La Crosse Virus in Southwestern Virginia: Role of Exotic Mosquito Species and Effect of Virus Infection on Feeding

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ABSTRACT

The family *Bunyaviridae* is the largest of vertebrate diseases and includes the mosquito-borne disease La Crosse (LAC) virus. Vectors include the major vector *Aedes triseriatus* and two accessory vectors *Aedes albopictus* and *Aedes japonicus*. In the past several decades there has been an increase in the number of LAC cases, implication of new vectors, and the appearance of new foci of disease in the southeastern U.S. To better understand the vectors and the relationship between vectors and the virus, laboratory and field studies were conducted to determine oviposition preferences, effect of virus infection on blood-feeding behavior, and compare the efficacy of various methods to determine minimum infection rates of vectors.

In laboratory studies of oviposition preference, only *Ae. japonicus* demonstrated a preference when presented with preexisting eggs. They deposited more eggs in cups containing either conspecifics or *Ae. albopictus*. The presence of 1st instar larvae *Ae. albopictus* larvae deterred oviposition by *Ae. triseriatus* and *Ae. japonicus. Ae. japonicus* and *Ae. triseriatus* preferred cups containing larval rearing water (LRW) of conspecifics and *Ae. albopictus*. *Aedes albopictus* preferred LRW regardless of species compared to control cups. Field experiments with fresh egg papers and preexisting eggs did not show significant differences, although the unequal population densities of species in the study area confounded the analysis. More work is needed to elucidate the interaction among these species and its effect on oviposition in the field.

Blood-feeding experiments showed that LAC virus-infected *Ae. triseriatus* and *Ae. albopictus* imbibed significantly less blood compared to uninfected mosquitoes. Because blood meal size affects the subsequent inhibition of host seeking, experiments were done to ascertain the effect of virus infection on refeeding. Significantly more infected *Ae. triseriatus* mosquitoes refed but there was no effect on the refeeding rate of *Ae. albopictus*. Thus, the detrimental effect of virus infection, i.e., reduction in blood meal size, may lead to increased host exposure by *Ae. triseriatus*, enhancing horizontal transmission.

Collecting adult mosquitoes was more efficient to detect virus in field populations than the collection of eggs. Maximum likelihood estimation-infection rates (MLE-IR) were calculated using bias-corrected maximum likelihood estimation. Adult collections yielded significantly more positive pools compared with egg collections. Virus was isolated from pools from *Ae. canadensis*, *Ae. triseriatus*, and *Ae. albopictus*. These results are comparable to other studies.

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Chapter 1

Introduction and Literature Review

1.1.1 Introduction to La Crosse virus

In 1964 the death of a young Minnesota girl led to the first isolation of La Crosse (LAC) virus (Grimstad 1988). LAC virus is a mosquito-borne disease in the California serogroup of the family *Bunyaviridae*. This family is the largest of vertebrate diseases (Beaty et al. 2000). Children 15 years of age or younger are mainly affected and show signs of febrile illnesses, headaches, disorientation, vomiting, and even progressing to seizures or other neurological problems, even coma (McJunkin et al. 1998). In cases of hospitalized patients, the fatality rate may be as high as 1% (Tsai 1991). The majority of cases are subclinical with estimates as high as 300,000 infections per year (Rust et al. 1999, CDC 2005). Between the initial effects and its sequelae, or after effects, the disease can result in medical costs ranging from $48,775 to $3,090,798 (Utz et al. 2003) per case and have adverse consequences on the child's IQ and school performance (CDC 2005). Originally, LAC virus was found in the Great Lakes states, but in recent years, a number of cases have been reported in the mid-Atlantic states including southwestern Virginia. Between 1964 and 2007 approximately 81 confirmed and probable cases per year were reported in the U.S. to the Centers for Disease Control and Prevention (CDC 2008).

1.1.2 Genome

The LAC virus genome consists of tripartite negative sense RNA. These three segments are named according to their nucleotide (nt) size, small (984 nt), medium (4526 nt), and large (6980 nt). The large segment codes for the polymerase, the medium segment codes for the G1 and G2 glycoproteins, and the small segment codes for the nucleocapsid protein. Both the

medium and small segments also code for nonstructural proteins. All of the segments are encapsulated in a host-derived lipid envelope which contains virus-encoded glycoprotein spikes and measures approximately 90-100 nm. The virus is thought to enter the cell through receptor-mediated endocytosis and membrane fusion before the nuclecapsids are released into the cytoplasm and transcription is initiated. The endoplasmic reticulum is the site where glycosylation of the G1 and G2 glycoproteins begins and ends in the Golgi apparatus along with virus maturation. Nucleocapsids bud through the membranes and start to accumulate in the cisternae before being transported to the cell's surface through exocytosis (Elliot 1990, Bishop 1996).

1.1.3 Vectors: *Aedes triseriatus*

The major vector of LAC virus is the mosquito *Aedes triseriatus* (Say), the eastern treehole mosquito (Pantuwatana et al. 1974, Beaty and Thompson 1975, Watts et al. 1974, Miller et al. 1979). *Aedes triseriatus* has been implicated as the vector of LAC because it is commonly collected in areas where human cases have occurred (Berry et al. 1975, Balfour et al. 1976) and field collected specimens have tested positive for the virus (Thompson et al. 1972, Berry et al. 1974, Pantuwatana et al. 1974, Balfour et al. 1975). *Aedes triseriatus* breeds in artificial containers or treeholes, and is distributed across the eastern half of the U.S. (Grimstad 1988). These breeding sites allow the mosquito to come into close contact with humankind (DeFoliart and Lisitza 1980). Adult feeding patterns in Wisconsin were observed in the early morning and mid to late afternoon (Loor and DeFoliart 1970), although in Texas, peak feeding occurred at dusk (Aziz and Hayes 1987). Blood feeding studies determined that the mosquito engorged primarily on eastern chipmunks (*Tamias striatus*) and gray squirrels (*Sciuris carolinensis*) in rural and urban settings (Nasci 1985, Richards et al. 2006).

During a feeding preference study of several mosquito species, Wright and DeFoliart (1970) discovered that *Ae. triseriatus* fed on amplifying hosts of LAC virus more than other mosquito species collected. Although *Ae. triseriatus* will feed on humans, rates are low. Higher rates have been associated with eastern chipmunks, an important amplifying host (Yuill 1983). While using fencerows or rows of vegetation as a route to travel between habitats (Nasci 1982), the adults have been known to travel over large open areas (Mather and DeFoliart 1984). However, some adults will not travel outside of their woodland larval habitat (Sinsko and Craig 1970). A habitat preference study concluded that the mosquito preferred forested habitats compared to urban or residential habitats (Barker et al. 2003). *Aedes triseriatus* preferred to lay more eggs in deciduous forests with more understory vegetation than in evergreen forests (Ellis 2008). In the north it overwinters in the egg stage and may have several broods each year. Eggs are laid above the water line and hatch when covered with water at a favorable temperature with larval habitats usually being within 1 m of the ground in shaded areas (Scholl and DeFoliart 1977, Aziz and Hayes 1987, Nasci 1988). The eggs are photo-period sensitive and will go into diapause as day length shortens (Shroyer and Craig 1980) and will terminate diapause after going through long periods of cold temperatures (Shroyer and Craig 1980). Larvae may be found from May to September (Carpenter and LaCasse 1955) and are capable of undergoing diapause in areas of the country where freezing temperatures are uncommon (Sims 1982). Szumlas et al. (1996a) captured a large number of host-seeking females in June and July, when LAC virus is amplified in an area. In late summer, when the majority of LAC encephalitis cases have been reported, the authors collected mosquitoes with higher infection rates (Szumlas et al. 1996b).

1.1.4 Vectors: *Aedes albopictus*

A second and more recently discovered vector is *Aedes albopictus* (Skuse). The common name for this mosquito is the Asian tiger mosquito. It was most likely introduced from Asia and was brought to the southern U.S. through the transport of used tires (Hawley et al. 1987). In 1985, the Asian tiger mosquito was found to be the most abundant container breeding mosquito in Houston, Texas, and since then has spread rapidly throughout the United States (Hawley et al. 1987). Now it can be found throughout the U.S. (recently California) and Central and South America (Gratz 2004). The surplus of water collecting containers and used tires, have provided optimal larval habitats and have contributed to the spread of *Ae. albopictus* (Hawley 1988).

Aedes albopictus feeds on a wider variety of vertebrate hosts than *Ae. triseriatus* and is a more aggressive biter than the eastern treehole mosquito when feeding on mammalian hosts (Streit et al. 1993). Feeding studies for *Ae. albopictus* showed a preference for humans (Sullivan et al. 1971) but the mosquito has been found to feed on chipmunks, also (Cully et al. 1991). Bloodmeals of *Ae. albopictus* collected from both rural and urban sites from Florida, Indiana, Missouri, Illinois, and Louisiana showed that a majority of blood meals came from rabbits and rats. Only 2.7% came from sciurids, such as chipmunks and squirrels (Neibylski et al. 1994). A study conducted in suburban North Carolina concluded that 11% of the blood meals examined came from squirrels with the majority coming from cats and humans (Richards et al. 2006). Because of the Asian tiger mosquito's preference to breed in artificial containers, it is more commonly associated with residential and urban habitats, increasing the possibility of human contact (Barker et al. 2003, Gratz 2004). The adult female is photoperiodically sensitive and will lay diapausing eggs when exposed to short day lengths (Hawley 1988). Diapausing eggs of *Ae. albopictus* are better able to survive winter temperatures compared to non-daipausing eggs, even though *Ae. triseriatus* eggs showed better survivability overall (Hawley et al. 1989). *Aedes*

albopictus is a competent vector for 22 arboviruses and in the U.S., eight arboviruses have been isolated from the mosquito (Eastern Equine encephalitis, Cache Valley, Potosi, West Nile virus (WNV), Jamestown Canyon, Keystone, Tensaw, and LAC) (Gratz 2004). The first field isolation of LAC from *Ae. albopictus* came from naturally infected mosquito eggs in Eastern Tennessee (Gerhardt et al. 2001).

1.1.5 Vectors: *Aedes japonicus*

A third vector is *Aedes japonicus japonicus* (Theobald) (Sardelis et al. 2002), which was first recorded in the U.S. in the late summer of 1998 in New York and New Jersey, and was probably introduced through the used tire trade with Asia (Peyton et al. 1999). Genetic testing determined that some U.S. specimens were genetically close to specimens from western Kyushu and south Honshu, Japan (Fonseca et al. 2001), where *Ae. japonicus* is common (Tanaka et al. 1979). Since its introduction, *Ae. japonicus* has spread rapidly throughout the eastern U.S. and now has been reported in Connecticut (Andreadis et al. 2001), Pennsylvania, Ohio, Maryland (Fonseca et al. 2001), Virginia (Harrison et al. 2002, Grim et al. 2007), West Virginia (Joy 2004), Georgia, and South Carolina (Harrison et al. 2004). Falco et al. (2002) reported that *Ae. japonicus* was found at 97.4% of 39 sites in southern New York. In the northeast U.S., peak collections occur in September (Andreadis et al. 2001). In a study conducted in Southwestern Virginia, *Ae. japonicus* adults were collected in gravid traps from June to September with the largest collections coming during the month of September (Grim et al. 2007). Larvae can be found in both artificial and natural containers such as rock pools, surface water rain pools, subterranean catch basins, tree holes, spring-fed depressions, toys, porcelain bath tubs, wooden barrels, stone flower pots, bird baths, plastic milk cartons, used tires, and vinyl tarpaulins (Andreadis et al. 2001, Scott et al. 2001, Harrison et al. 2002, Kutz et al 2003, Oliver et al. 2003,

Joy 2004). Studies have shown that *Ae. japonicus* females lay desiccation-resistant eggs vertically above the water's surface which can undergo diapause (Juliano and Lounibos 2005, Oliver and Howard 2005). *Aedes japonicus* is an opportunistic feeder, utilizing hosts including avians and mammals such as deer, horses, and humans (Knight 1968, Miyagi 1972, Tanaka et al. 1979, Apperson et al. 2004). Laboratory studies have determined that *Ae. japonicus* is an efficient vector of several arboviruses including WNV (Sardelis and Turell 2001, Turell et al. 2001), Eastern equine encephalitis, and most notably LAC virus (Sardelis et al. 2002). Several field isolations of WNV have come from *Ae. japonicus* (CDC 2009). Recent field isolations of LAC virus from both adult collections and egg collections of *Ae. japonicus* mosquitoes have solidified its status as a vector of the disease and suggests that transovarial transmission occurs in nature (Harris et al. *in review*).

1.1.6 Maintenance cycle

The LAC virus maintenance mechanism and transmission cycle is well documented and the best understood of the CAL viruses. The transmission cycle occurs in two ways, vertically and horizontally (Grimstad 1988). In horizontal transmission the infected mosquito will bite a host and infect it with the virus. The host acts as virus amplifier and reservoir. Two such hosts include the eastern chipmunk (Gauld et al. 1975) and the gray squirrel (Moulton and Thompson 1971). As mentioned previously, *Ae. triseriatus* feeds readily on these amplifying species (Nasci 1985, Richards et al. 2006). Analysis of blood meals from field collected *Ae. albopictus* mosquitoes indicated that feeding on eastern chipmunks did occur which increased the chances of the virus reaching amplifying hosts (Cully et al. 1992). In the lab, *Ae. albopictus* is a more competent vector of LAC virus than *Ae. triseriatus* (Grimstad et al. 1989) but it is unlikely to be efficient in horizontal amplification because it has such a large host range. Unlike *Ae.*

triseriatus, Ae. albopictus will feed on most any mammal, many of which are not reservoir species (Niebylski et al. 1994). However, *Ae. albopictus'* aggressive biting behavior and abundance in urban and suburban habitats may make it an important bridge vector. Lab results for *Ae. japonicus* showed similar transmission rates to that of *Ae. triseriatus* except at low viral titers when *Ae. japonicus* did not develop a disseminated infection (Sardelis et al. 2002). A second form of horizontal amplification involves an infected male mosquito transmitting the virus venereally to an uninfected female mosquito through the semen (Thompson and Beaty 1977).

In vertical transmission, an infected female mosquito transmits the virus transovarially from herself to her progeny (Miller et al. 1977). *Aedes triseriatus* is an important vector in the vertical transmission of the virus; although, Miller et al. (1979) were able to show that an infected female could not pass the virus onto her progeny until the second ovarian cycle. Studies on transovarial transmission rates describe rates as high as 71% and 98%, and filial infection rates ranging between 46% and 71% (Miller et al. 1977, Hughes et al. 2006). When the larvae of transovarially infected *Ae. triseriatus* mosquitoes were raised on nutritionally inadequate diets, the adults showed an increase in infection and transmission rates compared to larvae that were fed on optimal diets. These nutritionally deprived mosquitoes also showed a reduced reproductive capacity and produced a mean of 48.9 eggs compared to 73.3 for the optimal diet mosquitoes (Patrican et al. 1985). Transovarial transmission is important because it allows the virus to survive the winter in the egg (Miller et al. 1979), and can lead to the virus surviving in a population for at least four years without horizontal amplification in vertebrate hosts (Miller et al. 1977). In the lab, *Ae. albopictus* shows high rates of transovarial transmission, but these rates are lower than those of the eastern treehole mosquito (Tesh and Gubler 1975, Cully 1992). Rates

for transovarial transmission were calculated to be 52% and 18% for filial infection rates (Hughes et al. 2006). However, *Ae. albopictus* may contribute to the persistence of LAC virus in a location and possibly increase the rate of incidence.

1.1.7 Infection of vectors

The LAC virus infection cycle begins with virus uptake when a mosquito feeds on an infected host. The extrinsic incubation period begins from ingestion of the virus to the time the mosquito is capable of transmitting the virus. Virus first infects and replicates in the anterior portion of the midgut of the mosquito, and can be found in this region within three days after the initial digestion (Higgs and Beaty 2005). At this point the mosquito may or may not develop a disseminated infection depending on whether the virus makes it through the midgut escape barrier (Paulson et al 1991). Research by Paulson et al. (1989) demonstrates that the majority of *Ae. triseriatus* females develop a midgut infection; however a significant proportion may fail to develop a disseminated infection. Research also indicates that nutritionally deprived *Ae. triseriatus* larvae will increase the likelihood of the adult developing a disseminated infection because of physical differences in the basement membrane in the midgut, which normally acts as a physical barrier to the virus (Grimstad and Walker 1991). The numbers for *Ae. albopictus* are relatively the same as *Ae. triseriatus* when developing a disseminated infection (Grimstad et al. 1989). After the virus disseminates into the hemocoel it can then infect secondary organs. Within nine days the virus can be found in the heart, fat body, nervous tissue, and ovaries of the mosquito (Higgs and Beaty 2005). Approximately twelve days after, the virus disseminates into the salivary glands and the extrinsic incubation period ends. From day fifteen through the end of life, the mosquito may transmit the virus to vertebrate hosts during blood feeding.

1.1.8 Infection of mammals

To demonstrate replication in mammals, the laboratory mouse has been used during experimentation. As with humans, younger mice are more susceptible to infection (Johnson 1983). After subcutaneous injections, the virus undergoes an extraneural phase of replication when it first invades and replicates in muscle tissues, including striated, cardiac, and smooth. Following replication in the skeletal muscles the virus invades the plasma where it can reach a high titer before somehow crossing the blood-brain barrier and invading the neurons. The subsequent invasion of the central nervous system can lead to La Crosse encephalitis (Borucki et al. 2002). Methods for detecting infection include neutralization, hemagglutination-inhibition, complement fixation, or indirect fluorescent antibody assays. Different tests allow for the virus to be detected during infection or post-infection. Some tests work best within the first week while others may be able to detect the virus for several years (Rust et al. 1999).

1.1.9 Clinical studies

Jones et al. (1999) studied 27 patients, in Tennessee, with illnesses consistent with LAC encephalitis and found that a minimum of 10 to be positive. Seven of the ten patients were boys with ages ranging between 3 and 14 years. Signs and symptoms associated with patients included headaches, seizures, altered consciousness, and abnormal electroencephalograms. Several of the patients' homes were assessed for vector habitats and both permanent and disposable containers were found in the yards.

A study of six patients in Ohio suggested that neurologic sequelae may be more commonly associated with the encephalitis than originally thought. All six patients were boys and ranged in age from 9 months to 15 years. Neurological examinations during admittance to the hospital included disorientation, unsteady gait, lethargy, and seizures. Information on neurological problems were recorded one year later and found that several patients still suffered

from seizures and one had been diagnosed with attention deficit hyperactivity disorder (Balkhy et al. 2000).

McJunkin et al. (2001) studied 127 patients in Charleston, West Virginia between 1987 and 1996. Patients age ranged from 5 months to 15 years with a mean of 7.8 and 71% were boys. September was the month that 35% of the cases were reported, which was the most of any month. Upon admission, presented with 83% headaches, 70% vomiting, 42% disorientation, and 46% seizures. A total of 90 patients underwent electroencephalograms with 71% producing some form of abnormal tracings. Patient care included hospital admission to the pediatric intensive care unit, including intubation. The average number of days of hospitalization required was 6.2.

Humans are considered to be a dead end host. They can become infected, but do not develop sufficient viremia to allow for the passage of the virus to uninfected mosquitoes. Because humans are a "throw-away society," leaving artificial containers near their dwellings, they create a perfect habitat for the vectors. This environment and increased outdoor activities place humans into direct contact with LAC virus vectors (Grimstad 1989).

1.2.1 Vector collection methods

Mosquito based arboviral surveillance relies on the ability to collect virus infected mosquitoes through various trapping methods at different stages of the insect's life. Collections can come from simple techniques of sampling for larvae and adults or through the use of expensive traps using attractants such as light, CO_2, various chemicals, or organic infusions. Gravid traps require a battery operated fan and the use of an infusion to attract mosquitoes but have an advantage because the trap is collecting female mosquitoes that have presumably taken a blood meal increasing the likelihood of isolating an arbovirus (Reiter 1983). Light traps require

the use of a battery operated fan and generally use an incandescent light bulb as an attractant in combination with CO_2 or octenol (Burkett et al. 2001). Traps baited with CO_2 have been demonstrated to be effective at collecting host-seeking females (Reisen et al. 1990). The disadvantage with light trap collections is the majority of the specimens are nulliparous females and thus are unlikely to be infected with a virus unless through transovarial transmission (Reiter 1983). Since gravid traps and light traps attempt to collect mosquitoes at different biological stages of adulthood, both trap types can be operated concurrently at the same site (Meyer 1991). With any traps that use fans during operation, problems arise with damaged specimens, which make identification problematic (Saul et al. 1977, Apperson et al. 2002). Since all three vectors of LAC virus are container breeders, the use of ovitraps are an effective way to collect these species (Beehler 1991, Barker et al. 2003, Sardelis and Turell 2001). By placing 400 ml beverage cups, containing an oviposition substrate and water, at suitable habitats, an artificial oviposition site is created. These sites can be removed weekly so that eggs can be counted and reared to adults (Loor and DeFoliart 1969).

