Transmission of La Crosse Virus in Southwest Virginia:

Role of Accessory Vectors, Microfilarial Coinfection, and Canine Seroprevalence

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Abstract

Southwest Virginia has recently become an emerging focus of activity for La Crosse (LAC) virus, a mosquito-transmitted arbovirus in the California serogroup of Bunyaviruses. In 2005 and 2006, ovitrap surveys were conducted to access the spatiotemporal oviposition activity of LAC virus vectors *Aedes triseriatus*, *Ae. albopictus* and *Ae. japonicus* across a wide region of southwest Virginia. Egg abundance and oviposition patterns of these vectors were significantly different across the three study areas. The primary LAC virus vector, *Ae. triseriatus*, was collected in the greatest abundance from all three areas, and favored forested habitats. *Aedes albopictus* was the second most abundant species collected, and was found to favor urban environments. *Aedes japonicus* also has a preference for urban habitats, and is actively expanding its range throughout southwest Virginia.

Dogs were used to determine their efficacy as sentinels for assessing the distribution of LAC virus in southwest Virginia. Canine serum samples were tested using plaque reduction neutralization (PRNT) assays. Of the 436 collected canine serum samples, 21 (4.8%) were positive for LAC virus antibodies. LAC virus seroprevalence was evident in dogs from each study region, including areas where LAC virus human cases and LAC virus positive mosquito isolates have not been reported. As a result, this study provided documentation of horizontal

transmission of LAC virus throughout southwest Virginia, demonstrating that dogs make useful sentinels for assessing the distribution of LAC virus in an area.

The final objective examined the effects of coinfection with *D. immitis* microfilariae and LAC virus in three species of *Aedes* mosquitoes. No significant differences were found between mosquitoes fed dually infected bloodmeals (i.e. *D. immitis* microfilariae and LAC virus) and those fed bloodmeals containing LAC virus only. A follow-up study found low mosquito midgut penetration rates by *D. immitis*, despite using biologically significant doses of microfilariae. Failure to demonstrate enhancement of LAC virus in vector mosquitoes suggests that *D. immitis* does not have a significant impact on LAC virus epidemiology in areas where these organisms co-exist.

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Table of Contents

List of Figures

List of Tables

1. Introduction and Literature Review

1.1 La Crosse Virus

La Crosse (LAC) virus is an arbovirus in the California serogroup of the family Bunyaviridae. It was first isolated in 1960 in La Crosse, Wisconsin from brain tissue of a child who died after developing encephalitis (Thompson et al. 1965). LAC virus is the leading cause of pediatric encephalitis in the U.S. In addition, children infected with LAC virus can show neurological sequelae such as cognitive and behavioral deficits (Balkhy and Schreiber 2000, Mc Junkin et al. 2001, Utz et al. 2003).

In nature, LAC virus is maintained through mammalian host reservoirs, most commonly chipmunks (*Tamias striatus* Linnaeus) (Gauld et al. 1975), grey squirrels (*Sciurus carolinensis* Gmelin) (Ksiazek and Yuill 1977), and red foxes (*Vulpes fulva* Linnaeus) (Amundson and Yuill 1981). The eastern treehole mosquito, *Aedes triseriatus*, is the primary vector of LAC virus (Berry et al. 1974, Pantuwatana et al. 1974, Watts et al. 1974). LAC virus is maintained through vertical transmission from a female to her offspring, allowing the virus to overwinter in infected eggs (Watts 1973 and 1974). In addition, LAC virus can be transmitted venereally between adult mosquitoes. Humans are considered dead end hosts, as they do not maintain prolonged viremias and can therefore not serve as amplifying hosts for reinfection of the mosquito vector (Mc Junkin et al. 1998).

According to the Centers for Disease Control (CDC), LAC encephalitis has been reported from 27 states (CDC 2007), at a rate of about 70 cases per year. Historically, most cases have been reported from midwestern states, particularly Wisconsin, Ohio, and Minnesota (Calisher 1983). However, in the past decade, an increase in LAC encephalitis cases has been reported from the Appalachian region of mid-Atlantic and southeastern states, with the majority of the

cases occurring in West Virginia (Nasci et al. 2000). Additional reports show LAC virus as an emerging disease in Tennessee, North Carolina, and Virginia (Gerhardt et al. 2001, Barker et al. 2003a,b).

These increased reports of LAC virus may represent a new southeastern expansion of an endemic focus in the Appalachian region (Gerhardt et al. 2001). Southwest Virginia has recently emerged as a significant focus of LAC virus activity (Barker et al. 2003a,b). Between 1975 and 1993, only one case of LAC encephalitis was reported from the state of Virginia. However between 1994 and 1998, the CDC reported 13 cases of LAC encephalitis from areas in southwest Virginia in the Appalachian Mountains. An additional 12 cases were reported in Virginia between 2003 and 2008 (CDC 2007, VA Dept. of Health 2008).

This increase in LAC virus activity in the Appalachian region may be related to the relatively recent invasions of the mosquito species, *Aedes albopictus* and *Ae. japonicus*, which are capable LAC virus vectors in laboratory settings (Tesh and Gubler 1975, Sardelis et al. 2002). The role of these invasive mosquitoes as LAC virus accessory vectors will be investigated in this study.

1.2 LAC Virus Structure and Replication

LAC virus is a single stranded, negative sense RNA virus with a spherical nucleocapsid. The LAC virion is 90–100 nm in length, with G1 and G2 surface glycoproteins that appear as 5–10 nm spikes. The G1 and G2 glycoproteins serve for host cell attachment. G1 attaches to mammalian hosts and G2 to invertebrate mosquito hosts (Ludwig et al. 1991). LAC virus is a tripartite virus, possessing three circular nucleocapsid segments termed “L”, “M”, and “S’ segments. The “L” segment encodes for the viral polymerase, “M” for the G1 and G2

glycoproteins and a nonstructructural protein NS_m, and "S" for nucleocapsid proteins and another nonstructural protein NS_s (Elliot 1990, Borucki et al. 2002).

LAC virus attaches to host cells via G1 binding in mammals, or G2 in mosquitoes, and the virus is brought into the cell via receptor mediated endocytosis. Once inside the host cell, the virus will undergo transcription to synthesize viral mRNA. Primary transcription yields viral mRNAs from each genome segment, which occurs in the cytoplasm (Wagner and Hewlett 2004). LAC virus also uses the host for snatching 5' caps and adjacent oligonucleotides as primers for viral mRNA synthesis. Translation of structural and nonstructural proteins occurs in the ribosomes. After primary transcription and translation, a second round of transcription takes place, providing additional replication of the viral genome. Virus maturation occurs in the Golgi complex. Vesicle transport will carry accumulated nascent virions from the cisternae to the plasma membrane for exocytosis (Elliot 1990, Borucki et al. 2002).

1.3 LAC Virus in the Mosquito Vector

After mosquito ingestion of an infectious blood meal, the virus will infect the cuboidal epithelial cells of the midgut, and begin to replicate. The rate at which LAC virus replicates and disseminates depends on the mosquito vector. After approximately 3–6 days post-infection (PI), the virus will begin to spread to neighboring tissues, such as the foregut. Following a heavy accumulation of antigen in the gut tissues, the virus will disseminate into the hemolymph where it will infect secondary target organs such as the fat body, pericardial cells, nervous, and ovarian tissues (Higgs and Beaty 2005). Disseminated infections are usually detected by days 10–14 PI (Beaty and Thompson 1978, Paulson and Grimstad 1989). The salivary glands are the last organ infected, at which time the mosquito is capable of virus transmission. Rates for the extrinsic

incubation period of LAC virus can vary, and are generally 7–16 days PI (Borucki et al. 2002). Infection apparently has no ill effects on the vector and a life-long persistent infection usually develops (Elliot 1990).

1.4 Pathogenesis, Diagnosis and Treatment in Humans

An infected mosquito can transmit LAC virus to a human via its saliva, which is injected into the host's subcutaneous tissue during the course of obtaining a bloodmeal. The virus will replicate in an adjacent muscle causing a systemic viremia, which can further amplify in chondrocytes and the reticuloendothelial system. After this time, the invasion of the central nervous system (CNS) occurs, probably via vascular endothelial cells followed by replication in neurons and glial cells. Lesions including neurodegeneration, patchy inflammatory lesions, and vasculitis have been noted in the frontoparietal and temporal brain lobes (McJunkin et al. 1998).

Although children are generally more susceptible to illness from LAC virus infection, the vast majority of LAC virus infections are asymptomatic or present as a minor febrile illness. The incubation period is 5–10 days, and the onset of clinical disease is usually acute with rapid recovery (Balkhy and Schreiber 2000). However a small amount of cases (<1%) will present with severe clinical manifestations including vomiting, seizures, altered mental status, aseptic meningitis, and fatal encephalitis. The cases with severe illness are usually followed by a high rate of neurologic sequelae, including recurrent seizures, attention deficit disorder with hyperactivity (ADDH), and learning disorders (Balkhy and Schreiber 2000, de los Reyes et al. 2008).

Because LAC virus is difficult to isolate from the cerebrospinal fluid (CSF) and brain tissue, serology is the method of choice for diagnosis. Serologic methods include seeking

viral-specific IgM in the CSF or serum (Romero and Newland 2006). A fourfold or greater rise in serum antibody titer is used to confirm a diagnosis of LAC virus. Additional testing used in conjunction with serology includes EEGs to track abnormal brain activity, and MRIs to detect areas of focal enhancement.

Treatment for severe LAC virus illness consists mainly of supportive care, which may include fluids to combat dehydration from vomiting, and medication to control fevers and manage seizures. Because many of the above symptoms as well as abnormal EEG findings associated with LAC virus can also indicate herpes simplex (Mc Junkin et al. 1998, Romero and Newland 2006), the antiviral acyclovir may be administered until a diagnosis can be obtained, although this is ineffective against LAC virus. However, an antiviral agent which may prove useful against LAC virus is ribavirin, which has a direct effect on LAC virus polymerase activity (Cassidy and Patterson 1989). In fact, ribavirin saved the life of a dying child when emergency permission was granted for its use by the FDA following a diagnosis of LAC encephalitis (McJunkin et al. 1997). At present, ribavirin is still undergoing clinical trials for regular approval of its use.

1.5 Principle LAC Virus Vector--- Aedes triseriatus

The eastern tree hole mosquito *Aedes triseriatus* (Say) is the principle vector of LAC virus (Watts 1972) and is also a capable vector of *Dirofilaria immitis* (Leidy), a filarial nematode commonly referred to as dog heartworm (Intermill 1973, Rogers and Newson 1975). In addition, recent laboratory findings suggest that Ae. *triseriatus* may serve as an enzootic vector of West Nile Virus among some mammalian populations (Erikson et al. 2006).

The distribution of *Ae. triseriatus* includes forested areas from Florida to Canada and west to the Dakotas. As a result, *Ae. triseriatus* is capable of living in a wide range of temperatures and conditions, and species have adapted locally (Walker 1992, Joy and Hildreth-Whitehair 2000). *Ae. triseriatus* is mainly a forest-dwelling mosquito, preferring isolated woodlots or forest edges. These include different types of forested habitats (i.e. mixed northern hardwood, hemlock mixed hardwood habitats and small reed maple habitats (Nasci et al. 2000). Within these habitats, *Ae. triseriatus* commonly develops in both natural containers such as tree holes, as well as artificial containers.

This species is a daytime biter, and particularly likes to feed on small forest dwelling mammals, such as chipmunks and squirrels (Wright and Defoliart 1970). However, it also feeds on a variety of hosts, including humans, other mammals, and birds.

In the early 1970's the incrimination of *Ae. triseriatus* as the primary vector for LAC virus was confirmed by several epidemiological studies (Thompson et al. 1972, Watts et al. 1972). Although LAC virus does not occur throughout the range of *Ae. triseriatus*, this species is native to the geographic areas in which LAC encephalitis has been reported (Erwin et al. 2002). Many of the LAC virus endemic areas are in temperate zones (Fig 4.6). A primary mechanism of LAC virus maintenance by *Ae. triseriatus* is transovarial transmission to overwintering eggs (Watts et al. 1975). Both female and male progeny are infected this way, and this vertical component of transmission is vital for LAC virus maintenance in endemic areas. Also, males may in turn transmit LAC virus to females venereally during mating.

1.6 LAC Virus in Reservoir Hosts

The LAC virus amplifying hosts including chipmunks, grey squirrels and red foxes, all live in close proximity with the primary vector, *Ae. triseriatus*. Horizontal transmission of LAC virus from these vertebrate hosts helps maintain virus prevalence among vector species. Successful horizontal transmission of LAC virus to the mosquito requires a sufficient viremia in the amplifying host. This is the infection threshold, which is "the virus concentration required to establish an infection in at least a proportion of the individuals ingesting it" (Chamberlain and Sudia 1961). Several studies have focused on infection thresholds for LAC virus vectors and the circulating viremias exhibited by amplifying hosts (Watts et al. 1972, Ksiazek and Yuill 1977, Patrican et al. 1985a,b). The period of sufficient viremia usually lasts 2–3 days in chipmunks and squirrels, and 4–5 days in foxes (Watts et al. 1972, Pantuwatana et al. 1972, Amundson 1981). When vectors feed on amplifiers with insufficient virus titers, lower infection rates and subsequent transmission rates result. A study conducted on chipmunks indicated that the age of the chipmunk host had no significant effect on the titer or the duration of circulating viremia (Patrican 1985a).

A LAC virus infected mosquito vector may infect a significant percentage of the amplifying host population (as evidenced by antibody prevalence rates) in endemic areas. Gauld et al. (1974 and 1975) found that there were foci of considerable transmission activity among chipmunks within LAC virus endemic areas of southern Wisconsin. In these areas, antibody prevalence in adult and spring-born chipmunks reached 100% by the end of the transmission season in September (Yuill 1983).

1.7 Aedes albopictus

Aedes albopictus (Skuse) is regarded as the fastest spreading mosquito in the world (Budiansky 2002). *Aedes albopictus* is also a competent vector of many viral diseases, including dengue and yellow fever (Gerhardt et al. 2001). In the U.S., 7 viruses have been isolated from wild populations of *Ae. albopictus*, including eastern equine encephalitis, West Nile virus, and LAC virus (Erwin et al. 2002). In addition, laboratory studies have found *Ae. albopictus* capable of *D. immitis* transmission (Kartman 1953, Webber and Hawkin 1955, Apperson et al. 1989, Nayar and Knight 1999, Tiawsirisup and Kaewathamasorn 2007).

