Dynamics of La Crosse virus: Surveillance, Control and Effect on Vector Behavior

Fan Yang

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Sally L. Paulson, Chair
Troy D. Anderson
Carlyle C. Brewster
Jianyong Li
Zhijian Tu
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ABSTRACT

Abstract (Academic)

La Crosse virus (LACV) encephalitis is the most common and important endemic mosquito-borne disease of children in the U.S. with an estimated 300,000 annual infections. The disease is maintained in a zoonotic cycle involving the eastern treehole mosquito, *Aedes triseriatus* and small woodland mammals such as chipmunks and squirrels. The objectives of this study were 1) to conduct surveillance of LACV and other mosquito-borne viruses; 2) to evaluate the effect of virus infection on mosquito host-seeking and neurotransmitter levels, and 3) to determine the effectiveness of barrier sprays to control infected mosquito vectors.

Our surveillance study demonstrated the involvement of an invasive species, *Aedes japonicus*, in the transmission cycle of Cache Valley virus (CVV). CVV is a mosquito-borne virus that is closely related to LACV. Thus, surveillance is a critical step in public health, providing pathogen distribution and frequency data as well as identifying and incriminating new vectors.

LACV infection did not affect the host-seeking behavior of *Ae. triseriatus* females. Using high performance liquid chromatography with electrochemical detection (HPLC-ED), the levels of serotonin and dopamine were measured in infected and uninfected mosquitoes. Serotonin is known to affect blood-feeding and dopamine affects host-seeking. Serotonin levels were significantly lower in LACV-infected mosquitoes but dopamine levels were unaffected by virus. A previous study found that LACV infection caused an alteration in mosquito blood-feeding in a way that could enhance virus transmission. This work showed that LACV infection can reduce the level of serotonin in the mosquito, promoting virus transmission through altered blood-feeding without impairing the vector's ability to locate a host.

Standard CDC bottle assays were used to evaluate the efficacy of two pyrethroids and two essential oil sprays on LACV infected and uninfected mosquitoes. LACV-infected *Ae. triseriatus* females were more susceptible to both pyrethroids than uninfected ones. Infection status did not affect the susceptibility of *Ae. albopictus* to either pyrethroid. The essential oils were inconsistent in their effects. These results demonstrate that barrier sprays may be a viable part of a mosquito control program, not just to reduce the biting rate but to potentially reduce the virus-infected portion of the vector population.

Abstract (general):

La Crosse virus (LACV) encephalitis is the most common and important endemic mosquito-borne disease of children in the U.S. with an estimated 300,000 annual infections. The disease is maintained in a zoonotic cycle involving the eastern treehole mosquito, *Aedes triseriatus* and small woodland mammals such as chipmunks and squirrels. The objectives of this study were 1) to conduct surveillance of LACV and other mosquito-borne viruses; 2) to evaluate the effect of virus infection on mosquito host-seeking and neurotransmitter levels, and 3) to determine the effectiveness of barrier sprays to control infected mosquito vectors. The surveillance study demonstrated the involvement of an invasive species, *Aedes japonicus*, in the transmission cycle of Cache Valley virus (CVV). CVV is a mosquito-borne virus that is closely related to LACV. Thus, surveillance is a critical step in public health, providing pathogen distribution and frequency data as well as identifying and incriminating new vectors. Our study of the effects of LACV infection on host-seeking and neurotransmitter levels showed that LACV can manipulate *Ae. triseriatus* females in a way that could facilitate transmission of the virus. Lastly, we showed that barrier sprays may be a viable part of a mosquito control program, not just to reduce the biting rate but to potentially reduce the virus-infected portion of the vector population.

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Chapter 1. Literature Review

1.1 Introduction to the Bunyaviridae.

The family Bunyaviridae is the largest group of RNA viruses, containing more than 300 distinct viruses organized into 5 genera (Barrett and Shope 2005; Nichol 2001) (Table 1.1). Bunyaviruses can infect vertebrates, invertebrates and plants and are primarily vectored by arthropods such as mosquitoes, ticks and thrips. A number of factors including the use of a wide variety of arthropod vectors and vertebrate hosts, changing global distribution and genetic reassortment to have made the *Bunyaviridae* important as emerging and re-emerging infectious disease agents (Lambert and Lanciott 2009).

Table 1. Genera of Bunyaviridae.

Genus	Arthropod Vector	Hosts
Nairovirus	Ticks	Sheep, other livestock, birds, humans
Phlebovirus	Mosquitoes, sand flies, ticks	Livestock, humans
Orthobunyvirus	Mosquitoes, midges, ticks	Livestock, small mammals, humans
Hantavirus	None	Rodents, humans
Tospovirus	Thrips	Plants

1.2 Introduction to the Orthobunyaviruses.

Orthobunyavirus virions are enveloped, pleomorphic particles with a tripartite, single- stranded, negative-sense RNA genome. At least 30 orthobunyaviruses cause disease in humans (Elliott 2014). Common disease syndromes include febrile illness, including acute but self-limiting febrile illness, e.g., Itaya virus (Hontz et al. 2015), encephalitis, e.g., La Crosse virus (LACV), and hemorrhagic fever, e.g. Ngari virus (Gerrard et al. 2004). Orthobunyaviruses such as Cache Valley virus and Schmallenberg

virus cause abortion and teratogenic effects in sheep and cattle (Edwards 1994; Doceul et al. 2013). However, the true potential for detrimental effects of these viruses on human and animal health is not known because few labs test for them.

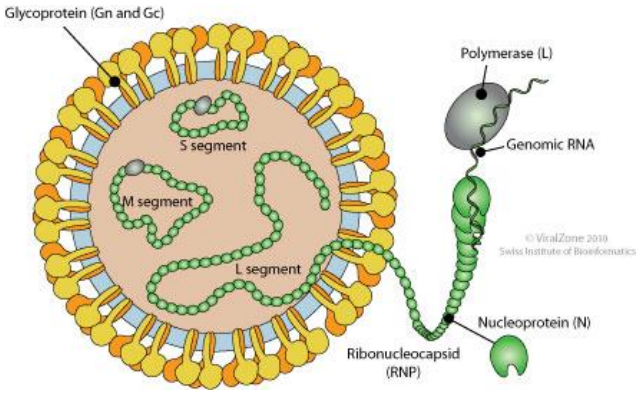

Figure 1.1. Orthobunyvirus virion structure. Reprinted with permission from SIB Swiss Institute of Bioinformatics, ViralZone. S: small; M: medium; and L: long.

1.2.1 La Crosse Virus

In 1964, La Crosse virus (LACV) was first isolated from a 4-year old girl who died from encephalitis in La Crosse County, Wisconsin (Thompson and Evans 1965). Since then, 80 to 110 cases of neuroinvasive disease have been reported annually (Rust et al. 1999; Soldan et al. 2010; Gaensbauer et al. 2014). However, the disease is significantly under-diagnosed and under-reported (CDC). It has been estimated that approximately 300,000 systemic LACV infections occur annually, although less than 1.5% of these present with clinical symptoms (Kalfayan, 1983). Approximately half of the children with LACV encephalitis have seizures during the acute illness, and about 10% will develop epilepsy. Approximately half of the children with LACV encephalitis have seizures during the acute illness, and about 10% will develop epilepsy. Most of the severe cases are found in children under 16, while adults may have asymptomatic or mild cases and recover fully in a few days (Borucki et al. 2002; Haddow 2009). Historically, most

cases have occurred in the Midwest but recently the incidence of LACV cases has increased in the Appalachian Region (Gerhardt et al. 2001; Jackson et al. 2012).

1.2.2. La Crosse Virus Structure and Replication

LACV is a single-stranded, negative-sense, segmented RNA virus in family *Bunyaviridae*, genus *Orthobunyavirus*. In its 90-100 nm pleomorphic virion, LACV has three RNA genome segments: L (large, 6980 nt) encoding the RNA-dependent RNA polymerase (RdRp) protein, M (medium, 4526 nt) encoding two glycoproteins (Gc and Gn) and a nonstructural protein (NSm), and S (small, 984 nt) encoding the nucleocapsid protein (N) and another nonstructural protein (NSs) (Beaty and Bishop 1988; Borucki et al. 2002; Horne and Vanlandingham 2014). The RNAs are encapsidated by N protein to form a helical structure. The viral RNAs are contained in a host-derived lipid envelope studded with virus-encoded glycoprotein spikes (Borucki et al. 2002). The Gc glycoprotein binds with mammalian cell receptors and the Gn glycoprotein binds with mosquito midgut proteins (Ludwig et al. 1989; Ludwig et al. 1991).

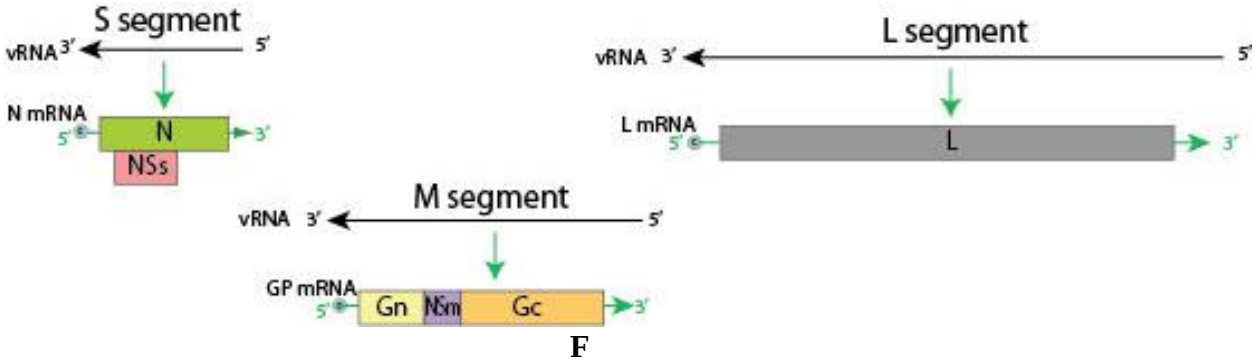

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Figure 1.2. La Crosse virus has asegmented, negative-sense RNA linear genome . Reprinted with permission from SIB Swiss Institute of Bioinformatics, ViralZone. Gn and Gc are two glycoproteins on LACV surface. NSs: nonstructural protein from small segment; NSm: nonstructural protein from medium segment. S: small; M: medium; and L: long.

The virus enters a host cell through receptor-mediated endocytosis and membrane fusion, the viral RNA is released into the cytoplasm of the cell, and transcription commences. The RdRp cleaves the 5' caps from host mRNA, a process called cap snatching, to act as primers for transcription of the negative-sense genomic RNA to positive-sense mRNA (Patterson and Kolakofsky 1984). Arboviruses are capable of replicating in physiologically divergent hosts, vertebrate and insect. Virus replication in vertebrate hosts results in acute infections with relatively high circulating viremia to ensure infection of insects while feeding. Infections in the insect are persistent so that the vector remains infective for life, facilitating transmission to more vertebrate hosts (Paulson et al. 1989; Bennett et al. 2008). Differences in gene expression between the 2 cell types have been seen (Rossier et al. 1988). The cytopathic effect of LACV virus infection in BHK cells (mammalian) is correlated with reduced host protein synthesis and host mRNA instability (Raju and Kolakofsky 1988). However, protein synthesis in C6/36 (mosquito) cells does not reduce dramatically because of LACV infection. Rossier et al (1988) suggested the methylated cap-dependent viral endonuclease was responsible for this difference in replication (Rossier et al. 1988). In BHK cells, LACV mRNA 5'-terminal sequence is heterogeneous, whereas in C6/36 cells, the 5'-terminal sequence was heterogeneous, but become homogeneous in later infection (Dobie et al. 1997). The virus matures in the Golgi apparatus where Gc and Gn glycoproteins bind with nucleocapsids and assemble into a complete virion. From the Golgi, virions are transferred to cell surface and released by exocytosis (Borucki et al. 2002).

1.2.3. The Transmission Cycle of La Crosse Virus

The primary vector of LACV is *Aedes triseriatus*, but *Aedes albopictus* and *Aedes japonictus* are emerging as adjunct vectors (Kitron et al. 1998; Grim et al. 2007).

Horizontal transmission acts to amplify the virus and vertical, or transovarial, transmission provides the overwintering mechanism (Figure 1.1) (Hughes et al. 2006). Squirrels and chipmunks have been shown to be important reservoir hosts (Ksiazek and Yuill 1977). Humans are dead-end host for LACV, because they do not develop a sufficiently high viremia to infect mosquitoes. In the laboratory, all three vector species (*Ae. aegypti*, *Ae. albopictus*, and *Ae. japonictus*) showed high rates of transovarial transmission rates, 71%, 52%, and 44% respectively (Hughes et al. 2006). The virus can survive the winter in infected eggs in diapause.

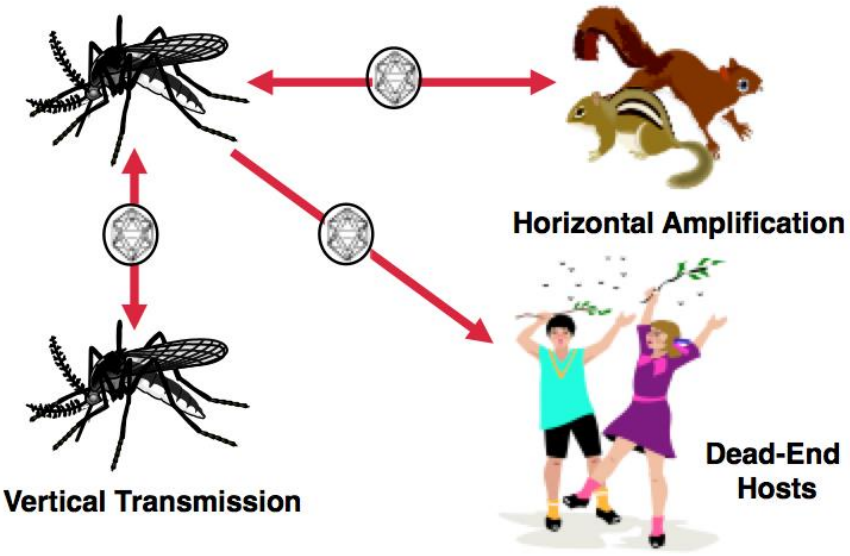

Figure 1.3. The transmission cycle of La Crosse virus.

1.2.4. Cache Valley Virus

Cache Valley virus was named after the location in northern Utah where it was first isolated from *Culiseta inornata* mosquitoes (Holden and Hess 1959). It is the most widespread Bunyumwera serogroup virus and is widespread throughout North and Central America (Calisher et al. 1986). The virus infects many species of domestic ungulates such as sheep and cattle (Sahu et al. 2002; Meyers et al. 2015), but white-tailed deer are likely the natural reservoir (Blackmore and Grimstad 1998). Although it has

been isolated from more than 30 species of culicine or anopheline mosquitoes in several genera, the principal mosquito vectors are unknown (Campbell et al. 2006). However, based on the number and frequency of field isolations and laboratory transmission studies, *Anopheles quadrimaculatus* and *Anopheles punctipennis* probably play important roles in the transmission cycle in nature (Saliba et al. 1973; Blackmore and Grimstad 1998).

CVV infection is common in sheep and can cause spontaneous abortion, stillbirth and congenital defects, making it a significant pathogen during lambing season (Edwards 1994). The virus is neuroinvasive in humans but there have been only 3 confirmed human cases, with a single mortality, in the U.S. (Nguyen et al. 2013). However, serological studies have reported infection rates as high as 18% in endemic areas (Buescher et al. 1970; Blitvich et el. 2012) and medical laboratories rarely test for it, so the true incidence and impact on human health of CVV is unknown.

Multiyear studies of CVV in mosquitoes have shown that virus activity was highly variable with no consistent year-to-year pattern and was not related to mosquito abundance (Buescher et al. 1970; Ngo, Maffei et al. 2006; Andreadis et al. 2014). One hypothesis to explain these fluctuations is may result from emergence, extinction and re-introduction of new virus strains into an area (Armstrong et al. 2015). This has been seen with other arboviruses, such as West Nile virus (Mann al. 2013). In the Northeast, a new lineage of CVV emerged in 2010 showing a common ancestry with viral stains from Mexico. By 2014, this new stain, was the dominant.

