Guillain-Barre syndrome in adults [Schuler-Faccini, 2016; Campos; 2015; Goodfellow, 2016]. In addition to being vectored by *Aedes* mosquitoes, the virus can also be sexually transmitted [Atkinson, 2016; Musso, 2015]. LACV (Peribunyaviridae, Orthobunyavirus) is another arbovirus vectored by *Aedes* mosquitoes. LACV infection is the leading cause of pediatric encephalitis in the United States with severe cases resulting in neurological sequelae in children [Balkhy, 2000; McJunkin, 1998].

Due to the potential effects of GST on xenobiotic and ROS detoxification, it is important to know if virus infection can significantly increase oxidative stress, and subsequently increase GST activity. Viral infection can induce several cellular pathways that promote the production of oxidants. Once the virus is recognized by the host cell and viral replication is initiated, stress signaling by the host cell initiates production of superoxides. Protonation of superoxides produce hydrogen peroxide that react with redox active metals to generate hydroxyl radicals [Ayala, 2014]. The resulting hydroxyl radical is a ROS that causes oxidative damage by attacking cellular proteins, lipids, and nucleic acids [Rehman, 2018]. Lipid peroxidation is caused by oxidative degradation of lipids, especially in polyunsaturated fatty acids (PUFA), a major component of cellular membranes [Yin, 2011]. Lipid peroxidation of PUFA can result in several byproducts, such as malondialdehyde (MDA) and 4-hydroxynoneal (4-HNE), that can be used as biomarkers for oxidative stress [Niki, 2008]. MDA has been widely used as a biomarker for measuring lipid peroxidation due to its high reactivity with thiobarbituric acid (TBA) and forms the basis of the thiobarbituric acid reacting substances tests (TBARS) [Esterbauer, 1990; Esterbauer, 1991]. Studies have shown that GSTs are involved in the detoxification of oxidative compounds and byproducts of lipid peroxidation [Berhane, 1994; Hubatsch, 1998].

Increase of GST activity has been linked to insecticide resistance in mosquitoes by facilitating xenobiotic metabolism and excretion. [Che-Mendoza, 2009]. For example, increased GST activity within the malaria mosquito, *Anopheles gambiae*, and the yellow fever mosquito, *Ae. aegypti*, has been associated with resistance to dichlorodiphenyltrichloroethane (DDT) [Grant, 1991; Lumjuan, 2005; Prapanthadara,1993; Ranson, 2001]. GSTs are also responsible for organophosphate resistance in houseflies and the diamondback moth [Oppenoorth, 1979; Kao, 1989] and pyrethroid resistance in *An. gambiae* [Awolola, 2018]. In addition, GSTs may

contribute to pyrethroid resistance by detoxification of lipid peroxidation byproducts induced from pyrethroid exposure. This increase in GST activity could significantly impact available vector control and management methods with pesticides and repellents [Enayati, 2005]. GST and oxidative stress may also play a role in pathogen development with implications that mosquitoes with higher GST activity can serve as more efficient vectors [Kumar, 2003]. Although no studies have looked at the effects of virus infection on detoxification activity in mosquitoes, there have been *in vitro* studies using mosquito cell lines. It has been shown that C6/36 cell lines infected with dengue virus exhibited increased GST activity [Chen, 2011] and that GSTs play a major role in reducing apoptosis in DENV-infected C6/36 cell lines [Chen, 2012].

In this study, the effects of virus infection on oxidative stress and GST activity was determined using two biochemical assays. First, the thiobarbituric acid reactive substances (TBARS) method was used to assess oxidative stress levels by fluorometrically measuring MDA-TBA adduct from homogenized mosquito tissue. Second, homogenized mosquito tissue was used to determine GST activity by the conjugation of GSH and 1-chloro-2, 4-dinitro-benzene (CDNB). This study will allow us to better understand the relationship between physiological responses and virus infection within mosquitoes by quantifying oxidative stress levels and GST activity in ZIKV and LACV-infected *Ae. albopictus.*

MATERIALS AND METHODS

Mosquito collection and rearing.

Aedes albopictus were collected from Blacksburg, VA using gravid traps. After laying eggs, the mothers were screened for virus using Vero cell plaque assay. The mosquitoes were reared according to the methods of Jackson et al. 2012. The larvae were reared at a density of approximately 250 larvae per

container (33 × 17.5 × 11 cm) with 1,600 ml deionized water and bovine liver powder solution (7.5 g/500 ml). Adult mosquitoes were placed into 30 x 30 x 30 cm cages and provided cotton balls soaked in a 10% sucrose solution. Environmental chamber conditions were set at 24°C with 75% RH and 16L: 8D photoperiod.