The introduction of *Ae. albopictus* into the U.S. was traced to a Houston, Texas tire shipment yard in 1985. Though the exact date and means of entry of *Ae. albopictus* into Houston, Texas is unknown, this species was likely introduced by a used tire shipment from Asia. Although *Ae. albopictus* had been collected on three previous occasions in the United States, the Houston, Texas tire yard is the first record of established breeding populations in the U.S. (Sprenger and Taweesak 1986). *Aedes albopictus* has since spread to at least 30 states and is extensively distributed along the east coast and throughout the southeastern and midwestern United States (Richards et al. 2006). A study by Erwin et al. (2002) reported *Ae. albopictus* as the most commonly encountered mosquito in Tennessee during a study in 1998–2000. *Aedes albopictus* shares a common ecological niche with *Ae. triseriatus* but is also found in less forested areas (Erwin et al. 2002). Furthermore, *Ae. albopictus* has the ability to adapt to a wide variety of rural, urban, and suburban habitats (Francy et al. 1990, Rai 1991). *Aedes albopictus* breeds in containers and commonly inhabits areas near human dwellings (Barker et al. 2003a,b). This species is an aggressive daytime biter of humans, domestic and wild animals, and birds (Swanson et al. 2000, Budiansky 2002).

In 1999, the first isolations of LAC virus from naturally infected *Ae. albopictus* mosquitoes was reported in Tennessee and North Carolina (Gerhardt et al. 2001). Transovarial transmission of LAC virus by *Ae. albopictus* has also been successfully demonstrated in the laboratory but at lower rates than *Ae. triseriatus* (Tesh and Gubler 1975, Hughes et al. 2006). These studies implicate *Ae. albopictus* as an accessory vector of LAC virus.

Because *Ae. albopictus* and *Ae. triseriatus* have ecological similarities, interspecific competition has been reported **(**Ho et al. 1989, Livdahl and Willey 1991, Edgerly et al. 1999, Lounibos et al. 2001). There is little evidence, however, to support competitive exclusion between *Ae. albopictus* and *Ae. triseriatus*, due to the microhabitat preferences of both of these species. While both *Ae. triseriatus* and *Ae. albopictus* can be found in the same environments, *Ae. triseriatus* has a greater treehole occupancy, while *Ae. albopictus* has an affinity for breeding in artificial containers such as used tires and cemetery vases (Lounibos 2001).

An additional mosquito species, *Ae. aegypti*, also shares the same habitat as both *Ae. albopictus* and *Ae. triseriatus*. Due to the success of *Ae. albopictus* as an invasive species, extensive studies have been conducted evaluating larval competition between *Ae. albopictus* and *Ae. aegypti* (Ho et al. 1989, Edgerly et al. 1999, Lounibos et al. 2001, Juliano 2004). In the southeast U.S. there is evidence of competitive displacement of *Ae. aegypti* by *Ae. albopictus*, as previous studies have shown increasing numbers of *Ae. albopictus* and declining numbers of *Ae. aegypti* in these regions (Hobbs and Hughes 1991, O'Meara 1995). However, the effects of larval competition between *Ae. albopictus* and *Ae. aegypti* are reduced when resources are abundant (Ho et al. 1989).

Based on evidence of interspecific competition, vector competence among *Ae. albopictus*, *Ae. triseriatus*, and *Ae. aegypti* becomes a vital issue if one of these species is

replacing another, and is a better vector of LAC virus. Hughes et al. (2006) conducted a study comparing vector competence and LAC virus transovarial transmission rates by *Ae. triseriatus*, *Ae. albopictus* and *Ae. aegypti*. Included in this study was an overall transmission amplification potential (TAP) score, which represented permissiveness for midgut infection, dissemination, transovarial transmission, and filial infection rates. While *Ae. triseriatus* received the highest TAP score (i.e. has the highest vector competence for LAC virus), *Ae. albopictus* had an overall TAP score that was approximately 10 fold greater than that of *Ae. aegypti* (Hughes 2006). Since *Ae. albopictus* is a better LAC virus vector and may be replacing *Ae. aegypti* in certain areas, the importance of studying this mosquito as a LAC virus accessory vector should not be underestimated. *Aedes albopictus* could potentially spread and maintain LAC virus in southern areas of the U.S. that were previously uninhabited by competent LAC virus vectors, particularly in urban habitats. Although *Ae. triseriatus* is found in southern areas, its habitat preference is for forested areas, while both *Ae. albopictus* and *Ae. aegypti* prefer more urban settings, particularly around human dwellings. It is likely that *Ae. albopictus* is serving as a bridge vector for LAC virus in urban areas, carrying the virus from forested to urban environments where *Ae. triseriatus* is usually found in lesser amounts.

1.8 Aedes japonicus

Aedes japonicus (Theobald) is a relatively recent invasive species that continues to expand its range throughout the U.S. Although laboratory studies have shown it to be a competent vector of LAC virus (Sardelis et al. 2002b), West Nile virus (Sardelis and Turell 2001), eastern equine encephalitis (Sardelis et al. 2002a), and St. Louis encephalitis (Sardelis 2003), there have been no field isolates to date.

Ae. japonicus was first reported in the U.S. from specimens collected in New Jersey and New York in 1998 and 1999, respectively (Peyton et al. 1999). In the U.S., this species has since been found from Maine to as far south as Georgia (Gray et al. 2005) and northwest to Washington (Roppo et al. 2004). *Aedes japonicus* was first reported in Virginia in Prince William County in July of 2000 (Harrison 2002), and populations have been found at several locations in southwest Virginia (Grim et al. 2007). Habitat for *Ae. japonicus* consists of a variety of natural and artificial settings, including rock holes in stream beds, tree holes, bird baths, plastic containers, and tire castings. *Aedes japonicus* is regarded as an opportunistic feeder on birds and mammals, including humans (Tanaka et al. 1979). Because *Ae. japonicus* occurs in LAC virus endemic areas, feeds on amplifier hosts, and has been shown to be a competent vector in the lab, further investigation of *Ae. japonicus* as a possible LAC virus accessory vector is warranted.

1.9 Aedes canadensis

Aedes canadensis (Theobald) has a broad distribution throughout the United States, has repeatedly been cited as an efficient vector of *D. immitis* (Magnarelli 1977, Arnott and Edman 1978). *Aedes canadensis* has also been implicated as a potential accessory vector of LAC virus (Sudia et al. 1971, Watts et al. 1973, Berry et al. 1986, Nasci et al. 2000). *Aedes canadensis* is commonly found in the eastern U.S. in a variety of habitats including shallow, leaf-lined pools in wooded areas, deep snow pools, roadside ditches, vernal pools in open fields, along the edges of permanent swamps, and in acid water bogs (Rutgers University 2006). This species has a univoltine lifecycle in northern states. Larvae can be found in large numbers in early spring as they hatch from overwintering eggs. Adults can be aggressive biters in shaded areas, and will

feed on a broad range of animals, including large and small mammals, birds, and reptiles (Rutgers University 2006).

Laboratory studies of LAC virus transmission by *Ae. canadensis* have shown that the mosquito is capable of developing high infection rates, but has poor virus multiplication and transmission rates (Watts et al. 1973a,b). Watts et al. (1973a) reported that this species appears to be "readily infectible", which may explain the high infection rates found in field studies. Two field studies found LAC virus infection rates in host-seeking *Ae. canadensis* females comparable to those of *Ae. triseriatus* females collected in the same areas (Berry et al. 1986, Nasci et al. 2000). Also, in a 2002 study in southwestern Virginia, a pool of nine field collected *Ae. canadensis* mosquitoes tested positive for LAC virus (Paulson, unpublished data). These data suggest that *Ae. canadensis* may be a significant contributor to the maintenance of LAC virus in endemic areas.

1.10 Aedes aegypti

Aedes aegypti is one of the most medically significant insects in the world. Its notoriety is based on its reputation as the principle vector of urban yellow fever virus, as well as the primary vector of dengue, which continues to threaten human populations with regular outbreaks in tropical and subtropical zones of the Americas (Smith 1956, Gould et al. 1968, Chan et al. 1971).

The distribution of *Ae. aegypti* is worldwide, and this species can be found in warmer regions of both eastern and western portions of the U.S., with greatest densities in the Gulf Coast areas. *Aedes aegypti* breed in artificial containers as well as natural water retaining cavities, such

as tree holes, and the eggs can resist desiccation for up to a year (Ho et al. 1989, Juliano et al. 2004).

In the U.S., this mosquito is seasonal in the north, becoming active in the warm summer months. *Ae. aegypti* cannot overwinter in the egg stage in colder climates, and the adults are killed by temperatures below freezing (Womack 1993). In southern states, this species remains reproductively active all year long.

Aedes aegypti is an urban mosquito that prefers to feed in the early morning, late afternoon, or at night in locations with artificial illumination. Humans are their preferred host, and they tend to target areas around the ankle during feeding (Womack 1993).

Although capable of acquiring LAC virus infections in the laboratory (Hughes et al. 2006), *Ae. aegypti* is not generally considered to be vector of LAC virus in the field. The distribution of *Ae. aegypti* within the U.S. lies mainly outside of known locations of LAC virus endemicity. Specifically, the highest amounts of LAC virus cases are reported from West Virginia and midwestern states, which have relatively few or no populations of *Ae. aegypti* mosquitoes. Furthermore, *Ae. aegypti* prefer urban habitats (Gilotra et al. 1967), (which contain relatively fewer LAC virus reservoir hosts, i.e. chipmunks, grey squirrels, red foxes), and human hosts (Womack 1993, Ponlawat and Harrington 2005).

Regardless of all of the above reasons, it is still prudent to mention *Ae. aegypti* in the context of LAC virus due to the possibility that it could be replaced by *Ae. albopictus* (Hobbs and Hughes 1991, O'Meara 1995). Because *Ae. aegypti* is a poor vector of LAC virus while *Ae. albopictus* is a competent vector, significant epidemiological consequences could result. Both mosquito species share habitats and overlapping ranges in many places throughout the world. Several studies have concluded that *Ae. albopictus* is the superior competitor and interspecific

competition between these two species might explain the range reduction of *Ae. aegypti* in several southern states (Hobbs and Hughes 1991, Braks et al. 1994, O'Meara 1995).

1.11 Mosquito Barriers to Infection

The arbovirus infection cycle in a mosquito vector begins when a mosquito ingests a virus during a bloodmeal. After ingestion, the virus must infect and replicate in the cells of the midgut epithelium in order to invade the hemocoel. Once inside the hemocoel, the virus can disseminate throughout the mosquito and infect the salivary glands, where it can replicate in high titers and be successfully transmitted in the mosquito's saliva during subsequent feedings (Chamberlain and Sudia 1961). In order for the infection cycle to occur, however, the virus must overcome several physiologic barriers within the mosquito. The midgut is the only portion of the mosquito not surrounded by a chitinous layer and therefore it is susceptible to infection. However, the midgut is also the first physiological barrier encountered by the virus, because it possesses several defenses. The midgut barriers include both the midgut infection (MI) barrier and the midgut escape (ME) barrier. The MI barriers include cell surface charge, digestive enzymes, and the presence of receptors on the midgut epithelium for viral attachment. The spatial distribution of cell surface charges are thought to affect viral absorption, digestive enzymes could inactivate virus particles by damaging the viral envelope, and the absence of receptor sites could block viruses at the attachment/ penetration stage (Hardy et al. 1983). Formation of the peritrophic matrix (PM) also serves as a barrier to arbovirus infections. The virus must invade the epithelium before PM maturation occurs because the PM may block penetration (Devenport and Jacobs–Lorena 2005).

The ME barrier is dose dependent and the virus must replicate in the midgut epithelium at a high enough titer to traverse the epithelium and enter the hemocoel. An ME barrier has been reported in strains of *Ae. triseriatus* for LAC virus. For example, in locations where LAC virus is endemic, there are greater numbers of mosquitoes with ME barriers among local strains of *Ae. triseriatus* (Paulson and Grimstad 1989). The ME barriers among the local strains are thought to be an adaptation of the mosquito to evade the virus. The size of the mosquito can also affect the ME barrier. In a study using LAC virus and *Ae. triseriatus*, Grimstad and Walker (1991) found that smaller females (due to nutritional deprivation in the larval stage) were more likely to develop disseminated infections than larger females. Electron microscopy revealed that the basement membrane of the midgut contained far fewer laminae in smaller females compared to the larger females. Fewer laminae reduces the membrane's thickness, which increases the efficiency of the virus to cross the ME barrier.

Once the virus is in the hemocoel, it can disseminate to various tissues of the body via the hemolymph. The hemolymph is the most likely source of salivary gland infection. In the salivary glands the virus will encounter additional barriers; the salivary infection barrier (SGI) and the salivary escape barrier (SGE). The virus must be able to infect and replicate in the salivary gland tissue in order to overcome the SGI barrier. Failure to overcome the SGI barrier is could be due to an inadequate amount of viremia in the mosquito (Chamberlain and Sudia 1961). The existence of the SGE barrier is well documented. After infection salivary gland tissue, the virus must cross this barrier to escape into the saliva in order for transmission to occur. Paulson and Grimstad (1989) documented an SGE barrier in *Aedes hendersoni*, which made *Ae. hendersoni* an incompetent vector for LAC virus. LAC virus was detected in high titers in the salivary glands of *Ae. hendersoni*, yet very few mosquitoes could actually transmit it, indicating

the presence of an SGE barrier (Paulson and Grimstad 1989). It has been suggested that the SGE may also have an infection threshold, making it dose dependent like the ME (Hardy et al. 1983).

1.12 Enhanced Transmission of an Arbovirus

When a mosquito ingests a bloodmeal dually infected with an arbovirus and filarial nematodes, enhanced transmission of an arbovirus can occur (Mellor and Boorman 1980, Zytoon et al. 1993a,b, Turell et al. 1997, Vaughan and Turell 1996, Vaughan et al. 1999). Enhanced transmission is accomplished through compromise of the mosquito's midgut barriers by filarial nematodes (microfilaria). The midgut barriers are normally overcome when a virus infects and replicates in the cells of the midgut epithelium. However when a mosquito ingests microfilaria, the microfilaria will puncture the mosquito's midgut epithelium to enter the hemocoel, creating holes in which a concurrently ingested virus could escape through (Mellor and Boorman 1980, Turell et al. 1984, Zytoon et al. 1993a). Direct escape into the hemocoel by a virus bypasses the need for the virus to infect and replicate in the mosquito midgut epithelium, and is the basic mechanism of microfilaria enhancement.

Microfilaria enhanced arbovirus transmission can affect arbovirus epidemiology in several ways, including a shortened extrinsic incubation period, and lowered infection thresholds, thus increasing vector competence (Turell et al. 1997, Vaughan and Turell 1996, Vaughan et al. 1999). The extrinsic incubation (EI) period is the amount of time between the ingestion of an infective bloodmeal and the time when the virus can be transmitted orally while refeeding. The duration of the EI period is extremely important, as it determines how long a mosquito must survive after ingestion of an infective bloodmeal before it is a competent transmitter of virus (Hardy et al. 1983). Turell et al. (1984) dually infected mosquitoes with Rift

Valley Fever virus and *Brugia malayi* and compared dissemination rates. Dually infected mosquitoes had disseminated infections 4 to 5 days post ingestion in contrast to the 18+ days in mosquitoes infected with Rift Valley Fever virus alone. A decrease in the EI could significantly impact the epidemiology of the arbovirus because prolonged vector survival times or higher vector population densities would not be required to maintain the arbovirus in locations endemic to the reservoir hosts (Hardy et al. 1983).