1.3. Mosquito Vectors

1.3.1. *Aedes triseriatus*

Aedes triseriatus, the eastern treehole mosquito, has been found in the upper Midwest and Eastern U.S, extending from Florida, north to Ontario and Quebec and west to the Dakotas and Texas (Paulson et al. 1989; Walker 1992). The larvae are mostly found in hardwood treeholes but because of its larval capability to live in poor nutrition condition, it can take advantage of artificial containers such as discarded tires (Kling et al. 2007; Williams et al. 2007). In a West Virginia study *Ae. triseriatus* larvae were found from Mar. to Oct. (Joy and Hildreth-Whitehair 2000) and is multivoltine. The population density reaches a peak in July and then declines in Southwest Virginia (Barker et al. 2003). The species may overwinter either as eggs or as larvae (Holzapfel and bradshaw 1981). Blood meal analysis showed that squirrels, chipmunks, and humans are the main hosts for *Ae. triseriatus* in nature (Ksiazek and Yuill 1977). It has been incriminated as the primary vector of LACV for several reasons. First of all, the seasonal occurrence of human LACV cases coincides with the phenology of *Ae. triseriatus* (Berry et al. 1975; Reese et al. 2010). Second, chipmunks and squirrels, the preferred hosts of the mosquito, are the amplifier hosts for LACV in nature (Gauld et al. 1975; Ksiazek and Yuill 1977). The titer of LACV in chipmunks can reach to 10^6 plaque-forming unit (PFU) which are high enough to infect mosquitoes. Third, laboratory and field studies have demonstrated that LACV can overwinter in diapausing eggs and larvae stage in field and the virus can be efficiently transmitted horizontally and vertically (Pantuwatana et al. 1974; Watts et al. 1974; Hughes et al. 2006). In addition to LACV, *Ae. triseriatus* is a competent vector of West Nile virus, Venezuelan equine virus, eastern equine virus, western equine virus,

dengue (type I), St. Louise encephalitis virus, and yellow fever virus (Davis et al. 1966; Freier and Grimstad 1983; Styer et al. 2007; Unlu et al. 2010).

1.3.2. *Aedes albopictus*

Aedes albopictus, the Asian tiger mosquito (ATM), is an aggressive, strongly anthropophilic and container-dwelling mosquito (Rai 1991). Since its discovery in India in 1894 by Skuse, the geographic distribution of *Ae. albopictus* has expanded from Asia to Europe, Africa, the Middle East, North and South America and the Caribbean, largely through the transport of eggs through the used tire trade (Gratz 2004). The first report of the species in the U.S. was a population found in Harris County, Texas, in 1985 (Rai 1991; Moore 1999). Now, 26 states in the continental USA have been infested with the ATM. This rapid mosquito invasion has led to the re-emergence of dengue and chikungunya in Africa and Asia (Tsetsarkin et al. 2007; Rochlin et al. 2013).

Females prefer to lay eggs just above the surface of the water in a container. Depending on available food, space and temperature, ATM larvae can complete their life cycle in 5-10 days. The adult female is the photosensitive stage; short day length induces females to lay diapause eggs whereas long day length leads to non-diapause eggs. Most ATM are domestic and peridomestic, living in urban and suburban areas in close proximity to humans. They are an aggressive daytime biter (Novak 1992). *Ae. albopictus* is an opportunistic blood feeder utilizing a variety of hosts like rabbits, rats, dogs, cows, humans, deer, sciurids, turtles, raccoons, passeriform birds, and cats (Niebylski et al. 1994). The ATM is a competent vector of at least 22 arboviruses (Gerhardt et al. 2001; Gratz 2004). Field and laboratory work have shown that *Ae. albopictus* can transmit

LACV horizontally and vertically (Gerhardt et al. 2001; Hughes et al. 2006), making it an important accessory vector.

1.3.3. *Aedes japonicus*

Aedes japonicus, the Asian bush or Asian rock pool mosquito, is another invasive species and container-dwelling mosquito originally from Asia (Kaufman and Fonseca 2014). In the mid-1990's, *Ae. japonicus* was found in New Zealand in used tires (Laird et al. 1994). Since then, the species has invaded the U.S. (first in New Jersey, 1998), Canada, and central Europe (Kampen and Werner 2014). It now found in all states east of the Mississippi River (except Florida and Louisiana) and Iowa, Missouri, Minnesota, Arkansas, Washington, Oregon, and Hawaii (Kaufman and Fonseca 2014). This wide distribution in the US is mainly due to land-based transportation. However, because the larvae can utilize natural rock pools and is tolerant to cold temperatures, *Ae. japonicus* can use river corridors as a means of dispersing. Another method of movement has been the Standardbred horse trade, with *Ae. japonicus* utilizing horse trailers as resting sites (Fonseca et al. 2001; Bevins 2007). *Ae. japonicus* is a multivoltine and its larvae can hatch out in cold temperature (4^{o}C water) (Andreadis et al. 2001). Some observations suggest that *Ae. japonicus* can overwinter as eggs, even larvae in cold water (Kampen and Werner 2014).

Because of its container-dwelling habit, *Ae. japonicus* can be found in both rural and urban areas. Female adults will feed on deer, horses, opossums, chipmunks, birds and humans. Vector competence studies have shown that *Ae. japonicus* can be a bridge vector for Japanese encephalitis virus, WNV, LACV, and SLEV (Takashima and Rosen 1989; Sardelis and Turell 2001; Sardelis et al. 2002; Sardelis et al. 2003; Harris et al. 2015).

Mosquito surveillance has consistently found LACV infected *Ae. japonicus* from Tennessee and Appalachian Mountain regions (Harris et al. 2015).

1.3.4. Interactions Among Vectors

Aedes albopoctus and *Ae. japonicus*, are sympatric with *Ae. triseriatus* in parts of the eastern U.S. and utilize the same breeding habitats. Several field studies have shown that these invasive species are outcompeting the native *Ae. triseriatus* in artificial containers and rock pools, but not in natural treeholes (Livdahl and Willey 1991; Joy and Sullivan 2005; Bevins 2007; Andreadis and Wolfe 2010; Kaufman et al. 2012). Although hypothesized that intraspecific competition, because of limited living space and food resources, could lead to displacement of the native species by exotic species in field, there is no strong evidence from lab to support this idea. Under laboratory conditions, larval interspecific competition studies showed that the exotic *Ae. japonicus* was inferior to native species, *Ae. triseriatus* (Alto 2011; Hardstone and Andreadis 2012). Although several characteristics, like pupation rate, development time, and survival rate, favors *Ae. albopictus* than *Ae. triseriatus* (Ho et al. 1989; Novak et al. 1993; Teng and Apperson 2000), in rural areas and forest, the native species has strong advantages (Lounibos et al. 2001). However, while *Ae. albopictus* may better adapted to urban and suburban areas, *Ae. triseriatus* and *Ae. japonicus* are dominant in rural and forest areas. There is probably another reason other than direct competition for native species decline in areas where invasive species have become established.

1.4. Vector Competence and Vectorial Capacity

To maintain an arbovirus in nature requires 3 components: infectious pathogens, competent vectors, and susceptible hosts. The relationship among those factors can be

evaluated by vectorial capacity and vector competence. Vectorical capacity quantifies how efficiently pathogens are transmitted by vectors. Vector competence, as a component of vectorial capacity, describes the capability of vectors to transmit pathogens (Garrett-Johnes and Shidrawi 1969; Hardy et al. 1983; Beerntsen and James 2000).

Vector competence is genetically controlled by midgut infection and escape barriers and salivary gland infection and escape barriers, which are time and dose dependent factors blocking the dissemination of a pathogen within the vector (Romoser et al. 2005). To be successfully transmitted through saliva during blood feeding, an arbovirus must infect, multiply, and disseminate to develop a systematic infection in every organ, especially salivary glands. When a mosquito takes an infectious blood meal with a sufficient titer, the meal enters vector posterior midgut and establishes an infection in the epithelial cells. Many physiological and genetic factors in arbovirus and vectors, even temperature and nutrition, can affect the midgut infection and escape barriers (Reeves 1965; McLintock 1978; Paulson and Hawley 1991). Viruses on the edge of blood mass start infecting epithelial cells on midgut (Chamberlain and Sudia 1961). Proteolytic enzymes, which are secreted to digest blood mass, have different effects on vector competence, for example increasing La Crosse virus, but decreasing dengue virus midgut infection rate (Ludwig et al. 1989; Ludwig et al. 1991; Molina-Cruz et al. 2005; Brackney et al. 2008). The peritrophic membrane in mosquitoes has only a limited function to block arbovirus from microvilli of midgut epithelial cells (McLintock 1978). The interaction between viral glycoproteins and cellular receptor proteins facilitate arbovirus to infect, multiply in, and escape the midgut (Ludwig et al. 1991; Ludwig et al. 1996; Hung et al. 2004; Kuno and Chang 2005). After escaping into the hemolymph,

virus is delivered to almost all tissues of the mosquito vectors including the salivary glands, the ultimate target for virus to be transmitted to animal hosts by feeding. As an organ to facilitate mosquito blood feeding and transmit the disease, salivary glands have six lobes, with three on each side (Ciano et al. 2014). Salivary infection and escape barriers have been reported that can influence the transmission of virus (Paulson et al. 1989; Romoser et al. 2005). After infect two organs and penetrate four barriers in a competent vector, the pathogen-vector-vertebrate host cycle can be completed.

In addition to horizontal transmission, vertical transmission is critical to maintain pathogen in nature during dry and cool seasons (Leake 1984; Lequime and Lambrechts 2014). Female mosquitoes can horizontally transmit to hosts and infected males can horizontally transmit virus to female mosquitoes sexually (Thompson and Beaty 1977). When infected with virus horizontally, a female mosquito will produce infected eggs starting with the second batch (Chandler et al. 1998; Romoser et al. 2011). Several arboviruses have a high rate of transovarial transmission, which also enhances its distribution in nature.

The vectorial capacity formula was originally devised to evaluate mosquito control s for malaria (Garrett-Jones 1964). However, vectorial capacity has also been applied on other pathogens, such as arboviruses (Anderson and Rico-Hesse 2006; Christofferson and Mores 2011). Initially proposed by Ross in 1911, this formula was modified by many scientists to reflect how specific pathogens and vectors can be transmitted to humans (Ross 1910; Macdonald 1957; Garrett-Jones and Shidrawi 1969). Vectorial capacity is calculated by the following formula:

$$VC = \frac{ma^2 p^N b}{-\ln(p)}$$

(Christofferson and Mores 2011)

In this formula, a is the man biting rate; m is the vector density; m is vector density per host; p is the daily survival of vectors after infected by pathogens; n is extrinsic incubation period (EIP) that a pathogen needs to infect a vector, disseminate to salivary gland, and be transmitted through a bite by vectors; b is the vector competence that the proportion of vectors with disseminated infection compare with total vectors exposed to pathogens; 1/(-ln p) is the duration of vector's life in days after the EIP (Kramer and Ebel 2003; Christofferson and Mores 2011). Perhaps the most powerful components in the formula are p^N and (a) the biting rate. Vector competence (b) and density (m) have a linear effect, and thus are weak contributors in the formula. Pathogens that can establish an efficient persistent infection with a shorter EIP, can cause more problems to public health (Anderson and Rico-Hesse 2006). In addition to the intrinsic factors listed, extrinsic factors, like temperature, larval density, nutrition, rainfall, host availability, and host immunity can indirectly affect the vectorial capacity (Kramer and Ebel 2003). For example, high temperature can increase biting frequency (a), shorten EIP (n), mortality rate (p) (Chamberlain and Sudia 1961; Dye 1992; Van Handel et al. 1994). Vectorial capacity is a robust tool to evaluate and understand the interaction between a pathogen and its vector.

1.5. Pathogen Manipulation of the Vector

Many different pathogens have been shown to manipulate vector behaviors; an alteration is believed to facilitate transmission (Hurd 2003; Lefèvre and Thomas 2008; Libersat et al. 2009). Pathogens can exert control on the vector directly on nervous system and indirectly on immune, endocrine, or metabolism systems. Infection can result in changes on several stereotype behaviors, like feeding, host-seeking, and mating,

resulting in an increase in vectorial capacity (Platt et al. 1997; Gabitzsch et al. 2006; Reese et al. 2009; Stafford et al. 2011; Ingwell et al. 2012; Jackson et al. 2012; Smallegange et al. 2013).

1.5.1. Alterations in Feeding Behavior

In a field study, 21% of *Anopheles gambiae* infected with *Plasmodium falciparum* took blood from two different hosts in one night, compared to only 10% of uninfected mosquitoes (Koella et al. 1998). Dengue-2 virus-infected *Ae. aegypti* show a 10-mintue longer blood feeding period than uninfected mosquitoes (Platt et al. 1997). This extended feeding time could easily be interrupted by host defensive behaviors, potentially leading to more host exposures. In 2012, Jackson *et al* found that LACV-infected *Ae. triseriatus* took significantly smaller blood meal and fed multiple times during a 24-hour period compared to uninfected siblings (Grimstad et al. 1980; Jackson et al. 2012). Probing rate in *Tomato spotted wilt virus* (TSWV) infected male *Frankliniella occidentalis* (thrips) is three folds higher uninfected thrips (Stafford et al. 2011) which increases the odds of vectors finding the right sites in plants. Changing the biting rate could dramatically increase vectorial capacity.

1.5.2. Alterations in Mating Behavior

Ae. triseriatus infected orally or transovarially with LACV showed a mating advantage comparing to uninfected mosquitoes. The insemination rate in the orally infected group is higher than uninfected mosquitoes after a 6-day mating period. Although the transovarially infected group did not demonstrate any advantage in insemination rate at the end of experiment, they showed an earlier insemination than uninfected mosquitoes. These alterations in mating strategy can enhance the transovarial

transmission and venereal transmission rates of the virus (Gabitzsch et al. 2006; Reese et al. 2009). Thus, the abundance of infected vectors would be increased.

1.5.3. Alterations in Host-seeking Behavior

Ingwell (2012) found that aphids (*Rhopalosiphum padi)* infected with barley yellow dwarf virus (BYDV) preferred non-infected wheat, while uninfected aphids fed more frequently from BYDV infected wheat (Ingwell, Eigenbrode et al. 2012). *Plasmodium falciparum*-infected *An. gambiae* show an increased host-seeking rate compared to uninfected mosquitoes (Smallegange et al. 2013). These modifications of vector host-seeking behavior would enhance virus transmission.

1.6. Mosquito Host-seeking and Blood Feeding Behavior

The host-seeking and blood feeding behaviors of mosquitoes are innate behaviors causing millions of death and enormous economic losses every year (McIver 1982; Bowen 1991; Potter 2014). These stimulus-response behaviors are complex procedures that depend on mosquito vision, host warmth, host odors, moisture, CO2 from hosts, and wind speed (Hocking 1971; Friend and Smith 1977; Allan et al. 1987; Bohbot et al. 2014).

New emerged female adult mosquitoes require 24 hours for the neurons innervating four types of sensilla on the maxillary palps (microtrichia, chaetica, capitate pegs, and campaniform) five types of sensilla on the antennae (chaetica, small/large coeloconica, ampullaceal, grooved pegs, trichodea) and sensilla on the proboscis to mature (Bohbot et al. 2007; Hill et al. 2009). Although the exact mechanisms of mosquito perception is not clear, a hungry adult female mosquito uses visual cues, senses the wind speed, warmth, and moisture by mechano-, thermos-, and hygrosensitive neurons, detects CO_2 and host

odorants through olfactory and gustatory receptor neurons (ORNs and GRNs) under mosquito sensilla (McIver 1982; Sutcliffe 1994). After collecting enough stimuli, the female flies and lands on the host. Their contact chemosensory on the labium and tarsi help mosquitoes verify the host odorants on skins before blood ingestion (Sparks et al. 2014). Following the host-seeking behavior, mosquitoes start blood feeding by insert their stylets into host's skin (Klowden 1995). After a full blood meal, there are two mechanisms to inhibit mosquito host seeking behavior in one gonotrophic cycle: one is stretch receptors on the abdomen, other one is a humoral factor produced during oogenesis (Davis 1984; Klowden 1988). Peripheral sensory neurons are unaffected by the stretch receptors but the humoral factor renders the mosquito the insensitive to lactic acid (Klowden et al. 1987). Nutritional status in larvae and adults affects the mosquito host-seeking behavior. Poor nutrition reared *Ae. aegypti* larvae result in a less likely in adults to take blood from host; however, sugar-deprived *Ae. aegypti* adults are more likely obtain blood from hosts (Klowden et al. 1988; Bowen 1991). Aging in mosquitoes generally lowers the threshold blood volume to trigger abdomen distension, extends the recovery time for host-seeking behavior after distension inhibition, and delays oocyte-induced inhibition (Bowen 1991).

1.6.1. Olfactory System and Sensilla

Olfaction plays a critical role in mosquito host-seeking behavior (Carey and Calson 2011). Sensilla on the antennae and maxillary palps bear three kinds of olfactory receptors: odorant receptors (ORs), gustatory receptors (GRs), and ionotropic receptors (IRs) that interact with environmental odorants (Potter 2014).