Cells and Virus

Vero cells (American Type Culture Collection, Manassas, VA, USA) were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Corning, Corning, NY) with 10% fetal bovine serum (FBS), 100 U/mL of penicillin and 100 μg/mL of streptomycin, and maintained at 37 °C with 5% CO_2. The Asian lineage of ZIKV, PRVABC59 (GenBank accession no. KU501215) and LACV strain VA0921075 were used for this study. PRVABC59 was isolated from the serum sample of a patient traveling from Puerto Rico in 2015. VA0921075 was isolated from *Ae. triseriatus* mosquitoes collected from Wise County, VA (Barker et al. 2003). The viruses were amplified and maintained in Vero cells and stored at -80^{o}C.

Infection

Two to three-day old female mosquitoes were cold anesthetized and intrathoracically inoculated with 0.2 μl of ZIKV (8.75 x 10^7 PFU/mL), LACV (2.25 x 10^5 PFU/mL), or cell culture media using methods from Rosen and Gubler (1974). After infection, mosquitoes were placed into 30 x 30 x 30 cm cages and held in the environmental chamber with a 10% sucrose solution. At 0-, 3-, 7-, and 10-days post-exposure (DPE), mosquitoes were removed from cages using a hand-held aspirator and placed into 2mL microcentrifuge tubes and stored at -80C.

Oxidative stress assay

Oxidative stress was determined by using a thiobarbituric acid reactive substances (TBARS) kit (Cayman Chemicals, Ann Arbor, MI) to measure malondialdehyde (MDA) content. Each sample contained three female adult mosquitoes and were prepared in RIPA buffer using the tissue homogenates procedure and heated to 90-100°C for one hour. The assay was measured fluorometrically at an excitation wavelength of 530 nm and an emission wavelength of 550 nm using a Synergy H1 Hybrid Multi-Mode Reader (BioTek, Winooski, VT). Each sample was normalized to the concentration of total protein using the bicinchoninic acid assay with bovine serum albumin as the standard.

GST activity assay

GST levels were detected using a modified spectrophotometric assay protocol outlined by the World Health Organization test procedure for insecticide resistance monitoring in malaria mosquitoes [WHO, 1998]. Each sample contained five female adult mosquitoes and was homogenized in 0.1 M sodium phosphate (pH 7.8) containing 0.3% Triton X-100 buffer. Mosquito homogenates were centrifuged at 10,000 x g for 10 minutes at 4°C. Supernatant was added to a microplate well along with a working solution containing 63mM CDNB and 10mM reduced glutathione. Absorbance was measured continuously at 340nm for 10 minutes at 1 minute intervals using a Molecular Devices SpectraMax M2 multimode microplate reader (Sunnyvale, CA). Each sample was normalized to the concentration of total protein using the bicinchoninic acid assay with bovine serum albumin as the standard.

Plaque assay and virus detection

Viral titers of individual mosquitoes and virus inoculum were determined by Vero cell plaque assay. Individual mosquitoes were homogenized in 1mL of DMEM and centrifuged at

1500 x g for two minutes. Supernatant was collected and plated onto confluent 6-well cell culture plates. The plates were incubated for one hour at 37°C and methyl cellulose was added as an overlay. The plates were incubated at 37°C with 5% CO2 for four to six days, or until plaques were visible. Plates were stained with crystal violet and virus titers were determined following the methods of Barker et al. 2003.

Statistical analysis

Comparison of oxidative stress and GST levels between infected and uninfected mosquitoes was analyzed using a two-way analysis of variance (ANOVA) followed by a Dunnett's multiple comparison test. Significant differences in oxidative stress, GST levels, and whole body mosquito titers on 0-, 3-, 7-, and 10-DPE were analyzed using a two-way ANOVA followed by a Tukey's multiple comparison test. The datasets were analyzed using GraphPad Prism 6.0 (La Jolla, CA).

RESULTS

TBARS in virus-infected and uninfected Ae. albopictus

Figure 4.1 shows the mean TBARS level of ZIKV-infected, LACV-infected, and uninfected *Ae. albopictus* 0-, 3-, 7-, and 10-DPE. TBARS levels was not significantly different between virus-infected and uninfected controls ($P>0.05$, Dunnett's multiple comparison test). There were no significant differences in TBARS levels 0-, 3-, 7-, and 10-DPE for all treatments and controls ($P>0.05$, Tukey's multiple comparison test). Although Figure 4.1 showed higher levels of TBARS in infected mosquitoes at 7- and 10-DPE, there were no statistically significant differences.