Ingestion of definite concentrations of virus is required in order to achieve the infection threshold. Since the ME barrier is dose dependent, successful disseminated infections are based largely upon ingesting viruses with a sufficient titer. However, if the virus could escape into the hemocoel via holes created by microfilaria without having to replicate first, then perhaps less virus would be required to establish an infection in the mosquito. Hence, mosquitoes feeding on amplifiers with lower viremias could still develop infections. Vaughan and Turrell (1996) investigated the effects of differing levels of host viremia and microfilaremia on *Aedes* mosquitoes with eastern equine encephalitis (EEE) virus and *Brugia* microfilaria. Microfilaria enhancement was found in two of three *Aedes* species, and the authors concluded that minimum infectious virus doses vary among species. Also, delivery of a minimum infectious dose is dependent on numbers of midgut holes induced by penetrating microfilaria and the extent of the induced midgut pathology such as rips and tears in the midgut (Vaughan and Turell 1996).

Additional targets of arboviral enhancement involve compromise of the salivary gland barriers, and can occur from dual infection with malaria parasites and an arbovirus. Malaria parasites compromise the salivary gland barriers via tissue destruction from their passage through the salivary glands. Paulson et al. (1992) dually infected the incompetent vector *Aedes hendersoni* with LAC virus and avian malaria, *Plasmodium gallinaceum*. As a result of the dual

infection, virus transmission dramatically increased from 8% (LAC virus only) to 72% (Paulson et al. 1992).

Dual infection can create a competent vector out of a mosquito species or strain previously considered to have little vector potential. Mellor and Boorman (1980) showed multiplication of bluetongue virus in the incapable vector *Culicoides nubeculosus* by simultaneously infecting this arthropod with bluetongue virus and *Oncherca cervicalis* microfilaria. Zytoon (1993a,b) showed increased viral multiplication and dissemination of Chikungunya (CHIK) virus in the *Ae. albopictus* Miki strain, using *D. immitis* microfilaria. Without microfilaria, this strain of *Ae. albopictus* is an incompetent CHIK virus vector.

In locations where filarial nematodes exist, microfilarial enhancement may be very important in maintaining arboviral transmission cycles. In tropical regions, filariasis can be greater than 70% in vertebrates, thus increasing the likelihood that many viremic bloodmeals are obtained from dually infected hosts (Vaughan and Turrell 1996). In the U.S., filarial nematodes such as *D. immitis* are found in all 50 states, with the highest prevalence in the southeastern states and Gulf Coast regions (Otto 1969, Knight 1977, Haddock 1987, AHS 2005). Coexistence with arboviruses in many of those areas is entirely possible. For example, the geographic range of *D. immitis* overlaps with LAC virus throughout the eastern U.S., including several mid-Atlantic and southern states where there is a high prevalence of *D. immitis*. Based on this information, one may hypothesize that LAC virus transmission is enhanced in mid-Atlantic and southern states due to the impacts of *D. immitis* on the mosquito vector. Additional arboviruses that exist in areas with high *D. immitis* prevalence include EEE and St. Louis Encephalitis (SLE). The coexistence of filarial nematodes, arboviruses, and mosquito vectors in the U.S. provides potential for dual infection, which in turn can impact arbovirus transmission cycles. Therefore,

further research is warranted on enhanced arbovirus transmission in order to accurately predict disease epidemiology and risk models for arboviruses in the U.S.

1.13 Canine Sentinels for LAC Virus

Southwest Virginia may have several endemic regions of LAC virus foci, as evidenced by reported human cases and vector surveillance studies (Barker et al. 2003a,b, CDC 2007, Virginia Dept. of Health 2008). However, because the majority of LAC virus cases are mild to asymptomatic, the disease is under–reported. Therefore, little is actually known about the distribution of LAC virus throughout southwest Virginia. One method that may help to determine locations of endemic foci is the use of sentinel animals.

Using dogs as sentinels for infectious disease surveillance is a common practice among researchers (Olson et al. 2000, Coffey et al. 2006, Diniz et al. 2007, Salb et al. 2008). Dogs live in close proximity to their owners which often provides the advantage of using them to obtain valuable human disease information such as incidence rates, risk factors, and sources of exposure before the occurrence of human disease outbreaks (Diniz et al. 2007). Dogs also make ideal sentinels because many spend much of their time outdoors, and are not likely treated with any type of mosquito repellent.

Based on the reasons mentioned above, it may be useful to utilize canine sentinels for investigating LAC virus in southwest Virginia. Dogs are particularly ideal because they are not amplifier hosts but do develop detectable serum antibody levels in response to LAC virus (Godsey et al. 1988). In addition, mosquito vectors of LAC virus readily feed on dogs. For example, a 1996 study in western North Carolina found that 40.4% of collected, blood-fed *Ae. triseriatus* fed on canines (Szumlas et al. 1996). Also, *Ae. japonicus* is regarded as an

opportunistic feeder on mammals (Tanaka et al 1979), and *Ae. albopictus* is an aggressive mammalian feeder (Francy et al. 1990, Tempelis et al. 1970.). Lastly, *Ae. canadensis* can be an aggressive biter, feeding on a broad range of animals, including large and small mammals (Rutgers University 2006).

1.14 Research Objectives

Southwest Virginia has become an emerging focus of LAC virus activity (Barker et al. 2003a,b, CDC 2007, VA Dept. of Health 2008). Reasons for the rise in human cases of LAC encephalitis in southwest Virginia may include the widespread occurrence of the primary LAC virus vector, *Aedes triseriatus* and the relatively recent invasions of mosquito species such as *Aedes albopictus* and *Aedes japonicus*. An important first step for improving our understanding and ability to predict the risk of LAC encephalitis in the southwestern region of Virginia is to develop a knowledge-base of information on the spatiotemporal dynamics of the vector-species. Therefore, my first objective (Objective I) was to investigate the spatiotemporal activity of vectors in order to assess their abundance, dispersion patterns, and species co-occurrence and displacement.

Using dogs as sentinel animals for the detection of LAC virus may also help to determine locations of LAC virus foci. My second objective (Objective II) is to measure the efficacy of canine sentinels to determine the distribution of LAC virus in southwest Virginia. Additionally, I will investigate the usefulness of dogs as indicators for other vector–borne diseases in southwest Virginia, including *Borrelia burgdorferi*, *Anaplasma phagocytophilum*, and *Ehrlichia canis*.

My final objective (Objective III) will investigate the occurrence of microfilaria enhanced transmission for LAC virus by assessing the effects of coinfection with *D. immitis* and LAC virus in *Ae. triseriatus*, *Ae. albopictus and Ae. aegypti.* I will evaluate mosquitoes for microfilaria enhanced LAC virus transmission by measuring infection, dissemination, and transmission rates, as well as extrinsic incubation periods and lowered virus infection thresholds.

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2. Oviposition Activity of *Aedes triseriatus*, *Aedes albopictus* and *Aedes japonicus* in a La Crosse Virus Endemic Region of Virginia

2.1 Introduction

La Crosse (LAC) virus, an arbovirus in the California serogroup of Bunyaviruses, is the most prevalent arboviral infection in children in North America (McJunkin et al. 2001). While most cases of LAC virus are mild to asymptomatic, a small number of cases will result in severe clinical manifestations such as encephalitis, followed by a high rate of neurologic sequelae (Balkhy and Schreiber 2000, de los Reyes et al. 2008). In nature, LAC virus is maintained through mammalian host reservoirs—most commonly chipmunks (*Tamias striatus*) (Gauld et al. 1975), grey squirrels (*Sciuris carolinensis*) (Ksiazek and Yuill 1977), and red foxes (*Vulpes fulva*) (Amundson and Yuill 1981). The primary vector of LAC virus is the eastern treehole mosquito, *Aedes triseriatus* (Berry et al. 1974, Pantuwatana et al. 1974, Watts et al. 1974).

Historically, most cases of LAC virus have been reported from midwestern states, but in the past decade there have been increasing reports of the disease in Tennessee, North Carolina, and Virginia (Gerhardt et al. 2001, Barker et al. 2003b). The increased reports of LAC virus may represent a new southeastern expansion of endemic focus (Gerhardt et al. 2001).

Areas in and around counties in far southwest Virginia (Fig. 2.1) have recently emerged as a significant focus of LAC virus activity (CDC 2007, Barker et al. 2003b, Va. Dept of Health 2008). Between 1975 and 1993, only one case of LAC encephalitis was reported from the state of Virginia. However between 1994 and 1998, the CDC reported 13 cases of LAC encephalitis from areas in southwest Virginia in the Appalachian Mountains. Additionally, 8 cases have been reported in Virginia between 2003 and 2005 (CDC 2007). Most recently in July 2008, a case was reported from the northwestern Virginia county of Rockingham (VA Dept of Health 2008).

Reasons for the rise in human cases of LAC encephalitis may be an increase in vector presence, specifically the widespread occurrence of the primary LAC virus vector, *Ae. triseriatus* (Say) and the relatively recent invasions of mosquito species such as *Aedes albopictus* (Skuse) and *Aedes japonicus* (Theobald). All three species share an ecological niche as artificial and natural container breeders (Tanaka 1979, Barker 2003b).

Aedes triseriatus has a broad U.S. distribution, ranging from Florida to Canada, and west to the Dakotas. This species is mainly a forest-dwelling mosquito, preferring isolated woodlots or forest edges. Preferred habitats include different types of forests (i.e. mixed northern hardwood, hemlock mixed hardwood habitats and small reed maple habitats (Nasci et al. 2000). In the early 1970s, the incrimination of *Ae. triseriatus* as the primary vector for LAC virus was confirmed by several epidemiological studies (Thompson et al. 1972, Watts et 1972, 1973, 1974). LAC virus is maintained in *Ae. triseriatus* through vertical transmission from a female to her offspring, allowing the virus to overwinter in infected eggs (Watts et al. 1972, Watts et al. 1974). In addition, LAC virus can be transmitted venereally between adults (Thompson and Beaty 1978). Although LAC virus does not occur throughout the range of *Ae. triseriatus*, this species is native to the geographic areas in which LAC encephalitis has been reported (Erwin et al. 2002).

Aedes albopictus entered the U.S. in a used tire shipment yard in 1985 (Sprenger and Taweesak 1986). It has since spread to at least 30 states and was the most commonly encountered mosquito in Tennessee during a study in 1998–2000 (Erwin et al. 2002). *Aedes albopictus* was first detected in Virginia at Virginia Beach in 1991, and since then has become a major cause for mosquito complaints in many areas of Virginia (Harrison et al. 2002). North American strains of *Ae. albopictus* have been found in both urban and rural environments, and

particularly thrive in artificial containers especially in urban environments (Hawley et al. 1987, Ali and Nayar 1997, Barker 2003a,b). In 1999, the first isolations of LAC virus from naturally infected *Ae. albopictus* mosquitoes were reported in Tennessee and North Carolina (Gerhardt et al. 2001). Transovarial transmission of LAC virus among *Ae. albopictus* has also been successfully demonstrated in the laboratory, but at lower rates than reported for *Ae. triseriatus* (Tesh and Gubler 1975). *Aedes albopictus* has since been implicated as a LAC virus accessory vector based on these findings.

Aedes japonicus was first identified from specimens collected from New Jersey and New York in 1998 and 1999, respectively (Peyton et al. 1999). In the U.S., this species has since been found from Maine to as far south as Georgia (Gray et al 2005) and northwest to Washington (Roppo et al 2004). It was first reported in Virginia in Prince William County in July of 2000 (Harrison 2002). Populations of *Ae. japonicus* continue to spread, and their numbers are increasing in southwest Virginia (Grim et al. 2007). Laboratory studies have shown *Ae. japonicus* to be a competent vector of LAC virus; however, there have been no field isolates to date (Sardelis et al. 2002).

A unique opportunity emerged to study the regional occurrences and spread of the vectors of LAC virus in southwest Virginia. In particular, we have been able to study the spatiotemporal dynamics of LAC vectors in three areas of southwest Virginia (Fig. 2.1) that differ in the number of human cases of LAC encephalitis and LAC virus positive mosquito isolates. The Wise area (Wise County) has had both human cases of LAC encephalitis, as well as LAC virus positive mosquito isolates; the New River Valley area (NRV – Montgomery and Pulaski counties) has had LAC virus positive mosquito isolates but no reported human cases; the

Roanoke area (Roanoke County and Roanoke City) has had no reported human cases of LAC encephalitis nor LAC virus positive mosquito isolates.

Previous studies have investigated the spatiotemporal dynamics of local mosquito species in order to predict disease transmission potential in specific locations (Kitron et al. 1989, Focks et al. 1999, Barker et al. 2003a,b, Richards et al. 2006, Diuk-Wasser et al. 2006, Dalatte et al. 2008). An important first step for improving our ability to predict the risk of LAC encephalitis was to investigate the spatiotemporal dynamics of the local vector species in southwest Virginia. The studies by Barker et al. 2003a and 2003b are particularly noteworthy because they focused on the spatiotemporal dynamics of two LAC virus vectors (*Ae. triseriatus* and *Ae. albopictus*) in the Wise County area where human cases of LAC encephalitis have been reported.

The current study is an extension of the study by Barker et al. 2003a,b, and will assess the spatiotemporal oviposition activity of the primary vector of LAC virus, *Ae. triseriatus*, and two potential accessory vectors *Ae. albopictus* and *Ae. japonicus,* across a broader region of southwest Virginia. Although the use of ovitrap data to characterize the size of a vector population has been criticized because the relationship between egg–laying females and the number of eggs collected is not straightforward (Focks 2003, cited in Richards et al. 2006), ovitrapping was still used in this study. Ovitrapping is a relatively efficient sampling method that can be employed over a large area, and because the data collected from the ovitraps have been frequently used as a surrogate measure for adult mosquito activity in previous studies (e.g., Kitron et al. 1989, Barker et al. 2003a,b, Braks et al. 2003, Richards et al. 2006). Information regarding the spatiotemporal activity of vectors can be used to assess the abundance of vector species, their rate of spread, and species co-occurrence and displacement (Juliano 1998, Braks et al. 2003, 2004). Improving our knowledge of these factors will provide a better understanding of

the LAC virus transmission risk potential that is currently surrounding the human population in southwest Virginia.

2.2 Materials and Methods

2.2.1 Study Area

Three areas in southwest Virginia were selected for surveying based on a 2002 study that determined the status for reported human cases of LAC encephalitis and/or LAC virus positive mosquito isolates (Paulson, unpublished data). As indicated above, the areas selected for the study were Wise County, New River Valley (NRV—Montgomery and Pulaski counties), and Roanoke County and Roanoke City (Fig. 2.1).