Three GRs on capitate pegs (CPs) sensilla are sensitive to CO_2 (Kent et al. 2008). There are at least 29 CPs on the fourth segment of the maxillary palp of *Ae. aegypti* (McIver and Charlton 1970). Each peg has numerous pores with an external orifice of about 150 Å diameter for *Ae. aegypti*, enlarging to spherical chamber region of about 1000 Å in diameter. There are 12 pores per square micrometers (μ^2) in the *Ae. aegypti* antenna (McIver 1972). Each CP is innervated with three chemosensory neurons (cpA, cpB, and cpC). In 1969, Kellogg found that thin-walled CPs in *Aedes* and *Culex* species respond to CO_2 (Kellogg 1970; McIver 1982; Grant et al. 1995). Based on morphology, three neurons are divided into two types: one with a lamellate dentrite and the other two with branching digitiform dendrites (Sutcliffe 1994). Two gustatory receptors (GR1&GR3), locating on cpA neuron, produce high action potential activity when exposed to CO_2 (Robertson and Kent 2009; Erdelyan et al. 2012). The chemoreceptors cpB and cpC sensitive to human odorants (Bohbot et al. 2014). Although both sexes express cpA to detect vertebrate or plant hosts, the expression of GR1&GR3 is sex-biased. *Ae. aegypti* males, which prefer to swarm near vertebrate hosts to find a mate, express more GR1 than female mosquitoes (Erdelyan et al. 2012).

Odorant receptors (ORs) are mainly responsive to host odorants. Bioinformatics analysis identifies about 100 ORs genes in *An. gamibae*, *Ae. aegypti*, and *Cx. pipiens quinquefasciatus* (Hill et al. 2002; Bohbot et al. 2007; Arensburger et al. 2010; Carey et al. 2010; Pelletier et al. 2010). Most of ORs are expressed on mosquito proboscis and antenna: sensilla grooved pegs and trichodea (McIver 1982; Bohbot et al. 2007). Sensilla grooved pegs are short, thick-walled, and nonarticulated thorn-shaped hairs and can be found on almost every flagellar. A single pore occurs at the tip of the grooved peg with 3,

4 or 5 morphologically similar neurons. Based on length, two types of grooved pegs are found on adult mosquitoes (Bowen 1995). Lactic acid, a component of human sweat, can excite or inhibit the neurons under grooved pegs. No or very weak response has been found in GP to repellents (Liu et al. 2013). Sensilla trichodea is most abundant on *Ae. aegypti* antennae (McIver 1978). Four types of sensilla trichodea are found: long sharp trichoid (LST), short sharp trichoid (SST), short blunt trichoid I (SBT-I), and short blunt trichoid II (SBT-II) (McIver 1978). In *Ae. aegypti*, all four types of sensilla trichodea have two neurons: A and B (Ghaninia et al. 2007). All types of sensilla trichodea have numerous pores; LST and SST have relatively thicker wall and unbranched dendrites to the tip of the hair; SBT-I&II have thin wall and branched dendrites (McIver 1978). LST has been reported to respond to human odorants; SBT-I is excited by longer chain fatty acids, but inhibited by shorter chain fatty acids; SBT-II has been found to respond odorants from oviposition sites; SST has been reported to be excited by sweat and oviposition odorants (McIver 1978; Ghaninia et al. 2007).

Ionotropic receptors (IRs) are the most recently identified receptors that are ionotropic glutamate receptor (iGluR) related genes (Benton et al. 2009; Rytz et al. 2013). On the distal end of the antenna (13rd flagellar segment) of anopheline and culicine mosquitoes, there are 2 or 3 coeloconica which were misidentified as campaniform organs (McIver 1973). With a scalloped wall, sensilla coeloconica appear as a peg in a pit. Three neurons (A, B, and C) are found under sensilla coeloconica in *Ae. aegypti* and *Cx. pipiens*. Neuron A and B have unbranched dendrites; neuron C has lamellae-shaped dendrite (McIver 1973). IRs have a lot of information deserve scientist to explore (McBride 2016).

Although male mosquitoes do not feed on vertebrate blood, they swarm around vertebrate hosts as a method of to find a female mosquito (McIver and Siemicki 1979; Erdelyan et al. 2012). In 1979, McIver found that male *Ae. aegypti* has same types of sensilla and similar ratio of sensory receptors, but four times fewer receptors than female *Ae. aegypti* (McIver and Siemicki 1979). The same type of sensilla has a different function (Davis 1977). Since male mosquitoes have fewer sensilla, it may be useful to compare the less complicated male s olfactory system with that of the female mosquitoes to identify the essential odor coding requirements to find a host.

1.6.2. Olfactory Receptors and Nervous System

Modern molecular techniques enable scientists to study the decision-making aspect of insect innate behaviors (Robinson et al. 2011; Alivisatos et al. 2013; Ito et al. 2014). Due to rapid progress in sensory neuroscience, scientists have demonstrated the mechanism of host-seeking behavior much more clearly than before (Matthews et al. 2016).

Through genome sequencing of *An. gambiae*, *Ae. aegypti*, and *Cx. pipiens quinquefasciatus*,79, 131, and 180 OR genes have been identified, respectively (Arensburger et al. 2010). Unlike vertebrate animals, which have metabotropic signaling, insects have ionotropic signaling (Kaupp 2010). Two GRs genes (GR21 and GR63) form heterodimers that are sensitive to CO_2 (Kaupp 2010). Similar heterodimer structures have been found in ORs, which require conserved co-receptors (Larsson et al. 2004). GRs and ORs are seven-transmembrane domain proteins, that lack a sequence similar to known G protein-coupled receptors (GPCRs) and are topologically reversed compared to canonical GPCRs: an intracellular N terminus and an extracellular C terminus (Bento et al. 2006;

Yao and Carlson 2010). G-proteins ($G\alpha_q$ and $G\gamma30A$) play a role in CO_2-sensing neurons to transduce signals to brains. Although ORs have ligand-gated ion channels and cyclic-nucleotide-gated channels, it is not clear whether GPCRs contribute to insects signal transduction or not (Sato et al. 2008; Wicher et al. 2008; Kaupp 2010; Yao and Carlson 2010). *Drosophila* has been used as a model to study IRs (Benton et al. 2009; Rytz et al. 2013). Compared to ORs and GRs, IRs have distinctive gene sequences, an extracellular N-terminus, a short cytoplasmic C-terminal region, and a bipartite ligand-binding domain (LBD) (Rytz et al. 2013). Up to five IRs genes can form a multimeric unit on one ORN to detect environmental odorants (Rytz et al. 2013). IRs strongly respond to amines and acids, while ORs respond to ester and alcohols (Silbering et al. 2011). The diversification between ORs and IRs show that they evolved as two separate olfactory systems.

The nervous system of mosquitoes composes of three parts: central nervous system (brain and ventral nerve cord) stomodaeal nervous system innervating the internal organs, and the peripheral nervous system composed of motor and sensory neurons. Generally, the brain can be divided into antennal and optic lobes, mushroom bodies, and central bodies (corpora pedunculata) (Howse 1975; Ito et al. 2014). Like mammals, insect ORNs that express the same ORs project their axons to a single glomerulus in antennal lobe (AL) of the brain (Fishilevich, and Leslie 2005). Electrical signals from chemosensory receptors are sent to antennal lobes through axons. Projection neurons next to antennal lobes receive those signals through synapses, sending other signals to two higher organs: mushroom body (MB) and lateral horn (LH), which are related with olfactory learning and memory and innate olfactory behaviors (Carey and Carlson 2011).

1.6.3. Chemosensory Synapses and Olfactory Neurotransmitters

Chemosensory synapses that convert environmental stimuli through the peripheral nervous system by sending signals to central nervous system are similar to neuronal synapses, which is the physical structure to process information between neurons and neurons (Shaham 2010). Presynaptical and postsynaptical similarities between chemosensory synapses and neuronal synapses make it easier for us to understand the olfactory perception in the insect brain by comparing these two synapses. Upon receiving environmental stimulus, bipolar ORNs initial the action potential, which is generated by voltage-gated ion channels that pump 3 Na^+ out and 2 K^+ inside cells (Bean 2007). Based on structure and function, ORNs are divided into reception, integration, conduction, and transmission: dendrites and cell body are used to convert environmental stimulus; the axon hillock and first segment of myelination are the region to initiate the action potential; the rest of axon is used to conduct the electrical signal; the end of ORNs are the transmission parts to release chemical or electrical signals (Bender and Trussell 2012). Membrane potential has been found in almost every cell. Normally, cells stay in a stable state called resting potential: inside of cell exits a negative voltage, comparing to positive voltage outside the voltage (Hille 2001). Once triggered to threshold from rest potential in a typical action potential, Na^+ gated channel opens at first causing depolarization, in which Na^+, outside of cells, moved in. Until membrane potential jumps from almost -70 mV to +40 mV, repolarization starts by closing Na^+ gated channel and opening K^+ gated channel. After dropping below resting level, membrane potential remains refractory for a while, then jump back to -70 mV. Ca^{2+} gated ion channel opens for chemical synapses.

Chemical and electrical synapses are two main wiring transmissions between neurons (Südhof and Malenka 2008; Pereda 2014). Since there is a few report about electrical synapses and olfactory, we just talk about chemical synapses in here. Several neurotransmitters, like Acetylcholine (ACH), γ-Aminobutyric acid (GABA), biogenic amines, neuropeptides, and nitric oxide, have been found in antennal lobe which is the first integration center for odorants in insects (Schachtner et al. 2005). Odorant activated sensory neurons trigger biogenic amines immunoreactive neurons to release these two neuromodulators, which affect a group of synapses around releasing sits (Ellen and Mercer, 2012; Kaissling 1986; Wang 2012; Hoyle 1985). Serotonin and dopamine are two well studied neurotransmitters, neuromodulators, and neurohormones that are related with not only circadian rhythms but also olfactory learning and sensitivity in insects (Dacks et al. 2006; Suh et al. 2004). Previous research demonstrated that the oscillating of serotonin and dopamine in insects can change the neuronal circuits and modulate the threshold of odor evoked behavior (Dacks et al. 2009; Ellen and Mercer 2012). In mosquitoes, blood feeding and host-seeking behaviors have been related with serotonin and dopamine (Novak and Rowley 1994; Fukumitsu et al. 2012).

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Chapter 2. Discovery of Cache Valley virus in *Aedes japonicus japonicus* mosquitoes in the Appalachian Region, United States

Authors: Fan Yang, Kevin Chan, Paul E. Marek, Philip M. Armstrong, Pengcheng Liu, Jacob E. Bova, Joshua N. Bernick, Benjamin E. McMillan, Benjamin G. Weidlich, Sally L. Paulson

Affiliations: Virginia Tech, Blacksburg, Virginia, USA (F. Yang, K. Chan, P.E. Marek, P. Liu, J.E. Bova, J.N. Bernick, B.E. McMillan, B.G. Weidlich, S.L. Paulson); The Connecticut Agricultural Experiment Station, New Haven, Connecticut, USA (P.M. Armstrong)

2.1 Abstract: Cache Valley virus (CVV) was found in an Appalachian forest from *Aedes japonicus japonicus*, a widely distributed invasive mosquito species. The forest includes abundant white-tailed deer, an important host of the mosquito and virus. Vector competence trials indicated that *Ae. j. japonicus* can transmit CVV in this region.

2.2 Introduction

Cache Valley virus (CVV; family *Bunyaviridae.* genus *Orthobunyavirus*) is widespread throughout North and Central America and infects many species of domestic ungulates (sheep and cattle), but white-tailed deer are a likely reservoir (*1*). Although it has been isolated from more than 30 mosquito species in several genera, the principal mosquito vectors remain unknown (*2*). However, based on empirical field isolations and laboratory transmission studies, *Anopheles quadrimaculatus* and *Anopheles punctipennis* mosquitoes probably play important roles in its transmission cycle (*1*).

CVV infection is common in sheep, triggering spontaneous abortion, stillbirth and congenital defects (*3*). The virus is neuro-invasive in humans and there have been 3 confirmed cases, with a single mortality in the U.S (*4*). Medical laboratories rarely test for CVV, belying its true incidence and impact on human health, but serological studies have reported high infection rates (≤18%) in endemic areas (*5*). Here we report the discovery of CVV from the invasive mosquito, *Aedes japonicus japonicus*, from Blacksburg, Virginia, USA. This study shows the first occurrence of CVV in Appalachia and demonstrates that *Ae. j. japonicus* is a competent vector of the virus.

2.3 The Study

Adult mosquitoes were collected 3 or 4 times/week from 1 Jun to 21 Aug 2015 using 8 gravid traps in a forested area (196,115 m^2) (Figure 1.). In total, 1197 *Ae. triseriatus* and 690 *Ae. j. japonicus* adult females were collected and identified to species based on morphology (Table 1). Mosquitoes were pooled according to species, trap number, and dates. Pools consisted of 1-50 mosquitoes, and all specimens were stored at -80^{o}C until assay. Samples were screened on Vero cells for cytopathic effect (CPE) and presence of CVV virus was confirmed in CPE positive samples by plaque assay according to Barker et al. 2003 (*6*).

Virus isolates were amplified on Vero cells to 10^5 PFU/mL. Viral RNA was extracted from infected cell supernatants using QIAamp Viral RNA Mini Kit (Qiagen, Valencia, CA) (*7*). Reverse transcription polymerase chain reaction (RT-PCR) using Bunyaviridae-specific universal primers, BCS82C and BCS332V, was used to produce a 251 bp amplicon of the small (S) segment that was sequenced (*8*). A BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) query was then performed indicating the isolates

were Cache Valley virus. The large (L) segments were amplified by RT-PCR using the CVV-specific primers CVV_L (5'-AGTCAGCCAAAACAGCCACT-3') and CVV_R (5'-TACAAATCTAGGGGGCATGG-3') and the medium (M) segments were amplified using the primers M14C and M4510R (*8, 9*). The resulting amplicons were sequenced and identified as CVV by performing a BLAST query. The M segment encoding the Gc protein were amplified using the primer pair CVV_M_L (5'-CTGTCACGGTGCTAGTAGGAAAGATGTG-3') and CVV_M_R (5'-AGTAGTGTGCTACCGGTATCAAAAACAGC-3') and sequenced.

Cache Valley virus was detected in two pools of *Ae. j. japonicus* collected from different traps on 7 Aug (Table 1). For the week of Aug. 4-11, the CVV minimum infection rate was calculated to be 11.5/1,000 mosquitoes (173 mosquitoes were tested individually).

We used the M segment to infer phylogeny with CVV isolates from Armstrong et al. (2015) (*9*). Sequences were translated to amino acid and aligned by eye, which was trivial due to invariant length and lack of indel events. The alignment comprised 1,803 bp from 100 isolates. Sequences were partitioned by codon position, and alternative models of nucleotide site substitution evaluated. Phylogenetic trees were inferred using a Markov chain Monte Carlo method in MrBayes 3.2.5 with a simultaneous estimation of topology, branch lengths, and other parameters (*10*). Stabilization of four concurrent chains occurred at 1 million generations, and the first 250,000 trees discarded as a burn-in. Branch lengths and other parameters were averaged and a consensus tree calculated from the posterior distribution. The tree is shown in Figure 2 with support values for each clade in posterior probabilities. Cache Valley virus isolates grouped into two clades, or

lineages (Figure 2) (*9*). Lineage 1 consisted of viruses from U.S. and Canada from 1952-2011, whereas lineage 2 included more recent strains from the northeastern U.S. Virus isolates from Virginia were genetically similar to each other (3 bp different) and occurred in the newly emergent lineage 2 of CVV.

To determine the vector competence of local mosquitoes for CVV, a laboratory strain was established from uninfected *Ae. j. japonicus*. Week-old female mosquitoes were offered an infectious blood meal (IBM) in a membrane feeder. The IBM contained 1 mL of the CVV-4B isolate in 9 ml sheep blood (Colorado Serum Company, Denver, CO.). After 1-hour of feeding, engorged mosquitoes were transferred to 0.7 L cages and held for 14 days in an insectary (25^{o}C, 75% relative humidity, and 16:8 (L:D) photoperiod) and provided 10% sucrose. A 1 mL sample of the IBM was saved for virus titer by plaque assay on Vero cells. Rates of non-disseminated and disseminated infection, and oral transmission were measured using the methods of Harris et al. (2015) (*7*). The experiment was replicated 3-fold. The IBM titers ranged from 1.6 x 10^5 to 4.6 x 10^6 PFU/mL (Table 2). *Aedes j. japonicus* females were susceptible to oral infection with CVV and capable of transmitting the virus (Table 2). There were no significant differences among the 3 replicates for infection or transmission rates (χ^2, $P > 0.05$).

2.4 Conclusions

Aedes j. japonicus is an invasive species that has spread throughout most of the eastern US and is a competent vector of several endemic viruses (*11*). Although CVV was previously isolated from *Ae. j. japonicus* in the Northeast (*12, 13*), we report the first isolation of CVV from this species in the Mid-Atlantic, and show that it is a competent vector of the virus. In the laboratory, vector competence of *Ae. j. japonicus* was

equivalent to other species believed to be part of the CVV transmission cycle. For example, transmission rates for *An. quadrimaculatus* ranged from 20-33% after imbibing infectious blood meals with virus titers similar to those used in our study (*1*).