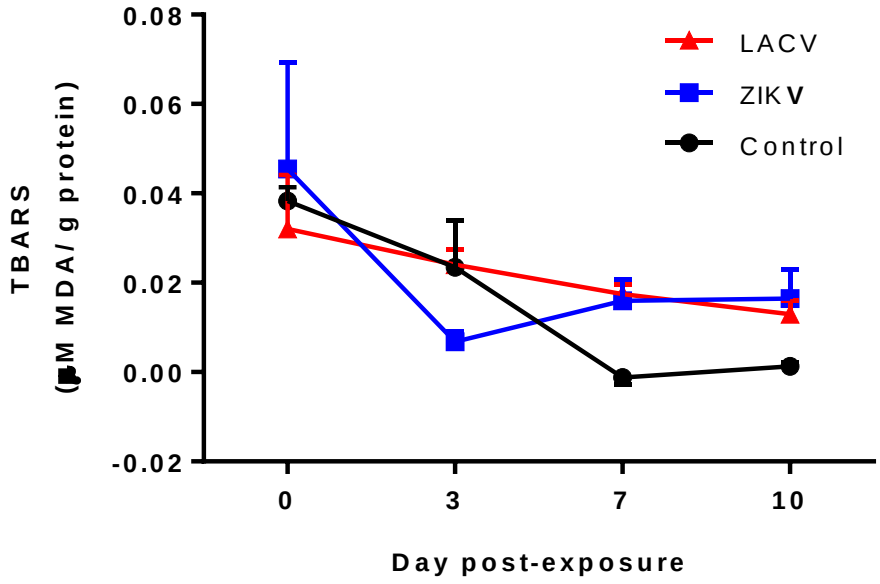

Figure 4.1. Thiobarbituric acid reactive substance (TBARS) levels of infected and uninfected mosquitoes. Mosquitoes were intrathoracically inoculated with ZIKV (N=3) or LACV (N=3), or uninfected (N=3), and tested 0-, 3-, 7-, and 10-DPE. Results were analyzed using a 2-way ANOVA followed by Dunnett's multiple comparison test for treatments and Tukey's multiple comparison test for days post-exposure, α=0.05.

GST activity in virus-infected and uninfected *Ae. albopictus*

Figures 4.2, 4.3, and 4.4 show GST activity of ZIKV, LACV, and media-inoculated *Ae. albopictus* compared to untreated controls. There were no significant differences in GST activity between controls (mosquitoes that were not subjected to intrathoracic injection) and cell culture media-injected mosquitoes (P>0.05) (Figure 4.2). This indicated that there were no effects of the injection process or cell culture media on GST enzyme activity. Mosquitoes infected with ZIKV (91.64 nmol/ min/ mg protein) (Figure 4.3) or LACV (91.24 nmol/ min/ mg protein) (Figure 4.4) had significant differences in GST activity 3-DPE (ZIKV: P = 0.0351, LACV: P = 0.0383, Dunnett's multiple comparison test) when compared to uninfected controls (63.77 nmol/ min/ mg protein). No differences were seen on 0-, 7-, or 10-DPE. All mosquito treatments showed an increase of GST activity over time. Mean GST activity for uninfected controls at 0-DPE (66.51

nmol/ min/ mg protein) and 3-DPE (63.77 nmol/ min/ mg protein) were significantly lower (P< 0.05, Tukey's multiple comparison test) than 10-DPE (107.3 nmol/ min/ mg protein) (Figure 4.2). Mean GST activity for ZIKV-infected mosquitoes at 0-DPE (65.35 nmol/ min/ mg protein) and 3-DPE (91.64 nmol/ min/ mg protein) was significantly lower (P< 0.05, Tukey's multiple comparison test) than 7-DPE (104.1 nmol/ min/ mg protein) and 10-DPE (105.8 nmol/ min/ mg protein) (Figure 4.3). Mean GST activity for LACV-infected mosquitoes at 0-DPE (63.77 nmol/ min/ mg protein) was significantly lower (P< 0.05, Tukey's multiple comparison test) than 10-DPE (105.3 nmol/ min/ mg protein).