Additional collection methods have been used to collect mosquitoes including resting catches of adults in red boxes and natural shelters (Loomis and Green 1959, Edman et al. 1968, Komar et al. 1995), battery powered aspirators for adults (Bailey 1966, Davis and Gould 1973, Meek et al. 1985, Perdew and Meek 1990), and larval sampling (Pantuwatana et al. 1974, Beaty and Thompson 1975, Service 1976, Kappus et al. 1982, Barker et al. 2003).

During mosquito collections, LAC virus isolations have come from gravid traps (Scheidler et al. 2006), light traps (Berry et al. 1975), CO_2-baited traps (Thompson et al. 1972), aspiration (Thompson et al. 1972, Berry et al. 1975, Nasci et al. 2000, Barker et al. 2003), oviposition traps (Clark et al. 1982, Szumlas et al. 1996c, Nasci et al. 2000, Gerhardt et al.

2001), and larval sampling (Pantuwatana et al. 1974, Beaty and Thompson 1975, Kappus et al. 1982, Barker et al. 2003). Each trapping method varies in the amount of time required to operate the trap, collect specimens, and time spent in the laboratory identifying the specimens. Traps that collect adults, such as light traps, gravid traps, and CO_2-baited traps, involve collections every 24 hours and large amounts of equipment. The collection of adults require less laboratory time because they can be immediately identified to species and tested for virus. In contrast, oviposition traps require less field time, with collections once a week or less but require more laboratory time and space to rear adults from eggs.

1.2.2 Calculation of infection rates

Collections of mosquitoes are often large and require species to be pooled together and tested for virus to determine the infection rate of field populations. The minimum infection rate (MIR) determines infection rates from pooled samples by calculating the ratio of the number of positive pools to the total number of mosquitoes tested. A second method is the maximum likelihood estimation (MLE) (Chiang and Reeves 1962). The MLE's calculations are similar to MIR but the results may be more accurate, depending on samples, and relaxes the constraints of the MIR (Walter et al. 1980). The MIR generally requires a sample size of >1,000 mosquitoes and when the sample size is <1,000 mosquitoes, it is suggested that MLE be used (Condotta et al. 2004). Gu et al. (2003) noted that the MIR estimates the lower bound of the infection rate and the MLE estimates the infection rate itself. To correct for these problems the authors wrote a computer program, maximum likelihood estimation of infection rates (MLE-IR) which takes into account unequal pool sizes. Biggerstaff (2006) developed the pooled infrate program for the Centers for Disease Control which is similar to Gu et al. (2003) program. Without the ability to test every mosquito individually, it is not possible to determine how many positive mosquitoes

there are in a positive pool. These programs assume that there is at least one positive mosquito in a positive pool. The use of computers allows for input into the equation that may not have been possible if calculated by hand.

1.3.1 Mosquito blood-feeding behavior

The normal sequence in mosquito blood-feeding behavior is host seeking, probing to locate blood, and then imbibing blood. Each of these steps is a separate behavior and may be subject to different modes of control. Once a female reaches a critical volume of blood it will stop feeding. Stretch receptors in the abdomen play a key role in determining when a mosquito is fully engorged and to stop feeding (Gwadz 1969). This was discovered in an experiment using *Ae. aegypti* females when the ventral nerve cord was cut anterior to the abdominal ganglia. The study found that females took unusually large blood meals when the ventral nerve cord was cut anterior to the second abdominal ganglion. The amount of blood ingested was significant but less pronounced than the amount imbibed when the nerve cord was cut. From these results it was determined that cutting the ventral nerve cord anterior to the second abdominal ganglia did not allow a signal to make it to the brain signifying a full blood meal had been imbibed and to stop feeding.

Once the mosquito reaches a threshold volume of blood it will start the ovarian cycle. This critical volume of blood will cause the mosquito to stop host-seeking and find a secluded area while its eggs are being produced. Klowden and Lea (1978) found that *Ae. aegypti* mosquitoes would continue host-seeking one hour after a partial blood meal was taken. Their data showed that the mosquitoes would continue host seeking until a critical volume of blood was ingested. For larger mosquitoes a blood meal of 2.5 μl or larger showed a sharp decline in the tendency of the mosquitoes to respond. At a blood meal of 4.0 μl or more, virtually none of

the mosquitoes were attracted to the host. In the case of *Ae. aegypti*, once a female has taken a blood meal, changes in hormone levels leads to the inhibition of host seeking (i.e., becoming less sensitive to host seeking cues) and the initiation of ovisite selection (i.e., increased sensitivity to oviposition site attractants) (Davis and Takahashi 1980).

1.3.2 Blood-feeding behavior of parasite infected vectors

Numerous accounts of parasite-vector systems have had effects on the vector's flight ability, fecundity, or gonotrophic concordance (Molyneux and Jeffries 1986). Different parasites have been shown to affect the blood-feeding ability of the vector. For instance, Jenni et al. (1980) observed differences in the feeding behavior of tsetse flies infected with salivarian trypanosomes compared to non-infected flies. They found that the trypanosome-infected flies fed more voraciously and probed more frequently than the non-infected ones. It was thought that the infection disrupted the function of the labral mechanoreceptors to detect the rate of blood flow. Añez and East (1984) looked at the effect of infection with *Trypanosoma rangeli* Tejeraon triatomine bugs. They found that infected bugs probed more (approx. 6X more) and engorged less than uninfected controls. They also showed that engorgement was not necessary for transmission of the parasite when two infected bugs did not take any blood. The mice they probed showed high parasitaemia. Thus, infected bugs were more likely to feed on multiple hosts, increasing parasite transmission.

1.3.3 Blood-feeding behavior of mosquitoes infected with malaria

Malaria infection has been shown to affect both blood-feeding and host seeking in mosquitoes. When *Ae. aegypti* mosquitoes were infected with malaria they found an increased intradermal probing time by the infected mosquitoes during later feedings. The sporozoites destroyed segments of the salivary glands impairing the mosquito's ability to locate blood. This

led to increased probing time and increased the probability of host contact (Rossignol et al. 1984). Wekesa et al. (1992) had the same results with naturally infected *Anopheles* mosquitoes. Infected mosquitoes probed more often and for a longer time than did the uninfected ones. When *Ae. aegypti* infected with *Plasmodium gallinaceum* were placed in an olfactometer, Rossignol et al. (1986) found the infected group exhibited a significant increase in olfactometer response compared to uninfected controls. They found this by comparing the relative daily biting rates of the mosquitoes for five days and concluded that transmission was enhanced because infected mosquitoes may take more attempts at probing before being successful when host contact is limited.

1.3.4 Blood-feeding behavior of mosquitoes infected with dengue

Virus infection also has been documented to affect mosquito blood-feeding. Platt et al. (1997) found that *Ae. aegypti* mosquitoes, infected with dengue 3, took significantly longer to complete feeding than did ones not infected. The infected mosquitoes took approximately 700 seconds longer to complete feeding when compared to the non-infected. Eight days after the infection, the virus had disseminated to tissues throughout the body. Infection of certain tissues may have affected the feeding behavior by extending the periods of probing which can lead to enhanced transmission of the virus.

1.3.5 Blood-feeding behavior of mosquitoes infected with LAC virus

For LAC virus, transmission trials for 20 different strains of *Ae. triseriatus* were studied to examine their feeding behaviors. While the females refed, the number of probes and the amount of blood imbibed was recorded. It was found that infected mosquitoes tended to engorge less and probe more than non-infected mosquitoes. In one group of infected mosquitoes 79% made multiple probes to obtain a partial blood meal and 21% took a partial blood meal with only

one probe. In contrast, 52% of uninfected mosquitoes fully engorged with only one probe and 48% took multiple probes to become fully engorged (Grimstad et al. 1980). However, Paulson et al. (1991) showed that when *Ae. triseriatus* was infected with LAC virus it had no effect on its ability to locate blood. So the effect is probably neurological and not due to the impairment of salivary gland functions. Appelbaum (Thesis, 1992) demonstrated that *Ae. triseriatus* mosquitoes, infected parentally with LAC virus, resulted in mean weights after blood feeding, that were significantly lower than the uninfected mosquitoes.

1.4.1 Pre-oviposition behavior of mosquitoes

Pre-oviposition behavior of gravid female mosquitoes includes ranging flight, orientation, encounter, acceptance, and surface evaluation (Clements 1999). Oviposition site selection by a gravid mosquito is determined by three major factors, physical, chemical, and physiological (Bentley and Day 1989). Physical factors include color, presence of vegetation, reflectance, and nature of the substrate (Kennedy 1942, Fay and Perry 1965, Millar et al. 1992). Insemination and nutritional status are physiological factors which could determine ovipositional behavior (Bentley and Day 1989). Chemical attractants or stimulants include those from decaying organic matter, mosquito eggs, or mosquito larvae (Bentley et al. 1981, Millar et al. 1992, Ganesan et al. 2006). All of these factors fall into one of four categories; attractant, arrestant, deterrent or stimulant (Clements 1999).

1.4.2 Oviposition attractants

Oviposition attractants and stimulants that may elicit oviposition site selection are generally due to an interaction of chemical and physical factors. Most of the studies come from laboratory experiments focusing on chemical attractants emanating from the break down of

organic matter, egg substances, larval substances, pupal substances or other organic compounds (Bentley and Day 1989).

1.4.3 Eggs as ovipositional attractants

Studies have demonstrated that an oviposition pheromone exists which is associated with the egg rafts of some *Culex* mosquitoes (Osgood 1971, Starratt and Osgood 1972, 1973, Dadd and Klienjan 1974). It was later determined that the attraction was associated with an apical droplet of the egg, which formed on the top of the egg after it was deposited (Bruno and Laurence 1979, Laurence and Pickett 1982). Gubler (1971) found that *Aedes polynesiensis* preferred waters that had previously contained eggs of *Ae. albopictus* rather than its own, but this may be due to an attractant released by the ovipositing female. In laboratory experiments *Ae. aegypti* females laid significantly more eggs on papers containing conspecific eggs or *Ae. albopictus* eggs when compared to papers without eggs, while *Ae. albopictus* showed no preference (Allan and Kline 1998). In contrast, Chadee (1993) conducted experiments in the field and found that *Ae. aegypti* females laid significantly more eggs in ovitraps without conspecific eggs and preferred substrates with less than 25 eggs when compared to those containing more. Ganesan et al. (2006) isolated and identified several compounds, through gas chromatography-mass spectrometry, which showed a positive ovipositional response by *Ae. aegypti*, with a significant response due to dodecanoic and (Z)-9-hexadecenoic acids. In laboratory experiments a significant increase in oviposition by *Ae. triseriatus* was demonstrated when 69% of eggs laid were in cups containing conspecific eggs (Beehler 1991). During field experiments, Kitron et al. (1989) found that *Ae. triseriatus* females showed a decline in oviposition when conspecific eggs were present, while Bentley et al. (1976) concluded that acetone extracts of eggs do not contain an oviposition attractant during laboratory experiments.

1.4.4 Larvae as ovipositional attractants

Water that contains or has contained immature mosquitoes has been shown to be highly attractive across many genera. This has been documented with the larvae of *Culex pipiens* (Ikeshoji 1966), *Ae. atropalpus* (Kalpage and Brust 1973, Maire 1985), *Ae. togoi* (Trimble and Wellington 1980), *Ae. aegypti* (Soman and Rueben 1970, Roberts and Hsi 1977), *Ae. albopictus* (Gubler 1971, Allan and Kline 1998), *Ae. triseriatus* (Bentley et al. 1976, McDaniel et al. 1976, 1979). In the case of *Culex pipiens quinquefasciatus* and *Culiseta incidens* field studies demonstrated that both species showed a significant ovipositional preference for containers containing conspecific larvae (Wilmot et al. 1987). In both field and laboratory experiments, *Ae aegypti* gravid females preferred to oviposit in water which contained conspecific 4th instar larvae when compared to control water, although when larvae were removed there was no preference (Soman and Reuben 1970). *Aedes albopictus* gravid females showed a 17.6% increase to conspecific larval water and a 15.1% increase to *Ae. aegypti* larval water when compared with controls (Allan and Kline 1998). Gubler (1971) had similar results with *Ae. albopictus* as well as *Ae. polynesiensis*, although when larvae and pupae where washed and placed in fresh water there was no effect on ovipositing females. Even when contact chemoreception was prohibited, *Ae. triseriatus* showed a significant preference for larval holding water (Bentley et al. 1976). While it was originally thought that attraction to larval water by gravid females was due to components of the gut contents or waste from the larvae, McDaniel et al. (1979) concluded that the attractant is a pheromone produced as a secondary metabolite. This was accomplished by keeping the larvae in water containing excess kaolin to flush the gut contents.

1.5.1 Introduction of invasive species

Aedes japonicus is the latest of several successful mosquito invasions in the U.S. from human transport of the immature stages including that of *Ae. albopictus* and *Ae. bahamensis* (Lounibos 2002). A species must first be introduced into a region, then establish itself, and finally be able to spread before it is considered a biological invasion (Lounibos 2002). *Aedes japonicus* has demonstrated all three of the above mentioned stages (Falco et al. 2002). A consequence of invasion by a species may be an impact on human and animal health (Juliano and Lounibos 2005).

In order for a species to become established, it must first overcome several possible negative effects on its population growth. These negative effects include resource competition, chemical or physical interference, mating interference, and predation (Juliano and Lounibos 2005). Both resource competition and predation are important limiting factors and can allow the introduced species to flourish, possibly taking over the niche of native species, or be added as another local species. Either of these situations could have a major effect on disease transmission by either introducing a more efficient vector or by replacing the major vector with a less efficient one (Juliano and Lounibos 2005).

1.5.2 Introduced species and competition

Since *Ae. japonicus* has become established on the East coast of the U.S. (Andreadis et al. 2001, Fonseca et al. 2001, Harrison et al. 2002, Joy 2004), it is likely that *Ae. japonicus* will compete for resources with other native or introduced species. Other introduced species have fared well when challenged with interspecific competition, the most well known being the introduction of *Ae. albopictus* which has since excluded *Ae. aegypti* from some areas of the U.S. (Barrera 1996, Juliano 1998, Juliano et al. 2004, Yee et al. 2004).

After the introduction of *Ae. albopictus* in 1985 (Hawley et al. 1988) there has been a decline or even extinction of *Ae. aegypti* mosquitoes in some areas of the U.S. (Juliano et al. 2004). Early studies showed that *Ae. aegypti* was a better resource competitor than *Ae. albopictus* when given artificial diets (Black et al. 1989, Ho et al. 1989). More recent studies have contradicted those conclusions when experiments were conducted in the field or in the lab with nutrients, like leaf litter, commonly found in the mosquito's habitat (Barrera 1996, Juliano 1998, Daugherty et al. 2000, Braks et al. 2004). Field studies conducted in South Florida using water-filled tires showed a competitive advantage for *Ae. albopictus* over *Ae. aegypti* at lower oak leaf litter amounts and at higher combined population densities (Juliano 1998). Barrera (1996) had similar results when using leaf litter in laboratory experiments. As part of the former study, the hypothesis, more successful larvae store larger energy reserves and resist the lack of food longer, was tested and showed that leaf litter fed *Ae. albopictus* faired better than *Ae. aegypti* and *Ae. triseriatus* when starved. Braks et al. (2004) tested seven combinations: 0-20, 0-40, 0-60, 20-40, 40-20, 20-20, and 30-30, of *Ae. albopictus* and *Ae. aegypti* larvae as well as two food regimes of 0.25 and 0.50 g of leaf litter. Results of the experiment showed that for all treatments and leaf litter amounts, *Ae. albopictus* showed higher survivorship than *Ae. aegypti*. Survivorship studies of *Ae. albopictus* and *Ae. aegypti* was recently revisited when Yee et al. (2004) tested their survival of larvae under food-limited conditions and obtained results that agreed with the previous studies. One important finding of Yee et al. (2004) was that *Ae. albopictus* spent more time feeding on the leaf surface unlike *Ae. aegypti* which spent more time filtering the water. The foraging behavior of *Ae. albopictus* may contribute to its competitive advantage.

Livdahl and Willey (1991) hypothesized that after the exclusion of *Ae. aegypti*, *Ae. albopictus* would eventually have a similar effect on native mosquito species like *Ae. triseriatus* in certain habitats because the two would share larval habitats in tires and water-filled treeholes. To test this prediction under laboratory conditions, the authors varied densities of the two species in fluid from treeholes and tires obtained from the field. They determined that the two species would continue to exist in a stable coexistence in treehole communities but there may be a decline of *Ae. triseriatus* in tire habitats. Lounibos et al. (2001) tested the same theory in the field at sites with treeholes, tires, and cemetery vases in south Florida. From their observations, they concluded that *Ae. albopictus* did not exclude *Ae. triseriatus* from treeholes but did have a negative effect on *Ae. triseriatus* in used tires and cemetery vases, but not enough for exclusion of the species.

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Chapter 2

Oviposition Preferences of Three Container-Breeding Mosquitoes and the Possible Implications for La Crosse Virus Transmission

2. 1 Abstract

Studies were conducted on *Aedes japonicus*, *Aedes triseriatus*, and *Aedes albopictus* to determine their ovipositional preferences when presented with preexisting eggs, 1st instar larvae, and larval rearing water of conspecific and heterospecific mosquitoes. Laboratory experiments showed that *Ae. japonicus* deposited significantly more eggs in cups containing preexisting eggs of conspecifics and *Ae. albopictus*. *Ae. japonicus* laid significantly more eggs in cups containing conspecific 1st instar larvae compared with control cups and those of *Ae. albopictus*. When presented with larval rearing waters, *Ae. japonicus* showed a significant preference for the water used for rearing conspecifics and *Ae. albopictus*. *Ae. triseriatus* deposited significantly more eggs in cups containing conspecific 1st instar larvae compared to *Ae. albopictus* and controls and when presented with larval rearing waters showed a strong preference for conspecific and *Ae. albopictus*. *Ae albopictus* showed no significance, except for cups containing larval rearing waters of all three species compared to controls. Data collected in the field on species oviposition preferences using fresh oviposition papers and analyzed to quantify interspecific association showed there were significant positive associations between *Ae. triseriatus* and both *Ae. japonicus* and *Ae. albopictus*. However the positive associations may have been due to the fact that *Ae. triseriatus* comprised nearly 70% of the eggs collected and the other species may not have been able to locate sites void of *Ae. triseriatus*. In a second field study conducted with papers containing preexisting eggs no significant differences in oviposition preferences were detected. A trend was detected by *Ae. japonicus* in which fewer cups containing conspecific eggs were oviposited in, while the mean intensity of eggs deposited was still high. All three

species are vectors of La Crosse virus and all showed significant ovipositional preferences. Selection of oviposition sites based on the presence or absence of the other species could alter the distribution of certain species, inadvertently changing the range of La Crosse virus.

2.2 Introduction

La Crosse (LAC) virus infection is one of the most commonly reported arboviral diseases and is, therefore, an important public health problem in the United States (Rust et al. 1999). Most cases occur in children under the age of 15 who devlelop symptoms such as febrile illness, vomiting, and headache with some cases progressing to seizures (McJunkin et al. 1998). The disease is endemic in the eastern U.S. with approximately 81 confirmed and probable cases per year reported between 1964 and 2007 (CDC 2008). Because the mild flu-like symptoms associated with LAC virus are often subclinical or misdiagnosed, estimates have been as high 300,000 infections per year (Rust et al. 1999).

The primary vector of LAC virus is *Aedes triseriatus* (Say), commonly called the eastern treehole mosquito (Berry et al. 1974, Watts et al., 1974). *Aedes albopictus* Skuse, which was introduced to the U.S. in 1985 (Hawley 1988) is considered a possible accessory vector of the virus. This species has been shown to be a competent vector in the laboratory (Grimstad et al. 1989) and there have been isolations of the virus from naturally infected *Ae. albopictus* mosquitoes (Gerhardt et al. 2001). Another possible accessory vector is *Aedes japonicus* (Theobald), which laboratory tests have demonstrated can become infected and transmit LAC virus at rates similar to *Ae. triseriatus* (Sardelis et al. 2002). Isolations of LAC virus have also come from the recently introduced species, *Ae. japonicus* collected as eggs and adults (Harris et al., *in review*).