Aedes j. japonicus readily feeds on humans and large animals such as white-tailed deer (*11*). Consequently, it is probable that this species may help contribute to local transmission of CVV. The site from which the infected mosquitoes were collected is a small woodlot in close proximity to humans and pastured sheep, and is frequented by deer (Fig. 1). Therefore all of the components for the establishment of a focus of CVV are present. Moreover, if *Ae. j. japonicus* is capable of transovarial transmission, as is the case with La Crosse virus (LACV), another member of Bunyaviridae, (*14*), then it could also contribute to concentrating the virus within this limited geographical area. It has been suggested that the emergence of LACV in the Appalachian region of the US may be positively correlated with the invasion of *Ae. j. japonicus* and *Ae. albopictus* in the area (*15*). Further studies should be conducted to determine the role of *Ae. j. japonicus* in the transmission, maintenance and occurrence of CVV.

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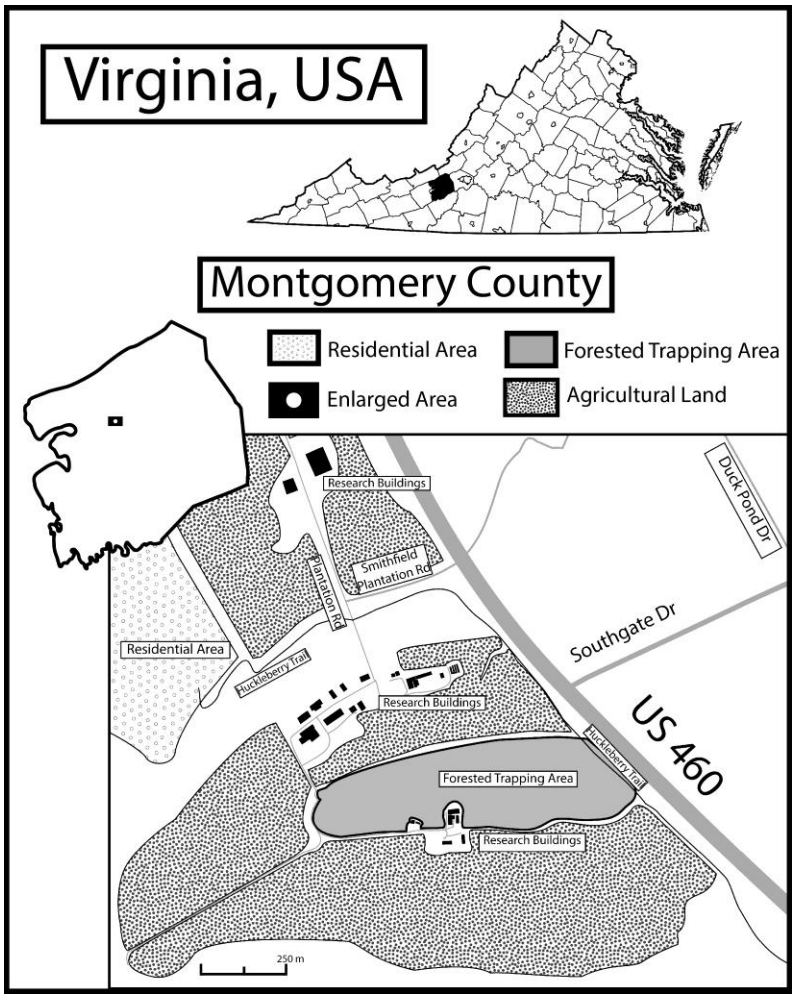

Figure 1. Map of study site.

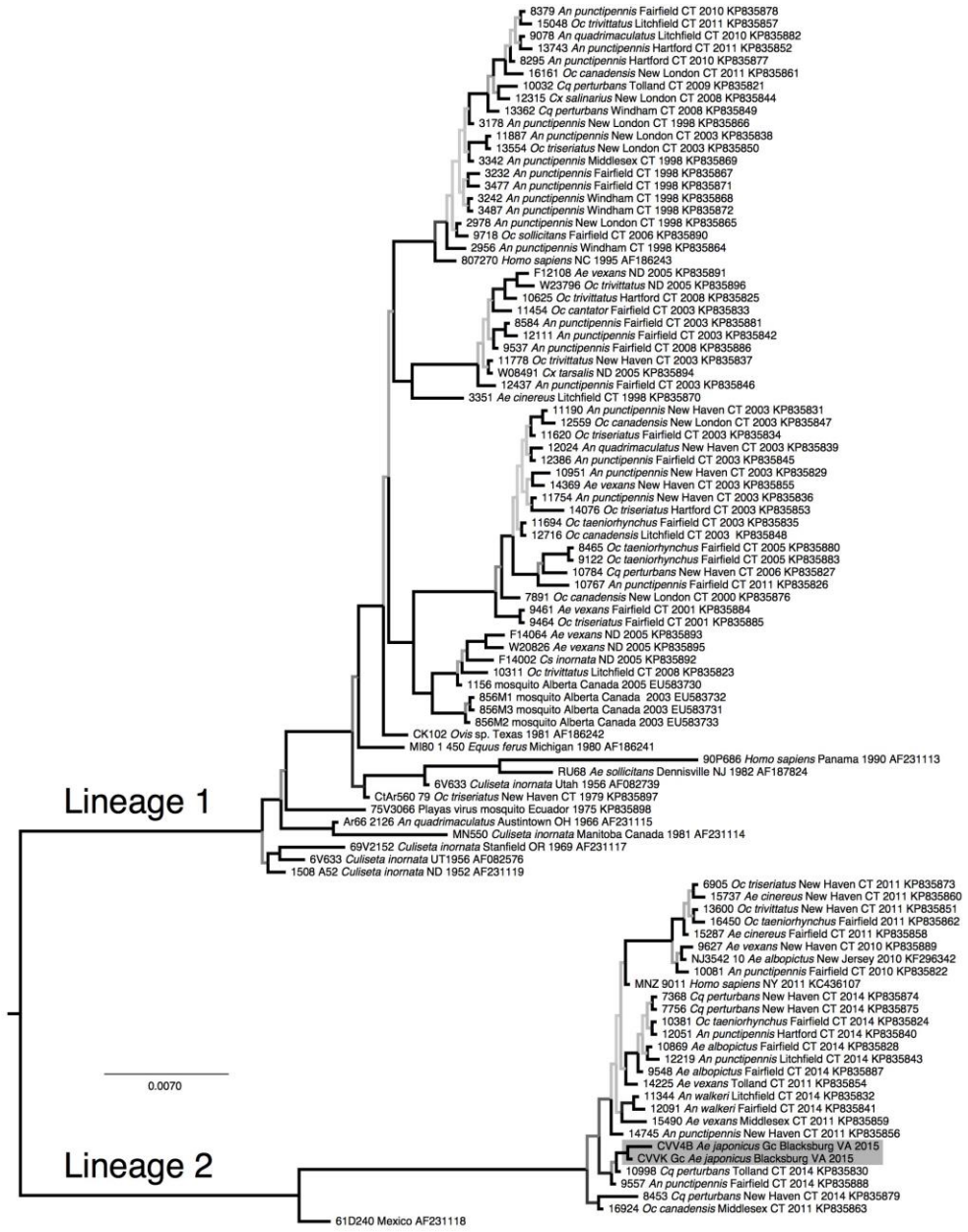

Figure 2. Phylogeny of Cache Valley virus (CVV) inferred based on the medium (M) segment of the viral polyprotein gene. Tree was estimated using a mixed model partitioned Bayesian analysis. State, year, host, and the National Center for Biotechnology Information (NCBI) accession numbers are listed for each isolate on the tree. The scale bar represents the expected nucleotide substitutions per site. Historical lineages of CVV are labelled 1 and 2. The 2015 isolates from Virginia group with lineage 2 viruses (NCBI accession nos. KX583998, KX583999). *Ae*., *Aedes*; *An*. *Anopheles*; *Cq*., *Coquillettidia*; *Cx*., *Culex*; *Oc*., *Ochlerotatus*. Tree rooted with the closely related Fort Sherman virus from Panama (NCBI AF234767), omitted from figure.

Table 1. Screening of *Aedes triseriatus* and *Aedes japonicus japonicus* adults from gravid traps for arthropod-borne virus using plaque assays

Collection dates	*Aedes triseriatus*		*Aedes japonicus japonicus*		Positive samples
	No. individuals	No. pools*	No. individuals	No. pools*	
6/1-6/30	569	14	196	26	0
7/1-7/31	383	367	257	240	0
8/1-8/19	245	245	237	176	2**
Total	1197	626	690	442	2**

*After 7/21, most of mosquitoes were tested individually rather than in pools.

**Two Cache Valley virus-infected adults *Aedes japonicus japonicus* were collected and tested individually on Aug 4th.

Table 2. Rates of midgut infection, dissemination and oral transmission of CVV by *Aedes japonicus japonicus* after a 2-week extrinsic incubation

Replicate	IBM* titer	No. tested	Non-disseminated infections (%) **	Disseminated infections (%)**	Transmission (%)**
1	1.2×10^6	18	44	39	33
2	1.6×10^5	26	42	42	27
3	4.6×10^6	30	37	33	27
Total		74	41	38	28

*Infectious blood meal

** Virus recovered from the midgut only was classified as a non-disseminated infection. Mosquitoes with virus-positive legs and wings were considered to have a disseminated infection. If virus was detected in salivary expectorate, it was classified as transmitting.

Chapter 3. Effects of La Crosse virus infection on the host-seeking behavior of and levels of two neurotransmitters in *Aedes triseriatus*

3.1 Abstract

Background: La Crosse virus (LACV) infection has been shown to manipulate the blood-feeding behaviors of its main vector, *Aedes triseriatus*, in ways that would favor its transmission. In investigations of the role of neurotransmitters, serotonin depletion has been shown to interfere with blood-feeding but not host-seeking of mosquitoes while dopamine depletion does not affect either blood-feeding or host-seeking. However, elevation of dopamine can inhibit host-seeking. The purpose of this study was to determine the effect of LACV infection on the host-seeking behavior of and neurotransmitter levels in *Ae. triseriatus*.

Methodology/Principal Findings: Host-seeking behavior was evaluated using a uni-port olfactormeter and a human hand and breath as bait. LACV infection did not affect the host-seeking behavior of *Ae. triseriatus* females. Levels of serotonin and dopamine in infected and control mosquito heads were measured using HPLC-ED. LACV-infected mosquitoes had lower serotonin levels than controls while the dopamine levels were not affected by infection status.

Conclusions: Our work suggests that virus-induced reduction of serotonin is related to the previously reported blood-feeding alterations in LACV-infected mosquitoes and could lead to enhanced transmission and increased vectorial capacity. Neither dopamine levels nor host-seeking behavior was affected by LACV infection.

3.2 Introduction

La Crosse encephalitis (Family *Bunyaviridae*, California serogroup, LACV) is the most important cause of arboviral neuroinvasive disease in the U.S.A. (Ludlow et al. 2016). The principal vector of LACV is *Aedes triseriatus* (Say). The virus is zoonotic, maintained in nature through horizontal transmission to small woodland mammals such as chipmunks and squirrels that act as amplifying hosts (Grimstad 1988).

Pathogen induced alterations of the blood-feeding behavior of insects resulting in enhanced transmission have been described for numerous parasite-vector systems (Schaub 2006). For example, *Ae. aegypti* infected with dengue virus displayed extended periods of probing compared to uninfected ones (Platt et al. 1997). Previous studies in our lab demonstrated that horizontal infection by LACV affected the blood feeding of *Ae. triseriatus* and *Ae. albopictus* mosquitoes (Jackson et al. 2012). Both species took smaller blood meals compared to uninfected siblings, and twice as many virus-infected *Ae. triseriatus* females feeding multiple times in a 24-hour period compared with controls (Jackson et al. 2012). This virus-induced feeding alteration likely results in multiple host contacts within one gonotrophic cycle, thereby increasing transmission of the LACV by its natural vector, *Ae. triseriatus*. However, little is known about the effects of virus infection on mosquito host-seeking behavior. Qualls et al. (2012) found that *Ae. aegypti* with disseminated infections took nearly 3 times longer to locate a bloodmeal than those of uninfected controls.

Many viruses have been shown to be neurotropic in the mosquito vector (Paulson et al. 1989, Salazar et al. 2007) and several neurotransmitters have been found to play a role in controlling mosquito host seeking, biting and feeding behaviors. For example,

elevation of dopamine levels can inhibit the host seeking behavior of *Ae. albopictus* (Fukumitsu et al. 2012) while depletion of serotonin inhibited feeding by *Ae. triseriatus* (Novak & Rowley 1994). Therefore, it is reasonable to hypothesize that virus-induced modulation of neurochemical levels may be a mechanism for altering blood feeding and/or host-seeking behaviors of infected mosquitoes.

The purpose of this study was to determine the effect of LACV infection on the host-seeking behavior of and neurotransmitter levels in *Ae. triseriatus*.

3.3 Materials and Methods

3.3.1 Virus Isolates and Assays

The VA0921075 isolate used in this study originated from adult *Ae. triseriatus* mosquitoes collected in 1999 in Wise County, VA (Barker et al. 2003). Prior to the study, the isolate was first amplified in adult female *Ae. triseriatus* and then Vero cells. The titer of the stock virus was 2.05×10^8 plaque forming units (PFU) /ml. Virus titers of the stock virus and individual infected mosquitoes were determined by plaque assay following the methods of Barker et al. (2003).

3.3.2 Mosquitoes

Eggs of *Ae. triseriatus* were collected from Blacksburg, VA in 2015. The eggs were hatched and held in an insectary maintained at 27.5 ± 1ºC, 75% relative humidity, and a 16:8 h L:D cycle. Mosquitoes were reared according to the methods of Jackson et al. (2012), a method that ensures uniformity in adult size.

3.3.3 Infection of Mosquitoes

Three to five day-old adult female mosquitoes were injected intrathoracically with 0.5 µl of LACV (2.05×10^8 PFU) or M199 medium for infected and control groups,

respectively (Rosen and Gubler 1974). After injection, mosquitoes were held under standard laboratory conditions for a 7-day extrinsic incubation period. During this time, they were provided with 10% sucrose ad libitum. To determine if virus titer varied in responding and non-responding mosquitoes, 10 mosquitoes from each category were assayed individually from 3 different trials.

3.3.4 Host-seeking Behavior

Host-seeking was measured using a uni-port olfactometer modified from a design by Cabrini and Andrade (2007). The device consisted of a 30 cm^3 holding cage, a 1 m x 20 cm polystyrene tube, and a 100 x 50 x 50 cm testing chamber. The testing chamber was divided by a mesh partition into two parts: the mosquito trap and the host compartment (Fig. 3.1). A 12V computer fan provided airflow from the attractant to the holding cage. Mosquitoes were aspirated into the holding cage and given a 30-minute acclimatization period. Assays were run between 9 to 11 AM because this is the peak time for feeding by *Ae. triseriatus* (Clark et al. 1985). A human arm and breath introduced via a latex tube were the attractants (DeGennaro et al. 2013). The same host was used for each assay. Mosquito response were determined after a 10-minute test period. Mosquitoes that left the holding chamber, travelled the length of the 1-m tube to enter the collection chamber at the end of the olfactometer tube were considered responders, those that left the holding chamber but did not enter the collection chamber were partial responders, and those that did not exit the holding chamber were non-responders. The experiment was replicated 10 times for both infected and uninfected control mosquitoes.

3.3.5 Measurement of Neurotransmitters

Two-week old female mosquitoes from control and infected groups were frozen on dry ice for 10 min. Heads from control or infected groups were dissected at 9 – 11am, the same time period as the host-seeking assays, and placed in groups of 5 for high performance liquid chromatography with electrochemical detection (HPLC-ED) measurement (Hardie and Hirsh 2006). All samples were stored at -70^{o}C immediately after collection. The heads were homogenized in 0.2 ml mobile phase pH 4.7 (sodium acetate 50 mM, citric acid 12.5 mM, EDTA 134 mM, octanesulfonic acid 230 mM, sodium chloride 2 mM, pH 4.7 and 12% methanol) by sonicator for 10 min on ice. Supernatant was collected by centrifuging homogenate for 15 min at 13,000 rpm at 4^{o}C and transferred into a new micro centrifuge tube for immediate analysis. The HPLC-ED system included the Agilent Technologies 1100 Series and an electrochemical detector (Waters 2465). Separation of electroactive species was achieved by a reverse-phase column (250 x 4.0, C18, with particle size 3µM) with a flow rate of 0.5 ml/min. The working electrode was 0.8V for serotonin and 0.6V for dopamine versus an Ag/AgCl working electrode.

3.3.6 Statistics

Individual host-seeking behavior trials were analyzed by two-tailed Fisher's exact tests and the overall host-seeking response was analyzed by the Wilcoxon matched-pairs signed rank test. The whole body virus titers of responding and non-responding mosquitoes were compared by a two-tailed unpaired t test. The levels of serotonin and dopamine in the heads of infected and uninfected mosquitoes were evaluated by two-tailed ratio paired t tests. All statistical analyses were done using Prism 7 for Mac OSX (GraphPad Software, Inc., 2016).