The Wise County area (hereafter referred to as Wise) has had both human cases of LAC encephalitis, as well as LAC virus positive mosquito isolates. It is a predominantly rural area (~1050 km^2) located in the far southwest corner of Virginia in the Appalachian Mountains (Fig. 2.1). Wise has an elevation range of 400–1287m, and consists of 61–80% mixed hardwood forest cover (i.e. oak-hickory, maple-beech birch, and pine-hemlock) with occasional clearings for pastures and strip mining for coal (Johnson 1992).

The NRV has had LAC virus positive mosquito isolates but no reported human cases to date. The NRV (~1860 km^2) is located between the Appalachian and Blue Ridge Mountains and is bisected by the New River. It is a combination of 50–60% oak hickory forest cover and suburbs, with an elevation range of 535–750m (Rose 2001).

To date there have been no reported human cases of LAC encephalitis nor LAC virus positive mosquito isolates in the Roanoke County and Roanoke City area (hereafter referred to as Roanoke). The area (~650 km^2) is located between the Appalachian and Blue Ridge Mountains, with a detached mountain (Mill Mountain) in its center. Elevation ranges from 269–1329m, with

a 40–61% oak-hickory forest cover that applies mainly to the mountainous portions of the area (Rose 2001). The valley areas consist of cities and suburbs, where most residents can be found.

2.2.2 *Trap Sites and Habitat Composition*

Between 30–49 trap sites were identified for collecting mosquito eggs within each study area with Wise, NRV, and Roanoke having 31, 49, and 30 trap sites, respectively. The majority of trap sites within each study area were located on public school property because schools are spatially dispersed throughout each of the areas and obtaining permission from the county school officials to set up traps was relatively easy. Traps that were not located on school property were placed in yards surrounding homes, as well as roadside trees on forest edges. The geographic position of each trap site was recorded in geographic coordinates (Latitude/Longitude) using a Garmin GPS receiver (Garmin International, Inc., Olathe, KS), which allowed the geographic position of each trap site to be located on a map and ensured that there was an even distribution of sites, as was possible, throughout the study areas.

Although school yards provided relatively uniform oviposition sites, the habitat surrounding the schools varied from urban to forested environments. Therefore, the landscape around each trap (collection) site was classified broadly as being either predominantly urban or forested to obtain a better understanding of the amount of habitat variability in each of the study areas.

2.2.3 Egg Collection and Processing

Mosquito eggs were collected at each trap site in 2005 and 2006 from mid-July to late August (International Organization for Standardization (ISO) weeks 30–35), using ovitrap

containers. At least three ovitrap containers were placed at each of the sites identified in the three study areas. Ovitrap containers are 450 ml black plastic cups with drain holes approximately halfway up each side (Barker et al. 2003a,b). Each container was nailed to a tree or post and a strip of seed germination paper was suspended on the inside of the cup. The seed germination papers were ≈5 cm wide and served as oviposition substrate (Steinly et al. 1991). Once in place, the ovitrap containers were filled with water up to their drain holes. Oviposition papers (ovistrips) were collected and replaced weekly, and water was added to the ovitrap containers. The ovistrips with eggs were brought to the Virginia Tech Med/Vet Entomology Lab and stored in plastic shoe box-type containers at 24°C, 80% RH, and 16:8 (L:D) photoperiod. Eggs from each ovistrip were counted using a dissecting microscope. All mosquito eggs were hatched and reared to adults according to the methods described by Munstermann and Wasmuth (1985). Adult mosquitoes were sorted by species and sex into pools of ≤50 mosquitoes, and stored at -70°C for virus testing.

2.2.4 Statistical and Spatial Pattern Analyses

Oviposition Data: Egg counts from individual ovistrips collected each week were recorded and the mean number of eggs at each trap site in an area was calculated by dividing the total eggs by the number of ovistrips per site. The mean number of eggs per trap-day at each sampling site was then calculated by dividing the mean numbers of eggs by the number of nights the ovistrips were left out in the ovitraps at the site.

Mean eggs per trap-day per trap site for both 2005 and 2006 were square root transformed ($\sqrt{y+0.1}$) to normalize the data before analysis using JMP (SAS Institute, Inc., Cary NC, 2005) and Graph Pad Prism (San Diego CA, 2007). A Repeated Measures Analysis of

Variance (ANOVA) was used to compare mean egg counts among the study areas. A sphericity test was used to check for the appropriateness of the unadjusted univariate F-tests being used. If the sphericity test was significant, the numerator and denominator degrees of freedom of the Repeated Measures ANOVA were adjusted using the H-F (Huynh and Feldt 1970) epsilon adjustment (Ott and Longnecker 2001) and the adjusted degrees of freedom values are reported. SAS software (SAS Institute, Inc., Cary NC, 2005) was used for the analysis of adult mosquito data. Adult mosquito data was obtained from rearing the collected eggs.

Habitat Preference: The classification of the habitat at each trap site in each of the study areas was combined with information on the seasonal mean number of eggs collected for the species to derive a selection index for each species based on its preference for oviposition in either forested or urban habitats. Krebs (1998, page 478), a selection (or forage) index for a species for either habitat type in a study area was calculated as,

$$w_i = \frac{o_i}{t_i} \qquad (1),$$

where w_i is the oviposition selection index for the species for ovitraps placed in habitat i (with i representing either forested or urban); o_i is the proportion of ovitraps in habitat i from which eggs above the seasonal median number for the species in an area were collected, and t_i is the proportion of all of the ovitraps in the study area in habitat i. The seasonal median number of eggs for a species was used Preference by a species for ovitraps in a particular habitat is indicated by a selection index > 1.0, while values < 1.0 imply avoidance.

Because the range of the selection index can be 0 to ∞, the index is often standardized as,

$$B_i = \frac{w_i}{\sum_{i=1}^{n} w_i} \qquad (2),$$

where B_i is the standardized selection index (range 0–1) and w_i is selection index of the species for habitat *i*. No preference is indicated when B_i = 1/number of habitats, which in this study is 1/2 = 0.50. Therefore, in this study selection indices > 0.50 indicate relative preference and values < 0.50 indicate relative avoidance.

Spatial Analysis: It was also important to examine the spatial pattern of oviposition with respect to the individual vector species. For this analysis I used the seasonal mean number of eggs per trap site per trap-day for each of the vectors. In addition to developing maps of the spatial distribution of mean seasonal egg counts for each species and year, I also analyzed the spatial data to assess the dispersion and spatial distribution patterns, and the association (or disassociation) between the oviposition activities for each of the species.

The dispersion pattern was assessed using the simplest method available, the variance-to-mean ($s^2 / \bar{x}$) ratio, which provides a measure of the index of dispersion, *D* (Elliot 1971, Krebs 1998). This analysis was done mainly so that the results could be compared with those obtained by Kitron et al. (1989) who used dispersion patterns to study the oviposition behavior of *Ae. triseriatus*. A random dispersion pattern is expected when $s^2 = \bar{x}$ ($D_I = 1$); regular and contagious (or aggregated) dispersion patterns are expected for $s^2 < \bar{x}$ ($D_I < 1$) and $s^2 > \bar{x}$ ($D_I > 1$), respectively. Significant differences from the random dispersion were tested using the methods described in Elliot (1971) and Krebs (1998) along with the χ^2 chart provided by Krebs (1998, page 120).

Because dispersion indices do not consider the spatial information, but rely on the frequency distribution of counts to make inferences about spatial patterns, other methods must be used to assess the spatial distribution patterns of oviposition. One such method is **S**patial **A**nalysis by **D**istance **I**ndic**E**s (SADIE) (Perry 1995, Thomas et al. 2001). For testing the spatial pattern of data, SADIE provides an index of aggregation (I_a) and P_a, the probability that the data are not distributed randomly. Values of $I_a > 1$, $I_a = 1$, and $I_a < 1$ indicate aggregation, randomness, and regularity respectively in the spatial distribution of the data. If the value of $I_a > 1$ is associated with a value of $P_a < 0.025$, the aggregated spatial distribution is considered significantly different from random; likewise, if the value of $I_a < 1$ is associated with a value of $P_a > 0.95$, the regular spatial distribution is considered significantly different from that expected from a random pattern.

The second measure that SADIE provides is the index of association or dissociation (I_m) between pair-wise spatial data sets (Thomas et al. 2001). Values of $I_m > 0$ indicate spatial association and negative values ($I_m < 0$) indicate disassociation between counts in the two data sets (Thomas et al. 2001). The probability (P_m) that there is significant association or disassociation between the two sets of spatial counts is indicated when $P_m < 0.025$ and $P_m > 0.975$, respectively.

2.3 Results

2.3.1 Habitat composition at Trap Sites

During the two-year study, in 2005 and 2006, 87% and 13% of trap sites in the Wise area were in forested and urban habitats, respectively. In the NRV area 47% of traps were in forested

areas and 53% in urban habitats. In the Roanoke area 14% and 86% of traps were in forested areas and urban habitats, respectively (Fig. 2.2).

2.3.2 Egg collection data

2005 Study: The total number of eggs collected during the sampling period at each of the study areas are shown in Table 2.1. The mean (± 95% CI) numbers of eggs per trap-day per trap site at each of the study areas during each week are presented numerically in Table 2.2 and graphically in Fig. 2.3. Collection weeks in Table 2.2 were recorded according to the International Standardization of Organization (ISO), in order to easily compare collection weeks between 2005 and 2006. The ISO calendar numbers each week of the year, beginning with the first week in January as week 1.

The results of the repeated measures analysis showed that there was a significant difference in mean egg counts ($F = 3.9835$; df = 2,105; $P = < 0.05$; Fig 2.3) among the three study areas. Orthogonal contrasts to compare mean egg counts between study areas showed that the mean egg counts in the NRV were significantly higher than in Roanoke ($F = 7.8538$; df = 1,105; $P = <0.01$), but not statistically different from Wise ($F = 1.9278$; df = 1,105 $P = 0.1679$). There was no significant difference in mean egg counts between Roanoke and Wise ($F = 1.6245$ df = 1,105 $P = 0.2053$).

The sphericity test of the repeated measures analysis on the transformed egg count data was significant ($\chi^2 = 42.20$, df= 14, $P < 0.001$) and, therefore, the numerator and denominator degrees of freedom from the analysis were adjusted. The results show that there was a significant week by area interaction for the mean egg counts ($F = 3.3720$; df = 9.2975, 488.12; $P = < 0.001$), indicating that the three areas had different temporal patterns of oviposition activity

across the collection weeks. Orthogonal contrasts showed that there were significant differences in the weekly patterns of mean egg counts between NRV and Roanoke (F = 2.5148 df = 5, 525; P = <0.05), and NRV and Wise (F = 5.8209 df = 5,525; P = <0.0001). However, there was no significant difference in the weekly oviposition patterns between Roanoke and Wise (F = 1.3028 df = 5, 525; P = 0.2610).

2006 Study: The patterns of mean egg counts per trap-day during each week of the study in 2006 are shown in Fig. 2.4 with the values presented in Table 2.3. There was a significant difference in mean egg counts among the study areas (F = 11.4780 df = 2, 93 P = <0.0001). Orthogonal contrasts showed that the differences were the result of significant differences in mean egg counts between NRV and Roanoke (F = 21.3032; df = 1, 93; P = < 0.0001) and between NRV and Wise (F = 8.1555; df = 1, 93; P = <0.05). There was no significant difference in mean egg counts between Roanoke and Wise (F = 1.5869; df = 1, 93; P = 0.2109).

The analysis also showed that the weekly patterns of oviposition activity differed significantly by areas in 2006 (F= 3.0950; df = 8.8899, 413.38; P <0.01). As in 2005, the H-F epsilon adjustment was applied based on the significance of the sphericity tests (χ^2 =44.66, df= 14, P < 0.001). Orthogonal contrasts showed that there were significant difference in the weekly patterns of mean egg counts between NRV and Roanoke (F = 4.5455 df = 5, 465; P = <0.001), and NRV and Wise (F = 2.2779 df = 5, 465; P = <0.05). Again, there was no significant difference in oviposition patterns between Roanoke and Wise (F = 2.0636; df = 5, 465; P = 0.0688).

2.3.3 Mosquito Species

The proportion of each of the species of vector at each trap site in 2005 and 2006 was estimated based on identification of the adults reared from the eggs collected each week at the site. At each study area, eggs of *Ae. triseriatus* were the most abundant, followed by *Ae. albopictus* and *Ae. japonicus*. A Tukey's Studentized (HSD) Range Test of the proportion of the vector species showed that there were significant differences in the occurrences of the three mosquito species within the study areas (Table 2.4).

The occurrences of the mosquito species based on the proportion of adults obtained were also compared between the study areas, using a Tukey's Studentized (HSD) Range Test for Percent. Again, there were significant differences ($P < 0.05$) in the mean proportion of mosquito species between the study areas (Table 2.5). A significantly higher proportion of the eggs collected in the NRV were from *Ae. triseriatus* compared with both Roanoke and Wise areas. Wise had significantly higher proportion of *Ae. triseriatus* eggs than Roanoke; and Roanoke had a significantly higher proportion of *Ae. albopictus* eggs than both the NRV and Wise areas. Finally, Wise had a significantly higher proportion of *Ae. albopictus* and *Ae. japonicus* eggs than the NRV. Therefore, in summary, the egg-laying by each of the three vectors in the study areas during the two-year study were as follows: Roanoke > Wise > NRV for *Ae. albopictus*; Wise > Roanoke> NRV for *Ae. japonicus*; NRV > Wise > Roanoke for *Ae. triseriatus*.

2.3.4 Habitat Preferences

The results of the analysis of habitat selection for oviposition among the three species in each of the study areas during the course of the study are shown in Table 2.5. Overall, the standardized selection indices for *Ae. albopictus* and *Ae. japonicus* indicate that these two

species have a stronger preference for oviposition sites in urban habitats compared with forested habitats. In contrast, Ae. *triseriatus* appears to have a strong preference for forested habitats.

2.3.5 Spatial Pattern of Oviposition

The spatial distribution of egg-laying for the three vector species are shown in the maps in Figs. 2.5–2.7 for the Wise, NRV, and Roanoke study areas, respectively. The analyses of the spatial pattern of the information in the maps are presented in Tables 2.6–2.8 for each of the three areas.

The values on the maps are scaled to the maximum seasonal mean number of eggs collected for the three species across the three regions during the two years of the study (69.3) eggs per trap-day for *Ae. triseriatus* in the NRV in 2006). The scaling was done to make it easier to visualize and confirm several things. First, the maps confirm that egg-laying was highest for *Ae. triseriatus*, followed by *Ae. albopictus,* and *Ae. japonicus.* The maps also reaffirm that that egg-laying by *Ae. albopictus, Ae. japonicus,* and *Ae. triseriatus* was greatest in the Roanoke, Wise, and NRV areas, respectively. Also, noteworthy is that *Ae. japonicus* was found in all three areas with generally a higher level of egg-laying occurring in 2006 compared with 2005.