All three vector species are container-breeders ovipositing in a wide range of natural and artificial container habitats (Hawley 1988, Beehler 1991, Andreadis et al. 2001), laying desiccation-resistant eggs, and overwintering as diapausing eggs in the more northern parts of their distributions (Shroyer and Craig 1980, Hawley 1988, Lounibos 2002). Also, the three

vectors are all daytime feeders that utilize a variety of hosts including humans (Nasci 1985, Hawley 1988, Andreadis et al. 2001). The two exotic species, *Ae. albopictus* and *Ae. japonicus*, as well as the native *Ae. triseriatus*, are known to occur throughout the Appalachian region of the U.S. (Joy 2004, Joy and Sullivan 2005, Bevins 2007).

While conducting larval surveys, Bevins (2007) found *Ae. japonicus* co-occurring with both *Ae. triseriatus* and *Ae. albopictus* in the same container; O'Meara et al. (1993) also found containers positive for both *Ae. triseriatus* and *Ae. albopictus*. During local ovitrap studies, eggs of all three species were found together on the same oviposition substrate (Jackson et al. unpublished data). The utilization of the same sites for oviposition can lead to competitive interactions among ovipositing females and the egg, larval, or pupal stages of heterospecific and conspecific mosquitoes. Oviposition site selection is influenced by both physical and chemical factors (Bentley and Day 1989) and chemical cues produced by different life stages of mosquitoes can lead to acceptance or avoidance of certain oviposition sites. For example, the presence of conspecific eggs acted as a deterrent to ovipositing *Ae. triseriatus* (Kitron et al. 1989), while *Ae. albopictus* females appear to be unaffected by the presence of either conspecific eggs or eggs of *Aedes aegypti* (Skuse) (Allan and Kline 1998). In laboratory experiments, *Ae. aegypti* was found to prefer oviposition substrates containing conspecific and *Ae. albopictus* eggs (Allan and Kline 1998); field studies have also shown that this species prefers sites with conspecific eggs (Chadee 1993). Another species, *Aedes polynesiensis* Marks had a preference for water that had previously held *Ae. albopictus* eggs (Gubler 1971). Finally, larval water from conspecifics or heterospecifics was shown to act as a stimulant for *Ae. triseriatus* (Bentley et al. 1976), *Ae. albopictus* (Allan and Kline 1998, Trexler et al. 2003), *Ae. aegypti* (Soman and Rueben 1970, Chadee 1993, Allan and Kline 1998), *Aedes atropalpus* (Coquillett) (Kalpage and

Brust 1973), *Aedes togoi* (Theoblad) (Trimbel and Wellington 1980), and *Aedes fluviatilis* (Lutz) (Consoli and Teixeira 1988).

The purpose of these studies was to determine whether oviposition site selection by *Ae. albopictus, Ae. japonicus,* and *Ae. triseriatus* is influenced by the presence or absence of other container breeding mosquito species. Ovipositional preferences could affect comparative distribution and abundance of the above mentioned species, as well as the impact the epidemiology of LAC virus.

2.3 Materials and methods

2.3.1 Laboratory studies. Experiments were conducted to determine the effect that the presence of preexisting eggs, 1st instar larvae, and larval rearing water (LRW) has on the oviposition behavior of *Ae. albopictus*, *Ae. japonicus*, and *Ae. triseriatus.* For each experiment, 20 to 30 blood fed females of each species were placed into individual 45.7 cm^3 cages and held for seven days. Black plastic cups (11 x 9 cm) (WNA, Inc. (SKU 16SPOLY), Covington, KY) lined with a 25 x 5 cm piece of seed germination paper (Anchor Paper Company (No. SD 3815L), Saint Paul, MN) placed horizontally around the inside of the cup were provided as an oviposition substrate. In each cage, oviposition cups containing one of four choices (*Ae. albopictus, Ae. japonicus, Ae. triseriatus* or a control cup) were randomly placed in the four corners of the cage and rotated daily for seven days. During the rotation of cups, each was inspected for the presence of dead adults, which were removed. Cups were marked for individual species' use, so no cross contamination would occur between experiments. A piece of apple placed in the middle of the cage served as a sugar source. Each experiment had three to four replications and was conducted in an environmental chamber maintained at 24°C, 75% RH and 16L:8D photoperiod.

Experiment 1 had the following four choices: Seed germination paper with 60 preexisting eggs of either *Ae. albopictus*, *Ae. japonicus* or *Ae. triseriatus,* and a control cup with no eggs. Papers were prepared by cutting a section of seed germination paper with 60 eggs obtained from a lab colony of the desired species and then stapling it to the test strip. A random size piece of seed germination paper without eggs was stapled to the control test strip. Each cup was filled with 150 ml of deionized water (DI) and 2.5 ml of a 500 ml/7.5 g bovine liver powder solution.

Experiment 2 had the following four choices: Cups filled with 150 ml of DI water, 2.5 ml of bovine liver powder solution, and 60 1st instar larvae of either *Ae. albopictus*, *Ae. japonicus* or *Ae. triseriatus,* and a control cup with no larvae.

Experiment 3 had the following four choices: Cups containing LRW of either *Ae. albopictus*, *Ae. japonicus* or *Ae. triseriatus*, and a control cup filled with DI water. Added to all four cups was 2.5 ml of bovine liver powder solution.

To create the LRW, two hundred larvae were reared in a 33 x 17.5 x 11 cm (L x W x H) plastic shoe box with 1600 ml of DI water and 45 ml of bovine liver powder solution. After 5 days an additional 30 ml of liver powder solution was added to each larval container. Once all the mosquitoes had reached the adult stage, the LRW water was then strained to remove any dead larvae or pupae and exuviae.

2.3.2 Field studies

New oviposition paper

During the summer of 2007, oviposition cups (Loor and DeFoliart 1969) were used to collect eggs of mosquitoes in Wise County, VA. Cups were similar to the ones used in the laboratory experiments with the addition of two holes drilled on opposite sides of cup to prevent overflow during rainfall. Oviposition cups contained a 12.5 x 5.0 cm piece of seed germination paper hung vertically and secured with a small binder clip. A total of 200 ml of DI water was added to each cup. Cups were attached to the top of 0.7 m wooden stakes. A total of 32 cups were placed at 10 sites in hardwood forests. Collections occurred three times throughout the summer (July 7, August 3, and September 7).

Papers were removed from the cups after seven days. Eggs were counted to species and hatched to confirm that species counts were correct.

Oviposition paper with preexisting eggs

This part of the study was conducted in September of 2008 in a hardwood forested area on university property, located in Blacksburg, VA. Forty-eight oviposition cups, similar to ones used in the 2007 field experiments, were placed along a road cutting through the forest. Cups were placed on 0.7 m high wooden stakes and divided evenly between each side of the road for a total of 26 within 0.6 m of the road and 24 cups ≈6.0 m from the edge of the road. Cups were ≈15 m apart. Seed germination papers (12.5 x 5.0 cm) containing either eggs of one of the three test species or a control paper, containing no eggs, were randomly placed in the cups, which were then filled with DI water. Papers with preexisting eggs came from lab colonies. Cups were checked daily to ensure that the water level was adequate. After nine days the papers were removed and the newly laid eggs were counted by species. Eggs on ten of the papers were hatched and reared to adults to verify species identifications. To determine whether the number

of eggs had any effect on oviposition, we used papers with a range of egg densities. *Aedes triseriatus* had papers with egg numbers ranging between 43 and 573, *Ae. japonicus* between 44 and 658, and *Ae. albopictus* between 89 and 374. At the start of the experiment, there were 12 egg papers from each species and 12 control papers. However, two of the cups containing papers with preexisting *Ae. triseriatus* eggs were destroyed in the field, probably by a small mammal.

2.3.3 Colonies. *Aedes albopictus* were derived from Wise County, VA in 2006, *Ae. triseriatus* from Blacksburg, VA, in 2007 and *Ae. japonicus* from a laboratory colony maintained at Rutgers University. Rearing conditions were 24°C, 75% RH, and 16L:8D photoperiod. All larvae were fed a bovine liver powder (500 ml/7.5 g) solution. Adults were fed slices of apple as a sugar source and mice or quail were used as a blood source.

2.3.4 Statistics. All laboratory experiments were analyzed using one-way analysis of variance (ANOVA) (Prism, GraphPad Software, Inc., San Diego, CA 2007) after the data were arcsin $\sqrt{Y}$ transformed because data were analyzed as proportions. Tukey's multiple comparison test was performed as a post-hoc test. The significance level for all test was set at $\alpha = 0.05$. For display purposes data are presented in the original form as percentages. The 2007 field data were analyzed using the coefficients of interspecific association (C_8), which assigns a value between +1 and -1 to indicate whether the association is positive or negative (Hurlbert 1969). Significant differences between the associations was determined using a χ^2 test (Prism, GraphPad Software, Inc., San Diego, CA 2007). The 2008 field data was analyzed to determine the mean intensity and was calculated by dividing the total number of eggs in traps by the number of traps positive for freshly laid eggs for a species (Kitron et al. 1989).

2.4 Results

2.4.1 Laboratory studies

Experiment 1: Preexisting eggs

Aedes japonicus females oviposited significantly more eggs in cups containing conspecific eggs and *Ae. albopictus* eggs than in cups containing *Ae. triseriatus* eggs ($P<0.05$) (Fig. 2.1a). A total of 1,303 eggs were deposited by *Ae. japonicus* females, with 67% in conspecific cups and cups with *Ae. albopictus* eggs. Females of *Ae. albopictus* and *Ae. triseriatus* showed no preference when ovipositing in cups containing preexisting eggs (Figs. 2.2a and 2.3a).

Experiment 2: 1st Instar larvae

The presence of *Ae. albopictus* larvae acted as a deterrent for both *Ae. japonicus* (Fig. 2.1b) and *Ae. triseriatus* (Fig. 2.3b) females when ovipositing. *Aedes japonicus* deposited 37% of the 2,578 total eggs in cups containing conspecific larvae, 19% in control cups, and 18% in cups containing *Ae. albopictus* larvae (Fig. 2.1b). A significant difference was detected between the number of eggs laid in cups containing conspecifics and control cups ($P<0.05$), and a highly significant difference in oviposition was observed between conspecifics and *Ae. albopictus* ($P<0.01$).

There was little variation in the number of eggs deposited by *Ae. albopictus* females in cups containing 1st instar larvae of any of the three species (Fig. 2.2b). A total of 6,645 eggs were deposited with only a 10% difference between cups containing the highest number of eggs (*Ae. japonicus*, 31%), and the cups containing the fewest eggs (*Ae. triseriatus*, 21%).

Of the 4,531 eggs laid by *Ae. triseriatus*, 63% were in cups containing 1st instar larvae of conspecifics and *Ae. japonicus* (Fig. 2.3b). Significantly more eggs were laid in *Ae. triseriatus* cups versus *Ae. albopictus*, and in *Ae. japonicus* cups versus control cups ($P<0.05$). *Aedes triseriatus* females also showed a strong preference for cups containing conspecifics when compared with control cups ($P<0.01$).

Experiment 3: LRW

Some of the strongest preferences by females were observed during oviposition experiments with LRW. *Aedes japonicus* females oviposited 1,081 eggs with the majority of eggs in conspecific cups (55%) (Fig. 2.1c). Significantly more eggs were laid in cups containing conspecific LRW compared to *Ae. triseriatus* LRW and control cups ($P<0.05$).

Over 5,300 eggs were deposited by *Ae. albopictus* females, with 89% coming from cups which contained conspecific and heterospecific LRW (Fig. 2.2c). Significantly more eggs were laid in both conspecifics and *Ae. japonicus* cups versus control cups ($P<0.01$). A highly significant difference was detected between the number of *Ae. albopictus* eggs in *Ae. triseriatus* cups versus control cups ($P<0.001$).

A total of 3,200 eggs were deposited by *Ae. triseriatus* females, with 44% coming from conspecific LRW and only 5% in the *Ae. japonicus* LRW (Fig. 2.3c). Significantly more eggs were laid in cups containing conspecific LRW compared with control cups ($P<0.05$) and *Ae. japonicus* LRW ($P<0.01$). *Aedes triseriatus* females deposited significantly more eggs in cups containing LRW of *Ae. albopictus* when compared with control cups and *Ae. japonicus* cups ($P<0.05$).

2.4.2 Field studies

New oviposition paper

A total of 10,622 eggs were identified to species with the majority of eggs coming from *Ae. triseriatus* (Fig. 2.4). Of the three C_8 comparisons, two had significant associations. Both *Ae. japonicus* and *Ae. albopictus* were positively associated with *Ae. triseriatus* (Table 2.1).

Oviposition paper with preexisting eggs

While we did not detect any significance differences in the intensity of oviposition with field collected data using paper with preexisting eggs, we did notice a trend, which was similar to our laboratory tests. The mean intensity of eggs laid by *Ae. japonicus* was highest in the control cup followed by *Ae. albopictus*, *Ae. japonicus*, and *Ae. triseriatus* (160, 144, 143, and 101, respectively) (Fig. 2.5a). Also, the range in numbers of preexisting eggs had no effect on the preferences of ovipositing females.

2.5 Discussion

Discrimination among oviposition sites by mosquito species can lead to changes in larval distribution, habitat overlap, and is considered an important component of most mosquito-borne diseases (Bentley and Day 1989). In this study, we examined the role of semiochemicals (chemicals that affect behavior of organisms) from all life stages of the mosquito on the oviposition behavior of *Ae. japonicus*, *Ae. triseriatus*, and *Ae. albopictus* both in the laboratory and the field.

In our laboratory experiments with preexisting eggs, *Ae. japonicus* was the only species to show any preference among the available oviposition cups. Significantly fewer eggs were laid

in cups containing preexisting *Ae. triseriatus* eggs (Fig. 2.1a). A chemical cue emitted from the egg or one deposited by the female when laying eggs, could have caused *Ae. japonicus* females to be deterred from ovipositing in these cups. Because *Ae. japonicus* is a newly introduced species, it has had little interaction over its evolutionary history with *Ae. triseriatus*, unlike *Ae. albopictus* which shares the same range as *Ae. japonicus* within its native land of Japan (Hubert and Tanaka 1972). We did not see any significant differences in the number of eggs laid by *Ae. triseriatus* females on papers with preexisting eggs of any of the three species, although more eggs were laid on papers with preexisting eggs compared to controls (Fig. 2.3a). Beehler (1991) reported a significant preference under laboratory conditions by ovipositing *Ae. triseriatus* females for surfaces containing preexisting conspecific eggs compared with controls. No oviposition attractants were found from acetone extracts of *Ae. triseriatus* eggs since adults showed no preference between egg extracts and controls (Bentley et al. 1976). This contradicts Kitron et al. (1989) who found that *Ae. triseriatus* females laid more eggs on surfaces without eggs; however, this could be explained by the surfaces having a carrying capacity since papers were left out in the field to accumulate eggs. We determined that preexisting eggs had no effect on the oviposition behavior of *Ae. albopictus*. Similar results were obtained when *Ae. albopictus* had to choose between papers containing conspecific eggs, *Ae. aegypti* eggs, or a control paper with no eggs (Allan and Kline 1998). Both *Ae. triseriatus* and *Ae. albopictus* females may not have been able to detect any chemicals emitted from the eggs or were unaffected by them when ovipositing. Ganesan et al. (2006) described a semiochemical from *Ae. aegypti* eggs that was dose specific, acting as an attractant or deterrent to gravid females. We placed 60 eggs in each cup, which may not have been sufficient to elicit a response.

Studies with 1st instar larvae demonstrated that the presence of *Ae. albopictus* larvae acted as an oviposition deterrent for the other two species (Figs. 2.1b and 2.3b). Avoidance could be due to the fact that *Ae. albopictus* larvae have been shown to be a superior competitor for resources and have been associated with the decline in abundance of other mosquito species (Juliano 1998). Also, the nature and intensity of chemical cues in the different cups may have varied by species. For instance, because *Ae. albopictus* larvae develop more quickly than the other species, some larvae in the cups reached 4th instar during the experiment while the larvae of the two other species did not molt past the 2nd instar.

Our studies with LRW demonstrated that LRW of conspecifics stimulated egg laying behavior by all three species (Figs. 2.1c, 2.2c, and 2.3c). *Aedes japonicus* and *Ae. triseriatus* preferred not to oviposit in cups containing the LRW of the other species. Preferential change exhibited by both *Ae. japonicus* and *Ae. triseriatus* between 1st instar larvae and LRW, may have been due to a change in semiochemicals, because LRW did not have larvae present and pupae had been present at one point. Similar results with LRW were observed with *Ae. triseriatus* when females preferred to oviposit in cups containing the holding water of 4th instars of both conspecifics and *Ae. atropalpus* (Bentley et al. 1976). McDaniel et al. (1979) determined that an oviposition attractant produced by *Ae. triseriatus* larvae, was a true pheromone since the gut contents of the larvae had been replaced with kaolin, ruling out any chemical cues associated with fecal material or microorganisms of the gut. *Aedes albopictus* preferred conspecific and heterospecific LRW's compared with controls. Our study suggests that *Ae. albopictus* females may recognize oviposition sites that are capable of providing enough nutrients for other mosquitoes to reach adulthood and, therefore, will prefer these sites over ones without such chemical cues, such as our control cups. These findings suggest that *Ae. albopictus* prefer areas

where other LAC vectors are already established increasing the number of potential vectors in an area. Allan and Kline (1998) saw similar results with *Ae. albopictus* when the females showed an increase in oviposition in cups with conspecific larval water and *Ae. aegypti* larval water when compared to controls. Trexler et al. (2003) found that *Ae. albopictus* laid significantly more eggs in cups containing conspecific LRW compared to control water. They were able to culture the bacteria, *Psychrobacter immobilis* from LRW, which was preferred by ovipositing females compared to control water without bacteria.

The presence of interspecifics had no impact on the oviposition of *Ae. albopictus*. This study, as well as others, have demonstrated that *Ae. albopictus* shows little selectivity when ovipositing in the presence of other species (Gubler 1971, Allan and Kline 1998). A number of studies have shown *Ae. albopictus* to have a competitive advantage over *Ae. triseriatus* (Livdahl and Willey 1991, Novak et al. 1993, Teng and Apperson 2000, Juliano and Lounibos 2005) and *Ae. japonicus* (Armistead et al. 2008). This could be one reason that *Ae. albopictus* has flourished and spread so rapidly in the U.S. Without an avoidance for oviposition sites containing other species, *Ae. albopictus* will readily oviposit at breeding sites overlapping with other species.

The 2007 collections using fresh oviposition papers without eggs in the field did not reproduce our laboratory findings. However, we were not able to control for the large population of *Ae. triseriatus* in our study area; *Ae. triseriatus* comprised almost 70% of the total egg collections and were detected on 62% of the egg papers (Fig. 2.4). Since we did not detect discrimination between oviposition sites in our field study and *Ae. triseriatus* can have multiple egg laying events in one gonotrophic cycle, it is plausible that a female may oviposit in more than one trap within one gonotrophic cycle (Kitron et al. 1989). Another complicating factor

was that we could not determine which species was first to oviposit at a site. Even if *Ae. japonicus* was the second species to oviposit, after *Ae. triseriatus*, oviposition papers that were absent of *Ae triseriatus* eggs would be minimal. The C_8 coefficient of interspecific association conducted on the data to determine any associations between species (Hurlbert 1969) indicated that two comparisons had significant positive associations. Both *Ae. albopictus* and *Ae. japonicus* were positively associated with *Ae. triseriatus* (Table 2.1). Had our population of *Ae. triseriatus* not been larger than the other two species, we may have seen relationships closer to zero.

A second field study was conducted using papers with preexisting eggs in a location that, based on previous collections, had a nearly equivalent level of oviposition activity for both *Ae. japonicus* and *Ae. triseriatus* (Jackson and Paulson, unpublished data). We did not find significant ovipositional preferences among the species. Oviposition by *Ae. japonicus* in the field (Fig. 2.5a) exhibited a similar trend when compared to our laboratory results (Fig. 2.1a). The mean intensity of eggs laid by *Ae. japonicus* was 143 eggs per cup in cups containing conspecific eggs, which was similar to *Ae. albopictus* and control cups (Fig. 2.5a). What should be noted is that even though the mean intensities were similar, *Ae. japonicus* used only 42% of the conspecific cups available compared with 67%, 70%, and 75% of the other cups (Fig. 2.5b). Interspecific competition may have prevented *Ae. japonicus* from depositing all the eggs in cups containing *Ae. triseriatus*; and therefore, *Ae. japonicus* used more of the *Ae. triseriatus* cups but laid fewer eggs. When *Ae. japonicus* did detect conspecific eggs, the eggs may have acted as a cue for an ovipositing female that the ovipositional site was suitable to deposit all her eggs. A female may prefer sites with conspecific eggs because the risk of interspecific competition would be too high for the larvae to reach adulthood. Cups with preexisting *Ae. triseriatus* eggs had a

low mean intensity of *Ae. japonicus* eggs (around 100) because ovipositing females may have encountered heterospecific eggs and, deposited a small batch of eggs, before moving on in search of other sites. Depositing eggs in small batches would have led to many cups being positive for *Ae. japonicus* but with a low mean intensity of eggs. *Aedes triseriatus* did not exhibit any preferences in the field when ovipositing in cups containing preexisting eggs. This was similar to our laboratory experiments with preexisting eggs since no ovipositional preferences were exhibited. The number of *Ae. albopictus* eggs collected from this field study were too low to make any assumptions for this species.