3.4 Results

3.4.1 Effect of Virus Infection on Host-seeking Behavior

LACV infection did not exert a consistent effect on the host-seeking behavior of female *Ae. triseriatus*. Out of 10 separate trials, infected mosquitoes showed a significantly lower response only twice (Table 3.1). When the overall host-seeking response was evaluated by comparing the percentage of responders in infected (52%) and control (60%) groups, there was no significant difference ($P > 0.05$) (Fig. 3.2). Most mosquitoes that exited the holding compartment moved down the tube all the way to the mosquito trap portion of the olfactometer regardless of infection status, with 2.4% of the infected and 1.4% of the controls showing a partial response. The exception was Trial 4 where 36% of the infected and 12% of the control mosquitoes were partial responders. As care was taken to ensure that all the conditions were identical for each replicate trial, it is unknown why there was an anomalous result. The level of virus titer in both responder and non-responder groups were equivalent (5.4 vs. 5.5 log10 PFU/ mosquito) ($t = 0.6042$; $df = 28$; $P > 0.05$) (Fig. 3.3).

3.4.2 Effect of Virus Infection on Serotonin and Dopamine Levels in Mosquito Heads

The mean level of serotonin in infected female *Ae. triseriatus* heads was significantly lower than in the control individuals (104.5 vs. 138.3 pg/head) ($t = 8.555$; $df = 2$; $P < 0.05$) (Fig. 3.4). However, levels of dopamine were not significantly different between infected and uninfected females ($t = 3.272$; $df = 2$; $P>0.05$) (Fig. 3.4).

3.5 Discussion

This study showed that LACV infection did not affect the host-seeking behavior of *Ae. triseriatus* females. Hamilton and Hurd (2002) describe a 4-step model of blood-feeding behavior: (i) the appetitive search; (ii) activation and orientation; (iii) attraction; and (iv) landing and probing. Using an olfactometer, we were primarily measuring activation, orientation and attraction. A combination of olfactory cues such as odor and CO_2, and physical stimuli such as heat and color are important in the activation and attraction of mosquitoes to hosts (Clements 1963, Takken 1991, Gibson and Torr 1999, Zwiebel and Takken 2004). There are hundreds of volatile compounds released in human breath and skin odor (Gallagher et al. 2008) but CO_2 has been shown to act as a behavioral stimulator (McMenimen et al. 2014).

Many different variables can influence host-seeking behavior, so care was taken to carefully control for as many as possible. Because newly emerged females are not host responsive while older mosquitoes show increased host-seeking (Bowen 1991, Grant and O'Connell 2007) all trials were done on mosquitoes of the same age (10-13 days) to control for changes in host-seeking activity with age. Individuals vary in a heritable way in their attractiveness to mosquitoes (Fernández-Grandon et al. 2015, Qiu et al. 2006) so the same host was used in all trials. All trials were run at the same time of day to control for endogenous host-seeking rhythms (Rund et al. 2013). As even body size can influence host-seeking (Farjana and Tuno 2013), the mosquitoes used in this study were reared using a method shown to produce *Ae. triseriatus* females of uniform size (Jackson et al. 2012). Even with these controls, no consistent effect of virus infection on the host-seeking behavior was seen.

A variety of pathogens have been shown to manipulate probing, engorgement and

other feeding behaviors of mosquitoes to enhance transmission, behaviors that occur in close proximity to the host. However, there have been few studies examined the effect of infection on the earlier steps of blood-feeding that occur at a distance, such as the initiation of host-seeking and location of a host (Cator et al. 2013, Hamilton and Hurd 2002). For example, *Plasmodium gallinaceum*-infected *Ae. aegypti* were significantly more attracted to guinea pig odors compared to uninfected ones (Rossignol et al. 1986) and *An. gambiae* infected with *P. falciparum* showed an increased attraction to human odors (Smallegange et al. 2013). In a study of *An. stephensi* infected with *P. yoelii*, the changes in attraction to a host were linked to changes in the responsiveness of the vector odorant receptors suggesting a possible neurophysiological mechanism (Cator et al. 2013). Our study is the first examination of virus effect on mosquito activation and host location. Qualls et al. (2012) reported a significant increase in the activation times of *Ae. aegypti* infected with Sindbis virus but this work was done in a small cage (20 cm^3) with a membrane feeder as an attractant rather than a living host. We tested host attraction over a distance of 1 m using a host frequently fed upon by *Ae. triseriatus* in nature.

The mechanism by which a pathogen enhances its transmission by a mosquito is unclear. Insect behavior is mainly driven by rewards and punishments, which are organized by a network of interacting circuits of several biogenic aminergic neurons (Perry and Barron 2013). Biogenic amines can act as neurotransmitters, neuromodulators or neurohormones in insects. The amines serotonin (5-HT) and dopamine act to control and regulate physiological functions such as circadian rhythms, endocrine secretion, cardiovascular control and even learning and memory (Blenau and Baumann 2001). Several studies have indicated a role of serotonin in controlling blood-feeding by

mosquitoes. The salivary glands of female *Ae. aegypti* demonstrate 5-HT-immunoreactive innervation, which is absent in male salivary glands (Novak et al. 1995). Also, when treated with *a*-methyl-tryptophan (AMTP, a chemical that depletes serotonin when injected into a mosquito), females secreted less saliva and that saliva contained less apyrase than control mosquitoes (Novak et al. 1995). Apyrase is an enzyme that inhibits ADP-dependent platelet aggregation, thus facilitating blood intake (Reno and Novak 2005). The AMTP treated mosquitoes probed longer and showed a lower blood-feeding success. In a similar study using *Ae. triseriatus*, AMTP treatment resulted in significantly reduced blood-feeding success but the host-seeking ability was not altered (Novak and Rowley 1994). Dopamine does not seem to be involved in controlling blood-feeding but rather with host-seeking. Injecting *a*-methyl-tyrosine (AMT), which causes dopamine reduction but does not affect serotonin, into *Ae. triseriatus* did not affect blood-feeding or host-seeking (Novak and Rowley 1994). However, elevation of dopamine levels reduced host-seeking activity in *Ae. albopictus* (Fukumitsu et al. 2012).

In this study, we found that LACV-infected mosquitoes had lower serotonin levels than controls while the dopamine levels were not affected by infection status. These data may explain why LACV infection did not affect host-seeking activity. However, a previous study by Jackson et al. (2012) showed that LACV-infected mosquitoes took smaller blood meals and fed more frequently than uninfected females. Perhaps the virus-induced reduction of serotonin is related to the observed blood-feeding alteration and could lead to enhanced transmission and increased vectorial capacity of infected mosquitoes. However, the enhancement of virus transmission through changes in blood-feeding ability could be cancelled out if the infected mosquitoes also showed

inhibition of host-seeking activity. It is interesting to note that several viruses in the family Bunyaviridae have been shown to affect the feeding behavior of the vector including LACV (Jackson et al. 2012), Rift Valley Fever (Turell et al. 1985) and tobacco spotted wilt virus (Stafford et al. 2011). Han et al (2015) speculated that this might be a conserved trait among the bunyaviruses. Thus, it is possible that bunyaviruses exert an effect on the levels of biogenic amines in the vector, which promotes virus transmission through altered blood-feeding without impairment of the vector's ability to locate a host.

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Table 3.1. Host-seeking response of infected and uninfected mosquitoes to a human host in a uni-port olfactometer. A mosquito that left the holding chamber but did not enter the collection chamber was considered a partial responder.

Trial	Treatment	N	% Response	% Partial	% No Response
1	Infected	63	51	2	43
	Control	58	52	2	47
2	Infected	83	43	2	54*
	Control	73	74	1	25
3	Infected	56	82	0	18
	Control	75	84	1	15
4	Infected	55	20	36	44*
	Control	68	50	12	38
5	Infected	127	65	9	27
	Control	137	65	4	31
6	Infected	105	21	6	73
	Control	92	35	1	64
7	Infected	85	51	0	49
	Control	84	64	1	35
8	Infected	97	49	1	49
	Control	91	48	1	51
9	Infected	98	71	2	27
	Control	86	71	1	29
10	Infected	126	44	1	55
	Control	79	38	1	61

*Statistically significant ($P < 0.05$), two-tailed Fisher's exact test comparing response vs. non-response.

Figure 3.1. The uni-port olfactometer modified after Cabrini and Andrade (2007). Plastic box on the right is the testing chamber; cage on the left is the cage with mosquito.

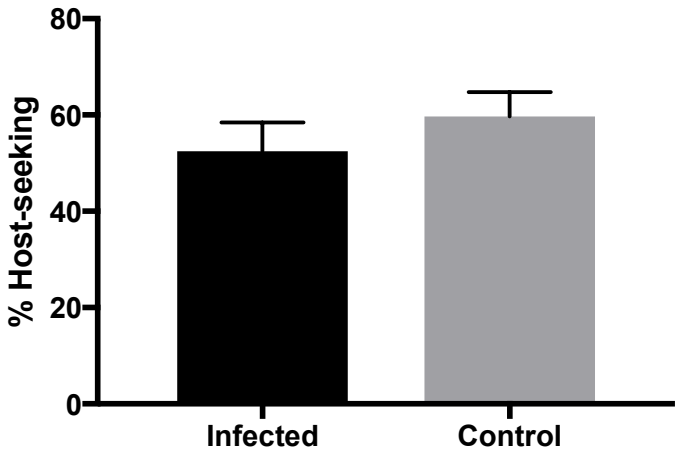

Fig. 3.2. Mean (± SEM) host-seeking response of infected and control mosquitoes (Wilcoxon matched-pairs signed rank test, P > 0.05).

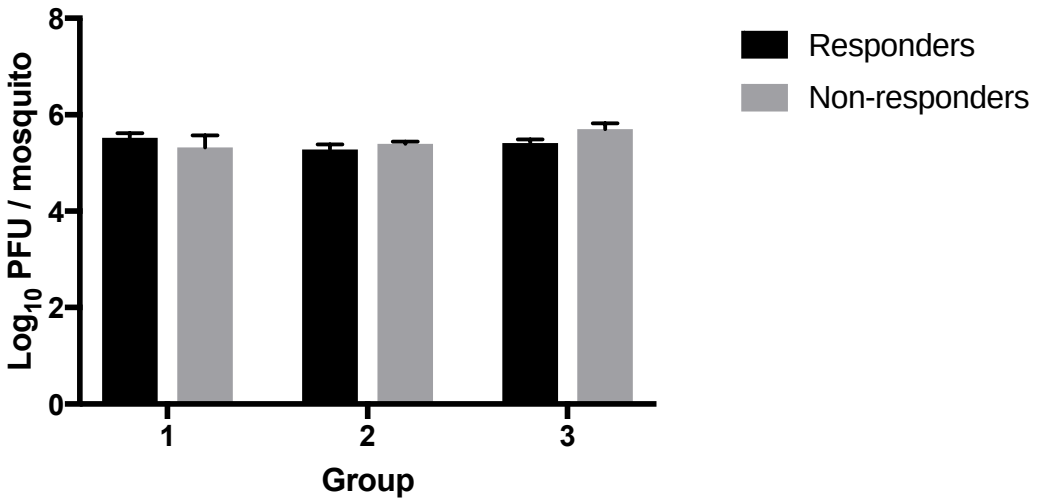

Fig. 3.3. Mean (± SEM, n=5) whole body titers of responders and non-responders.

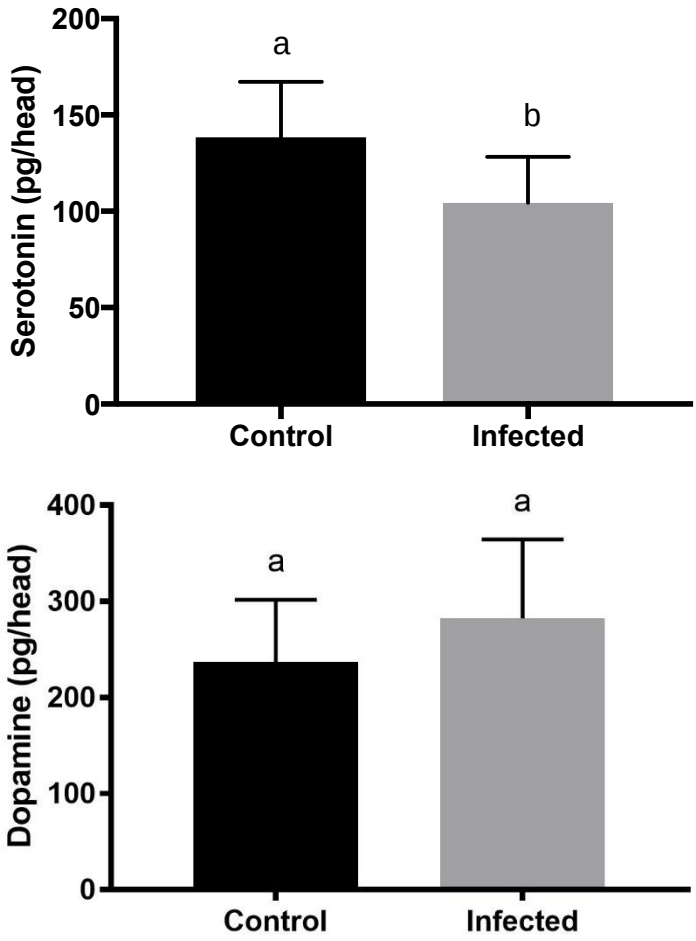

Fig. 3.4. Mean (±SEM) serotonin and dopamine levels in infected and control mosquito heads. For each graph, columns with different letters are significantly different (P > 0.05, ratio paired t test).

Chapter 4. Effect of La Crosse Virus Infection on the Sensitivity of *Aedes triseriatus* and *Aedes albopictus* to Barrier Sprays

4.1 Abstract

Background

La Crosse virus (LACV) encephalitis are the most common mosquito-borne disease of children in the U.S., responsible for an estimated 300,000 cases annually. A number of factors including climate change and utilization of new vectors have resulted in the expansion of the LACV distribution from primarily in the Midwest into the Appalachian region. The aedine vectors of LACV are notoriously difficult to control but barrier sprays are oftern recommended as an effective method to protect homeowners. Pyrethroids and essential oils are two popular types of insecticides permitted for use in our backyards. However, the efficacy of these sprays has not been evaluated for virus-infected mosquitoes.

Methodology/Principal Findings

Standard CDC bottle assays were used to evaluate the efficacy of two pyrethroids and two essential oil sprays on LACV infected and uninfected mosquitoes. LACV-infected *Ae. triseriatus* females were more susceptible to both pyrethroids than uninfected ones. Infection status did not affect the susceptibility of *Ae. albopictus* to either pyrethroid. The essential oils were inconsistent in their effects.

Conclusions

Our results support the use of barrier sprays as part of a mosquito control program, not just to reduce the biting rate but to potentially reduce the virus-infected

portion of the vector population. However, pyrethroids are much more effective than essential oils.

4.2 Introduction

La Crosse virus (LACV) encephalitis is an important mosquito borne disease that causes about 80 cases of neuroinvasive disease each year, primarily in children under 16. (Rust et al. 1999; Walker 1992). Because LACV is under-diagnosed in clinics, the actual infections are estimated to be approximately 300,000 annually (Kalfayan 1983). The main vector of this pathogen is *Aedes triseriatus* (Paulson et al. 1989). However, an invasive mosquito, *Ae. albopictus*, has emerged to be a new accessory vector in the transmission of LACV (Gerhardt et al. 2001). Several other mosquitoes can also transmit this disease under laboratory conditions (Harris et al. 2015). Recent studies indicated that LACV encephalitis has emerged as a serious problem in the Appalachia region (Bewick et al. 2016).

Aedes triseriatus is a container-dwelling mosquito that can utilize tree holes in forest areas and artificial containers in city areas (Williams et al. 2007). The invasive species, *Ae. albopictus* has expanded its distribution from Texas to 40 states in US (Hahn et al. 2016) and is also a container-inhabiting mosquito. It is a daytime feeder, showing aggressive biting behavior and a strong human preference that is competent for at least 22 arboviruses (Gratz 2004).

Barrier sprays are often used to control mosquito populations around homeowners' backyards, where vectors can rest (Perich et al. 1993; Trout et al. 2007). After spraying, insecticide residues on foliage can create a barrier to knockdown vectors directly (Martins and Valle, 2012). This knockdown is a critical factor to evaluate barrier

spray products. There are two kinds of barrier sprays available on market. Chemical residues like pyrethroids, are stable in the environment and have fast-acting and knock-down effects. Botanical residues are considered to be safer and have insecticide and repellent functions (Isman 2006). Both types of products are widely used in market.

However, LACV infection has been shown to alter mosquito feeding behavior (Jackson et al. 2012). Virus infection has led to altered vector reaction to repellents. For example, LACV-infected *Ae. triseriatus* were insensitive to several repellents (Chan & Paulson, unpublished data). And Sindbis-virus infected *Ae. aegypti* shown to be insensitive to Deet (Qualls et al. 2012). However, the effects of barrier sprays have not evaluated on virus-infected vectors.