Overall, the results of the dispersion pattern and spatial distribution analyses suggest that with a few exceptions (in the case of *Ae. japonicus*), egg-laying by the vectors was contagious (aggregated) among trap sites. Although the P_a values did not indicate significant differences of the spatial distributions from a random pattern ($P_a > 0.025$), the results of the dispersion analyses were generally supported by the SADIE analyses that examined the spatial distribution of egg-laying among trap-sites within each study area.

The SADIE analyses also showed that there was some level of disassociation in egg-laying among the three species, but particularly between *Ae. albopictus* and the two other vectors. In one case, in the Roanoke area in 2005, the disassociation in the spatial patterns of egg-laying between *Ae. albopictus* and *Ae. triseriatus* was highly significant (I_m = -0.54; P_m = 0.998; Table 2.8), and in another case, in the Wise area in 2005, the disassociation between *Ae. albopictus* and *Ae. japonicus* was nearly significant (I_m = -0.30; P_m = 0.941; Table 2.8). Not surprisingly, there were significant associations in the distribution of egg-laying among trap sites between years for *Ae. triseriatus* (Tables 2.7–2.9, last row), which suggest that the level of egg-laying by this species within a study area was consistent among the sites between years.

2.4 Discussion

The eggs collected in the three study areas in southwest Virginia were identified as belonging to three species of mosquitoes, *Ae. triseriatus*, the primary vector of the LAC virus (Thompson et al. 1972, Watts et 1972, 1973, 1974), *Ae. albopictus*, which has been shown to have potential as an accessory vector for the LAC virus (Tesh and Gubler 1975, Gerhardt et al. 2001), and *Ae. japonicus* for which the potential as a vector of the LAC virus has so far only been demonstrated in the laboratory (Sardelis et al. 2002).

The oviposition activity of the three species was found to differ significantly between years and among the study areas, with *Ae. triseriatus* being the dominant species followed by *Ae. albopictus* and *Ae. japonicus*. *Ae. triseriatus* is the most well established of the three species in southwest Virginia. The eggs deposited by this species comprised the majority of the mosquito eggs collected (≈70–93%) in each of the study areas during the two-year study. Ovitrapping studies by Barker et al. (2003a,b) in southwest Virginia also found *Ae. triseriatus* to be the

predominant species (90.1%) collected in artificial containers. Furthermore, studies in a LAC virus endemic area of western North Carolina by Szumlas et al. (1996a,b) reported that *Ae. triseriatus* was the dominant species collected (80.9%) from peridomestic containers.

Previous studies have also shown that *Ae. triseriatus* has a higher oviposition activity compared with *Ae. albopictus* (Barker et al. 2003 a,b). For example, Barker et al. (2003a) reported mean egg counts of *Ae. triseriatus* and *Ae. albopictus* of 20.4 and 3.7 eggs/trap-day, respectively. Because *Ae. japonicus* has only recently been reported within the study areas (Grim et al. 2007), very little information is available on the oviposition activity of this species in southwest Virginia. The data collected in this study, however, does confirm the presence and spread of *Ae. japonicus* throughout the region.

Although a large number of eggs were collected in both years, approximately 58,000 more eggs were collected in 2006 then in 2005. This increase in egg collection could have been due to differences in the amount of rainfall during the breeding season of the mosquitoes in 2005 and 2006 (Table 2.9). Rainfall produces many potential breeding sites for mosquitoes by filling natural and artificial containers with water. More rain reduces the need for mosquitoes to seek out artificial containers such as our ovitraps because more natural breeding sites become available. Hence in 2006, with less rain, mosquitoes may have favored our artificial containers as breeding sites, allowing us to collect significantly more eggs. Conversely, in 2005, more rainfall presumably produced a greater number of breeding sites, and therefore fewer eggs were oviposited into our artificial traps.

In spite of differences in the number of eggs collected between years, the patterns of egg-laying among sites with each year were similar. For example, in 2005, the major peak in oviposition activity at the three study sites occurred late in the breeding season (ISO week 34),

while in 2006 the major peak in activity at all three sites occurred two weeks earlier (ISO week 32). The timing of the peak in egg-laying between years could have been the result of differences in the amount of rainfall that occurred early and late in the season in each of the years. In 2005, the late peak in egg numbers was probably caused by the lack of suitable natural breeding sites because of lower rainfall during the period of natural increase in mosquito numbers (Table 2.9). For instance, the natural increase in populations of the dominant species in the study areas, *Ae. triseriatus*, usually occurs early in the season (Barker et al. 2003a). The low rainfall during the early season in 2006 could have made the ovitraps more attractive as breeding sites and hence the early peaks in egg-laying.

Although the three vector species found in the study areas share an ecological niche of natural and artificial containers, the egg-laying activity of each species tended to be influenced by the habitat in which oviposition sites were located. As such, clear preferences by each species were observed for the two main habitat types (forest or urban) defined for the study (Fig 2.8; Table 2.5). *Ae. triseriatus* showed a preference for forested areas, whereas *Ae. albopictus* showed a propensity for urban areas. A previous study in southwest Virginia also found that at the highest numbers of *Ae. triseriatus* eggs were collected from forested areas, while the highest number of *Ae. albopictus* eggs were collected from urban and residential areas (Barker 2003a,b). The habitat preferences of the recently introduced species, *Ae. japonicus,* are difficult to define, since this species appears to be still spreading within southwest Virginia. However, although *Ae. japonicus* was found in both forested and urban habitats in this study, the analysis of habitat selection suggests that this species has a preference for urban habitats similar to that of *Ae. albopictus* (Table 2.5).

We should keep in mind that although the analysis showed that each of the species has a preference for a particular type of habitat that each is capable of using other habitats. This is clearly the case since on many occasions all three species were found at the same collection site. *Aedes albopictus*, for example, was found throughout Wise County in spite of widespread forest habitat and the mosquito's usual preference for urban habitats. In fact, significantly more *Ae. albopictus* eggs were found in the Wise area than in the NRV, in spite of the fact that the latter has a higher proportion of urban landscapes. However, this should not be surprising because even though *Ae. albopictus* are mainly associated with urban habitats in North America, they are common throughout rural and forested areas in Asia (Hawley 1988). Mogi (1982) suggested that forest-breeding strains of *Ae. albopictus* in some parts of Asia are the result of individuals from rural environments that have readapted to a forest habitat. Therefore, it is possible that due to the predominance of forest in Wise County, populations of *Ae. albopictus* may be adapting to this habitat. The process of readaptation by *Ae. albopictus* could in part reduce the level of interspecific competition between this species and *Ae. japonicus* for the scarce urban habitats the Wise area, and might also explain the relatively high oviposition activity by *Ae. japonicus* in the Wise study area and the significant disassociation in the spatial egg-laying pattern between the two species in the area.

A study by Grim et al. (2007) found that over the course of a year, *Ae. japonicus* adults had become the second most dominant species collected from gravid traps in southwest Virginia. This species has been actively expanding its range (Grim et al. 2007, Harrison et al. 2002) and in this study it was found at many more sites in 2006 than in 2005 (Fig. 2.9). The expansion of *Ae. japonicus* can also be seen in the spatial maps of egg-laying between 2005 and 2006 within the study areas. Little is known about *Ae. japonicus* so until we can conduct in-depth studies of this

species we can only speculate about its ability to spread and to displace other mosquito species, such as, *Ae. albopictus*, which has itself been able to successfully invade and displace *Ae. aegypti* in many areas in the United States (Braks et al. 2004).

Previous studies have assessed the dispersion and spatial distribution patterns of egg-laying have been studied for *Ae. triseriatus* and *Ae. albopictus* in the current study. Similar to the results in this study, Kitron et al. (1989) found that the spatial dispersion of oviposition among trap sites by *Ae. triseriatus* was aggregated. Kitron et al. (1989) attributed the aggregated pattern to the heterogeneous deposition of "a large number of eggs per gonotrophic cycle in one or more clumps" by females that resulted in between 29–47 eggs deposited per oviposition cycle. The effect of this behavior (depositing a large number of eggs in multiple small clumps) is similar to the process known as skip oviposition where female mosquitoes deposit a small number of eggs among many oviposition sites as a means of species dispersal (Reiter 2007).

The analysis of spatial dispersion in the current study also indicated that in most cases the dispersion pattern of *Ae. albopictus* egg-laying was aggregated. In addition, the analyses suggested that the spatial distribution patterns of oviposition for both *Ae. albopictus* and *Ae. triseriatus* were aggregated, although the P_a values imply that the patterns are really not different from a random distribution. The studies by both Focks et al. (1999) and Richards et al. (2006), however, support the idea that the spatial distribution of *Ae. albopictus* egg-laying in ovitraps is aggregated. This type of ovitrap information on the spatial distribution of egg-laying for a species when combined with information that the pattern across years are associated (Table 2.7–2.9) can prove useful for tracking the spread of the species and for developing management programs.

An attempt to determine minimum field infection rates among the adults reared from our egg collections was unsuccessful due to a freezer failure in 2006. All adults from 2005 however, were tested for LAC virus via real time polymerase chain reaction (RT-PCR). LAC virus positive mosquito pools were found in both Wise and the NRV. This finding supports the results of a 2002 study (Paulson unpublished data) on which the study areas selected in the current study were based, where LAC virus positive mosquito isolates were found in both of these areas.

In summary, the study found that the egg abundance and oviposition patterns of *Ae. triseriatus*, *Ae. albopictus*, and *Ae. japonicus* varied across the three study areas. The connection between the risk of LAC virus infection in endemic areas and the density of breeding sites available to the vector (Defoliart et al. 1986) suggests that LAC virus endemic areas are sporadic and levels of risk probably vary throughout these areas. In addition, the primary LAC virus vector, *Ae. triseriatus*, was collected in the greatest abundance from all three study areas. *Aedes albopictus* was the second most abundant species collected, and this species was found to favor urban environments. Perhaps the role of *Ae. albopictus* in this new southwestern focus of LAC virus is to serve as a bridge vector, carrying LAC virus from the forests into urban areas where the primary vector is usually found in fewer numbers.

The range of *Ae. japonicus* seems to be expanding throughout southwest Virginia. The ability of this species to transmit LAC virus in laboratory settings heightens our awareness for its potential to contract LAC virus in the wild, thereby contributing to the maintenance and transmission cycle of the LAC virus endemic foci in southwest Virginia.

It is also important to note that the majority of our trap sites were at grade schools, many of which are adjacent to residential areas. Perhaps local public health officials could take this

into account and use this study to assess the risks and heighten public awareness of arboviruses in southwest Virginia, especially LAC virus.

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Tables and Figures

Table 2.1: Total number of mosquito eggs collected at three study areas in southwest Virginia during sampling periods in 2005 and 2006.

Study Area	2005	2006	Total
Wise	75,614	72,330	147,944
New River Valley	135,304	192,395	327,699
Roanoke	54,567	59,048	113,615
Total	265,485	323,773	589,258

Table 2.2: Weekly mean mosquito eggs collected per trap-day and 95% confidence intervals at three study areas in southwest Virginia in 2005. Collection weeks are recorded according to the International Standardization of Organization (ISO) calendar.

Collection Week	ISO Date	Wise	New River Valley	Roanoke
		Mean(± 95% C.I.	Mean (± 95% CI)	Mean (± 95% C.I.)
1	**30**	18.0 (5.91)	14.4 (3.39)	9.7 (2.26)
2	**31**	15.5 (5.18)	20.7 (4.49)	13.8 (4.65)
3	**32**	19.2 (5.62)	23.7 (5.08)	16.6 (4.71)
4	**33**	22.0 (5.64)	26.2 (5.60)	18.7 (6.13)
5	**34**	17.9 (6.15)	28.6 (5.04)	13.9 (4.42)
6	**35**	22.1 (7.52)	18.3 (4.07)	13.7 (4.65)

Table 2.3: Weekly mean mosquito eggs collected per trap-day and 95% confidence intervals at three study areas in southwest Virginia in 2006. Collection weeks are recorded according to the International Standardization of Organization (ISO) calendar.

Collection Week	ISO Date	Wise	New River Valley	Roanoke
		Mean (± 95% CI)	Mean (± 95% CI)	Mean (± 95% CI)
1	30	22.8 (6.80)	29.0 (6.62)	18.6 (3.95)
2	31	27.3 (8.63)	42.4 (8.17)	23.0 (6.39)
3	32	29.2 (9.42)	56.1 (13.10)	20.5 (5.71)
4	33	13.0 (3.77)	29.1 (6.20)	16.4 (4.66)
5	34	14.8 (3.67)	22.2 (4.46)	12.3 (3.90)
6	35	15.7 (3.84)	23.1 (5.34)	9.5 (4.25)

Table 2.4: Percentages of adult mosquitoes reared from eggs collected at three study areas in southwest Virginia.

Species	Wise		New River Valley		Roanoke	
	2005	2006	2005	2006	2005	2006
Ae. triseriatus	84.7*a*	69.9*a*	92.8*a*	92.7*a*	53.7*a*	75.1*a*
Ae. albopictus	11.9*b*	24.1*b*	7.2*b*	6.7*b*	44.8*a*	22.6*b*
Ae. japonicus	3.4*b*	6.0*c*	0.1*c*	0.5*c*	2.7*b*	2.3*c*

Means within a column followed by the same letter are not significantly different; P >0.05.

Table 2.5: Standardized selection indices for three mosquito species in three study areas in southwest Virginia indicating their preference for oviposition sites in forest or urban habitats.

Year	Study Area	Vector	Standardized Selection Index[a]	
			Forest	**Urban**
2005	Wise	*Ae. albopictus*	0.29	**0.71**
		Ae. japonicus	0.44	**0.56**
		Ae. triseriatus	**0.68**	0.32
	New River Valley	*Ae. albopictus*	**0.62**	0.38
		Ae. japonicus	0.00	**1.00**
		Ae. triseriatus	**0.67**	0.33
	Roanoke	*Ae. albopictus*	0.32	**0.68**
		Ae. japonicus	0.00	**1.00**
		Ae. triseriatus	**0.63**	0.37
2006	Wise	*Ae. albopictus*	0.29	**0.71**
		Ae. japonicus	0.37	**0.63**
		Ae. triseriatus	**1.00**	0.00
	New River Valley	*Ae. albopictus*	0.44	**0.56**
		Ae. japonicus	0.46	**0.54**
		Ae. triseriatus	**0.67**	0.33
	Roanoke	*Ae. albopictus*	0.00	**1.00**
		Ae. japonicus	**0.73**	0.27
		Ae. triseriatus	**0.71**	0.29

[a] A standardized index of 1/number of habitats (i.e. 1/2 = 0.50) indicates no preference. Indices > 0.50 indicate relative preference and indices < 0.50 indicate relative avoidance. Preference in the table is highlighted by values in bold font.

Table 2.6: Results of spatial pattern analysis and association of the seasonal mean number of eggs for trap-day per site collected in the Wise area for three LAC virus vector species.