In addition to the factors previously mentioned throughout this paper, several other attractants and repellents could affect the oviposition behavior of mosquitoes. Environmental conditions and physical features are also determinants in acceptance of oviposition sites. Temperature, humidity, color of the site, surface reflectance, substrate texture, surface moisture, and salinity are just a few of the factors shown to contribute the suitability of sites (Bentley and Day 1989). One other group of important attractants and repellents are the metabolites from the decomposition of organic matter (Millar et al. 1992). Bacteria produced during decomposition has been shown to be an attractant to female mosquitoes (Trexler et al. 2003). We attempted to counteract the possibility of any one of these factors affecting the ovipositional response of the mosquitoes by ensuring that these variables were consistent throughout experimentation.

Williams et al. (2008) described a hierarchy by *Ae. aegypti* females when it came to choosing oviposition sites and stated that alternate sites would have a stronger influence on ovipositing females compared to existing egg numbers. This hierarchy of suitable oviposition sites may have been a part of site selection for mosquitoes during our field experiments. Visual clues and the environment immediately surrounding cups were varied. Some cups were under

trees with a high canopy, others with a low canopy, while some were in thick brush. These variations, and others, may have been important factors to ovipositing females, even more important than which species' eggs were already present. We were able to control for these variations in the laboratory by limiting the conditions which an ovipositing female would have to choose between. Our laboratory experiments consisted of four choices in close proximity while field experiments had many choices, both artificial and natural, over a large area. Any number of factors at our field sites may have altered the suitability of sites, and therefore, possibly altered the oviposition preferences of females. Semiochemicals from mosquito eggs may attract ovipositing females to a site but may not be the deciding factor of whether oviposition would occur.

A further understanding of the effects of semiochemicals on oviposition may benefit the development of models for mosquito population dynamics. Because all three species are vectors of LAC virus, their ovipositional preferences could impact the epidemiology of the disease. *Aedes albopictus* will most likely continue to populate areas where the other vectors are already present. Both *Ae. triseriatus* and *Ae. japonicus* may discriminate between ovipositional habitats depending on the life stage and species already present, which could increase the number of vectors in a given area or cause movement of vectors into previously uninhabited areas.

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Figures

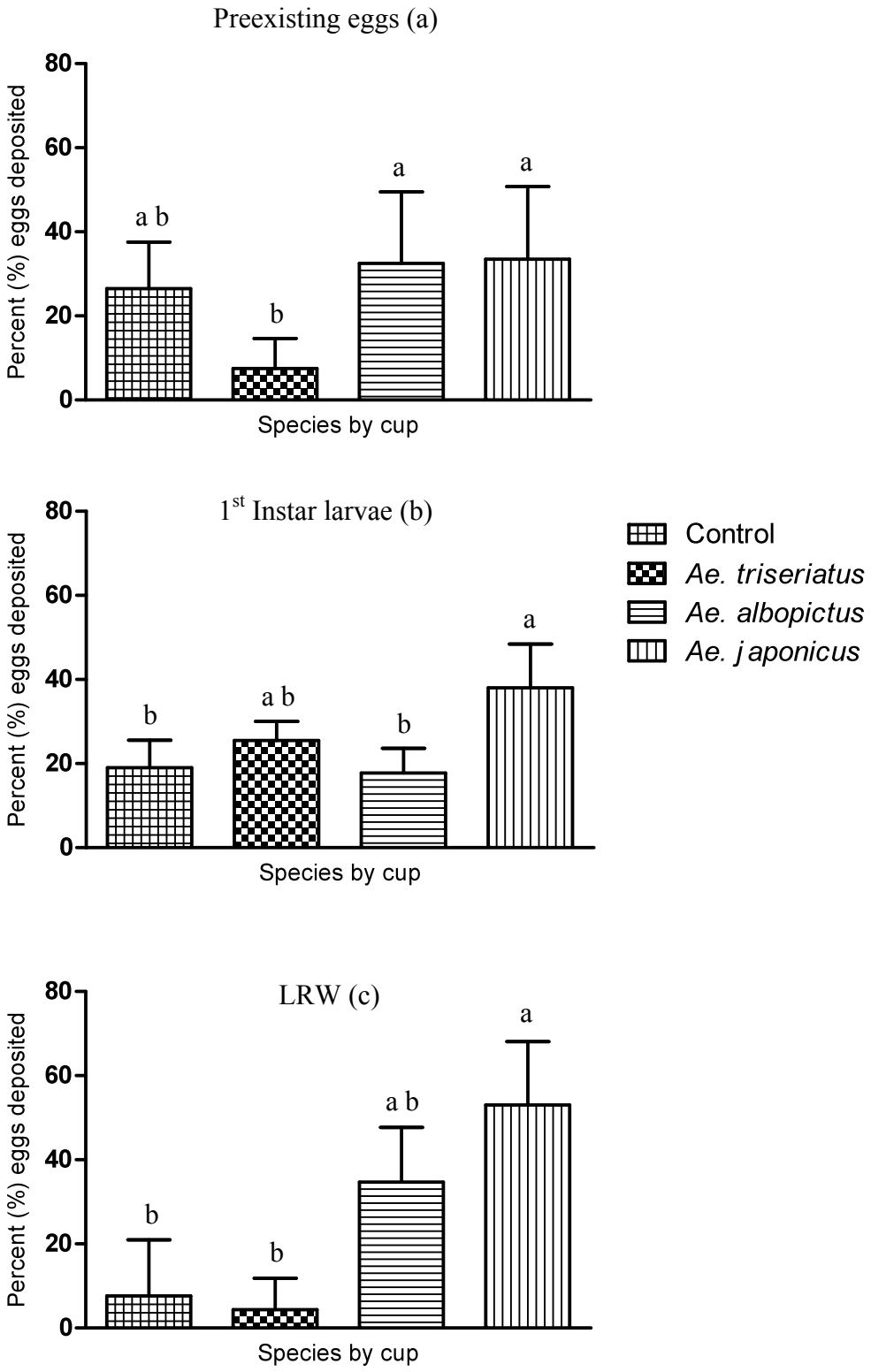

Fig. 2.1. Effect of species of preexisting eggs (a), 1st instar larvae (b), and larval rearing water (LRW) (c) on subsequent oviposition by *Aedes japonicus* under laboratory conditions. Treatments with the same letter are not significantly different (one-way ANOVA, Tukey's multiple comparison test, α=0.05).

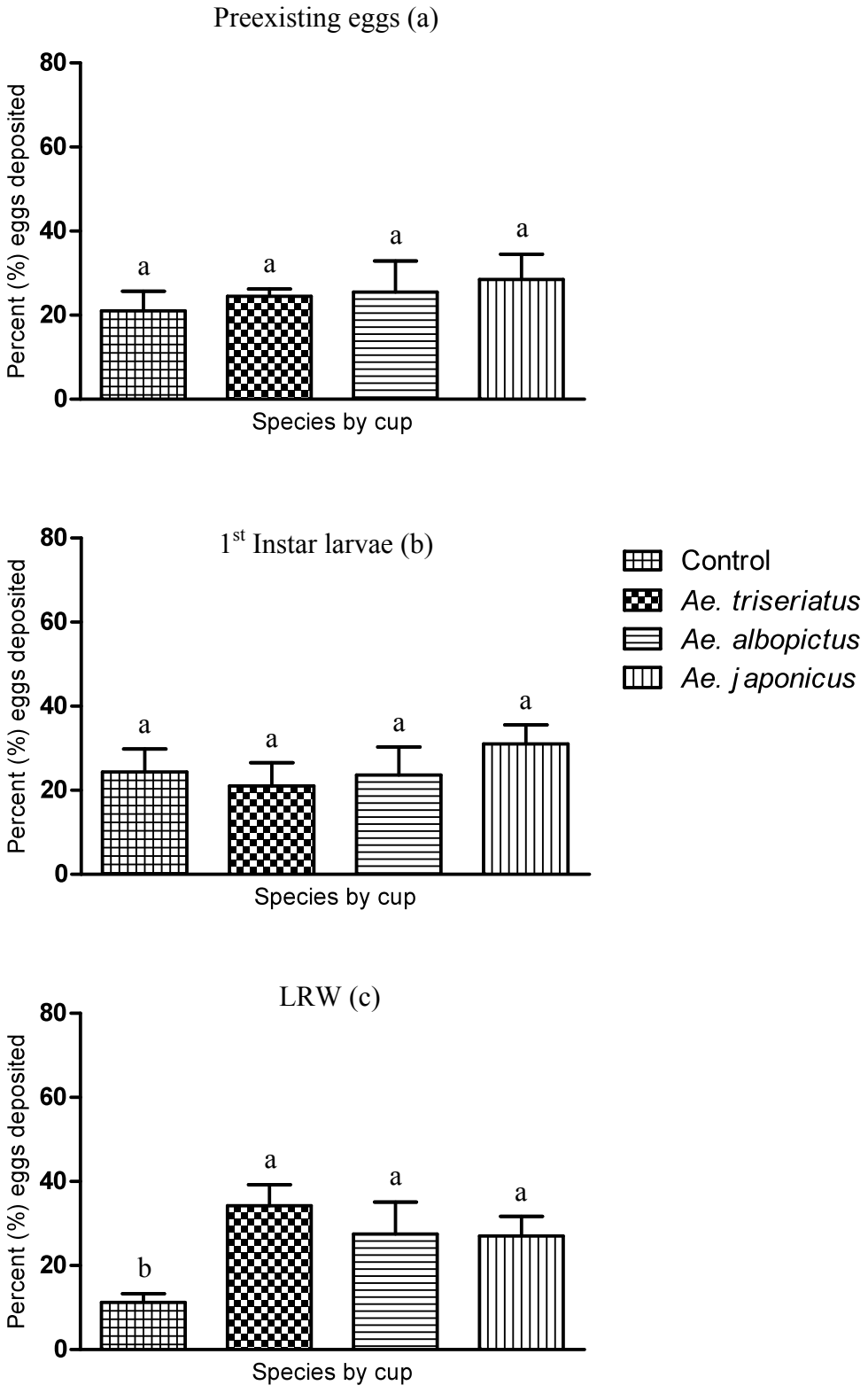

Fig. 2.2. Effect of species of preexisting eggs (a), 1st instar larvae (b), and larval rearing water (LRW) (c) on subsequent oviposition by *Aedes albopictus* under laboratory conditions. Treatments with the same letter are not significantly different (one-way ANOVA, Tukey's multiple comparison test, α=0.05).

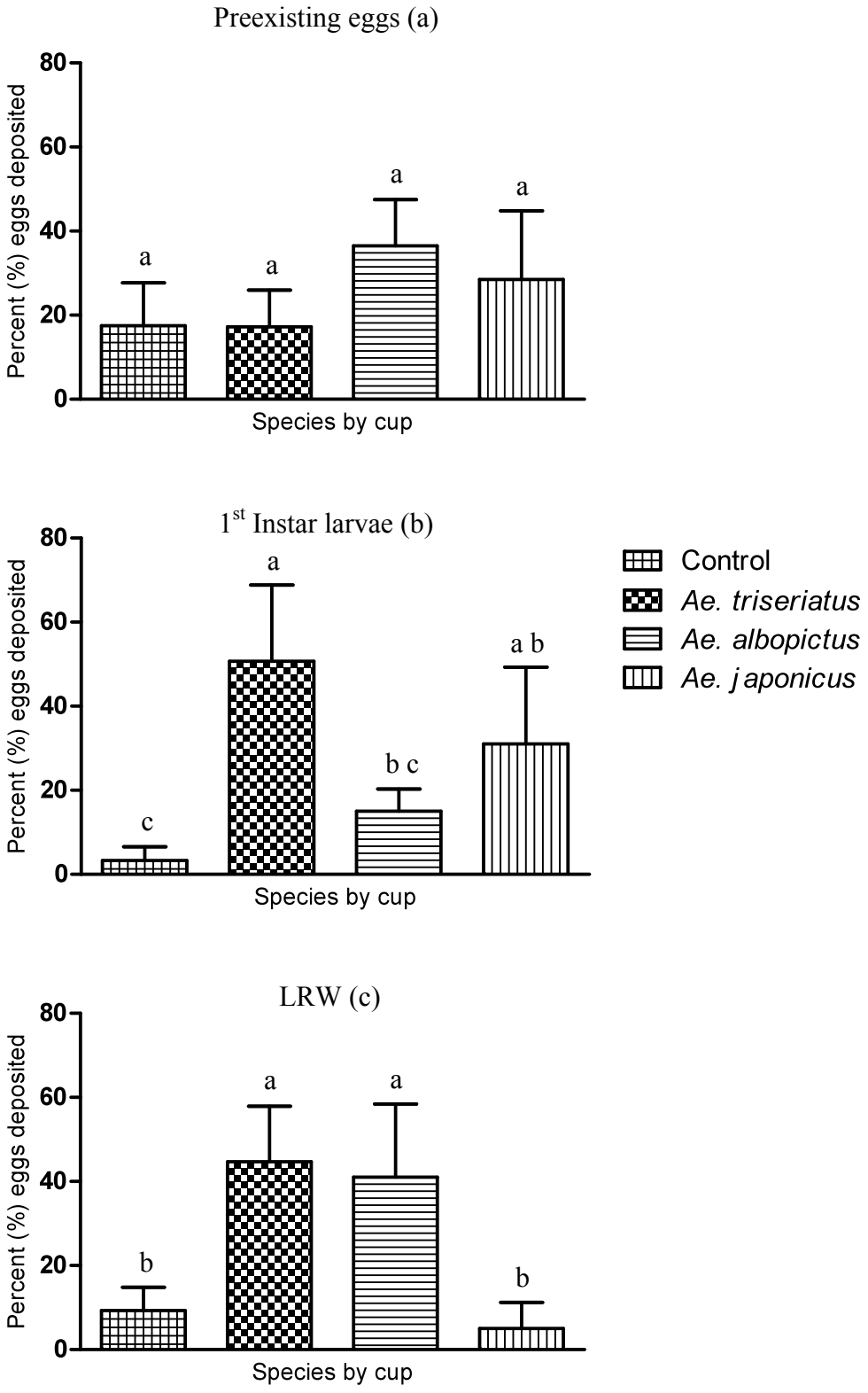

Fig. 2.3. Effect of species of preexisting eggs (a), 1st instar larvae (b), and larval rearing water (LRW) (c) on subsequent oviposition by *Aedes triseriatus* under laboratory conditions. Treatments with the same letter are not significantly different (one-way ANOVA, Tukey's multiple comparison test, α=0.05).

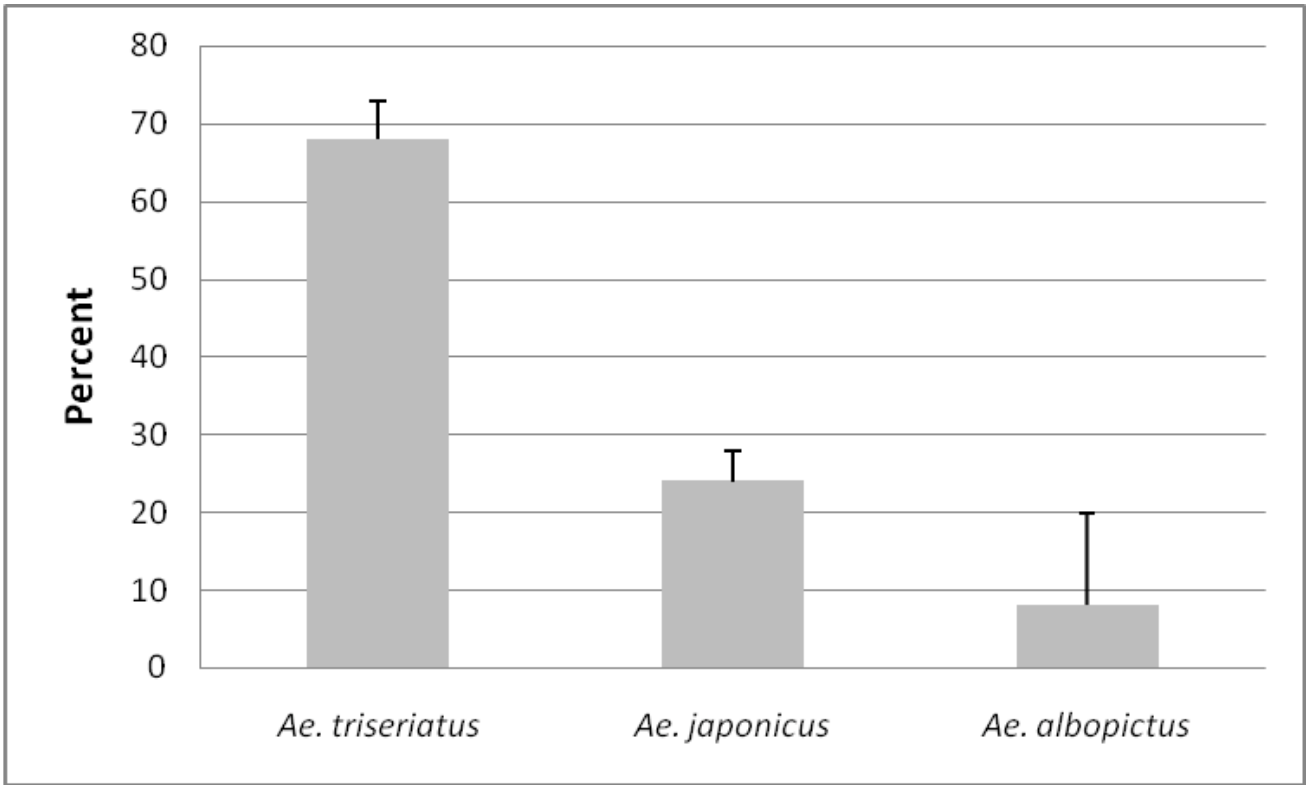

Fig. 2.4. Percent composition of total eggs collected from ovitraps in Wise County, Virginia during the summer of 2007.

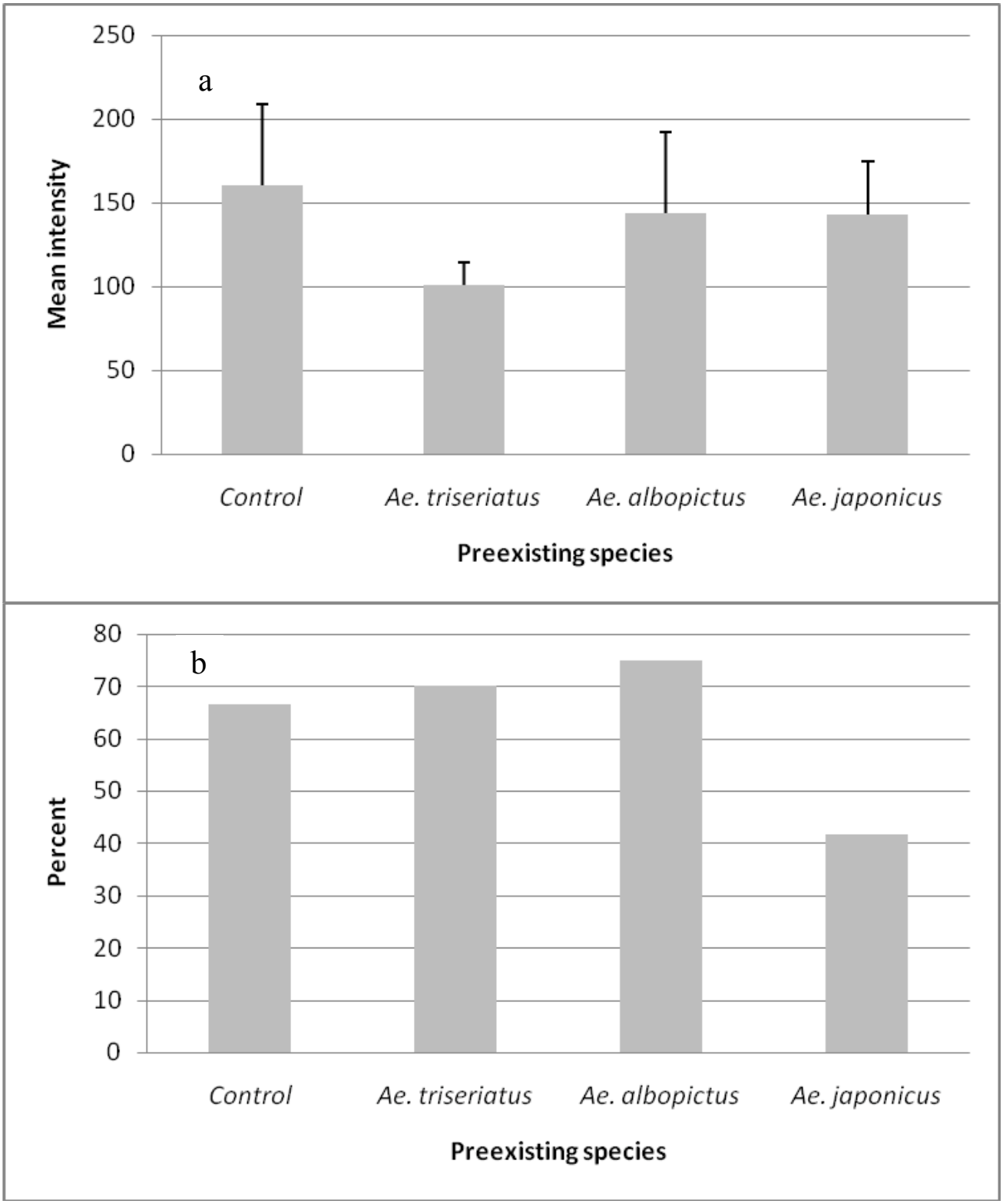

Fig. 2.5. Egg collections from oviposition traps with preexisting eggs from Blacksburg, Virginia in 2008. (a) Mean intensity of eggs laid by *Aedes japonicus* in cups (SEM). (b) Percent of cups positive for *Aedes japonicus* eggs.