In our study, we used CDC bottle assays to evaluate the efficacy of two pyrethroids and two essential oil sprays on LACV infected and uninfected mosquitoes. The products selected were commonly recommended online for homeowners to purchase and use to control container-inhabiting mosquitoes. The mosquito species selected, *Ae. triseriatus* and *Ae. albopictus*, are both important vectors of LACV as well as other important pathogens.

4.3 Materials and methods

4.3.1 Mosquitoes

Both *Ae. albopictus* and *Ae. triseriatus* were from colonies established from collections on the campus of Virginia Tech in Blacksburg, VA. The *Ae. triseriatus* colony was originated from eggs collected from a 196,115 m^2 forested area in 2015 and *Ae. albopictus* females were collected the Hahn Horticulture Garden in 2014. Mosquitoes were held in an insectary maintained at 27.5 ± 1^oC, 75% relative humidity, and a 16:8 h

L:D cycle. Rearing was done in plastic containers (33 X 17.5 X 11 cm) containing approximately 250 larvae in 1,600 mL deionized (DI) water and fed bovine liver powder solution (7.5 g/500 mL) ad libitum to guarantee the uniform size of adults (Jackson *et al.* 2012).

4.3.2 Virus Isolates and Assays

LACV isolate used in this experiment originated from adult *Ae. triseriatus* mosquitoes collected in 1999 in Wise County, VA (Barker et al. 2013). Before the study, 2 separate stock viruses were produced by passage in adult *Ae. triseriatus* or *Ae. albopictus* females and then amplified on Vero cells. Virus titers of these two stock viruses were measured following the methods of Barker et al. (2003) and found to be 2.05 X 10^8 and 3.3 X10^8 plaque forming units (PFU)/mL, respectively.

4.3.3 Infection of mosquitoes

Three to five-day old adult female *Ae. albopictus* and *Ae. triseriatus* were injected intrathoracically with 0.5 µl of LACV or diluent only for the controls following the methods of Jackson et al. (2012). Post infection, mosquitoes were held under standard laboratory conditions as described above for 7-day extrinsic incubation period. During this incubation time, 10% sucrose ad libitum was provided ad libitum for the mosquitoes as food resource.

4.3.4 Laboratory bioassays

Four commercial insecticides were evaluated for their contact toxicity to mosquitoes: two pyrethroids [lambda-cyhalothrin (Demand CS, Syngenta Crop Protection) and bifenthrin (Bifen I/T, Control Solution Inc)] and two botanical essential oils [Thyme Oil, 2-Phenethyl Propionate, Rosemary Oil (EcoVia, Rockwell Labs Ltd)

and Rosemary Oil, Geraniol, Peppermint Oil (essentria IC^3, Envincio LLC)]. Serial dilutions of the label working concentrations were made with acetone. The dilution range for the pyrethroids was 1:10, 1:100, 1: 1,000 and 1: 10,000 and for the botanicals was 1:10, 1:100 and 1: 1,000 (Table 1).

Regular 250 ml wheaton glass bottles (Sigma-Aldrich, St. Louis, MO) were pre-treated inside with the insecticide dilutions (Aïzoun et al. 2013; Denlinger et al. 2015). The actual amount of insecticide was determined to be x µg of active ingredient per bottle (CDC 2010). The method of coating inside bottle was as previously described (Denlinger et al. 2015). The amount of insecticide active ingredient in each bottle for the dilution series for each insecticide is shown in Fig. 4.1. One ml of acetone with insecticide was applied and coated to each bottle by swirling the solution on the bottom, sides, and lid. Then the bottle was placed on mechanical roller to evenly distribute the acetone and dried for half hour under chemical hood. During this half hour, the lid was loosened from the bottle and removed at the end for acetone to evaporate. Air-dried bottles were left open to dry overnight. Each test was triplicated.

Mosquitoes were chilled on wet ice to immobilize them and 10 individuals were carefully placed into the bottles. Each bottle was observed at several time points: 10, 20, 30, 40, 50, 60, and 120 min. Mosquitoes that could not fly or stand were counted as knocked down (Brogdon et al. 2010). The Add-Ins (Simple Probit) in JMP (JMP® Pro 12. SAS Institute Inc., Cary, NC) was used to generate the KD_{50} (knockdown), KD_{90} and KT_{50} (knockdown time), KT_{90} for each insecticide.

4.4 Results

The KD and KT values were determined for infected and uninfected *Ae. albopictus* and *Ae. triseriatus* for both Demand CS and Bifen I/T As the exposure time increased, the KD50 and KD90 values decreased substantially for both species and both insecticides (Figs. 4.1 and 4.2, Tables. 4.2 and 4.3).; as insecticide concentration decreased, the KT50 and KT90 increased for both species and both insecticides (Figs. 4.3 and 4.4, Tables. 4.4 and 4.5).

At shorter exposure times, LACV-infected *Ae. triseriatus* females were substantially more susceptible to either insecticide when compared to uninfected controls. For example, at the 10-minute time point, the KD50 for Demand CS was more than 4.2 times higher for the control (19.81 ug) than for the infected group (4.73 ug) and the KD90 was more than 6 times higher (45.5 ug for controls and 7.3 ug for infected) at the same time point (Figs. 4.1A & 4.2A). Although the pattern was the same, the magnitude of difference between infected and control mosquitoes was less for Bifen I/T. At the 10-minute exposure time, the KD50 value was 2.2 times higher for the controls (24.65 µg) than infected ones (11.09 µg) and the KD90 value was 2.7 times higher for controls (53.53 µg) than infected ones (20.2 µg) (Fig. 4.1 B, 4.2 B). *Aedes albopictus* was less susceptible, as measured by knockdown, to both insecticides than was *Ae. triseriatus* (Figs. 4.1 and 4.2). However, the KD50 and KD90 values for the virus-infected groups were higher than those for the controls for both Demand CS (Figs. 4.1C & 4.2C) and Bifen I/T (Figs. 4.1D & 4.2D). As dilution increased, the KT50 and KT 90 of both Demand CS and Bifen I/T increased for both *Ae. triseriatus* and *Ae. albopictus* but the effect of infection status was inconsistent (Figs. 4.3 & 4.4).

Two botanical commercial products were evaluated in this experiment, EcoVia and essentria IC^3. Only the most concentrated dilutions were evaluated in our data because lower concentrations were not effective. EcoVia did not show any effects on *Ae. triseriatus* control groups until 120-min time point; however, for infected *Ae. triseriatus*, EcoVia demonstrated an approximately 30% of knockdown rate from 10 to 120-min time points (Fig. 4.5 A). essentria IC^3 had a consistent, albeit low compared to the pyrethroids, knockdown rate against both control and infected *Ae. triseriatus* (Fig. 4.5 B). EcoVia knocked down more virus-infected *Ae. albopictus* at the first 3 time points but at longer exposure times it caused 100% knockdown for both groups. (Fig 4.5 C). Regardless of infection status, essentria IC^3 was not effective against *Ae. albopictus* (Fig. 4.5 D).

For KT50 in EcoVia and essentria IC^3, we only calculated effects of the most concentrated dilution. Both *Ae. triseriatus* control group and LACV-infected *Ae. triseriatus* need 120 and 173 min to knockdown 50% of mosquitoes. However, essentria IC^3 need 329 and 404 min to knockdown 50% of *Ae. triseriatus* in LACV-infected and control groups. EcoVia had a better effect on *Ae. albopictus* than essentria IC^3 (Fig 4.5, Table 4.4 and 4.5). For KT90, EcoVia were more efficient to *Ae. triseriatus* control group than LACV-infected group. However, there was no different between LACV-infected *Ae. triseriatus* and control in essentria IC^3. EcoVia had a better efficient on LACV-infected *Ae. albopictus* than control group. Both LACV-infected and control *Ae. albopictus* did not show any difference in essentria IC^3 (Figs. 4.6 and 4.7 and Tables. 4.4 and 4.5).

4.5 Discussion

The objectives of this paper were to evaluate the efficacy of barrier sprays to the important vector species, *Ae. albopictus* and *Ae. triseriatus*, and to determine if virus

infection alters susceptibility to the products. We chose products that are commonly marketed for use by homeowners. In this study, we found that the 2 pyrethroids tested, Demand CS and Bifen I/T, were highly toxic to both LACV-infected and uninfected *Ae. albopictus* and *Ae. triseriatus*. However, virus infection status did alter the susceptibility; LACV-infected *Ae. triseriatus* were more susceptible to the pyrethroids while infected *Ae. albopictus* were less susceptible. The botanicals were far less effective against both species, but infection status did affect the susceptibility of *Ae. triseriatus*.

Both Demand CS and Bifen I/T are broad spectrum, fast acting and stable. Our data indicated that Demand CS required a lower concentration and shorter exposure time than Bifen I/T, especially in *Ae. albopictus.* Pyrethroids affect both the central and peripheral nervous systems of insects by modification of the sodium channels, leading to repetitive discharges of neurons causing paralysis (knockdown) and, ultimately, death (Davies et al. 2007). However, Demand CS is a Type II pyrethroid while Bifen I/T is a Type I pyrethroid and therefore exert different physiological effects (Du et al. 2008). In general, Type I agents cause very rapid knockdown while Type II agents result in higher insect mortality (Davies et al. 2007). Various factors can affect the outcome of CDC bottle assays including the physiological state of the mosquitoes used, mosquito age and the environmental conditions, i.e., temperature and humidity under which the insecticide exposure is done (Chouaibou et al. 2012). In this study, all mosquitoes were of the same age and in the same physiological state and all tests were done under standard laboratory conditions.

Essential oils are a very common group of botanical insecticides (Isman 2006). However, these compounds volatilize readily, which may limit their efficacy (Regnault-

Roger et al. 2012). The active ingredients in EcoVia and essentria IC^3 include thymol which blocks GABA synapses, rosemary which has repellent activity, geraniol which interacts with acetylcholinesterase, and 2-phenethyl propionate and peppermint oil which are repellents (Priestley et al. 2003; Regnault-Roger et al. 2012). The neurotoxic activity of essential oils against insects is thought to be from binding to octopamine receptors (Enan 2001, Blenau et al. 2012) but in vertebrates octopamine is a trace amine whose function as a neurotransmitter is unclear (Grohmann et al. 2003). For this reason, essential oils that target octopamine receptors are marketed as safer alternatives to chemical controls. In this study, EcoVia was substantially more effective against *Ae. albopictus* than essentria IC^3 and virus infection did not result in an altered response to either product (Figs. 4.5 C & D). However, while essentria IC^3 was equally effective against infected and uninfected *Ae. triseriatus,* LACV-infected mosquitoes were much more sensitive to EcoVia than the uninfected ones (Figs. 4.5 A & B).

LACV is a neurotropic virus that causes changes in the feeding behavior of and serotonin production by *Ae. triseriatus* (Grimstad et al. 1980; Jackson et al. 2012; Yang et al. unpublished data). But, LACV infection does not affect *Ae. albopictus* to the same degree. While LACV infection reduced bloodmeal size in both *Ae. triseriatus* and *Ae. albopictus,* and it increased refeeding rates in only in *Ae. triseriatus* not *Ae. albopictus* (Jackson et al. 2012). Westby et al. (2015) also found no effect of LACV infection on the refeeding rate of *Ae. albopictus*. and suggest that LACV is more effective at modifying behavior of *Ae. triseriatus* than that of invasive *Ae. albopictus*. *Ae. albopictus* is an invasive species, first appearing in the U.S. in the mid-1980's and thus has little evolutionary history with LACV (Moore and Mitchell 1997). Chronic infection with

LACV may impose a metabolic stress on *Ae. triseriatus* making it less able to tolerate exposure to pyrethroids. In fact, nutritional stress has been shown to affect vector competence to LACV in *Ae. triseriatus* (Grimstad and Haramis 1984; Paulson and Hawley 1991). *Aedes albopictus* cells have been shown to use an antioxidant defense against infection with dengue virus that included significant elevation of glutathione S-transferase (GST) activity (Chen et al. 2011). Variation in resistance to insecticides can be modulated through metabolic detoxication (Hemingway et al. 1998; Terriere 1984) and upregulation of GSTs can lead to resistance to pyrethroids (Hemingway et al. 2004). It is possible that *Ae. albopictus* mosquitoes mount a more intense response against LACV infection than does the natural vector, *Ae. triseriatus*. Further studies are needed to determine the mechanism behind the differing effects of LACV infection on the susceptibility to pyrethroids and EcoVia by these 2 vectors.

Trying to control container-inhabiting mosquitoes presents a challenge. Standard mosquito control efforts utilizing spray trucks or aerial sprays are generally directed towards crepuscular species, in part because environmental conditions favor administration of insecticides in the early evening. Because *Ae. triseriatus* and *Ae. albopictus* are diurnal species this is not effective. Physical control by the elimination of breeding and resting sites are effective steps to reduce the mosquito population that individual homeowners can take to reduce the risk of disease transmission in their own yards, with the added benefit that resistance cannot develop to these efforts. Applying residual pesticides to mosquito resting sites in vegetation have been shown to reduce pest mosquito populations (Trout et al. 2007; Amoo et al. 2008; Doyle et al. 2009). Our results confirms the value of barrier sprays as part of a mosquito control program, not just

to reduce the biting rate but to potentially reduce the virus-infected portion of the vector population.

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Table 4.1. The 4 commercial barrier sprays, concentrations tested, and active ingredients used in this study.

Commercial Brand Name	Concentration (µg insecticide/ bottle)	Active Ingredient
Demand CS	60, 6, 0.6, 0.06	lambda-cyhalothrin
Bifen I/T	60, 6, 0.6, 0.06	bifenthrin
EcoVia	1310, 131, 13.1, 1.31	Thyme Oil, 2-Phenethyl Propionate, Rosemary Oil
Essentri IC[3]	400, 40, 4, 0.4	Rosemary Oil, Geraniol, Peppermint Oil

Table 4.2 JMP (Simple Probit) logistic regression parameters and lethal concentration (µg/bottle) values causing 50 and 90% knock down rate in La Crosse virus infected and uninfected *Aedes triseriatus* by Demand CS, Bifen I/T, EcoVia, and essentria IC^3.

Insecticide	Time (min)	Infected	KD50 (µg insecticide per bottle) [LL, UL]*	KD90 (µg insecticide per bottle) [LL, UL]*
Demand CS	10	Y	4.73[2.89, 6.57]	7.32[4.52, 10.13]
	10	No	19.81[5.74, 33.89]	45.48[18.75, 72.21]
	20	Y	1.2[-1.46. 4.71]	2.00[-2.04, 6.04]
	20	No	2.11[0.47, 3.74]	4.73[1.78, 7.69]
	30	Y	0.6[0.6, 0.6]	0.6[0.6, 0.6]
	30	No	0.57[0.25, 0.89]	1.01[0.3, 1.72]
	40	Y	0.6[0.12, 1.08]	1.22[-0.02, 2.47]
	40	No	0.57[0.2, 0.93]	1.07[0.21, 1.94]
	50	Y	0.33[0.045, 0.61]	0.81[0.23, 1.38]
	50	No	0.38[-0.0006, 0.76]	1.04[-0.0039, 2.08]
	60	Y	0.29[0.005, 0.57]	0.76[0.21, 1.31]
	60	No	0.29[0.06, 0.52]	0.65[0.27, 1.02]
	120	Y	0.06[0.06, 0.06]	0.06[0.06, 0.06]
	120	No	0.07[0.01, 0.12]	0.16[0.11, 0.22]
Bifen I/T	10	Y	11.09[-5.27, 27.46]	20.20[-13.47, 53.86]
	10	No	24.65[8.77, 40.53]	53.53[24, 83.07]
	20	Y	3.46[1.3, 5.62]	7.28[3.15, 11.42]
	20	No	3.46[1.09, 5.83]	7.81[2.88, 12.75]
	30	Y	2.23[-7.12, 11.57]	3.67[-12.86, 20.19]
	30	No	0.57[0.25, 0.89]	1.01[0.3, 1.72]
	40	Y	1.29[-1.33, 3.91]	2.34[-3.23, 7.91]
	40	No	0.57[0.25, 0.89]	1.01[0.3, 1.72]
	50	Y	1.89[-2.57, 6.34]	3.41[-9.63, 16.45]
	50	No	0.63[0.23, 1.03]	1.12[0.20, 2.04]
	60	Y	1.43[-1.77, 4.63]	
	60	No	0.52[0.11, 0.93]	1.13[0.05, 2.21]
	120	Y	0.61[0.6, 0.61]	0.62[0.61, 0.62]
	120	No	0.22[-0.003, 0.44]	0.54[0.20, 0.89]
EcoVia	120	Y	1549.92[-2197.45, 5297.28]	2886.18[-5397.07, 11169.43]
	120	N	5634.57[-33667.9, 44937.1]	10728.24[-67751.1, 89207.6]
essentria IC^3	120	Y	4250.94[-37585.2, 46087.1]	8530.24[-80829.5, 97890]
	120	N	3567.47[-22788.8, 29923.77]	6553.51[-45784.1, 58891.15]

*LL: lower 95% confidence limit; UL: upper 95% confidence limit.