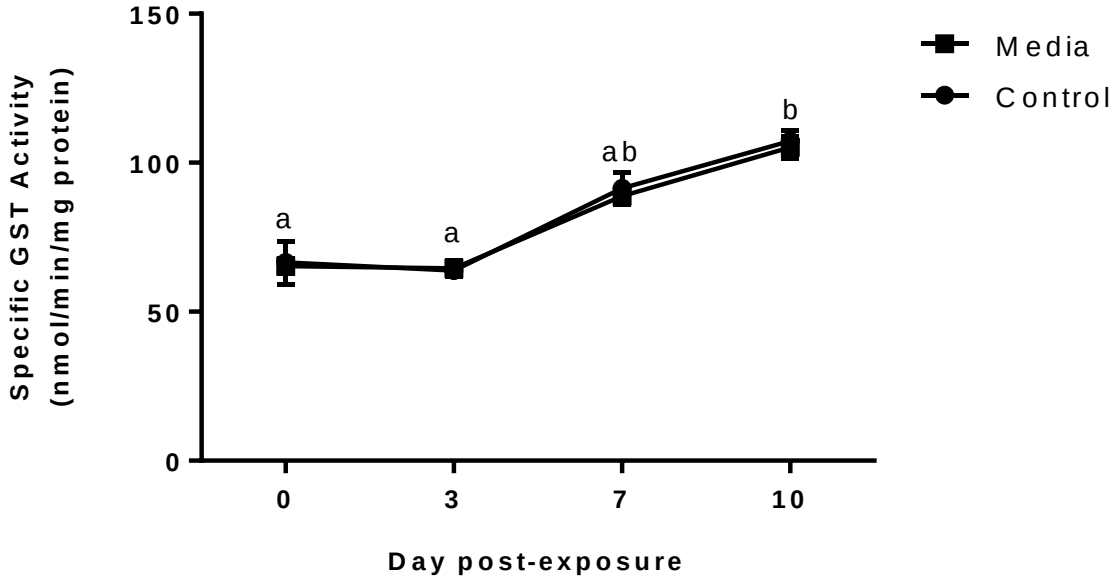

Figure 4.2. Mean GST activity of cell culture media-injected *Aedes albopictus.* Mosquitoes were tested 0-, 3-, 7-, and 10-DPE. Results were analyzed using a 2-way ANOVA followed by Dunnett's multiple comparison test for treatments and Tukey's multiple comparison test for days post-exposure, α=0.05, *N*= 3. Letters denote significance between days.

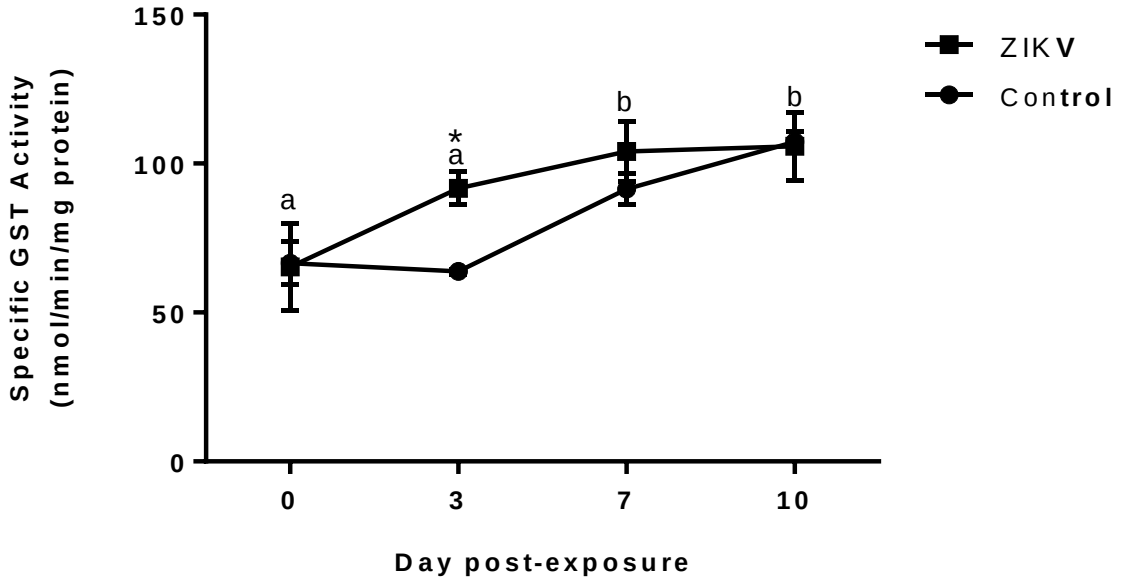

Figure 4.3. Mean GST activity of ZIKV-infected *Aedes albopictus.* Mosquitoes were tested 0-, 3-, 7-, and 10-DPE. Results were analyzed using a 2-way ANOVA followed by Dunnett's multiple comparison test for treatments and Tukey's multiple comparison test for days post-exposure, α=0.05, *N*= 3. Asterisk (*) denotes significance between treatments (P = 0.0351) and letters denote significance between days.