Table

	Coefficients of species association		
	Ae. triseriatus	*Ae. japonicus*	*Ae. albopictus*
Ae. triseriatus	-	0.25*	0.39*
Ae. japonicus	0.25*	-	0.18
Ae. albopictus	0.39*	0.18	-

Table. 2.1. Coefficients of association (C_8) between species of mosquitoes collected as eggs from oviposition traps in Wise County, Virginia during the summer of 2007. (* Chi-square determination of significance, $P<0.05$.)

Chapter 3

Altered Blood-Feeding Behavior of *Aedes triseriatus* and *Aedes albopictus* as Affected by La Crosse Virus

3.1 Abstract

Aedes triseriatus and *Aedes albopictus* mosquitoes were parentally infected with La Crosse virus and the blood meal sizes and refeeding habits were examined. Both species took significantly smaller blood meals when infected with the virus compared with controls. Uninfected *Ae. albopictus* blood meals had a mean of 3.34 µl compared to 3.06 µl for the virus-infected group. Mean blood meal size for uninfected *Ae. triseriatus* was 5.66 µl compared to 5.22 µl for the virus-infected group. Refeeding experiments demonstrated that significantly more virus infected *Ae. triseriatus* mosquitoes refed to obtain a full blood meal compared to control mosquitoes. Approximately 23% of the infected mosquitoes refed compared to only 9% of the control group. No significant differences were detected during refeeding experiments with *Ae. albopictus*. LAC virus infection of mosquitoes affects their ability to blood feed, possibly through neural disruption. Multiple feedings by LAC virus infected *Ae. triseriatus* mosquitoes within one gonotrophic cycle increases its vectorial capacity by increasing the number of host contacted. Increased host exposure would lead to an increase in horizontal transmission and a possible increase to the number of human contacts. Decreased blood meal size in *Ae. albopictus* infected mosquitoes is a detrimental effect for both the virus and the mosquito but this may change as the two evolve to form a more benign relationship.

3.2 Introduction

La Crosse (LAC) virus encephalitis (Bunyaviridae: California serogroup) is the most common and important endemic mosquito-borne disease of children in the U.S. (Rust et al. 1999). It affects mainly school-aged children, causing a febrile illness with central nervous system involvement. Neurologic deficits and electroencephalogram abnormalities can persist for months after discharge from the hospital (McJunkin et al. 2001). The majority of the human cases have occurred in the upper Midwestern states with more recent cases being reported in West Virginia and Tennessee (Jones et al. 1999). Estimates for the number of cases annually have been as high as 300,000, with the majority of the cases going undiagnosed because of the mild symptoms associated with the disease (Rust et al. 1999).

Transmission of LAC virus can occur both vertically and horizontally (Grimstad 1988). Vertical transmission allows the virus to survive the winter in the egg stage of the vector (Miller et al. 1979). Both the gray squirrel (*Sciuris carolinensis*) and the eastern chipmunk (*Tamias striatus*) have been implicated as playing a major role in the horizontal transmission cycle of the virus since LAC virus isolates have been obtained from both species and they have both been found to produce sufficient viremias to infect susceptible vectors (Ksiazek and Yuill 1977).

The principal vector of LAC virus is *Aedes triseriatus* (Say), commonly called the eastern treehole mosquito (Pantuwatana et al. 1974). *Aedes triseriatus* has been found to feed on eastern chipmunks and gray squirrels (Nasci 1985, Richards et al. 2006) and in the laboratory, this species has demonstrated high transovarial transmission and filial infection rates (Miller et al. 1977, Hughes et al. 2006). Recent findings of naturally infected *Aedes albopictus* (Skuse) mosquitoes have implicated this species' as a possible accessory vector of LAC virus (Gerhardt

et al. 2001). During laboratory studies, *Ae. albopictus* was a more competent vector of LAC virus than *Ae. triseriatus* (Grimstad et al. 1989) but it is unlikely to be efficient in horizontal amplification because of its broad host range (Niebylski et al. 1994).

Alteration of feeding behavior of virus-infected mosquitoes can result in enhanced transmission (Kuno and Chang 2005). For example, *Aedes aegypti* (Skuse) mosquitoes infected with dengue 3 virus required 10 minutes longer or more to complete feeding when compared to non-infected controls (Platt et al. 1997). LAC virus infection has also been shown to modify feeding behavior of *Ae. triseriatus*. Grimstad et al. (1980) described increased probing behavior and reduced rates of engorgement by several strains of orally infected *Ae. triseriatus*. The impairment seen in LAC virus infected mosquitoes is not the result of decreased salivary gland function (Paulson et al. 1992) since mosquitoes that probe without feeding successfully transmit virus (Grimstad et al. 1980). If this alteration in ability to blood feed results in increased host-seeking, host contact would be increased, enhancing horizontal transmission.

The purpose of this study was to determine the effect of LAC virus infection on the blood feeding behavior in *Ae. triseriatus* and the accessory vector, *Ae. albopictus*, by measuring blood meal size and host-seeking activity of infected mosquitoes.

3.3 Materials and methods

3.3.1 Virus. The LAC virus isolate (VA0921075), which was used for the study, came from adult *Ae. triseriatus* mosquitoes collected from Wise County, Virginia in 1999 (Barker et al. 2003). The isolate was maintained in the lab by alternate passage through *Ae. triseriatus* mosquitoes and VERO cells before being used in this study. The titer of the stock virus was 1.0 x 10^8 plaque forming units (PFU) / ml.

3.3.2 Virus assays. Virus titers of mosquitoes were determined by plaque assay on VERO cells following the methods of Barker et al. (2003). To determine the average dosage of virus delivered, several mosquitoes were frozen immediately after inoculations and assayed. The inoculated virus mean titers for *Ae. triseriatus* and *Ae. albopictus* were 3.7×10^3 and 3.4×10^3 PFU / per mosquito, respectively. To ensure that virus infection of inoculated mosquitoes was consistent, mosquitoes were frozen after the incubation period and later assayed for virus titer. All inoculated mosquitoes were positive for virus with mean titers of 2.2×10^6 PFU / per mosquito for *Ae. triseriatus* and 3.3×10^6 for *Ae. albopictus*. All control mosquitoes were negative for virus.

3.3.4 Mosquitoes. *Aedes triseriatus* mosquitoes were collected as eggs from Blacksburg, Virginia in 2006 and 2007 and the F_0 progeny were used for all experiments. *Aedes albopictus* adults were obtained from eggs collected in Wise County, Virginia in 2006 and subsequently maintained as a lab colony. Mosquitoes were maintained at 24°C, 75% RH, and 16L:8D photoperiod. To ensure uniformity in size, larvae were reared at a density of 250 per container (33 x 17.5 x 11 cm) with 1600 ml deionized water (DI) and fed a bovine liver powder solution (7.5g/500 ml).

3.3.5 Virus infection of mosquitoes. Approximately 50 three-day old adult female mosquitoes were injected intrathoracically with 0.5 µl of virus or control media according to the methods of Rosen and Gubler (1974). Inocula consisted of LAC virus and M199 cell medium (500 ml M199, 27 ml fetal bovine serum, 10 ml penicillin/streptomycin (5000 I.U./ml), 0.5 ml gentamycin (50 mg/ml), and 2 ml Amphotericin B (250 µl/ml) or M199 cell media for the

controls. Mosquitoes were then held for one week under environmental chamber conditions (24°C, 75% RH and 16L:8D photoperiod) in 5,000 ml plastic buckets with a screened top and fabric sleeve on the side. Cotton balls soaked in a 10% sugar solution were placed on top of the cage as a food source. Two days prior to the start of the experiment, the sugar source was removed and replaced with cotton balls soaked in DI water.

3.3.6 Measurement of blood meal size. A laboratory mouse was anesthetized with 15 cc of a ketamine/xylazine solution (9 parts Ketamine (100 mg/ml), 9 parts Xylazine (100 mg/ml), and 3 parts Acepromazine (10 mg/ml) to 70 parts saline) and placed on top of the cage for 20 minutes. Separate mice were used for the virus and control cages for each replication. A damp paper towel was placed over the top of the cage and the mouse. After 20 minutes, the cage was checked to see if any mosquitoes were actively feeding. If any individuals were still feeding, then the mouse was left on the cage until all mosquitoes were finished feeding. *Aedes triseriatus* females were held in the cage for 20 minutes after feeding, while *Ae. albopictus* females were held for at least two hours, to allow the blood to become more congealed and less likely to escape the gut during dissections (M. Klowden, personal communication). Adults were then removed with a handheld aspirator and placed on ice until dissection.

The methods of Briegel et al. (1979) were modified and used to determine blood meal size through the conversion of hemoglobin into hemiglobincyanide (HiCN). Individual blood meals were removed and placed in a 1.5 ml eppendorf tube containing 1.0 ml of Drabkin's solution. After the blood meals were thoroughly ground and incubated at room temperature for at least 1 hour, a 200 µl sample was transferred to a FisherBrand 96-well flat bottom plate (Fisher Scientific, Pittsburgh, PA). Test samples were run in quadruplicate. A new standard

curve was prepared for each mouse by adding 20 µl of blood to 2 ml of reagent and producing a dilution series (10, 8, 7, 5, 2.5, 1.25, 0.625, and 0.3125 µl) that was run in triplicate. Plates were then read with a Dynex TRIAD Series Multimode Detector using Concert-TRIAD Series software (version 2.0.0.11) (Dynex Technologies, Inc., Chantilly, VA) at an absorbance scan of 540 nm to determine the optical density (OD) for each sample. Readings from the known volumes of blood were obtained using linear regression to develop a standard curve to calculate blood meal size, in µl, from the OD reading of the sample. All samples were run through the plate reader on the day of feeding; none were frozen for later testing. Because wing length is directly correlated with body weight (Christophers 1960), mosquito size was determined by measuring the wing length of each mosquito using a dissecting scope and an ocular micrometer.

3.3.7 Refeeding. After inoculation, the control group and virus group were separated into three cages each containing approximately 15 to 20 mosquitoes. At 7 days post-infection an anesthetized mouse was placed inside each cage. A moistened paper towel and piece of plexi glass were placed on top of the cage with a sufficient opening so that feeding could be observed. After a 15-minute feeding period, the mouse was removed from the cage and the mosquitoes were collected, chilled on ice, and examined under a dissecting scope. The number of mosquitoes that fed was recorded and the amount of blood each imbibed was scored according to Pilitt and Jones (1972). The mosquitoes were immediately placed back in the chamber. Blood feedings were repeated at 2 hour and 24 hour intervals. Any mosquito with fresh blood in the abdomen that had taken a previous blood meal was recorded as having refed. New mice were used during each feeding.

3.3.8 Statistics. All data were analyzed using Prism (Version 5.0, GraphPad Software, El Camino, CA). All comparisons of blood meal size were made using one-tailed unpaired t-tests and Mann-Whitney tests. Wing lengths were analyzed using two-tailed unpaired t-tests and the data from the refeeding experiment were analyzed using a 2 × 2 contingency table and one-tailed Fisher's Exact Test. The level of significance for all test was set at $\alpha = 0.05$.

3.4 Results

3.4.1 Blood meal size. *Aedes albopictus* mosquitoes inoculated with LAC virus took significantly ($P<0.05$) smaller blood meals compared with the control group when the data were pooled (Table 3.1). The data showed that the mean blood meal size of 3.34 µl in the control group was significantly larger ($P<0.01$) than the virus infected mosquitoes which had a mean blood meal size of 3.06 µl. Insignificant variation was seen between the wing lengths of the control and virus groups of mosquitoes. A t-test performed on wing lengths determined that there were no significant differences in wing lengths between the two groups ($P>0.05$). A positive linear relationship was seen between wing length and blood meal size in the control group (Fig. 3.1a) with the infected group having a lower r^2 value (Fig. 3.1b). The majority of blood meals in the control group were 3.0 µl or larger while the majority of blood meals by the virus group were 3.0 µl or smaller (Fig. 3.2). Even though the difference between the median blood meal size for both groups was small, the difference was significant ($P<0.01$). The range of blood meal sizes for *Ae. albopictus* mosquitoes was very narrow with several of the smallest blood meals coming from LAC virus infected mosquitoes.

Virus inoculated *Ae. triseriatus* mosquitoes had a mean blood meal size of 5.22 µl which was significantly smaller ($P<0.01$) than the mean blood meal size of the control group, 5.66 µl

(Table 3.2). There were no significant differences between the wing lengths of the control group and the virus infected group (P>0.05). A positive linear relationship between wing length and blood meal size was observed in the control group (Fig. 3.3a). Several of our data points in the LAC virus group did not fall near our regression line because the blood meals were small for the size of the wing length (Fig. 3.3b). The range of blood meals was broader compared with that of *Ae. albopictus* and the difference between the medians was larger (Fig. 3.4). Approximately 30% of the infected mosquitoes had a blood meal size of 4.5 µl or smaller compared to only 17% of the uninfected control group

3.4.2 Refeeding. No significant differences were detected between the number of virus-free (control) *Ae. albopictus* mosquitoes that refed versus the number of virus-infected mosquitoes that refed (P>0.05; Fig. 3.5). Of the total 109 control mosquitoes that took a blood meal, 5% refed, and of the 130 virus-infected mosquitoes that took a blood meal 8% refed.

With respect to *Ae. triseriatus,* significantly more (P<0.05) virus-infected mosquitoes refed on a mouse compared to the control group (Fig. 3.5). Of the 107 mosquitoes that took a blood meal, 9% refed, and of the 93 virus-infected mosquitoes that took a blood meal 23% refed.

3.5 Discussion

Our data show that LAC virus infection altered the feeding behavior of both *Ae. triseriatus* and *Ae. albopictus* mosquitoes by decreasing blood meal size. Infection also increased the probability that *Ae. triseriatus* mosquitoes will take multiple blood meals within one gonotrophic cycle. The idea that mosquito feeding behavior can be altered by pathogens is supported by several laboratory experiments (Grimstad et al. 1980, Rossignol et al. 1984,

Rossignol et al. 1986, Wekesa et al. 1992, Platt et al. 1997). Virus altered feeding behavior by mosquitoes may lead to increased transmission rates and may also cause detrimental effects to the vector.

LAC virus infection of *Ae. triseriatus* altered the mosquitoes' feeding behavior by causing the insect to take significantly smaller blood meals than uninfected controls. Grimstad et al. (1980) also reported a negative effect of LAC virus infection on the blood-feeding by *Ae. triseriatus* but they visually graded the amount of blood imbibed. We used hemoglobinometry because this method provides a quantitative measure of blood meal size, is unaffected by digestion or urination by the mosquito, and provides accurate readings for up to 12 hours post feeding (Briegel et al. 1979).

In our studies with both species, LAC virus infection disrupted feeding behavior, causing the mosquito to stop feeding before it had become fully engorged. Grimstad et al. (1980) stated that, "The engorgement reduction may simply be an inability to engorge - perhaps the result of neural disruption caused by virus infection" and not the result of salivary damage. LAC virus is neurotropic in both the vertebrate and insect hosts. Using immunofluorescence microscopy Beaty and Thompson (1976), demonstrated that the abdominal ganglia of adult *Ae. triseriatus* mosquitoes were infected with LAC virus, along with other organs. Neural disruption of the abdominal ganglia may be related to early termination of feeding because stretch receptors in the abdomen send a signal to the brain that an adequate amount of blood has been imbibed to produce a batch of eggs (Gwadz 1969). These same stretch receptors also inhibit host-seeking behavior (Klowden and Lea 1979).

Aedes triseriatus and *Ae. albopictus* mosquitoes took significantly smaller blood meals when infected with LAC virus compared to uninfected siblings probably because the virus-

infected mosquitoes terminated feeding before a full blood meal was imbibed. Because we found little variation in wing length between the virus-infected and control groups, any differences in blood meal size that we observed could not be the result of differences in size variation of the mosquitoes. The smaller blood meals taken by virus-infected mosquitoes would not cause a change in the virus transmission rates, but does result in fewer progeny. Because virus infected *Ae. albopictus* took small blood meals and did not refeed, these mosquitoes are imbibing less blood than control mosquitoes within one gonotrophic cycle. Smaller blood meals would be deleterious for both the virus and the mosquito. This alteration in feeding behavior would decrease the number of progeny and concomitantly decrease transovarial transmission of the virus.

Our refeeding experiments with infected *Ae. triseriatus* mosquitoes demonstrated that significantly more infected mosquitoes refed compared to controls. In pooled data from three sets of paired replicates, infected mosquitoes were more than twice as likely to refeed as uninfected controls. This refeeding behavior would increase the number of host contacts within one gonotrophic cycle and, because *Ae. triseriatus* is able to transmit the virus with only one probe (Grimstad et al. 1980) this species has the potential to increase horizontal amplification.

Most likely, virus infection impacts one or both of the endogenous regulatory mechanisms controlling host-seeking behavior after a blood meal (Klowden 1981, Klowden and Lea 1979). First, the distention caused by the blood meal triggers abdominal stretch receptors and later a humoral factor is produced by vitellogenic females. Together, these factors result in inhibition of host-seeking between a feeding event and subsequent oviposition. If the reduced blood-feeding and multiple probing induced by LAC virus causes increased host-seeking, the results would be an increase in the number of hosts contacted by a vector.

Aedes albopictus showed no difference in the number that refed when control and virus infected mosquitoes were compared. The lack of significant differences in refeeding behavior may be due to the fact that even though the blood meals of virus infected mosquitoes were significantly smaller, these mosquitoes still may have imbibed a sufficient volume of blood to inhibit further host-seeking. The narrow range of blood meal sizes and the limited number of *Ae. albopictus* mosquitoes taking a small blood meal (Fig. 3.2), may have been the reason we did not see a significant number of mosquitoes refeeding. Had the histogram been similar to *Ae. triseriatus* with a larger percentage of mosquitoes taking a smaller blood meal (Fig. 3.4), then there may have been more individuals refeeding.

LAC virus infection of *Ae. albopictus* is evolutionarily recent and this may have been the reason the mosquitoes did not refeed. This is unlike *Ae. triseriatus* and LAC virus, which have a long evolutionary history. Mosquitoes that have only recently become vectors of viruses are often not well adapted to the virus. This new relationship could result in detrimental effects of the vector (Scott and Lorenz 1998). As time progresses, the association between a vector and a pathogen will develop into one with less detrimental effects (Burnet and White 1972). LAC virus may have changed over time to form a more benign relationship with *Ae. triseriatus* mosquitoes compared to *Ae. albopictus* mosquitoes.

An increase in the number of hosts a mosquito will feed on in one gonotrophic cycle will ultimately lead to an increase in its vectorial capacity. Vectorial capacity is defined as the number of new infections disseminated per case per day by a vector and is estimated in a *posteriori* fashion from parameters describing vector abundance, survival, host feeding pattern, and blood feeding rate (Reisen 1989). By increasing the biting rate of an infected mosquito we would expect to see, as an end result, an increase in vectorial capacity. Our refeeding

experiments demonstrated an increase in the biting rate, which ultimately may mean increased numbers of new infections disseminated per case per day by *Ae. triseriatus*. The increase in horizontal amplification would, presumably, compensate for any reduction in transovarial transmission resulting from reduced fecundity.