Spatial Data Set	D_I	$\chi 2$	Dispersion Pattern	I_a	P_a	Spatial Distribution	I_m	P_m	*Association/ Disassociation*
2005									
Ae. albopictus	4.32	120.84	Aggregated*	1.19	0.229	Aggregated			
Ae. japonicus	3.22	90.10	Aggregated*	0.72	0.865	Regular			
Ae. triseriatus	7.96	222.77	Aggregated*	0.59	0.987	Regular*			
Ae. albopictus vs. Ae. japonicus							-0.30	0.941	Disassociation
Ae. albopictus vs. Ae. triseriatus							0.02	0.465	Association
Ae. japonicus vs. Ae. triseriatus							0.01	0.472	Association
2006									
Ae. albopictus	1.50	42.10	Aggregated	1.03	0.397	Aggregated			
Ae. japonicus	1.23	34.33	Aggregated	0.99	0.474	Regular			
Ae. triseriatus	9.93	278.10	Aggregated*	1.13	0.252	Aggregated			
Ae. albopictus vs. Ae. japonicus							-0.06	0.621	Disassociation
Ae. albopictus vs. Ae. triseriatus							-0.16	0.790	Disassociation
Ae. japonicus vs. Ae. triseriatus							-0.09	0.696	Disassociation
2005 vs. 2006									
Ae. albopictus vs. Ae. albopictus							0.12	0.345	Association
Ae. japonicus vs. Ae. japonicus							0.09	0.321	Association
Ae. triseriatus vs. Ae. triseriatus							0.62	0.001	Association*

* Indicates significant difference of dispersion pattern ($P < 0.05$) or distribution pattern ($P_a < 0.025$ for aggregation; $P_a > 0.95$ for regularity) from randomness, and significant association ($P_a < 0.025$) or disassociation ($P_m > 0.975$) between two spatial distributions.

$\boldsymbol{D_I}$ = Dispersion Index = $s^2 / \bar{x}$; $D_I > 1$(contagious), $D_I = 1$(random), and $D_I < 1$(regular) dispersion patterns

$\boldsymbol{\chi 2} = \frac{s^2(n-1)}{\bar{x}}$, the value used with the df, $(n - 1)$, to test for significant difference of the dispersion pattern from random dispersion using Table 4.5, page 120 in Krebs (1998).

$\boldsymbol{I_a}$ = Aggregation index; $I_a > 1$ (aggregated), $I_a = 1$ (random), and $I_a < 1$ (regular) spatial distribution.

$\boldsymbol{P_a}$ = Probability associated with I_a; $I_a > 1$ and $P_a < 0.025$, aggregation significantly different from random; $I_a < 1$ and $P_a > 0.95$, regular pattern that is significantly different from random.

$\boldsymbol{I_m}$ = Index of Association; $I_m > 0$ indicates spatial association; $I_m < 0$ indicates spatial disassociation

$\boldsymbol{P_a}$ = Probability associated with I_m; $P_m < 0.025$ indicates significant association; $P_m > 0.975$ indicates significant disassociation.

Table 2.7: Results of spatial pattern analysis and association of the seasonal mean number of eggs for trap-day per site collected in the New River Valley for three LAC virus vector species.

Spatial Data Set	D_I	$\chi 2$	Dispersion Pattern	I_a	P_a	Spatial Distribution	I_m	P_m	*Association/ Disassociation*
2005									
Ae. albopictus	3.94	173.26	Aggregated*	1.06	0.327	Aggregated			
Ae. japonicus	0.34	14.93	Regular*	---	---	---			
Ae. triseriatus	6.26	275.27	Aggregated*	1.21	0.137	Aggregated			
Ae. albopictus vs. Ae. japonicus							---	---	
Ae. albopictus vs. Ae. triseriatus							-0.02	0.560	Disassociation
Ae. japonicus vs. Ae. triseriatus							---	---	
2006									
Ae. albopictus	1.76	77.34	Aggregated*	1.05	0.372	Aggregated			
Ae. japonicus	2.10	91.67	Aggregated*	0.80	0.872	Regular			
Ae. triseriatus	10.50	461.20	Aggregated*	1.21	0.158	Aggregated			
Ae. albopictus vs. Ae. japonicus							-0.12	0.762	Disassociation
Ae. albopictus vs. Ae. triseriatus							-0.23	0.897	Disassociation
Ae. japonicus vs. Ae. triseriatus							0.01	0.493	Association
2005 vs. 2006									
Ae. albopictus vs. Ae. albopictus							0.23	0.075	Association
Ae. japonicus vs. Ae. japonicus							---	---	
Ae. triseriatus vs. Ae. triseriatus							0.47	0.001	Association*

* Indicates significant difference of dispersion pattern ($P < 0.05$) or distribution pattern ($P_a < 0.025$ for aggregation; $P_a > 0.95$ for regularity) from randomness, and significant association ($P_a < 0.025$) or disassociation ($P_m > 0.975$) between two spatial distributions.

$\boldsymbol{D_I}$ = Dispersion Index = $s^2 / \bar{x}$; $D_I > 1$(contagious), $D_I = 1$(random), and $D_I < 1$(regular) dispersion patterns

$\boldsymbol{\chi 2} = \frac{s^2(n-1)}{\bar{x}}$, the value used with the df, (n – 1), to test for significant difference of the dispersion pattern from random dispersion using Table 4.5, page 120 in Krebs (1998).

$\boldsymbol{I_a}$ = Aggregation index; $I_a > 1$ (aggregated), $I_a = 1$ (random), and $I_a < 1$ (regular) spatial distribution.

$\boldsymbol{P_a}$ = Probability associated with I_a; $I_a > 1$ and $P_a < 0.025$, aggregation significantly different from random; $I_a < 1$ and $P_a > 0.95$, regular pattern that is significantly different from random.

$\boldsymbol{I_m}$ = Index of Association; $I_m > 0$ indicates spatial association; $I_m < 0$ indicates spatial disassociation

$\boldsymbol{P_a}$ = Probability associated with I_m; $P_m < 0.025$ indicates significant association; $P_m > 0.975$ indicates significant disassociation.

Table 2.8: Results of spatial pattern analysis and association of the seasonal mean number of eggs for trap-day per site collected in the Roanoke area for three LAC virus vector species.

Spatial Data Set	D_I	$\chi 2$	Dispersion Pattern	I_a	P_a	Spatial Distribution	I_m	P_m	*Association/ Disassociation*
2005									
Ae. albopictus	5.34	149.66	Aggregated*	1.17	0.135	Aggregated			
Ae. japonicus	6.50	183.14	Aggregated*	0.89	0.573	Regular			
Ae. triseriatus	5.15	144.31	Aggregated*	1.23	0.092	Aggregated			
Ae. albopictus vs. Ae. japonicus							0.14	0.245	Association
Ae. albopictus vs. Ae. triseriatus							-0.54	0.998	Disassociation*
Ae. japonicus vs. Ae. triseriatus							-0.19	0.774	Disassociation
2006									
Ae. albopictus	1.25	57.76	Aggregated*	1.35	0.026	Aggregated			
Ae. japonicus	1.43	3.26	Regular*	1.07	0.291	Aggregated			
Ae. triseriatus	4.72	325.69	Aggregated*	1.51	0.013	Aggregated*			
Ae. albopictus vs. Ae. japonicus							-0.08	0.640	Disassociation
Ae. albopictus vs. Ae. triseriatus							-0.10	0.699	Disassociation
Ae. japonicus vs. Ae. triseriatus							0.21	0.140	Association
2005 vs. 2006									
Ae. albopictus vs. Ae. albopictus							0.04	0.427	Association
Ae. japonicus vs. Ae. japonicus							0.36	0.054	Association
Ae. triseriatus vs. Ae. triseriatus							0.70	0.0001	Association*

* Indicates significant difference of dispersion pattern ($P < 0.05$) or distribution pattern ($P_a < 0.025$ for aggregation; $P_a > 0.95$ for regularity) from randomness, and significant association ($P_a < 0.025$) or disassociation ($P_m > 0.975$) between two spatial distributions.

$\boldsymbol{D_I}$ = Dispersion Index = $s^2 / \bar{x}$; $D_I > 1$(contagious), $D_I = 1$(random), and $D_I < 1$(regular) dispersion patterns

$\boldsymbol{\chi 2} = \frac{s^2(n-1)}{\bar{x}}$, the value used with the df, (n – 1), to test for significant difference of the dispersion pattern from random dispersion using Table 4.5, page 120 in Krebs (1998).

$\boldsymbol{I_a}$ = Aggregation index; $I_a > 1$ (aggregated), $I_a = 1$ (random), and $I_a < 1$ (regular) spatial distribution.

$\boldsymbol{P_a}$ = Probability associated with I_a; $I_a > 1$ and $P_a < 0.025$, aggregation significantly different from random; $I_a < 1$ and $P_a > 0.95$, regular pattern that is significantly different from random.

$\boldsymbol{I_m}$ = Index of Association; $I_m > 0$ indicates spatial association; $I_m < 0$ indicates spatial disassociation

$\boldsymbol{P_a}$ = Probability associated with I_m; $P_m < 0.025$ indicates significant association; $P_m > 0.975$ indicates significant disassociation.

Table 2.9: Total rainfall (in centimeters) in three study areas in southwest Virginia during the 2005 and 2006 sampling periods.

Study Area	2005		2006	
	July	Aug	July	Aug
Wise	21.97	7.31	7.42	13.33
NRV	13.89	9.50	8.86	5.69
Roanoke	12.60	9.45	4.88	5.97

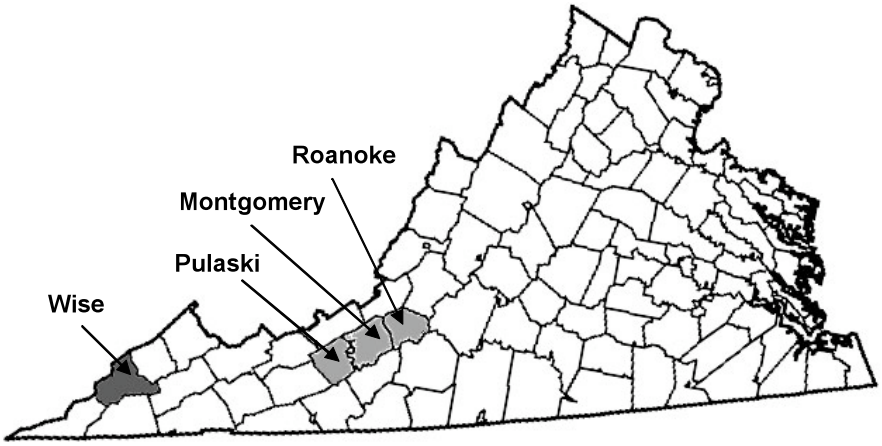

Fig. 2.1: Southwestern Virginia areas targeted by the study.

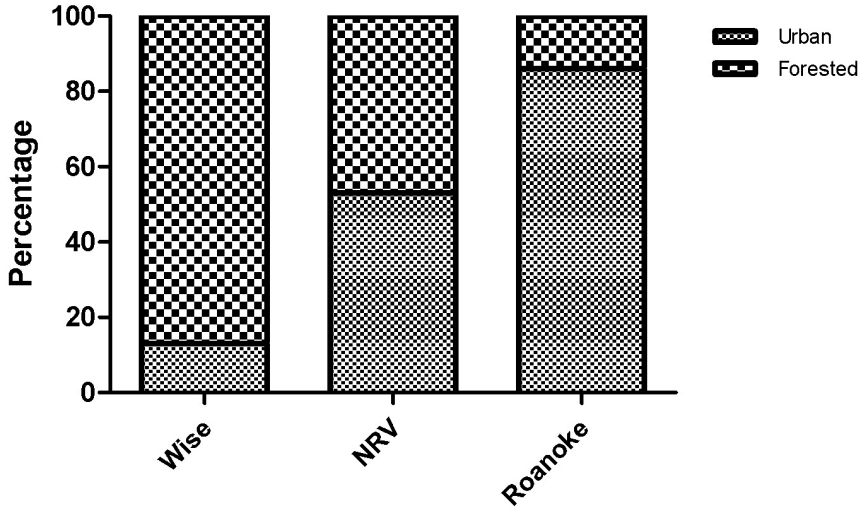

Fig. 2.2: Percentages of forested and urban areas surrounding the trap sites.

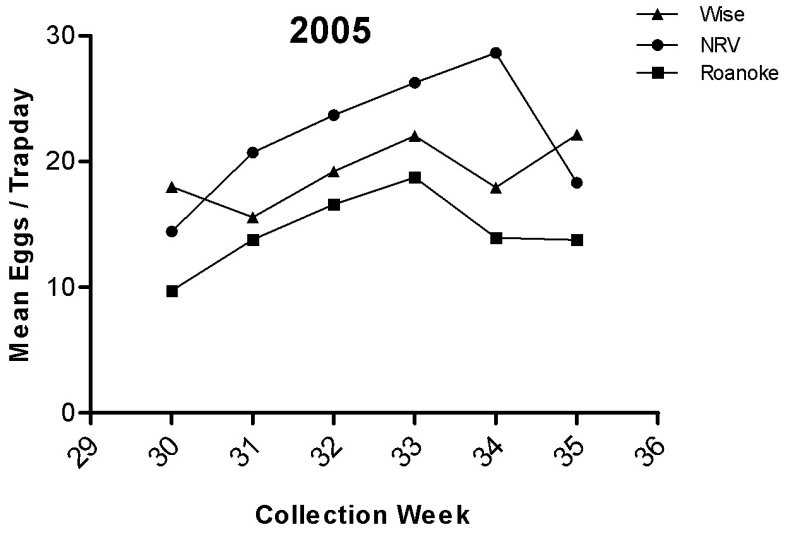

Fig. 2.3: Line graph depicting the mean mosquito eggs per trapday collected across the three southwestern Virginia study areas in 2005.

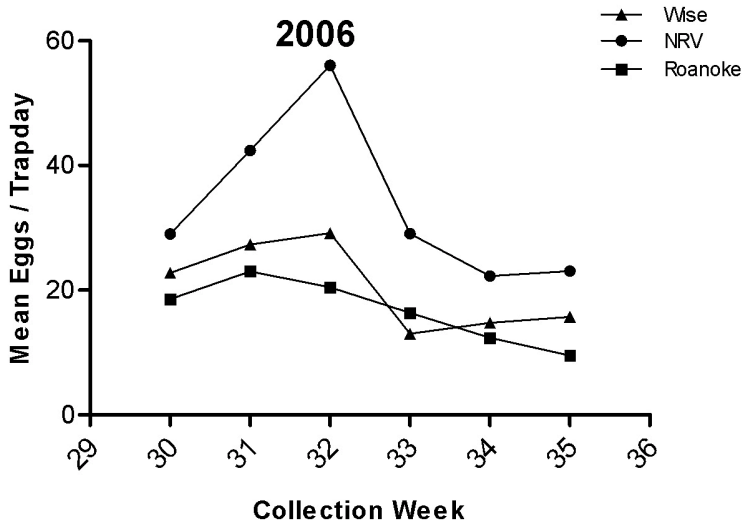

Fig. 2.4: Line graph depicting the mean mosquito eggs per trapday collected across the three southwestern Virginia study areas in 2006.