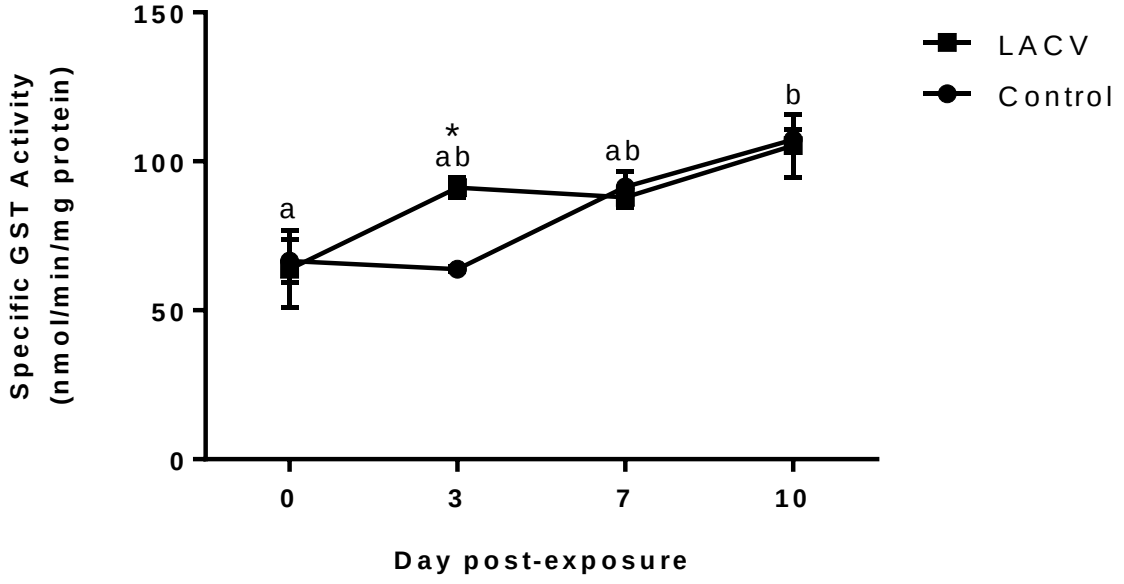

Figure 4.4. Mean GST activity of LACV-infected *Aedes albopictus.* Mosquitoes were tested 0-, 3-, 7-, and 10-DPE. Results were analyzed using a 2-way ANOVA followed by Dunnett's multiple comparison test for treatments and Tukey's multiple comparison test for days post-

exposure, α=0.05, *N*= 3. Asterisk (*) denotes significance between treatments and letters denote significance between days.

In vivo dynamics of ZIKV and LACV infection in *Ae. albopictus*

Figure 4.5 shows the mean virus titers of ZIKV and LACV-infected *Ae. albopictus*. Mean titers of ZIKV-infected mosquitoes were 9.55 x 10^3 PFU/mL (0-DPE), 5 x 10^3 PFU/mL (3-DPE), 2.2 x 10^5 PFU/mL (7-DPE), and 1.15 x 10^6 PFU/mL (10- DPE). With the exception from 0 to 3-DPE, titers of ZIKV-infected mosquitoes increased significantly over time. There was significant increase in ZIKV titers from 0 to 7-DPE (P = 0.0009), 0 to 10-DPE (P < 0.0001), 3 to 7-DPE (P = 0.0008), 3 to 10-DPE (P < 0.0001), and 7 to 10-DPE (P < 0.0001). Mean titers of LACV-infected mosquitoes were 1.14 x 10^4 PFU/mL (0-DPE), 8.5 x 10^4 PFU/mL (3-DPE), 9 x 10^4 PFU/mL (7-DPE), and 1.75 x 10^5 PFU/mL (10-DPE). Although virus titers of LACV-infected mosquitoes increased over time, significant increases were only seen from 0 to 10-DPE (P = 0.0038) and 3 to 10-DPE (P = 0.0260). There were no significant differences in virus titers from 0- to 3-DPE, 0- to 7-DPE, 3- to-7 DPE, and 7- to 10-DPE.