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Tables

Replicate	Treatment	*n*	Mean Wing Length (mm)	Mean Blood Meal (µl)	Range Blood Meal (µl)
1	Control	46	3.31 (a)	3.44 (±0.79) (a)	2.38-7.07
	Virus	46	3.30 (a)	2.92 (±0.75) (b)	0.69-4.35
2	Control	18	3.27 (a)	3.37 (±0.59) (a)	2.33-4.63
	Virus	29	3.29 (a)	3.12 (±0.95) (a)	0.30-4.70
3	Control	31	3.29 (a)	3.35 (±0.56) (a)	2.38-4.59
	Virus	30	3.30 (a)	3.08 (±0.64) (b)	1.05-4.45
4	Control	38	3.24 (a)	3.19 (±0.71) (a)	1.82-4.71
	Virus	39	3.27 (a)	3.18 (±0.76) (a)	1.17-4.59
Pooled Data	Control	133	3.28 (a)	3.34 (±0.69) (a)	1.82-7.07
	Virus	144	3.29 (a)	3.06 (±0.78) (b)	0.30-4.70

Table 3.1. Mean blood meal size (±Standard Deviation) and mean wing length of *Aedes albopictus* mosquitoes parenterally infected with La Crosse virus. Treatments with different letters were found to be significantly different ($P<0.05$).

Replicate	Treatment	*n*	Mean Wing Length (mm)	Mean Blood Meal (µl)	Range Blood Meal (µl)
1	Control	30	4.14 (a)	5.32 (±1.17) (a)	2.19-7.45
	Virus	41	4.16 (a)	4.70 (±1.74) (b)	0.39-7.32
2	Control	14	4.17 (a)	6.50 (±0.87) (a)	4.58-7.37
	Virus	18	4.17 (a)	5.82 (±0.97) (b)	3.85-7.10
3	Control	30	4.17 (a)	5.39 (±1.09) (a)	2.44-7.05
	Virus	40	4.19 (a)	5.48 (±1.40) (a)	2.48-8.21
4	Control	32	4.11 (a)	5.78 (±1.00) (a)	3.66-7.34
	Virus	35	4.16 (a)	5.19 (±1.66) (b)	1.16-7.84
Pooled Data	Control	106	4.14 (a)	5.66 (±1.09) (a)	2.19-7.45
	Virus	134	4.17 (a)	5.22 (±1.57) (b)	0.39-8.21

Table 3.2. Mean blood meal size (±Standard Deviation) and mean wing length of *Aedes triseriatus* mosquitoes parenterally infect with La Crosse virus. Treatments with different letters were found to be significantly different ($P<0.05$).

Figures

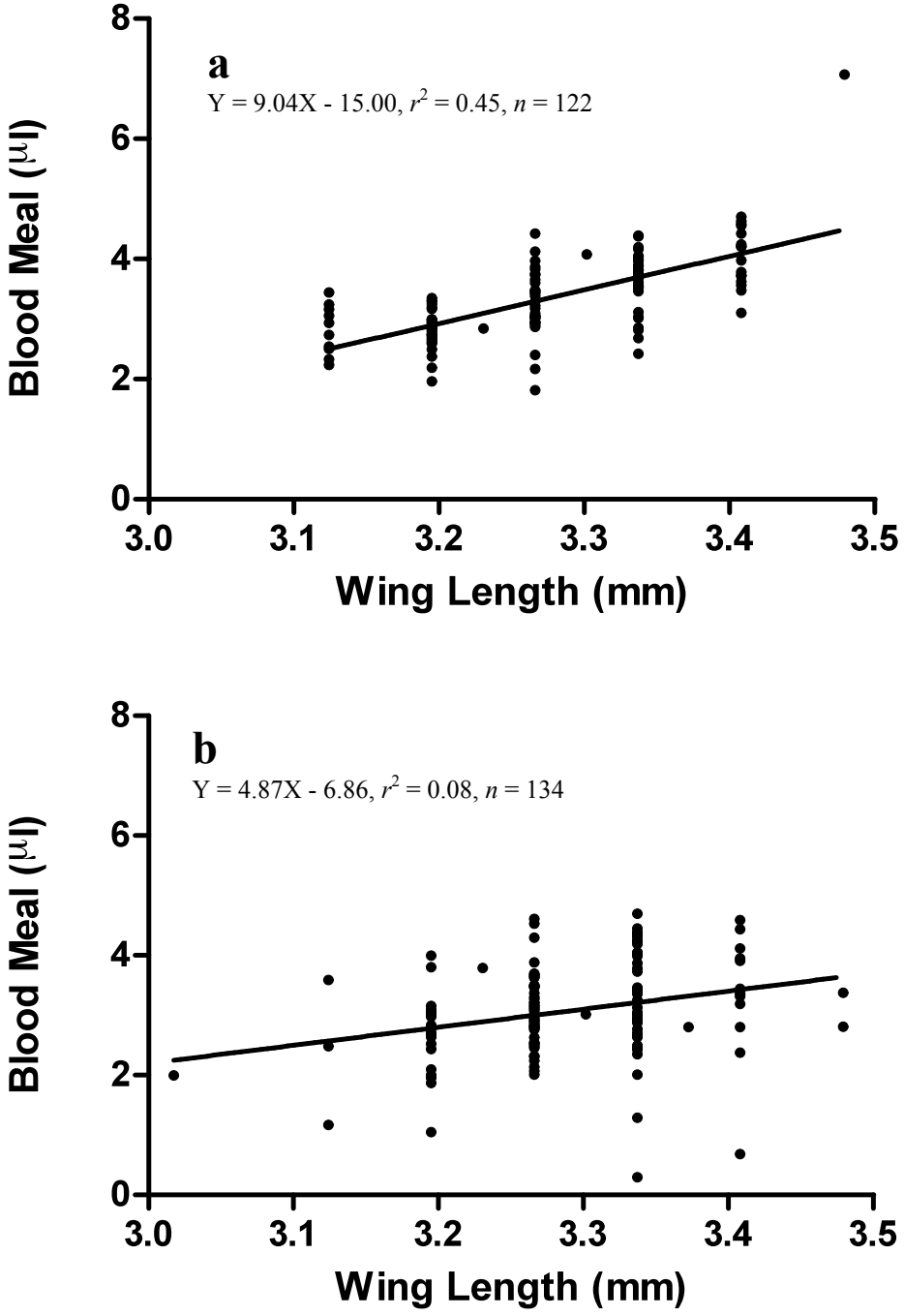

Fig. 3.1. Linear relationship of blood meal size (µl) and size of mosquito as measured by wing length (mm) for *Aedes albopictus* injected with control media (a) or La Crosse virus (b).

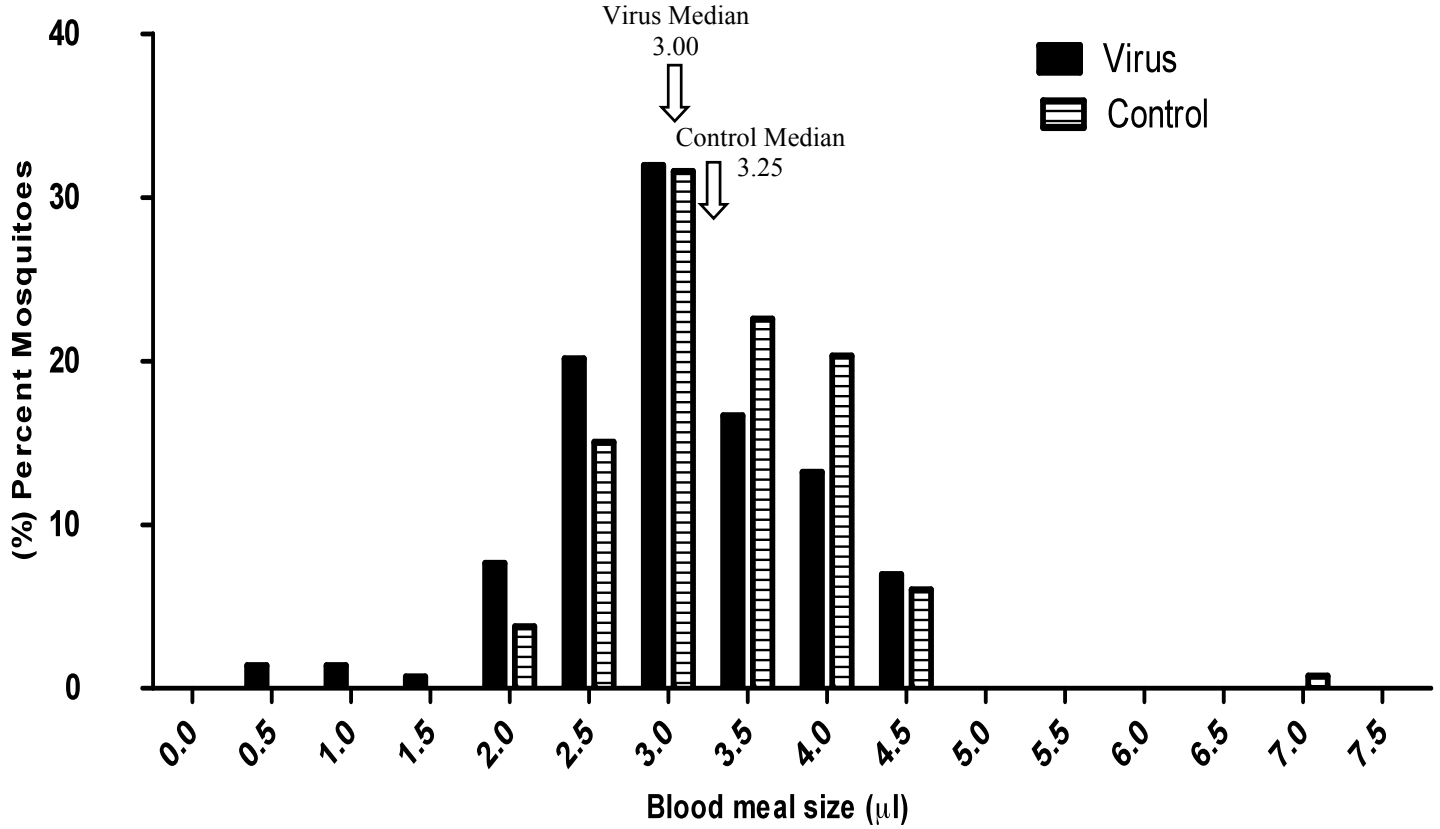

Fig. 3.2. Histogram of percent blood-meal size for *Aedes albopictus* mosquitoes injected with LAC virus or control media. Medians were found to be significantly different ($P<0.05$).

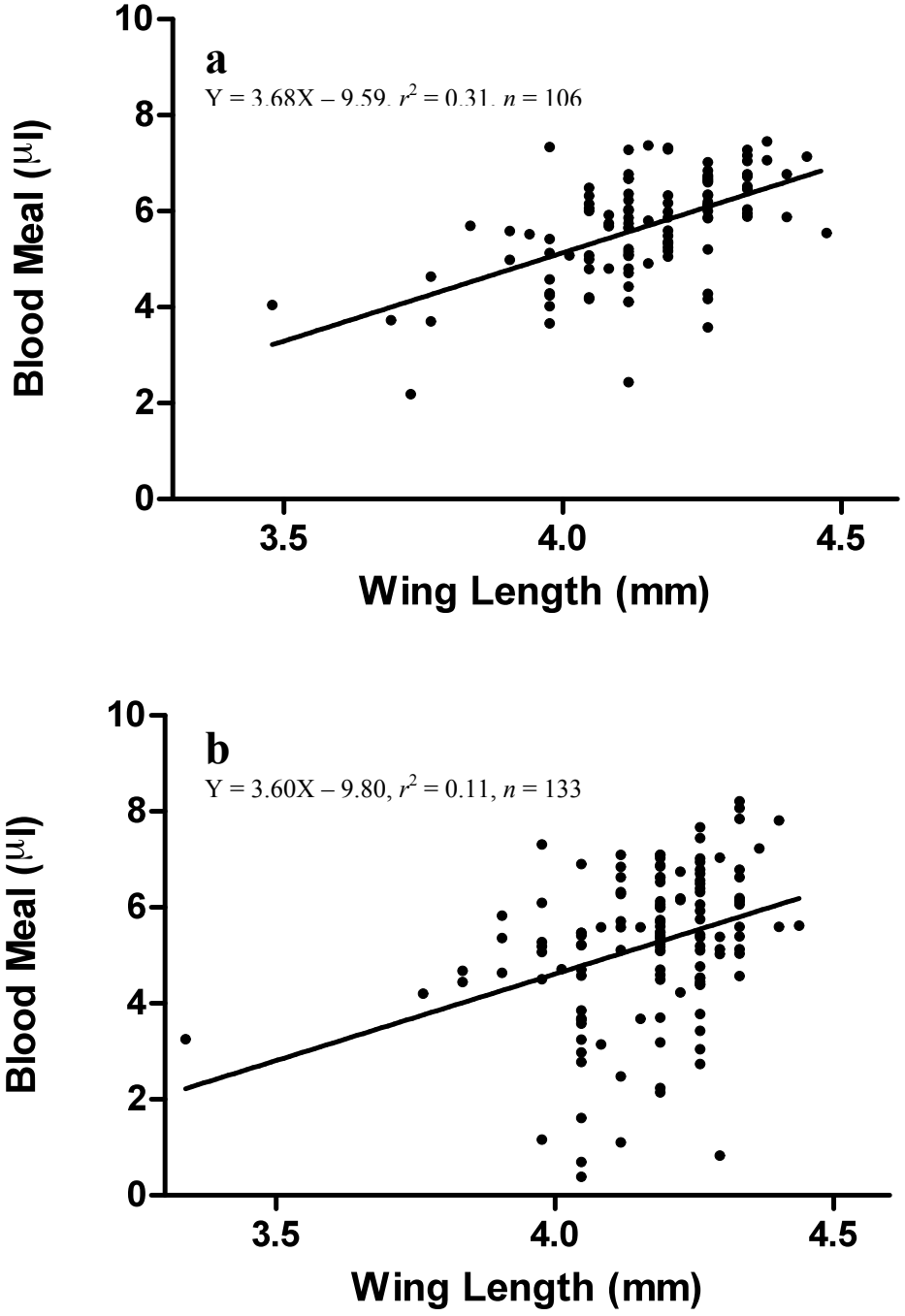

Fig. 3.3. Linear relationship of blood meal size (µl) and size of mosquito as measured by wing length (mm) for *Aedes triseriatus* mosquitoes injected with control media (a) or La Crosse virus (b).

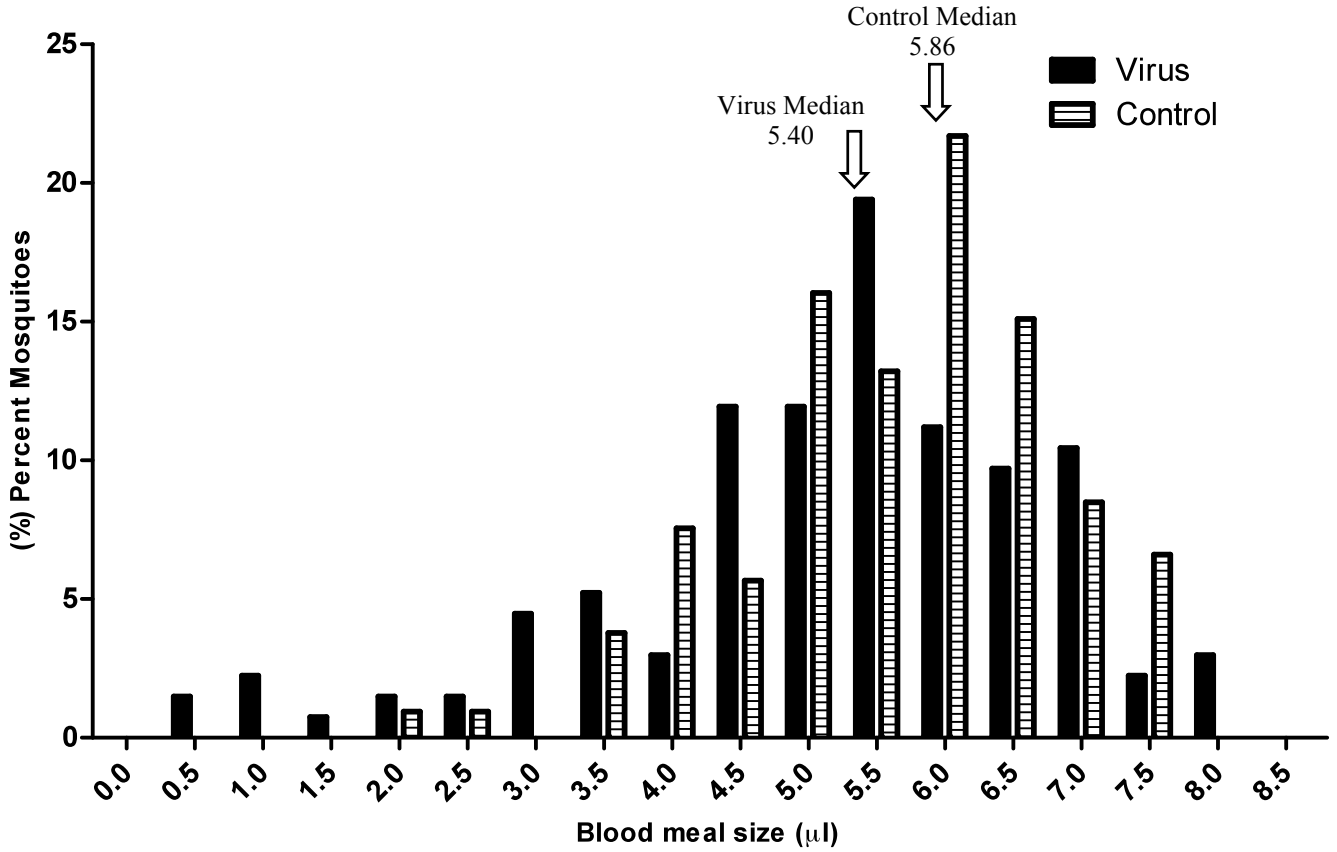

Fig. 3.4. Histogram of percent blood-meal size for *Aedes triseriatus* mosquitoes injected with LAC virus or control media. Medians were found to be significantly different ($P<0.05$).

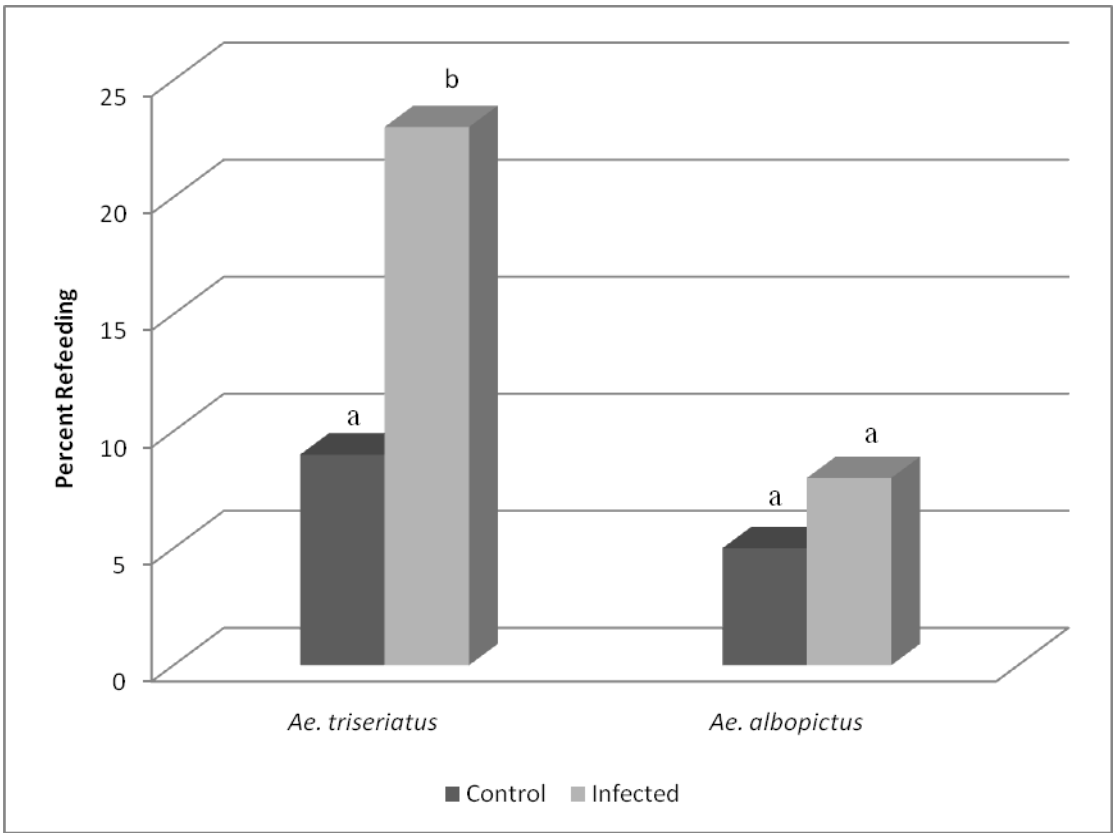

Fig. 3.5. Effect of La Crosse virus infection on the refeeding behavior of *Aedes triseriatus* and *Aedes albopictus*. Treatments with different letters were found to be significantly different within species and between species ($P<0.05$).