Table 4.3 JMP (Simple Probit) logistic regression parameters and lethal concentration (µg/bottle) values causing 50 and 90% knock down rate in La Crosse virus infected and uninfected *Aedes albopictus* by Demand CS, Bifen I/T, EcoVia, and essentria IC^3.

Insecticide	Time (min)	Infected	KD50 (µg insecticide per bottle) [LL, UL]*	KD90 (µg insecticide per bottle) [LL, UL]*
Demand CS	10	Y	44.61[23.37, 65.86]	79.49[40.4, 118.57]
	10	No	48.04[26.57, 69.52]	81.48[42.97, 120]
	20	Y	5.64[2.15, 9.13]	10.48[3.07, 17.9]
	20	No	6.23[2.98, 9.49]	10.28[3.73, 16.84]
	30	Y	4.05[1.72, 6.38]	7.99[3.48, 12.51]
	30	No	2.95[1.26, 4.63]	5.46[2.82, 8.1]
	40	Y	3.07[1.16, 4.97]	6.37[2.99, 9.76]
	40	No	2.17[0.63, 3.71]	4.63[2.01, 7.24]
	50	Y	1.99[0.24, 3.74]	5.23[2.01, 8.46]
	50	No	0.6[0.6, 0.6]	0.6[0.6, 0.6]
	60	Y	0.6[0.31, 0.89]	0.98[0.34, 1.61]
	60	No	0.55[0.31, 0.8]	0.9[0.4, 1.4]
	120	Y	0.21[0.01, 0.41]	0.48[0.17, 0.8]
	120	No	0.18[-0.06, 0.42]	0.54[0.16, 0.92]
Bifen I/T	10	Y	60[60, 60]	60[60, 60]
	10	No	60[60, 60]	60[60, 60]
	20	Y	43.05[26.28, 59.82]	65.29[42.82, 87.77]
	20	No	34.64[16.97, 52.3]	52.16[29.84, 74.47]
	30	Y	11.94[-5.47, 29.35]	18.85[-12.85, 50.55]
	30	No	14.09[-13.35, 41.54]	23.58[-27.31, 74.46]
	40	Y	6.46[3.39, 9.54]	10.09[3.89, 16.3]
	40	No	6.57[2.48, 10.67]	11.33[2.81, 19.85]
	50	Y	4.57[2.77, 6.37]	7.07[4.45, 9.69]
	50	No	4.23[2.4, 6.06]	6.89[4.14, 9.64]
	60	Y	3.47[1.75, 5.2]	5.6[3.27, 7.93]
	60	No	2.89[1.21, 4.57]	5[2.48, 7.52]
	120	Y	0.6[0.6, 0.6]	0.6[0.6, 0.6]
	120	No	0.6[0.6, 0.6]	0.6[0.6, 0.6]
EcoVia	120	Y	2.2**	14.23**
	120	N	11.9**	32.6**
essentria IC^3	120	Y	170.55**	276.82**
	120	N	238.50**	378.1**

*LL: lower 95% confidence limit; UL: upper 95% confidence limit.
** LL and UL could not be calculated.

Table 4.4 JMP (Simple Probit) logistic regression parameters and lethal time (min) values causing 50 and 90% knock down rate in La Crosse virus infected and uninfected *Aedes triseriatus* by Demand CS, Bifen I/T, EcoVia, and essentria IC^3.

Insecticide	Dilution	Infected	KT50 (min) [LL, UL]*	KT90 (min) [LL, UL]*
Demand CS	1/10	Y	**	**
	1/10	No	10[10, 10]	10[10, 10]
	1/100	Y	9.78[9.58, 9.99]	10.23[10.02, 10.44]
	1/100	No	11.21[8.63, 13.8]	17.35[13.29, 21.4]
	1/1000	Y	39.34[33.98, 44.7]	64.36[53.65, 75.08]
	1/1000	No	33.55[26.36, 40.75]	69.56[52.03, 87.08]
	1/10000	Y	134.22[81.73, 186.71]	236.07[120.96, 351. 18]
	1/10000	No	118.78[81.7, 155.86]	205.38[123.91, 286.85]
Bifen I/T	1/10	Y	**	**
	1/10	No	9.98[9.97, 9.99]	10[9.99, 10.01]
	1/100	Y	14.56[11.77, 17.34]	22.27[17.31, 27.23]
	1/100	No	14.06[10.94, 17.18]	23.03[17.15, 28.92]
	1/1000	Y	19938.99[-3035615, 3075493]	43349.77[-6608297, 6694997]
	1/1000	No	49.27[37.46, 61.08]	110.26[79, 141.52]
	1/10000	Y	-163.52[-708.75, 381.7]	-310.43[-1258.12, 637.27]
	1/10000	No	179[71.64, 286.35]	309.11[93.04, 525.18]
EcoVia	1/10	Y	173.04[-21.14, 367.23]	455.08[-159.73, 1069.9]
	1/10	N	120[120, 120]	120[120, 120]
essentria IC^3	1/10	Y	329.79[-235.2, 894.79]	646.49[-553.56, 1846.53]
	1/10	N	404.27[-659.36, 1467.9]	857[-1558.75, 3272.75]

*LL: lower 95% confidence limit; UL: upper 95% confidence limit.
** could not be calculated.

Table 4.5 JMP (Simple Probit) logistic regression parameters and lethal time (min) values causing 50 and 90% knock down rate in La Crosse virus infected and uninfected *Aedes albopictus* by Demand CS, Bifen I/T, EcoVia, and essentria IC^3.

Insecticide	Dilution	Infected	KT50 (min) [LL, UL]*	KT90 (min) [LL, UL]*
Demand CS	1/10	Y	9.79[9.55, 10.03]	10.31[10.07, 10.55]
	1/10	No	9.8[9.53, 10.07]	10.39[10.13, 10.66]
	1/100	Y	21.09[15.5, 26.68]	42.08[34.71, 49.45]
	1/100	No	19.9[16.44, 23.36]	31.26[26.61, 35.91]
	1/1000	Y	59.56[50.28, 68.85]	97.71[78.5, 117.32]
	1/1000	No	58.57[49.83, 67.31]	99.27[82.43, 116.11]
	1/10000	Y	185.3[79.3, 291.29]	300.33[101.35, 499.3]
	1/10000	No	134.23[107, 161.46]	178.31[124.8, 231.82]
Bifen I/T	1/10	Y	14.62[12.02, 17.22]	21.53[17.17, 25.88]
	1/10	No	16.89[14.18, 19.61]	24.31[19.06, 29.56]
	1/100	Y	42.1[38.3, 45.9]	56.76[50.53, 63]
	1/100	No	41.31[37.67, 44.94]	54.8[48.83, 60.77]
	1/1000	Y	148.13[100.14, 196.12]	219.85[131.96, 307.73]
	1/1000	No	118.25[90.06, 146.44]	182.71[127.64, 237.79]
	1/10000	Y	**	**
	1/10000	No	**	**
EcoVia	1/10	Y	2.2[-11.66, 16.07]	14.23[8.51, 19.95]
	1/10	N	11.09[3.2, 18.98]	31.06[22.03, 40.09]
essentria IC^3	1/10	Y	170.55[81.27, 259.82]	276.82[104.05, 449.6]
	1/10	N	238.5[60.64, 416.36]	378.1[67.36, 688.83]

*LL: lower 95% confidence limit; UL: upper 95% confidence limit.
** could not be calculated.

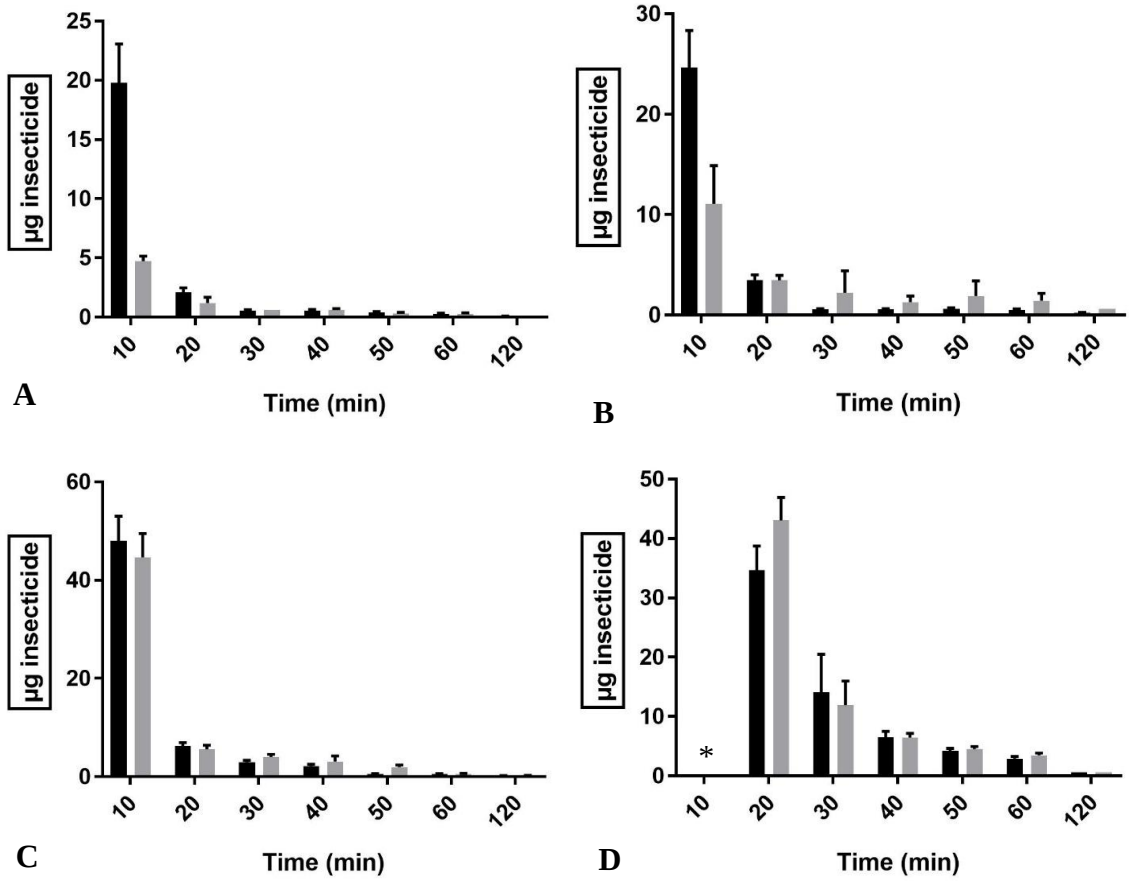

Figure 4.1. Knock down 50% (± SEM) rates of La Crosse virus-infected and uninfected *Aedes triseriatus* and *Aedes albopictus* by Demand CS and Bifen I/T. A: Demand CS, *Ae. triseriatus*; B: Bifen I/T, *Ae. triseriatus*; C: Demand CS, *Ae. albopictus*; D: Bifen I/T, *Ae. albopictus*. * could not be calculated. Black bars are controls, gray bars are infected.

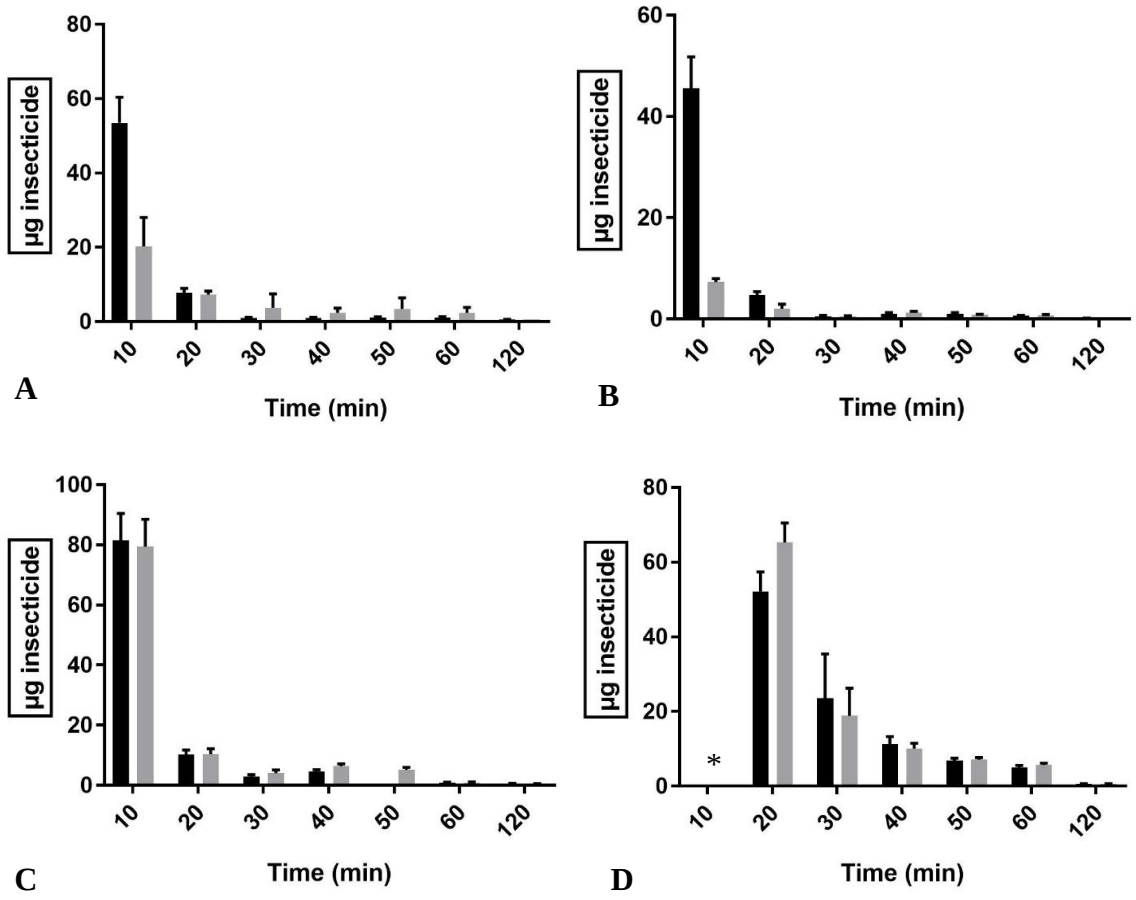

Figure 4.2. Knock down 90% (± SEM) rates of La Crosse virus-infected and uninfected *Aedes triseriatus* and *Aedes albopictus* by Demand CS and Bifen I/T. A: Demand CS, *Ae. triseriatus*; B: Bifen I/T, *Ae. triseriatus*; C: Demand, *Ae. albopictus*; D: Bifen I/T, *Ae. albopictus*. * could not be calculated. Black bars are controls, gray bars are infected.

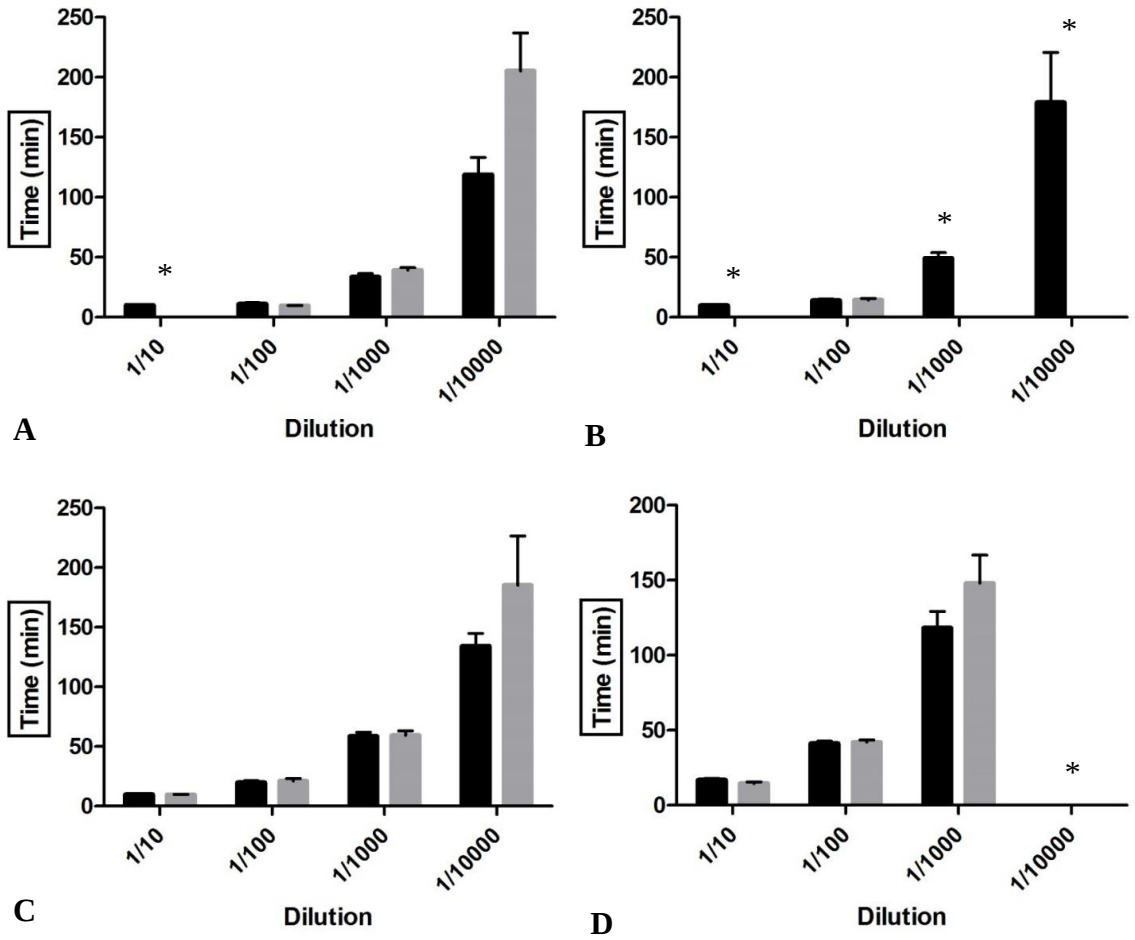

Figure 4.3. Time to knock down 50% (±SEM) rates of La Crosse virus infected and uninfected *Aedes triseriatus* and *Aedes albopictus* by Demand CS and Bifen I/T. A: Demand CS, *Ae. triseriatus*; B: Bifen I/T, *Ae. triseriatus*; C: Demand CS, *Ae. albopictus*; D: Bifen I/T, *Ae. albopictus*. * could not be calculated. Black bars are controls, gray bars are infected.