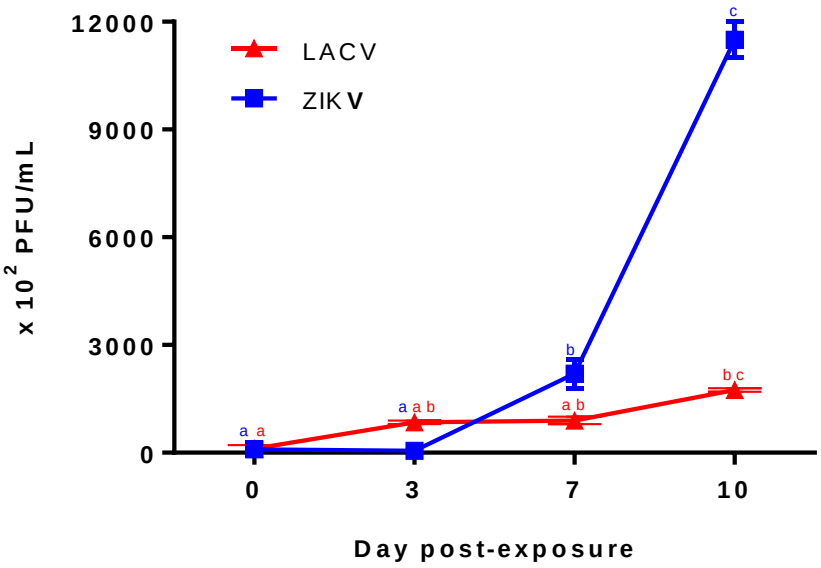

Figure 4.5. Mean plaque assay titers (x 10^2 PFU/mL) of homogenized *Aedes albopictus* intrathoracically infected with ZIKV or LACV. Differences in mean titers on 0-, 3-, 7-, and 10-DPE were analyzed using 2-way ANOVA followed by Tukey's multiple comparison test, α=0.05, *N*= 2. Letters denote significance between days.

DISCUSSION

Virus infection is known to cause oxidative stress within an organism which, in turn, may increase the production of detoxification enzymes to prevent further damage. In addition, virus infection causes apoptosis in various organs, contributing to oxidative stress [Clem, 2016]. This occurrence changes the permeability and fluidity of the cell membrane, resulting in cellular damage [Dix, 1993]. By using the TBARS and GST assays, we can determine if virus infection significantly increases oxidative stress levels within mosquitoes, which in turn increases detoxification enzyme activity for protection. Here, we performed an *in vivo* study by directly infecting *Ae. albopictus* with ZIKV or LACV and then quantified levels of MDA-TBA adduct and total GST activity.

TBARS assays are often used as sensitive tests for MDA which is a byproduct of lipid peroxidation. Measuring MDA levels can be used to assess the current state of oxidative stress and factors that may contribute those changes. In the case of MDA, higher levels from a sample is associated with more lipid peroxidation [Tsikas, 2017]. We found that there were no significant differences in MDA-TBA levels between infected and uninfected mosquitoes. Overall MDA-TBA levels decreased over time while GST activity increased, indicating that GSTs may play an important role in controlling oxidative stress that is independent of virus infection. A few limitations that can be addressed for future studies may optimize the experimental results. This study looked at MDA-TBA levels in whole mosquitoes, but performing the assay with different body parts, such as only the head or body, may influence the results. Mosquito heads contain a higher amount of nervous tissue, which include the brain and sensory nerves of the olfactory and gustatory systems. The brain and nervous tissue consume a higher amount of oxygen and are comprised of membranes rich in PUFA, which are prone to degradation by ROS [Chen, 2008; Shichiri, 2014]. This, along with the neurotropism of some *Flaviviruses* and *Orthobunyaviruses*, makes neural cells potentially more susceptible to damage by ROS [Ludlow, 2016; Maximova, 2018].

GST activity between controls and cell culture media-injected mosquitoes were not significantly different, indicating the cell culture media and injection process had no effect on GST activity. GST activity increased over time, with 3-DPE as the only time point with significant differences in GST activity between virus-infected and uninfected mosquitoes. Because there were no differences in GST activity for controls and virus-infected mosquitoes on 7- and 10-DPE, the increase in activity may be a result of the aging process opposed to infection. A study looking at the effects of aging on GST activity in *Ae. aegypti* showed the highest GST

activity from the larval stage and throughout metamorphosis, reaching a plateau from 5 to 20 days of adulthood [Hazelton and Lang, 1978]. The same study also found that GST activity in the thorax and head were significantly higher than the thorax or whole body for 40 day-old mosquitoes, with no differences in 10 day-old mosquitoes. Most research on insect GSTs focuses on detoxification organs such as the midgut, fat body, and Malpighian tubules [Huang et al. 2011], but the olfactory organs have also been shown to express GSTs in various insect species [Huang et al., 2017; Leal et al., 2009; Rogers et al., 1999; Tan et al., 2014]. A study by Gui et al., 2009 showed that *Bombyx mori* larvae infected with *Bombyx mori* nuclear polyhedrosis virus and *Bombyx mori* densonucleosis virus showed varying GST activity associated with strain, tissue, and developmental stages. Our study looked at GST activity with whole mosquitoes, so future studies can be conducted using GST-rich body regions. In addition, we can refer to the study by Hazelton and Lang to look at GST activity in mosquitoes past 10-DPE, where they found that activity plateaus after eclosion and significantly decrease for 30 day-old senescent adults.