Chapter 4

Effect of Method of Collection on Estimations of La Crosse Virus Minimum Infection Rates

4.1 Abstract

Adult collections and egg collections of La Crosse vectors, from within urban and rural landscapes, were tested to determine LAC virus infection rates. Gravid traps and CO_2-baited light taps were used to collect adults while oviposition cups were used to collect eggs. Comparisons were made among species, trapping methods, and settings. A total of 88 pools consisting of 420 adults collected from rural areas were tested. Seven of these pools tested positive for the virus with a maximum likelihood estimation of infection rate (MLE-IR) of 17.66/1,000 mosquitoes. Three species tested positive, *Ae. triseriatus*, *Ae. albopictus*, and *Ae. canadensis*. Egg collections from our rural areas produced one virus positive pool of *Ae. triseriatus* with a MLE-IR of 0.15/1,000 mosquitoes. In contrast to the adult collections, 282 pools from 7,152 mosquitoes were processed from egg collections. No collections from the urban setting were positive for LAC virus. Virus may not be circulating at the urban setting or levels were too low to be detected through our trapping methods. *Ae. albopictus* had the highest MLE-IR of 18.86/1,000 from 58 pools processed for testing. *Ae. albopictus* comprised the majority of adult collections because most of the traps were placed in residential areas. Adult trapping methods have several advantages compared with egg collections in our study. Fewer mosquitoes from adult collections produced more virus positive pools. Fewer specimens decreases the chances of cross contamination among samples and less money spent on testing. Also, adult collections require less time before processed for testing compared to egg collections which need time to be reared to adults. The only drawback to adult trapping is the costs

associated with running traps and need to check these traps daily. This study demonstrated that the trapping of adults is a better method to collect LAC virus positive mosquitoes.

4.2 Introduction

La Crosse (LAC) virus accounts for the majority of the California serogroup viruses detected in children in the eastern half of the United States (CDC 2001). Children under the age of 15 are most commonly infected and exhibit signs of vomiting, febrile illness, and headaches with some cases progressing to seizures (McJunkin et al. 1998). When hospitalized, fatality rates may be as high 1%, but overall rates are less than 1% (Tsai 1991). Between 1964 and 2007, approximately 81 confirmed and probable California serogroup viral (mainly La Crosse) encephalitis cases were reported each year, with a total of 596 coming from Virginia and West Virginia (CDC 2008). In Virginia, 27 human cases have been reported since 1994, with the majority of the cases coming from the southwestern region of the state (VDH 2009). The actual number of cases throughout the U.S. may be as high as 300,000 per year, but this approximation cannot be confirmed since many cases are undiagnosed or misdiagnosed (Rust et al. 1999).

The virus is maintained in the environment by two different transmission cycles of the mosquito vector, horizontal and vertical (Grimstad 1998). In horizontal transmission, an amplifying host becomes infected and develops sufficient viremia to infect susceptible vectors. In the case of LAC virus, two amplifying hosts have been identified: tree squirrels (*Sciuris niger* and *Sciuris carolinensis*) and the eastern chipmunk (*Tamias striatus*) (Moulton and Thompson 1971, Gauld et al. 1975). Both amplifying hosts overlap in range with the primary vector and the disease (Moulton and Thompson 1971). Chipmunks, trapped in Wisconsin during September, had seroprevalence rates as high as 100% (Gauld et al. 1974) while tree squirrels trapped in the state demonstrated rates as high as 53% (Moulton and Thompson 1971).

Through vertical transmission the virus overwinters while in the egg stage of the vector (Pantuwatana et al. 1974). Mosquitoes hatching and emerging the following spring can then

initiate horizontal amplification. La Crosse virus infected larvae and eggs have been collected from several states including Minnesota (Balfour et al. 1975), Wisconsin (Pantuwatana et al. 1974, Beaty and Thompson 1975), Illinois (Clark et al. 1982), North Carolina (Kappus et al. 1982, Szumlas et al. 1996), West Virginia (Nasci et al. 2000), Tennessee (Gerhardt et al. 2001), and Virginia (Barker et al. 2003). In the absence of horizontal transmission, the virus is still able to persist for up to eight generations by means of vertical transmission (Miller et al. 1977).

The primary vector associated with LAC virus is *Aedes triseriatus* (Say), as evidenced by its frequent collection in areas with human cases (Berry et al. 1975, Balfour et al. 1976) and numerous virus isolations from field-collected mosquitoes (Sudia et al. 1971, Berry et al. 1974, Pantuwatana et al. 1974, Balfour et al. 1975). *Aedes triseriatus* is an integral part of the maintenance cycle of the virus due to its high transovarial transmission rates, vector competence, and its feeding preferences. Transovarial transmission rates as high as 71% and 98% and filial infection rates of 46% and 71% have been reported (Miller et al. 1977, Hughes et al. 2006). Several field studies on the blood-feeding behavior of *Ae. triseriatus* have shown that the mosquito engorged primarily on gray squirrels and eastern chipmunks in both rural and urban settings (Nasci 1985, Richards et al. 2006). In fact, a feeding preference study comparing several mosquito species determined that *Ae. triseriatus* fed on the amplifying hosts more than other mosquito species (Wright and DeFoliart 1970). Also, the mosquito's habitat preference for forested habitats rather than urban or residential settings allows for more contact with amplifying hosts (Barker et al. 2003).

The exotic mosquito, *Aedes albopictus* (Skuse), is another potential vector of LAC virus. Research has shown that infection and oral transmission rates for *Ae. albopictus* are equal to or higher than *Ae. triseriatus* (Grimstad et al. 1989, Cully et al. 1992). Laboratory studies

determined that the transovarial transmission rates and filial infection rates for *Ae. albopictus* are 52% and 18%, respectively, which were significantly lower than *Ae. triseriatus* (Hughes et al. 2006). Virus isolations from field-collected eggs in North Carolina and Tennessee (Gerhardt et al. 2001) solidify *Ae. albopictus* as a natural vector and demonstrate its ability to transmit the virus transovarially in nature. While *Ae. albopictus* has been shown to feed on amplifying hosts, it displays a lack of host specificity. Neibylski et al. (1994), identified blood meals of *Ae. albopictus* collected from a mix of rural and urban sites from Missouri, Florida, Indiana, Illinois and Louisiana, and found that the majority of blood meals came from rabbits and *Rattus* spp. with only 2.7% coming from sciurids, such as chipmunks and squirrels. In suburban North Carolina, 11% of the blood meals came from squirrels but the majority came from humans and cats (Richards et al. 2006). In a habitat preference study, Barker et al. (2003) found that *Ae. albopictus* were more commonly associated with residential and urban habitats, which would allow more contact with its preferred hosts. The opportunistic feeding behavior and habitat preference of *Ae. albopictus* likely limits its potential as an efficient vector of LAC virus.

Various methods have been employed in surveillance to determine the occurrence and distribution of arboviral vector populations in an area. An additional benefit of these methods is that they also allow us to confirm the presence of one or more viruses in survey area. For example, LAC virus isolations have been obtained from eggs collected from oviposition traps (Clark et al. 1982, Szumlas et al. 1996, Nasci et al. 2000, Gerhardt et al. 2001), adults collected in light traps (Berry et al. 1975), CO_2-baited traps (Thompson et al. 1972), gravid traps (Scheidler et al. 2006), or by aspiration (Berry et al. 1975, Thompson et al. 1972, Nasci et al. 2000, Barker et al. 2003), and larval sampling (Pantuwatana et al. 1974, Beaty and Thompson 1975, Kappus et al. 1982, Barker et al. 2003). Each method has advantages and disadvantages

with respect to the time spent in the field sampling or rearing individuals in the laboratory. For example, light traps, gravid traps, and CO_2-baited traps all require collections every 24 hours and large amounts of equipment, but less laboratory time since adults are collected. In comparison, oviposition traps require less field time, with collections once a week or less, but more laboratory time and space is required for rearing adults from the eggs that are collected. Actively searching for larvae or aspirating adults requires a large amount of time in the field; some laboratory time is required with larval sampling.

We used data from previous research studies to assess the potential of different trapping methods for collecting mosquitoes from rural and urban landscapes that are endemic for LAC virus. Gravid traps and CO_2-baited light traps were used to collect adult mosquitoes and oviposition traps were used to collect eggs.

4.3 Materials and methods

4.3.1 Location

Trapping was conducted in portions of two regions, New River Valley (NRV) and Roanoke, located in southwestern Virginia, along the Blue Ridge Mountains. The NRV includes the Counties of Floyd, Giles, Montgomery, and Pulaski, and the City of Radford; combined these areas have an elevation ranging between 535 to 750 m. The approximate population for the NRV is 156,122 spread over 3,748 km^2 (Census 2008). The Roanoke region is a more urban area located east-northeast of the NRV and includes both Roanoke County and the City of Roanoke. The combined population is approximately 181,972 in 756 km^2 (Census 2008). Elevation ranges between 269 and 530 m with the higher elevations coming from peaks of several mountains. The dominant forest type throughout the area is oak-hickory (Johnson 1992).

4.3.2 Sampling techniques

Adult mosquitoes were obtained from collections made during the summer of 2002 using both gravid traps (Hausherr's Machine Works, Toms River, NJ) and CO_2-baited light traps (John W. Hock Co., Gainesville, Florida). Gravid trap collections were done as part of a congruent study (Jackson et al. 2005). For this study, the males were retained for testing and only data on LAC virus vectors are considered. Light traps were baited with 0.9 kg of dry ice and were set approximately 1.5 m above the ground. Traps were put out in the late afternoon and collected the next morning. Collections cups were placed on ice and returned to the lab where the mosquitoes were frozen and identified to species. Other species were collected and identified but were not processed for testing; only four species (*Ae. triseriatus*, *Ae. albopictus*, *Ae. japonicus*, and *Ae. canadensis*) were considered in this study. Mosquitoes were stored in a -80°C freezer until shipped for testing.

Both the gravid traps and light traps were rotated during the season so some sites had both trap types while others only had one trap type. A total of 9 sites in the NRV and 34 in Roanoke were used during this study year. Trapping in the NRV commenced on June 6 and continued until October 18, whereas trapping in Roanoke started on August 9 and ended October 17. Each site was trapped between one and four times each week, depending on the site.

In the summer of 2005, oviposition cups were used to collect the eggs of container breeding mosquitoes (Loor and DeFoliart 1969). Traps consisted of 450 ml black plastic cups (11 x 9 cm) (WNA, Inc. (SKU 16SPOLY), Covington, KY). Two holes were drilled on opposite sides of cup to prevent overflow. Cups were filled with approximately 200 ml of deionized water. A 12.5 x 5 cm piece of seed germination paper (Anchor Paper Company (No. SD 3815L), Saint Paul, MN) was hung vertically in the cup and secured with a small binder clip. Striations

on seed germination paper were in a vertical orientation. Cups were placed approximately 0.7 m above the ground on wooden stakes.

Cups with the germination paper were left in the field for seven days and were then removed and taken back to the lab. Eggs on papers were kept in an environmental chamber (24°C, 75% RH, and 16L:8D photoperiod) for seven days before being hatched and reared to adults. Adults were identified to species and stored in a -80°C freezer until they were shipped for testing. Eggs were collected between August 15 and August 29, from 49 sites in Montgomery County. Trapping in the Roanoke region occurred between August 16 and August 30 at 29 locations.

4.3.3 LAC virus detection

Mosquitoes were separated into pools of no more than 50 individuals according to species and location. Several gravid trap and light trap collections, from the same site, were combined because of the testing lab required a minimum number of specimens per pool. Mosquitoes were kept on a cold chain during processing, identification, and shipping. The Virginia Division of Consolidated Laboratory Services analyzed pools for LAC virus RNA following homogenization in BA-1 diluent, clarification by centrifugation, and nucleic acid extraction using the QIAamp Virus BioRobot system. Extracts were subjected to real time reverse transcription-polymerase chain reaction assays using FAM- and TAMRA-labeled probes and primers specific to LAC virus (sequences provided by Dr. Robert Lanciotti, CDC).

4.3.4 Infection rates and statistical analysis

Infection rates were determined using bias-corrected maximum likelihood estimation (MLE) (Biggerstaff 2006). Fisher's exact tests (two-tailed, $P<0.05$) were used to determine significant differences in the number of infected pools between years, between species, and within years using Prism (Version 5.0, GraphPad Software, El Camino, CA).

4.4 Results

During the summer of 2002, a total of 1,310 adult mosquitoes, in 171 pools, were processed for LAC virus testing. Of 890 mosquitoes collected in the Roanoke region, 97% were *Ae. albopictus*. The median pool size was 5.5 mosquitoes. No pools from Roanoke tested positive for LAC virus.

The NRV accounted for only 32% of the total mosquitoes tested, but there were more pools tested. The number of pools from the NRV was slightly higher than that of Roanoke because the median number of mosquitoes in each pool was only two. The most numerous of the two species was *Ae. albopictus* (56%) while *Ae. triseriatus* accounted for only 34% of the collections. Two pools of 39 *Aedes canadensis* (Theobald) were also collected, one of which, collected on June 19, tested positive for the LAC virus. The infection rate for *Ae. canadensis* was not calculated because this species was not common in the traps.

In addition to the positive pool of *Ae. canadensis*, six other pools of mosquitoes tested positive, two pools of *Ae. triseriatus* and four pools of *Ae. albopictus* mosquitoes. The overall maximum likelihood estimation of infection rate (MLE-IR) for adult mosquitoes collected with gravid traps and light traps in the NRV was 17.66/1,000 (95% CL = 8.12–34.12) (Table 4.1). *Aedes albopictus* had a higher MLE-IR at 18.86 (95% CL = 6.10–46.28) compared to 12.41 (95% CL = 2.70–38.25) for *Ae. triseriatus*. The virus positive *Ae. albopictus* were collected on

July 31, August 14, August 24, and October 2 and came from pools of 2, 3, 10, and 43 mosquitoes, respectively. The two earliest pools were collected from light traps while the other two pools contained adults that were collected in both gravid and light traps. Both *Ae. triseriatus* pools were collected on August 21 with the number of adults in each pool being 1 and 2. One pool came from a gravid trap and other from a mix of the two adult traps.

A total of 423 pools from 9,802 adults reared from eggs collected in 2005 in Montgomery County and Roanoke were tested for LAC virus. Roanoke accounted for 27% of the total number of eggs reared to adults. *Aedes triseriatus* was the most abundant species comprising 70% of the total, followed by *Ae. albopictus* and *Ae. japonicus* with 29% and <1%, respectively. Median number of adults per pool was 13. No pools from Roanoke tested positive for LAC virus.

The majority (73%) of the adults reared from eggs came from Montgomery County. *Aedes triseriatus* was by far the most abundant mosquito processed with 93% followed by *Ae. albopictus* with 6% and *Ae. japonicus* with <1%. The median number of adults per pool was 20. The only positive came from one pool of *Ae. triseriatus* adults reared from eggs collected on August 22. The MLE-IR for *Ae. triseriatus* in Montgomery County was 0.15/1,000 (95% CL = 0.01–0.72) (Table 4.2) and the overall infection rate for the county in August of 2005 was 0.14 (95% CL = 0.01–0.68).

Significantly more infected pools were collected in 2002 from adult mosquitoes collected in gravid traps and light traps than from adults, which were reared from eggs collected in 2005 (P<0.001). There were significantly more pools of adult *Ae. triseriatus* infected with LAC virus collected in 2002 compared with adults reared from eggs in 2005 (P<0.05). The number of

infected pools was not significantly different between *Ae. albopictus* and *Ae. triseriatus* for the collections made in 2002.

4.5 Discussion

The decision to collect adult mosquitoes or eggs to detect and determine LAC virus infection rates is important because one method may offer a better chance of collecting virus isolates. Our data suggest that collecting adult mosquitoes with gravid traps or CO_2-baited light traps offers a better chance of collecting virus positive mosquitoes. In 2002, we collected 420 adult mosquitoes from the NRV and submitted 88 pools for testing. Seven of the pools (8%) tested positive for LAC virus. These virus positive mosquitoes were the first known collections of LAC virus from this region of Virginia. In 2005, we raised 7,152 mosquitoes to adults from eggs collected through ovitrapping in Montgomery County. We separated these mosquitoes into 282 pools and submitted them for testing. Of these pools, only one tested positive for LAC virus. Less than 0.01% of the pools of mosquitoes collected through ovitrapping were positive for the LAC virus. Ovitrapping, therefore, produced significantly fewer virus positive pools than collecting adults with gravid traps and CO_2-baited light traps. Nasci et al. (2000) also found differences in the infection rates of mosquitoes collected as adults and eggs in Nicholas County, West Virginia. The authors found that adult *Ae. triseriatus* landing collections had minimum infection rates between 0.0 and 27.0/1,000, while egg collections had rates ranging between 0.4 and 7.5/1,000.

Scheidler et al. (2006) collected adult *Ae. triseriatus* mosquitoes using gravid traps and CO_2-baited light traps in Ohio and recorded an overall MLE-IR of 3.22/1,000. With an MLE-IR

for *Ae. triseriatus* of 12.41/1,000 and an overall infection rate for all species of 17.66/1,000, our infection rate was higher than the rate found in Ohio but was similar to the rate in West Virginia.

There is a wide range of infection rates from the collection of eggs reported from studies in other states: 0.26/1,000 and 1.0/1,000 from North Carolina (Kappus et al. 1983, Szumlas et al. 1996), 12.3/1,000 in Ohio (Berry et al. 1975), 0/1,000–5.9/1,000 and 6.4/1,000 from Wisconsin (Beaty and Thompson 1975, Lisitza et al. 1977), and 1.2/1,000 in Illinois (Clark et al. 1983). Our infection rate of *Ae. triseriatus* eggs was 0.14/1,000 which falls within the range reported in other studies.

Three of the virus positive samples were *Ae. triseriatus*, two from adult collections and one from egg collected in ovitraps. The single virus positive pool of mosquitoes from the egg collections confirms transovarial LAC virus transmission by *Ae. triseriatus* in this area. Collecting LAC virus isolates from field collected eggs is not uncommon (Clark et al. 1982, Szumlas et al. 1996, Nasci et al. 2000). With high rates of transovarial transmission and filial infection, it is not surprising that one of our isolates came from eggs. Studies by Miller et al. (1977) and Hughes et al. (2006) found filial infection rates of 46% and 71% and transovarial transmission rates of 71% and 98%, respectively. Even with the high rates, we cannot assume that our isolates from field collected adults were from transovarially infected mosquitoes because the feeding habits of the mosquito often include a tree squirrel and chipmunk cycle (Beaty and Calsiher 1991). Several studies have determined that *Ae. triseriatus* mosquitoes prefers to feed on amplifying hosts, such as chipmunks and squirrels (Wright and DeFoliart 1970, Nasci 1985, Richards et al. 2006).

Four of our eight LAC virus positives came from adult *Ae. albopictus* mosquitoes. Mosquitoes collected as adults may have acquired LAC virus infection through horizontal

amplification or through transovarial transmission. Hughes et al. (2006) calculated the transovarial transmission rates and filial transmission rates of *Ae. albopictus* to be 52% and 18%, respectively, which are significantly lower compared to those of *Ae. triseriatus*. Given these low rates, it is unlikely that all of the positives came from transovarially infected mosquitoes. Studies on host blood from field collected *Ae. albopictus*, have shown that the mosquito will feed on many different hosts with very few blood meals coming from amplifying hosts, such as chipmunks and squirrels (Neibylski et al. 1994, Richards et al. 2006). The catholic feeding behavior of *Ae. albopictus* provides a means for the mosquito to become infected but also reduces its efficiency as a vector. The broad host range means that a large proportion of *Ae. albopictus* bites are "wasted" because the mosquito is not specifically feeding on competent hosts. Our data suggests that the mosquito plays some role in virus amplification but only a small part in the overwintering of the virus.