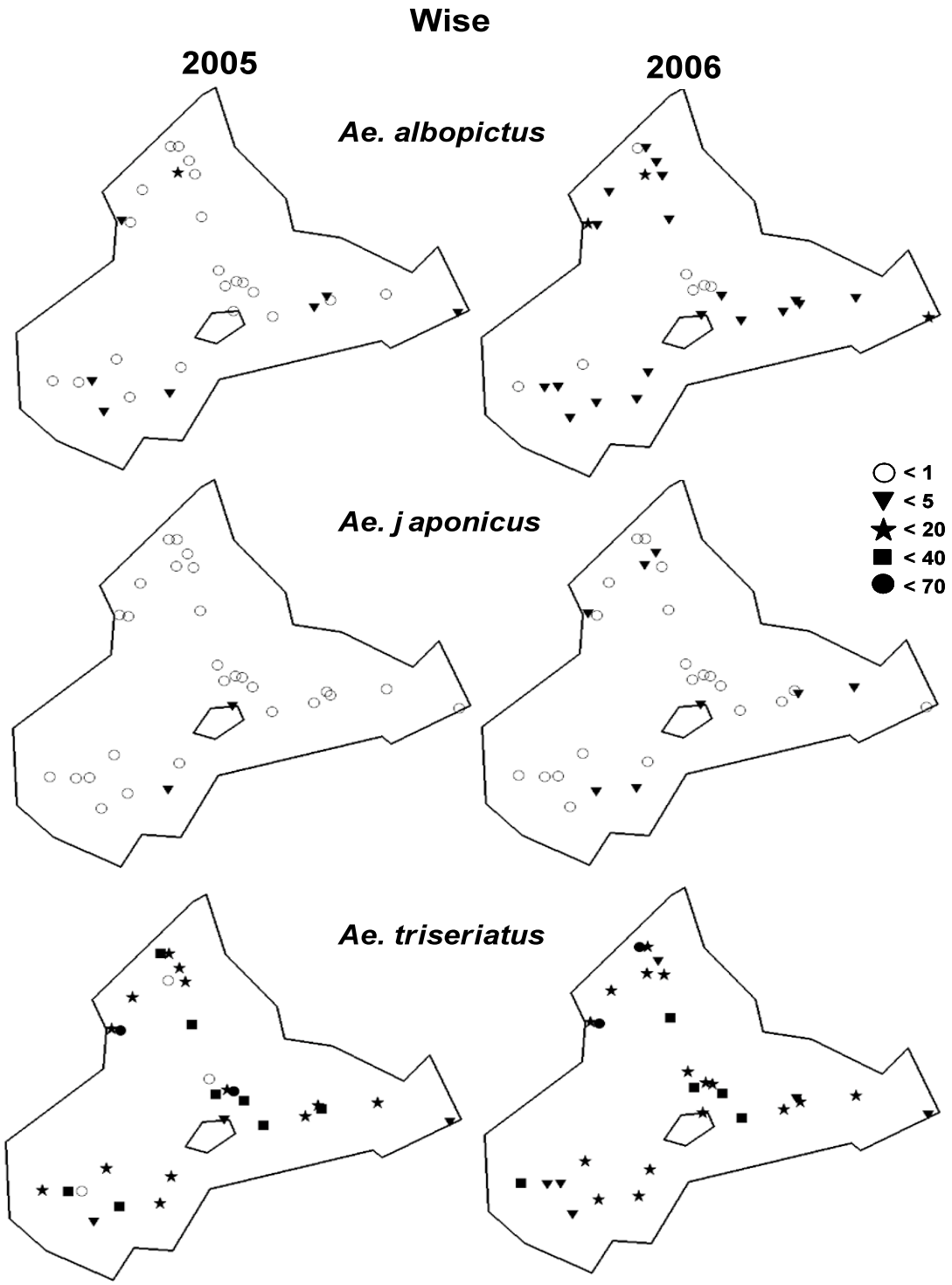

Fig. 2.5. Distribution of the **s**easonal egg-laying for three LAC virus mosquito vectors in the Wise area in 2005 and 2006. The mean value for each species was determined by pooling adults reared from eggs collected in ovitraps placed at each of the sampling sites from mid-July to late August of each year.

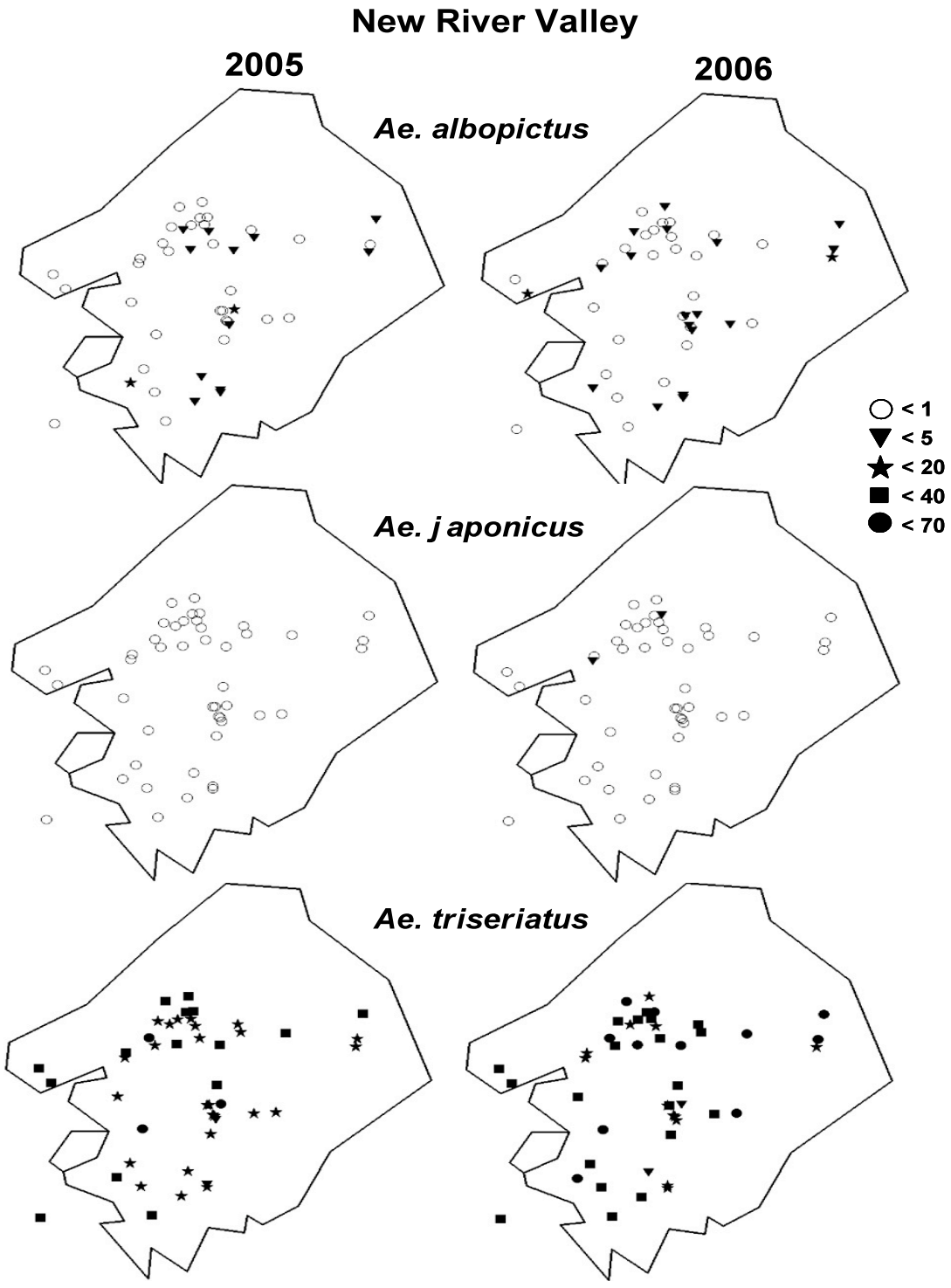

Fig. 2.6. Distribution of the seasonal egg-laying for three LAC virus mosquito vectors in the New River Valley area in 2005 and 2006. The mean value for each species was determined by pooling adults reared from eggs collected in ovitraps placed at each of the sampling sites from mid-July to late August of each year.

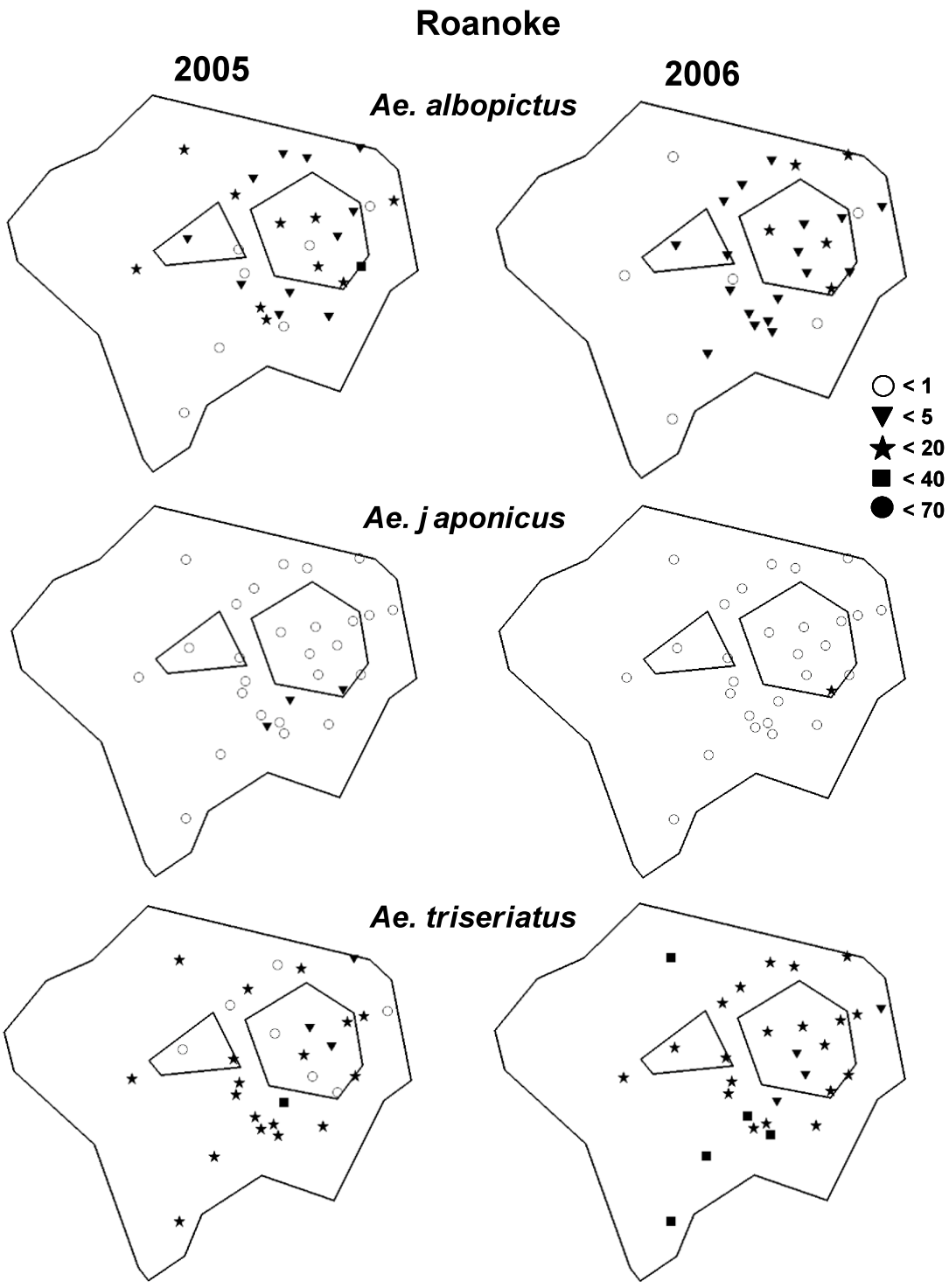

Fig. 2.7. Distribution of the seasonal egg-laying for three LAC virus mosquito vectors in the Roanoke area in 2005 and 2006. The mean value for each species was determined by pooling adults reared from eggs collected in ovitraps placed at each of the sampling sites from mid-July to late August of each year.

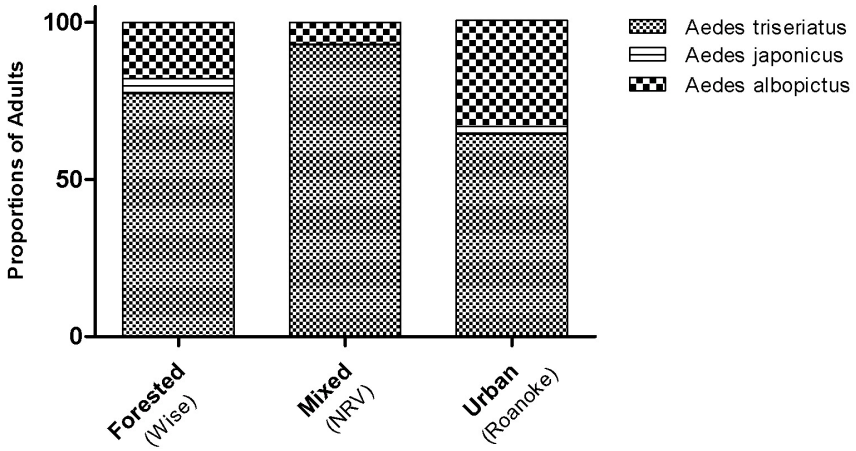

Figure 2.8: Percentages of adult mosquitoes (reared from collected eggs) from each habitat.

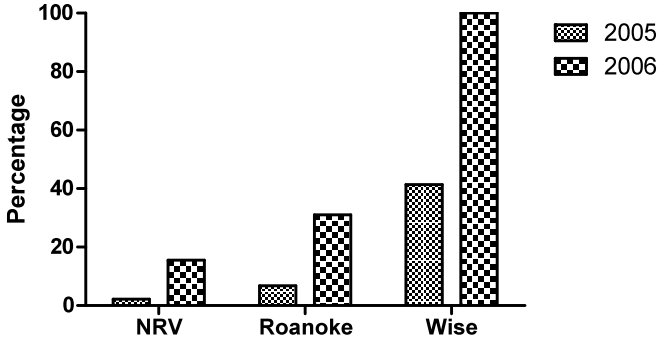

Fig. 2.9: Percentage of trap sites in three study areas of southwest Virginia with *Ae. japonicus* in 2005 and 2006.