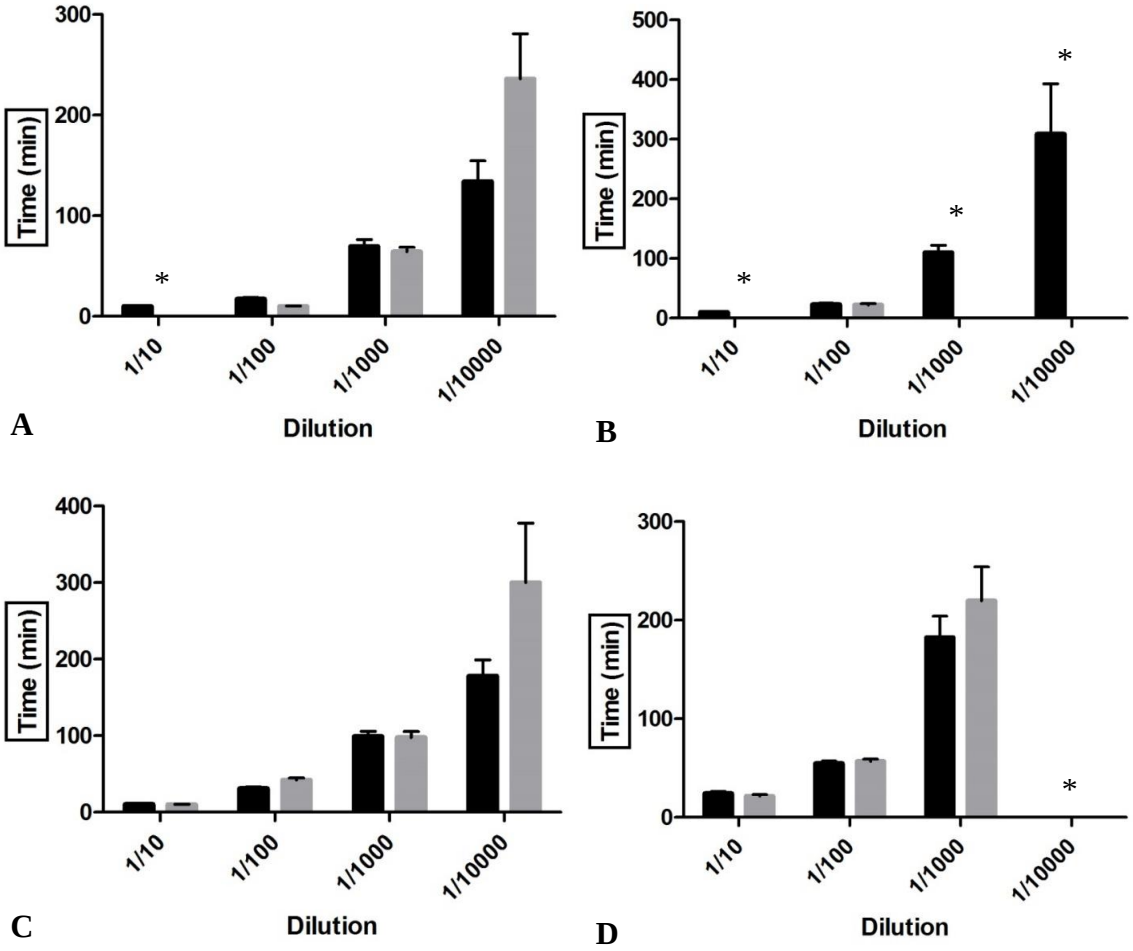

Figure 4.4. Time to knock down 90% (±SEM) rates of La Crosse virus infected and uninfected *Aedes triseriatus* and *Aedes albopictus* by Demand CS and Bifen I/T. A: Demand CS, *Ae. triseriatus*; B: Bifen I/T, *Ae. triseriatus*; C: Demand CS, *Ae. albopictus*; D: Bifen I/T, *Ae. albopictus*. * could not be calculated. Black bars are controls, gray bars are infected.

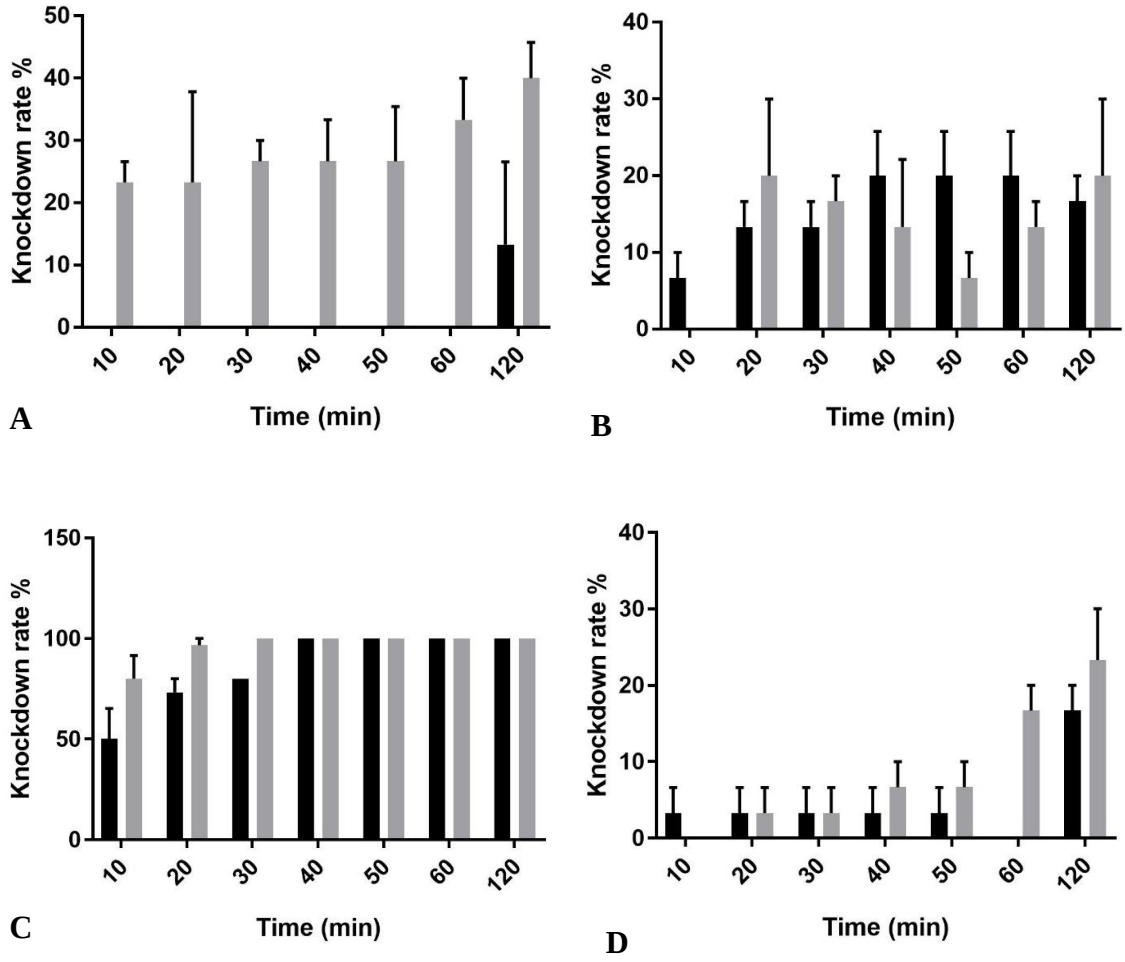

Figure 4.5. EcoVia and essentria IC^3 effects on *Aedes triseriatus* and *Ae. albopictus*. The working concentration was a 1:10 dilution of the commercial concentration. Error bar are standard errors determined by GraphPad Prism 7. Black bars are control; gray bars are infected. A: EcoVia on *Ae. triseriatus*; B: essentria IC^3 on *Ae. triseriatus*; C EcoVia on *Ae. albopictus*; D: essentria IC^3 on *Ae. albopictus*

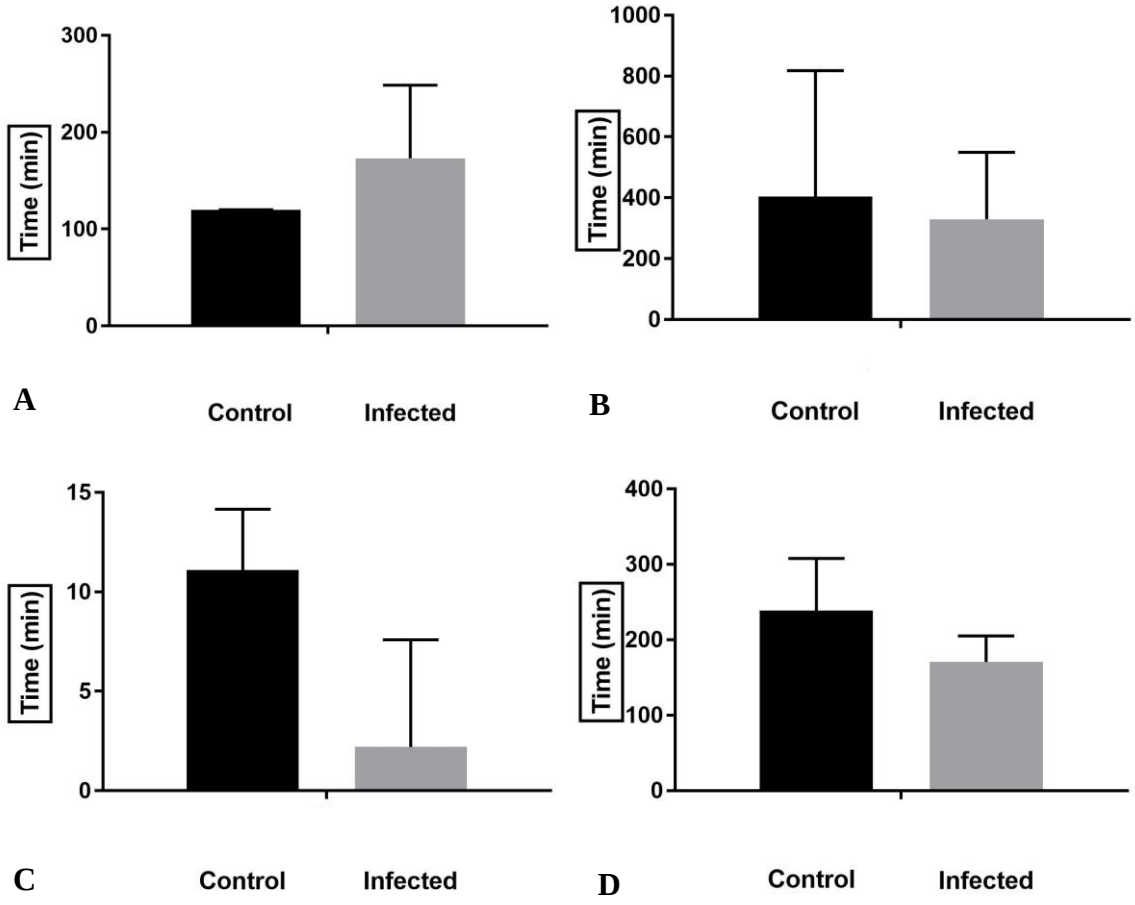

Figure 4.6. Time to knock down 50% (±SEM) rates of La Crosse virus infected and uninfected *Aedes triseriatus* and *Aedes albopictus* by EcoVia and essentria IC^3. The working concentration was a 1:10 dilution of the recommended label concentration. A: EcoVia on *Ae. triseriatus*; B: essentria IC^3 on *Ae. triseriatus*; C: EcoVia on *Ae. albopictus*; D: essentria IC^3 on *Ae. albopictus*.

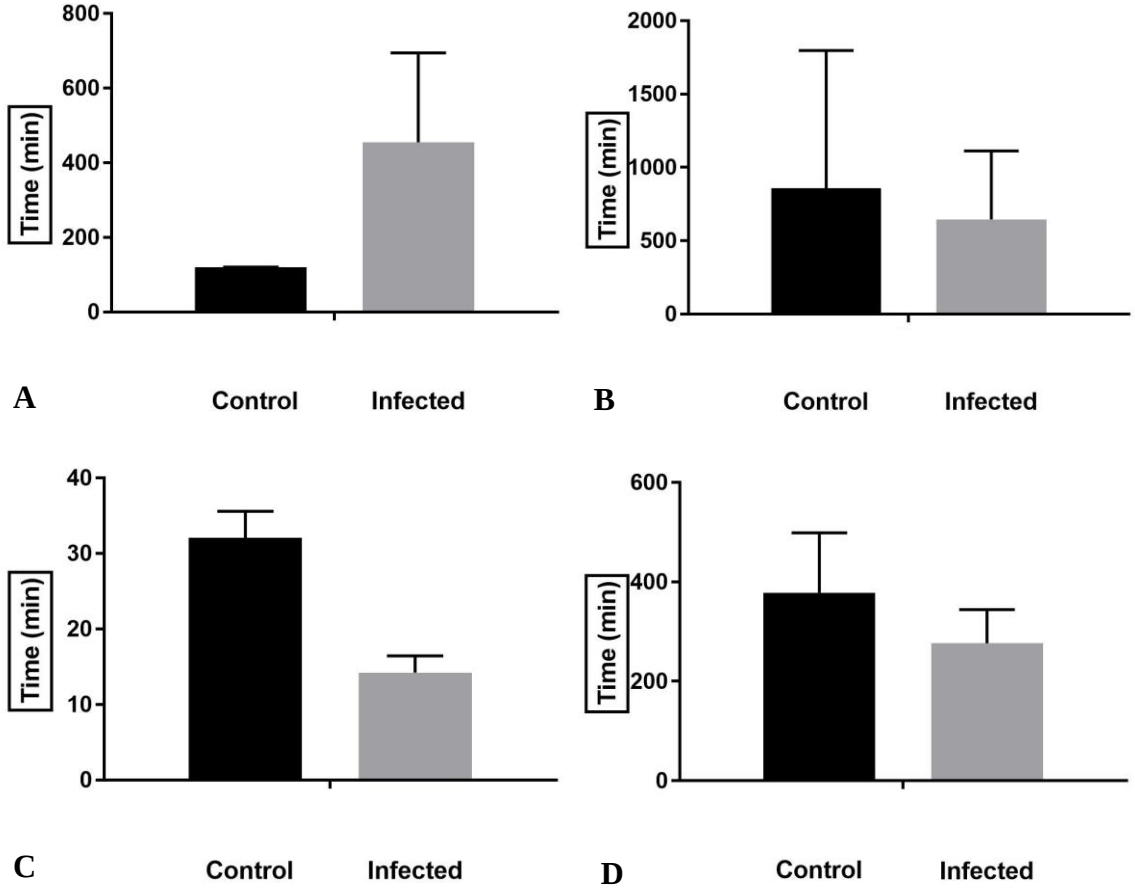

Figure 4.7. Time to knock down 90% (±SEM) rates of La Crosse virus infected and uninfected *Aedes triseriatus* and *Aedes albopictus* by Demand CS and Bifen I/T. The working concentration was a 1:10 dilution of the recommended label concentration. A: EcoVia on *Ae. triseriatus*; B: essentria IC^3 on *Ae. triseriatus*; C: EcoVia on *Ae. albopictus*; D: essentria IC^3 on *Ae. albopictus*.