Our earliest positive pool from adults collected in 2002, came from a pool of *Ae. canadensis* collected on June 19. *Aedes canadensis* is generally an early season mosquito with overwintering eggs hatching in late winter or early spring and larvae found from March to late June (Magnarelli 1977, Troyano 2009). This corresponds with our early positive for this species. Under laboratory conditions, *Ae. canadensis* is a competent vector of LAC virus, although it is not as efficient as *Ae. triseriatus*. Several virus isolations from the field have been made in Ohio and West Virginia (Berry et al. 1986, Nasci et al. 2000). The low number of adults collected in our study make it difficult to assess the role *Ae. canadensis* might play in the maintenance cycle of LAC virus in our study areas.

The ability to predict human outbreaks of arboviral diseases is dependent upon monitoring temporal differences in infection rates of vector populations (Moore et al. 1993). The

dates for our positive mosquitoes ranged from June 19 to October 2 with five isolations being collected in mid to late August. Areas such as Illinois, North Carolina, Tennessee, and Ohio have reported human cases in late August and early September (Berry et al. 1975, 1983, Clark et al. 1983, Szumlas et al. 1996, Jones et al. 1999). No human cases have ever been reported in our trapping region. Nationally, approximately 94% of 54 cases were reported in July, August, and September (Kappus et al. 1983). This time period is also when LAC virus antibodies peaked in chipmunks (Gauld et al. 1975).

In all likelihood, neither of our trapping methods provided an accurate indicator of species composition in the two regions. One reason for this is that generally adult traps were placed near homes while ovitraps were placed in more secluded wooded areas. From the adult collections, 97% of the mosquitoes collected in the Roanoke region were *Ae. albopictus* compared to 56% from the NRV. In contrast, in the 2005 egg study *Ae. triseriatus* comprised 70% of our Roanoke collections compared to 94% of the collections in Montgomery County. The ovitraps yielded lower *Ae. albopictus* numbers in part because there is a lower recovery of *Ae. albopictus;* some *Ae. albopictus* eggs hatched in the ovitraps while in the field and there is greater egg mortality for this species during storage. Thus, the abundance of *Ae. albopictus* is underestimated. Also, placement of the traps influences the species composition. By placing ovitraps closer to residences, Barker et al. (2003) found a greater number of *Ae. albopictus* than they did in more wooded areas.

Between 2005 and 2008, Troyano (2009) conducted a serosurvey for LAC virus antibodies from dog blood collected at veterinary hospitals. A total of 183 samples were tested from the NRV and 103 from the Roanoke region. Twelve samples (6.6%) from the NRV were positive and 6 samples (5.8%) from the Roanoke region were positive. Seropositive dogs from

the NRV confirm that horizontal transmission of LAC virus is occurring in this region. Although, Troyano (2009) found seropositive dogs in Roanoke, we did not find any virus positive mosquitoes in the area. There are several possible reasons for this apparent contradiction. First, the virus may be circulating at low levels so that the number of infected mosquitoes were too few to be detected in traps. Second, it is not known when these dogs acquired the virus, thus there was no temporal link between the dog exposure and our mosquito collections. A third reason is the absence of a complete and accurate travel history for the dog's lifetime, since trips to the NRV were not recorded.

In certain situations results are needed as soon as possible to indicate where a virus is occurring or to identify vector species. Because of the reduced processing time, collecting adults directly requires less time to provide answers to these questions. Once back in the lab, adult collections need only be separated by site and species before being assayed for virus. In contrast, egg collections require time to rear the eggs to adults before processing them for testing. For example, during our studies we allowed the eggs one week to embryonate before hatching and the time required to reach adulthood was between 8 and 20 days depending on the species. Thus, there is a minimum 2–3 week delay with egg collections before samples could be sent off for testing.

We obtained significantly more LAC virus infected mosquito pools using adult trapping methods. For the two regions we reared 9,802 eggs to adults and found one virus positive pool compared with 1,310 field-collected adults which produced seven virus positive pools. The smaller number of specimens collected and processed as adults also meant there was less possibility of contamination among samples. More resources were needed to operate adult traps, but less laboratory time was required to process the samples. A single adult trap can cost as

much as $150 dollars and both trap types require attractants on a daily basis as well as batteries and chargers. In our study, each CO_2-baited light trap was baited with 0.9 kg of dry ice per day, which was costly and not available in all locations. In contrast, oviposition cups are relatively inexpensive requiring only plastic drinking cups and seed germination paper. However, the laboratory facilities and technical assistance needed to hatch and rear the mosquitoes to adult should be considered. We conclude that studies to estimate LAC virus field infection rates should include adult trapping. Because adult traps collect both horizontally and vertically infected mosquitoes, they provide a more sensitive measure of virus activity. The relatively lower transovarial transmission rate of *Ae. albopictus* compared to *Ae. triseriatus* is an added advantage to using adult traps because egg collections may underestimate infection rate of *Ae. albopictus* (Hughes et al. 2006). During our study several gravid trap and light trap collections were combined to reduce total pool numbers, therefore we could not determine which trap type was more sensitive for collecting adults infected with LAC virus. Future studies should include keeping both trap type collections separate to determine which trap is more sensitive.

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Tables

Table 4.1. La Crosse virus bias-corrected MLE-IRs of infection rates for adult mosquitoes collected in gravid traps and CO_2-baited light traps in 2002.

	Number of Positive Pools	Number of Pools	Number of Adults	Infection Rate	Lower Limit	Upper Limit
NRV						
Ae. albopictus	4	58	237	18.86	6.10	46.28
Ae. triseriatus	2	28	144	12.41	2.70	38.25
*Total	7	88	420	17.66	8.12	34.12
Roanoke						
Ae. albopictus	0	72	861	0.00		
Ae. japonicus	0	1	1	0.00		
Ae. triseriatus	0	10	28	0.00		
Total	0	83	890	0.00		

(*) Two pools of *Aedes canadensis* were tested for virus, one of which was positive.

Table 4.2. La Crosse virus bias-corrected MLE-IRs of infection rates for adult mosquitoes collected as eggs from oviposition cups in 2005.

	Number of Positive Pools	Number of Pools	Number of Adults	Infection Rate	Lower Limit	Upper Limit
Montgomery						
Ae. albopictus	0	60	452	0.00		
Ae. japonicus	0	2	4	0.00		
Ae. triseriatus	1	220	6696	0.15	0.01	0.72
Total	1	282	7152	0.14	0.01	0.68
Roanoke						
Ae. albopictus	0	58	785	0.00		
Ae. japonicus	0	4	9	0.00		
Ae. triseriatus	0	79	1856	0.00		
Total	0	141	2650	0.00		

Chapter 5

SUMMARY

Approximately 81 confirmed and probable La Crosse (LAC) encephalitis cases have been reported each year, between 1964 and 2007, making it one of the most commonly reported arboviral diseases (Rust et al. 1999, CDC 2008). Cases from Virginia and West Virginia total 596 (CDC 2008). Symptoms include febrile illness, vomiting, and headache with some cases progressing to seizures and generally occur in children under the age of 15 (McJunkin et al. 1998). The mild flu-like symptoms associated with LAC virus are often subclinical or misdiagnosed. Estimates for the number of actual cases have been as high 300,000 infections per year (Rust et al. 1999). Three container breeding mosquito species have all been implicated as vectors of LAC virus (Pantuwatana et al. 1974, Gerhardt et al. 2001, Harris et al., *in review*). Both *Aedes albopictus* and *Aedes japonicus* are recent introductions and share habitat with the major vector, *Aedes triseriatus* (O'Meara et al. 1993, Bevins 2007). With the increased number of LAC encephalitis cases and the introduction of exotic accessory vectors, there is a need to have a better understanding of the interactions among vectors, the virus and its vectors, and methods to estimate virus activity in an area. The purposes of this study were: 1) to determine the ovipositional preferences of three LAC virus vectors when presented with heterospecifics and conspecifics, 2) to test the effect of LAC virus infection on *Ae. triseriatus* and *Ae. albopictus* blood-feeding behavior, and 3) to determine which lifestage of the mosquito, egg or adult, would be more efficient for calculating minimum infection rates (MIR).

Laboratory studies were conducted on the ovipositional preferences of *Ae. triseriatus*, *Ae. albopictus*, and *Ae. japonicus*. Four choice tests of heterospecific, conspecifics, and a control were presented to gravid female mosquitoes. Three separate experiments consisted of one of

species demonstrated ovipositional preferences in at least one of the choice tests. *Aedes japonicus* was the only species that showed any preference when ovipositing in cups with preexisting eggs. More eggs were deposited in cups containing preexisting eggs of conspecifics and *Ae. albopictus.* Both *Ae. triseriatus* and *Ae. japonicus* were deterred from ovipositing in cups containing1st instar *Ae. albopictus* larvae and the control cup. *Aedes japonicus* and *Ae. triseriatus* preferred cups containing LRW of conspecifics and *Ae. albopictus*. Bentley et al. (1976) had similar results when *Ae. triseriatus* preferred conspecific LRW. *Aedes albopictus* demonstrated ovipositional preferences for LRW. More eggs were laid in cups which had previously contained larvae and pupae of all three mosquito species compared to the control cups. Other studies showed that *Ae. albopictus* preferred LRW over control water (Allan and Kline 1998, Trexler et al. 2003).

Aedes japonicus appeared to be the only species that could distinguish between eggs of heterospecifics. *Aedes albopictus* 1st instar larvae acted as a deterrent for the other two species. A possible explanation for this behavior is that *Ae. albopictus* larvae have shown competitive advantages over other mosquito species (Juliano and Lounibos 2005, Armistead et al. 2008). Changes in the chemical cues coming from the oviposition sites and the absence or presence of larvae in the cups, may have contributed to the changes in oviposition preference among experiments. The apparent nondiscrimination by *Ae. albopictus* when depositing eggs has probably contributed to the rapid spread of this species throughout the country. Other studies have also noted that this superior competitor shows little selectivity when ovipositing in the presence of other species (Gubler 1971, Allan and Kline 1998).

As a follow up to our laboratory experiments, we conducted two field studies. In the first study, we placed fresh egg papers in the field and then counted the number of species' eggs. The

C_8 coefficient of interspecific association showed a positive relationship between *Ae. triseriatus* and the other two species. This finding was unexpected, because our laboratory results suggested that *Ae. japonicus* was deterred by *Ae. triseriatus* eggs. However, in the field site, *Ae. triseriatus* comprised nearly 70% of the total eggs collected. Thus, it would have been difficult for *Ae. japonicus* or *Ae. albopictus* to avoid egg papers that did not contain eggs of *Ae. triseriatus*. Also, it was impossible to determine the order of oviposition. In our second field study, we used egg papers with preexisiting eggs. Although we could not detect any differences between the number of eggs laid on the papers, *Ae. japonicus* had a high mean intensity of eggs in all the treatments. Only 42% of the available conspecific cups were used by *Ae. japonicus*. Environmental influences in our field studies, such as height of tree canopy, may have contributed to ovipositional site selection rather than the preexisting eggs.

Both *Ae. triseriatus* and *Ae. albopictus* mosquitoes infected with LAC virus took significantly smaller blood meals when compared to controls. The median blood meal size of *Ae. albopictus* control mosquitoes was 0.25 µl (3%) larger than infected mosquitoes and in *Ae. trisertiatus* the size difference was 0.46 µl (6%). While our experiments did not examine the cause of the disruption of feeding behavior, it is believed that infection of abdominal ganglia with LAC virus results in a neural disruption and ultimately the inability of the mosquito to fully engorge (Beaty and Thompson 1976, Grimstad et al. 1980).

Infected mosquitoes were then tested to see if they would refeed on a host 24 hours after the first feeding. More *Ae. triseriatus* mosquitoes infected with LAC virus did return to a host to take a second blood meal. Only 9% of the uninfected mosquitoes returned for a blood meal compared to 23% of the infected. Normally an uninfected mosquito would take a full blood

meal with one feeding attempt and exhibit a host seeking inhibition while developing eggs. A similar effect on feeding was not seen in infected *Ae. albopictus*.

An important part of any arbovirus surveillance program is the ability to estimate virus activity in field populations of mosquitoes. By comparing MLE-IRs of mosquitoes collected as adults (CO_2-baited light traps and gravid traps) and eggs (oviposition traps), we conclude that adult trapping provided a more efficient means of collecting virus positive mosquitoes. The MLE-IR for the New River Valley using adult trapping was 17.66/1,000 specimens. This rate was higher than our egg collections which produced an MLE-IR of 0.14/1,000. Almost 20X more mosquitoes from eggs were processed for testing compared to adult trapping. Egg collections yielded one virus positive compared to seven from adult trapping. Nasci et al. (2000) found differences in the infection rates of different lifestages, adult landing collections had minimum infection rates between 0.0 and 27.0/1,000, while egg collections had rates ranging between 0.4 and 7.5/1,000.

From our study, we concluded that adult trapping increases the chances of isolation virus from field-collected mosquitoes, although, the cost of purchasing and operating the traps is high. However, the cost of laboratory effort to rear the mosquitoes for the 2-3 weeks required for them to reach adulthood probably balances out this expense. Field collected mosquitoes can be tested for virus immediately, thus providing more rapid results. The higher MLE-IRs seen in adult collections were most likely due to the fact these mosquitoes may result from either transovarial or horizontal transmission. In contrast, mosquitoes collected as eggs could only become infected with virus transovarially.

In conclusion, since *Ae. albopictus*, *Ae. japonicus*, and *Ae. triseriatus* all share common habitats, there is an increased probability the mosquitoes will encounter one another when

ovipositing. Our laboratory studies suggest that certain species may avoid ovipositing at sites where other species are present, although, we were not able to recreate our results in the field. If mosquitoes use the absence or presence of certain mosquito species as an ovipositional cue, we may see a change in the distribution of certain species. These changes may lead to the expansion of LAC virus into areas that are not traditionally known to have the virus. Because *Ae. albopictus* demonstrated little preference, and when they did it was for cups with LRW of other vectors of LAC virus, there could be an increase in vectors in an area as *Ae. albopictus* moves into areas currently inhabited by other vectors. The addition of *Ae. albopictus* would increase the number of bridge vectors, thereby increasing the chances of humans becoming infected with the virus. Areas that are traditionally endemic for LAC virus may see an increase in human cases and this may be the reason we are seeing increased case numbers in Virginia. Our data does not suggest that any one species will become locally extinct from certain areas in the presence of other species. None of the species demonstrated a preference or deterrence for any other species in every experiment. Oviposition would depend on which lifestage a gravid female encountered as to whether or the female would choose to oviposit. If any of the three species is likely to be more commonly associated with another it would be *Ae. japonicus* and *Ae. albopictus*. *Aedes japonicus* may move from more forested areas and into areas traditionally inhabited by *Ae. albopictus*. As secondary vectors move into areas that are not endemic for LAC virus, it is not expected that these vectors will maintain the virus in new areas. The transovarial transmission rates and feeding habits of the accessory vectors would limit their ability to maintain the virus. Thus, the development of new foci of LAC virus would require the presence of the major vector, *Ae. triseriatus*, to become established. However, the two accessory vectors

have the potential to increase horizontal amplification and, subsequently, increase the incidence of human cases in LAC virus endemic areas.

Our blood-feeding and refeeding studies suggest that, if given the chance, virus infected *Ae. triseriatus* mosquitoes will take multiple blood meals. The implication for LAC virus infected mosquitoes taking an initial smaller blood meal and followed by a second blood meal, creating the possibility of increased feedings by infected mosquitoes within one gonotrophic cycle is that horizontal transmission may be enhanced. Infected mosquitoes may bite multiple hosts to obtain a full blood meal, therefore, increasing the number of infected hosts. This, in turn, increases the vectorial capacity of *Ae. triseriatus*. *Aedes albopictus* will most likely form a benign relationship with LAC virus so that infection is not detrimental to both. This relationship may take hundred and possibly thousands of years but eventually it is expected that infected *Ae. albopictus* mosquitoes will refeed within one gonotrophic cycle. An increase in feeding attempts will create more chances for the virus to infect amplifying hosts and humans but the increase will only be minimal because of the feeding habits of *Ae. albopictus*.

If adequate funds and staff are available for an arbovirus surveillance program, then adult trapping methods would be preferred. This method provides a more sensitive and rapid estimate of infected mosquitoes in a given area. However, egg collections should not be completely eliminated because they provide an efficient way of monitoring transovarial infection rates in field populations. If only one trap type could be used to collect LAC virus vectors, then the author would choose to operate gravid traps. The traps collect all three vector species and has demonstrated the ability to collect large numbers of *Ae. japonicus*. Gravid traps do not collect large amounts of trash insects, collections can be quickly processed for testing, and the infusions are generally cheap and only require a week or less to prepare. While upfront costs may be

expensive, running the traps and maintenance is often cheap. The only drawbacks to using gravid traps are the specimens may be damaged making identification more difficult and the traps must be checked on a daily basis.

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Appendix

Detection of La Crosse Virus in Field-collected *Ochlerotatus j. japonicus* in Southwestern Virginia

La Crosse encephalitis virus (LAC), a California serogroup bunyavirus, is the major cause of pediatric arbovirus encephalitis in the United States (US) (Mcjunkin et al. 1998). Since its 1963 discovery in Wisconsin, LAC has been identified in thirty-two states within the contiguous US (CDC 2008). The Appalachian Mountains are part of an emerging focus of LAC (Barker et al. 2003). *Aedes triseriatus*, the primary vector of LAC, is prevalent in our area but we have recently noted the emergence of two invasive species: *Ae. albopictus* (Barker et al. 2003) and *Oc. japonicus* (Grim et al. 2007). Both have been shown to be competent experimental LAC vectors (Grimstad 1989; Sardelis et al. 2002), however, their role in LAC dynamics is not fully understood. Here we report two independent field isolations of LAC from *Oc. japonicus* eggs and adults, suggesting a potential role for this invasive vector in LAC dynamics.

In 2005, mosquitoes were collected weekly in Wise County, Virginia. Ovitraps with seed germination paper as an oviposition substrate were used to collect eggs (Steinly et al. 1991). Larvae were reared to adults in a BSL-2 insectory at 24°C, 75% RH, and 16L: 8D photoperiod. Mosquitoes were morphologically identified to species and grouped in pools of ≤50 individuals according to species, location and collection date. The adults were stored at -80°C until viral testing could be performed. In 2008, adult mosquitoes were collected weekly from infusion-baited gravid traps (Jackson et al. 2005) at sites in Montgomery and Craig counties. After 24-h storage in a -80°C freezer, mosquitoes were sorted by species, collection site and date.

Viral RNA was extracted from homogenized mosquito pools with QIAamp Viral RNA Mini Kit (QIAGEN, Valencia, CA, USA). Real-time reverse-transcriptase polymerase chain reaction (RT-PCR) was used to detect LAC using the QuantiTect probe RT-PCR Kit (QIAGEN, Valencia, CA, USA). An ABI PRISM 7000 system and the following probe and primer sequences were used (R. Lanciotti, pers. communication): LAC2364 LAC Forward 2: CAATAATTGCGTGTGGTGAACC, LAC2387 LAC Probe 2: AATGGGCCAAGTGTGTATAGGAAACCATCA, LAC2448 LAC Reverse 2: GACCGATCAGTGCTAGATTGGAA. We present the mean crossing point (CP), defined as the point at which the fluorescence rises significantly above the background fluorescence, for each run.

In August 2005, 2,331 adults, reared from eggs, were analyzed for virus. LAC virus was isolated from one pool of nine *Oc. japonicus* females collected from Wise County, VA in August 2005. The average CP of two runs was 38.04. The bias-corrected maximum likelihood estimate (MLE) for LAC-infected *Oc. japonicus,* during the month the positive pool was collected, was estimated to be 8.59 (95% CL = 0.54 - 41) infected mosquitoes per 1,000 specimens (Biggerstaff 2006). None of the 1,965 (97 pools) of *Ae. triseriatus* adults tested positive. 1,188 mosquitoes were collected from Montgomery County in July 2008. The *Ae. triseriatus* density was approximately 1.5 times greater than *Oc. japonicus*. One pool of 22 *Oc. japonicus* females from Montgomery County, VA tested positive for LAC. A high amplitude signal (mean: 37.57 CP) was obtained from this pool and reproduced in three additional runs. The bias-corrected MLE for LAC-infected *Oc. japonicus*, during the month the positive pool was collected, was estimated to be 4.51 (95% CL = 0.26 - 22) infected mosquitoes per 1,000 specimens (Biggerstaff 2006).

Although *Ae. triseriatus* outnumbered *Oc. japonicus*, no positive pools were identified for this primary vector.

To the authors' knowledge, this is the first report of LAC in field-collected *Oc. japonicus*. The discovery of vertically-infected *Oc. japonicus* eggs in the field suggests that transovarial transmission of LAC occurs in this species. In this southern LAC focus, *Oc. japonicus* may play an important role in maintenance, transmission and expansion of LAC given its recent range expansion. Additional research is needed to confirm that *Oc. japonicus* is an important LAC vector in Virginia and to document potential impacts of this invasive vector on emerging LAC foci.

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