In summary, intrathoracic inoculation of ZIKV or LACV had no significant effect on oxidative stress in *Ae. albopictus*. Infection with the viruses did not significantly affect GST activity in the mosquitoes, but a significant difference between infected and uninfected mosquitoes were observed on the 3-day post-exposure. Future studies looking at the effects of additional antioxidative enzymes, such as superoxide dismutase (SOD) or glutathione peroxidase (GTPx), and oxidants, such as oxidized glutathione (GSSG), hydrogen peroxide, and superoxide radicals, may identify potential biomarker for oxidative stress with virus-infected mosquitoes.

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Chapter 5

Summary and Conclusion

Summary

Today, infectious diseases vectored by mosquitoes remain a global issue and contribute to significant human illness and death. Understanding the factors that contribute to arbovirus transmission provide crucial knowledge to strengthen control and prevention practices. Conducting basic and applied research on mosquito vectors and the pathogens they transmit is important for developing and implementing proper control techniques and to evaluate the effectiveness of current methodologies. I designed this study to answer imperative questions regarding the transmission of emerging and endemic arboviruses of public health concern where we focused on vector competence and the effects of virus infection on repellent response, oxidative stress, and glutathione-*S*-transferase activity.

What mosquito species in Virginia are competent vectors for Zika and Cache Valley viruses?

I found that the vector competence of a Virginia strain of *Ae. albopictus* was equivalent to that of a Florida strain of *Ae. aegypti*. Since *Ae. aegypti* is the primary ZIKV vector, any mosquito species that demonstrates equivalent ZIKV vector competency could contribute to autochthonous transmission, and should be managed by mosquito control and public health districts. *Ae. japonicus* from Virginia was capable of transmitting ZIKV, but at a much lower rate compared to *Ae. aegypti* and *Ae. albopictus*. This study with Virginia *Ae. triseriatus* was similar to a previous study where *Ae. triseriatus* only developed midgut infections after oral exposure [Aliota, 2016]. I also determined the presence of tissue barriers by intrathoracic inoculation of ZIKV. After intrathoracic inoculation, ZIKV was detected in the saliva of *Ae. japonicus*, but not

Ae. triseriatus, which indicated the presence of salivary gland barriers in *Ae. triseriatus*. Although transmission rates for *Ae. japonicus* increased following intrathoracic inoculation, the low transmission rates indicate that this species may not be significant in ZIKV transmission. Due to the abundance of *Ae. albopictus* in this region, in combination with high ZIKV competency and anthropophilic behavior, it is more likely for this species to contribute to ZIKV transmission in Virginia. Other laboratory transmission studies looking at various strains of *Ae. albopictus* have implicated that this species may serve as a secondary vector for ZIKV [McKenzie, 2019].

I also found that *Ae. albopictus, Ae. triseriatus, Ae. japonicus*, and *Ae. aegypti* were competent vectors for CVV. *Ae. albopictus, Ae. triseriatus*, and *Ae. aegypti* were susceptible to CVV infection and subsequently transmitted virus after oral exposure to low titer blood-meals. The existence of a dose-dependent infection or escape barrier can determine how certain mosquito species and strains are refractory to infection. With laboratory evidence of low titer vector competency and abundant distribution throughout North America, *Ae. aegypti*, *Ae. albopictus*, *Ae. japonicus*, and *Ae. triseriatus* could play major roles in CVV transmission.

Cx. pipiens and *Cx. restuans* were not competent vectors for ZIKV and CVV, indicating that neither species is significant in transmission of the viruses. (Chapter 2)

Does virus infection in mosquitoes affect their response to repellents?

Overall, LACV- and ZIKV-infected mosquitoes were more likely to land on repellent-treated surfaces compared to uninfected mosquitoes. There was lower percent protection (%*p)* against infected mosquitoes exposed to 5% DEET, picaridin, or PMD. To increase %*p* and reduce landing, probing, and blood-feeding by virus-infected mosquitoes, utilizing repellents

with a minimum of 10% active ingredient is recommended. The effectiveness of repellents can also vary depending on environmental factors and mosquito species, with the combination of the virus and mosquito species playing a larger role than the virus itself. [Barnard, 1998; Van Roey et al., 2014]. *Ae. albopictus* used for this study showed more aggressive host-seeking and feeding behaviors compared to *Ae. triseriatus* and *Ae. aegypti*. For example, *Ae albopictus* exhibited a higher mean landing, probing, and blood-feeding for the SRAMF compared to *Ae. aegypti* and higher mean landing for the human arm assay compared to *Ae. aegypti* and *Ae. triseriatus*. *Ae. albopictus* is an aggressive daytime biting mosquito that is emerging as a worldwide public health concern due to its rapid spread from globalization and ability to vector numerous diseases [Bonizzoni, 2013]. Using repellents in areas with high *Ae. albopictus* populations is an important preventative measure to protect against arbovirus transmission. (Chapter 3)

Does virus infection influence oxidative stress and GST activity in Aedes albopictus?

This entire study was based off the principle that virus infection results in oxidative stress, which causes the organism to restore its redox state via metabolic detoxification. We found that there were no significant differences in TBARS levels between infected and uninfected mosquitoes. Overall, TBARs activity decreased over time while GST activity increased. Although there were no statistical differences in the decrease of TBARS, biologically, GSTs may play an important role in controlling oxidative stress that is independent of virus infection. Virus-infected mosquitoes had significantly higher GST activity 3-DPE compared to uninfected mosquitoes. Various antioxidative enzymes, such as superoxide dismutase (SOD) or glutathione peroxidase (GTPx), and oxidants, such as oxidized glutathione (GSSG), hydrogen peroxide, and superoxide radicals, may serve as potential biomarker for future studies [Perrin-

Nadif, 1996; Ho, 2013]. The biomarker used for the oxidative stress portion of this study was malondialdehyde, but 4-hydroxynonenal (4-HNE) is equally expressed as a byproduct of lipid peroxidation [Ayala, 2014]. Because GSTs are involved in 4-HNE metabolism [Balogh, 2011], studying the interaction between GSTs and 4-HNE may provide additional insight on the relationship between virus infection, oxidative stress, and detoxification activity. Overall, the physiological effects between vector and virus interactions is complex. Virus infection can cause various forms of oxidative stress that is biologically restored by different detoxification mechanisms. Due to the association with increased detoxification activity and insecticide resistance, it is important to understand the effects of virus infection and physiological response of mosquitoes. (Chapter 4)

Conclusion

Based on my research, the risk of ZIKV, CVV, and LACV should be considered in Virginia. Mosquito control programs and public health departments should conduct surveillance to monitor primary and secondary vectors that can transmit the viruses to human populations and maintain the viruses in natural cycles. Due to the abundance of *Ae. albopictus* in Virginia, targeted control methods are encouraged in areas with ZIKV, CVV, or LACV detection. The mosquito's anthropophilic behavior, close association with human habitation, and vector competency of numerous mosquito-borne pathogens further supports the public health significance of *Ae. albopictus*. Knowing the potential of arbovirus transmission by local mosquito species and the global increase of disease cases, it is important to utilize all forms of personal protection against mosquito bites. Commercially available repellents are accessible and relatively inexpensive, and have been rigorously tested before granted an EPA registration. In

my research, I found that the three most common products, DEET, picaridin, and PMD, were effective against infected and uninfected mosquitoes. It is important for public outreach and education programs to promote the use of repellents, especially during peak mosquito activity. To achieve higher percent protection (%*p*), individuals have to also take into account of environment and types of activity. Concentrations of 10% can significantly increase %*p* against *Ae. albopictus, Ae. aegypti,* and *Ae. triseriatus,* regardless of infection status. With evidence of desensitize response to repellents, we wanted to figure out the potential physiological mechanism. Due to the fact increased concentrations was able to increase %*p* and decrease contact by infected mosquitoes, we aimed to look at the metabolic enzyme, glutathione-*S*-transferase (GST) as a possible mechanism. We found no significant differences in GST activity between virus-infected and uninfected mosquitoes, except for mosquitoes tested 3-days post-infection. During this time, high rates of virus infection could be occurring within the mosquito, resulting in the biological need for detoxification of harmful byproducts. This last chapter of my dissertation led to many questions regarding mosquito-virus interactions and additional research, such as organ-specific GSTs and additional oxidative stress byproducts metabolized by GSTs, is vital for developing and implementing effective control strategies.

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