3. Dogs as Sentinels for the Surveillance of La Crosse Virus and Other Vector-Borne Diseases in Southwest Virginia

3.1 Introduction

The use of dogs as sentinels for surveillance of infectious diseases is a common practice (Olson et al. 2000, Duncan et al. 2004, Coffey et al. 2006, Diniz et al. 2007, Salb et al. 2008). Dogs live in close proximity to their owners which often provides the advantage of using dogs to obtain valuable human disease information such as incidence rates, risk factors, and sources of exposure before the occurrence of human disease outbreaks (Diniz et al. 2007). An estimated 75% of recently discovered emerging infectious diseases are zoonotic (Taylor et al. 2001), and dogs are susceptible to a large number of emerging or re-emerging human vector-borne infections (Diniz et al. 2007). Dogs also make ideal sentinels because many spend much of their time outdoors, are more frequently exposed to ticks, fleas, and mosquitoes, and are unlikely treated with any type of mosquito repellent. Using dogs as sentinels is also practical for determining the prevalence of vector-borne disease over large areas (Guerra et al. 2001, Johnson et al. 2004), and at locations where outbreaks of emerging infectious diseases are reported.

Southwest Virginia may have several endemic areas of La Crosse (LAC) virus foci, as evidenced by reported human cases and vector surveillance studies (Virginia Dept. of Health 2008, Barker 2003a,b). LAC virus, a mosquito-transmitted arbovirus in the California serogroup of Bunyaviruses, is the most prevalent arboviral infection in children in North America (McJunkin et al. 2001). While the onset of clinical disease is usually acute with rapid recovery (Balkhy and Schreiber 2000), a small amount of cases (<1%) will present with severe clinical manifestations including seizures, altered mental status, and encephalitis, followed by a high rate

of neurologic sequelae, including learning disorders (Balkhy and Schreiber 2000, de los Reyes et al. 2008).

Because most LAC virus infections are mild to asymptomatic, many people with LAC virus infections go undiagnosed. The clinical manifestations of LAC virus infections in children under the age of 16 commonly present as flu-like symptoms, while most healthy adults lack any clinical symptoms. The absence of symptoms from LAC virus infections may result in poor case recognition by physicians (Grimstad et al. 1984). Furthermore, diagnosis of LAC virus can be problematic because isolation of the virus from the cerebrospinal fluid (CSF) and brain tissue is difficult. Serology is the method of choice for diagnosis, including detection of viral-specific IgM in the CSF or serum (Romero and Newland 2006). Additional testing is used in conjunction with serology, including EEGs to track abnormal brain activity, and MRIs to detect areas of focal enhancement.

Because the majority of LAC virus infections are undiagnosed, little is actually known about the distribution of LAC virus in endemic areas such as southwest Virginia. Knowledge of LAC virus distribution, therefore, is crucial to determine human risk for LAC virus transmission. Using dogs as sentinel animals for the detection of LAC virus may help to determine locations of LAC virus foci. Dogs are particularly ideal because they are not amplifier hosts but do develop detectable serum antibody levels in response to LAC virus (Godsey et al. 1988). In addition, the primary and accessory LAC virus vectors, *Aedes triseriatus* and *Ae. albopictus*, have shown an affinity for canine hosts. In fact, a mosquito blood meal analysis study determined that *Aedes triseriatus* fed predominantly on dogs in North Carolina (Szumlas et al. 1996). A similar analysis using *Ae. albopictus* reported that dogs are a common mammalian host for this mosquito species as well (Richards et al. 2006).

Lyme disease has also been reported as an emerging infectious disease in southwest Virginia (Virginia Dept of Health 2008). Lyme disease, vectored by the *Ixodes scapularis* tick in the eastern U.S., is caused by the bacterium *Borrelia burgdorferi.* Symptoms of Lyme disease in humans are flu-like, and can spread to the heart, joints, and nervous system if left untreated (CDC 2008). Recent reports suggest that *I. scapularis* has become increasingly common in southwest Virginia (Paulson pers. comm.), and the Virginia Dept of Health (2008) has reported several human cases of Lyme disease in the southwest portion of the state. In addition, a number of southwest Virginia veterinary practices have observed an increased incidence of Lyme disease in dogs.

Surveillance for Lyme disease in southwest Virginia is passive, relying mainly on reported human cases and submission of tick specimens to local health authorities for identification. This method of surveillance may be ineffective (Johnson et al. 2004), as cases oftentimes go unreported and tick submission rates depend on public awareness of Lyme disease and local concerns of individuals. As a result, passive surveillance for Lyme disease may say little about disease transmission, particularly in disease-emergent areas where infection rates may lag behind tick distribution (Stone et al. 2005). Using dogs as sentinels for *B. burgdorferi* may alleviate the drawbacks of passive surveillance, and provide useful information regarding the distribution of this disease in southwest Virginia (e.g., Johnson et al. 2004). Previous studies have determined that dogs are effective sentinels for Lyme disease surveillance (Lindenmayer et al.1991, Olsen et al. 2000, Stone et al. 2005;), and have also shown a correlation between Lyme disease prevalence in dog sentinels and cases of human infection (Lindenmayer et al.1990, Guerra et al. 2001).

Canine serologic screening for Lyme disease is routinely performed in veterinary clinics throughout the U.S. with commercially available testing kits that are easy to use and provide results in less than ten minutes. The ELISA-based Idexx ® Snap 4Dx ® test (Idexx Laboratories, Westbrook, ME, USA) screens for a variety of vector-borne infections, including *Borrelia burgdorferi*, *Anaplasma phagocytophilum*, *Ehrlichia canis*, and *Dirofilaria immitis* (heartworm). Diagnosis of *B. burgdorferi* infection is based on detection of antibody to the C6 peptide, which is located on an antigenically conserved region in the variable surface antigen of *B. burgdorferi*. The C6 peptide is not cross-reactive with antibodies induced by vaccination with the outer surface protein A or with a whole fixed spirochete vaccine (Liang et al 2000). A similar test, the Idexx R Snap 3Dx R test, had been used previously to determine canine seroprevalence of Lyme disease as an indicator of human health risk in Maine, Pennsylvania, Maryland, Virginia and North Carolina (Duncan et al. 2004, Stone et al. 2005).

While previous studies have used Idexx ® Snap 4Dx ® tests as a public health indicator of Lyme seroprevalence, little has been published regarding the use of these test kits to investigate public health risks for *A. phagocytophilum* and *E. canis*. Both *A. phagocytophilum* and *E. canis* are vector-borne diseases found sporadically throughout Virginia (Idexx Laboratories 2009). *Anaplasma phagocytophilum* (formerly *E. equi*, *E. phagocytophila*), is a bacterium vectored by *I. scapularis* in the eastern U.S., which causes flu-like symptoms that can lead to fatal complications if left untreated (CDC 2008). In 1990, *A. phagocytophilum* was recognized as a human pathogen, (although defined as a veterinary pathogen since 1932), and reported U.S. cases have markedly increased since then (Dumler et al. 2005). *Ehrlichia* is a bacterium that causes mild to moderately severe symptoms in humans, such as high fevers, confusion, muscle aches, and nausea. Several tick vectors are associated with *Ehrlichia*, depending on the species,

and include the Brown dog tick (*Rhipicephalus sanquineus*) and the Lone-Star tick (*Amblyomma americanum*). While *E. canis* primarily infects dogs, the antibodies used by the Idexx ® Snap 4Dx ® test for detection are cross-reactive with *E. chaffeensis*, an *Ehrlichia* species known to infect humans. A prior study in Virginia and North Carolina provided PCR evidence that healthy dogs can be naturally infected with *E. chafeensis* (Breitschwerdt et al. 1998). Additionally, human infections with *E. canis* have been described (Conrad 1998) and documented in several locations, including the southeastern portion of the U.S. (Maeda et al. 1987, Barton and Foy 1989). Thus correlations can be made between dogs positive for *E. canis* via the Idexx ® Snap 4Dx ® test and public health risks for *Erhlichiosis*.

The primary objective for this study was to determine the efficacy of canine sentinels for accessing the distribution of LAC virus in southwest Virginia. Additionally, the study also examined the usefulness of dogs as sentinels of *B. burgdorferi*, *A. phagocytophilum*, and *E. canis* infection in the region.

3.2 Materials and Methods

3.2.1 Study areas

Veterinary clinics were selected from the following three areas in the southwestern region of Virginia (Fig. 3.1): Wise County, New River Valley (NRV–Montgomery and Pulaski counties), and Roanoke County; (referred to as Wise, NRV, and Roanoke). These areas were selected based on a 2002 study that determined their status for reported human cases of LAC encephalitis and/or LAC virus positive mosquito isolates (Paulson unpublished data).

Wise County has had both human cases of LAC encephalitis, as well as LAC virus positive mosquito isolates. It is a predominantly rural area (~1050 km^2) located in the far

southwest corner of Virginia in the Appalachian Mountains. Wise County has an elevation range of 400–1287m, and consists of 61-80% mixed hardwood forest cover (i.e. oak-hickory, maple-beech birch, and pine-hemlock) with occasional clearings for pastures and strip mining for coal (Johnson 1992).

The NRV has had LAC virus positive mosquito isolates but no reported human cases to date. The NRV (~1860 km^2) is located between the Appalachian and Blue Ridge Mountains and is bisected by the New River. It is a combination of 50–60% oak hickory forest cover, farmland, and suburbs, with an elevation range of 535m to 750m (Rose 2001).

The Roanoke area includes Roanoke County and Roanoke City. In this area, there have been no reported human cases of LAC encephalitis and no mosquito isolates in 2002. The Roanoke area (~650 km^2) is located between the Appalachian and Blue Ridge Mountains, with a detached mountain (Mill Mountain) in its center. The elevation ranges from 269–1329m, with a 40–61% oak-hickory forest cover that applies mainly to the mountainous portions of Roanoke (Rose 2001). The valley areas consist of cities and suburbs, where most residents can be found.

3.2.2 Serum Collection

Serum was extracted from whole blood collected from 258 dogs in 2005–2006, and from 178 dogs in 2008. A minimum of 50 samples were obtained from veterinary clinics in each of the three study areas during both time periods. In Wise County, there was one participating clinic and in both NRV and Roanoke there were two participating clinics. The participating clinics were Pound Veterinary Hospital in Wise, Companion Animal Clinic and Dublin Animal Hospital in the NRV, and Roanoke Animal Hospital and Cave Spring Veterinary Clinic in Roanoke. Blood was taken from dogs during routine visits to veterinary clinics.

Veterinarians were provided with individual packets containing a serum separator tube, two 2 ml Eppendorf tubes, a disposable pipette, and a history/release form (Appendix A) for the dog owner to complete. Each item in the packet was prelabeled with a sample identification number. At the time of blood collection, dog owners were provided with general information regarding LAC virus and other vector-borne diseases in southwest Virginia. Dog owners who agreed to let their dog(s) participate in the study were then asked to provide the age, sex and breed of the dog, also its travel history, and the amount of time the dog spent outdoors. Puppies less than 12 weeks old were excluded from the study. In addition, the home addresses of participating dogs were recorded and the information was used to determine the geographic coordinates of the dog's place of residence within the study areas (Fig. 3.1).

Approximately 2 ml of whole blood were collected from each dog and transferred into a serum separator tube. The tubes were centrifuged in order to extract the serum, and the serum was pipetted into the 2 ml Eppendorf tubes. These serum samples were frozen until project personnel could transport them back to the Medical Entomology Laboratory in Latham Hall at Virginia Tech. All of the serum samples were stored at -20°C until assayed.

3.2.3 LAC Virus Antibody Assays

Each serum sample was tested for neutralizing antibodies to LAC virus using a plaque reduction neutralization test (PRNT). This method was chosen over hemagglutination inhibition (HI) and complement fixation (CF) tests because of its sensitivity and simplicity (Lindsey et al. 1976). Briefly, dog sera were first inactivated at 56°C for 30 minutes. Sera were diluted 1:5 in diluent (Medium 199 containing Earle's balanced salt solution, 5% fetal bovine serum, penicillin (20,000 units/ml)/ streptomycin (20,000 ug/ml), amphotericin B (250 ug/ml), and gentamycin

(50 ug /ml)). Approximately 100–200 plaque-forming units (PFU) of a Virginia isolate of LAC virus in a 0.2 ml volume was mixed with an equal volume of serum dilution and incubated at 37°C for 1 hour. The incubated mixture was applied in 0.2 ml amounts to Vero (African Green Monkey kidney) cell monolayers attached to 6-well plates. A methyl cellulose overlay was applied to the wells after an additional 1 hour incubation at 37°C.

Following a 5-day incubation period at 34°C, the plates were fixed and stained by crystal violet in buffered formalin (0.5g crystal violet / 500ml buffered formalin). Positive controls consisted of both mouse hyper immune ascitic fluid (CDC, Atlanta GA) and LAC virus antibody positive canine serum, while LAC virus antibody negative serum and LAC virus in M-199 diluent were used as negative controls. A reduction in the number of plaques by ≥80%, compared with negative controls, was considered positive. Antibody titers of positive sera were determined by PRNT using 2-fold dilutions of serum against LAC virus to an endpoint of the highest dilution of serum that inhibited at least 80% of plaques. The reciprocal of the final virus-serum dilution was recorded as the antibody titer.

3.2.4 Additional Vector-Borne Disease Testing

Canine sera were screened for additional vector-borne diseases using the Idexx ® Snap 4Dx ® test. The Idexx ® Snap 4Dx ® test is an enzyme-linked immunosorbent assay (ELISA) based in-vitro diagnostic device that screens for antibodies to *E. canis*, *B. burgdorferi* (Lyme disease) and *A. phagocytophilum*, as well as antigens of *D. immitis* (heartworm) in canine sera, plasma, or whole blood. The test results were interpreted 8 minutes after mixing a canine serum sample with a provided enzyme-antigen conjugate and applying it to the Idexx ® Snap 4Dx ® test device.

The Idexx ® Snap 4Dx ® test has a 99% sensitivity rate for *E. canis* and *A. phagocytophilum*, and a 96% sensitivity for *B. burgdorferi*. Specificity rates are 100% for all four of these vector-borne disease organisms (Idexx Laboratories, 2009). The detection of *B. burgdorferi* targeted antibodies to the C6 peptide, while the detection of *A. phagocytophilum* and *E. canis* used synthetic peptides for the detection of IgM and IgG antibodies. The C6 peptide used for the detection of *B. burgdorferi* is not cross-reactive with antibodies induced by vaccination with the outer surface protein A or with a whole fixed spirochete vaccine (Liang et al. 2000).

In total, 412 of the 436 collected canine serum samples were screened for additional vector-borne diseases using the Idexx ® Snap 4Dx ® test. Serum volumes in a few of the samples were inadequate and thus were omitted from this portion of the study.

3.2.5 Mapping and Data Analyses

The addresses provided by the owners of participating dogs were geocoded using GPS Visualizer (AdamSchneider.net ©2003-2009) to obtain geographic coordinates of the primary residence for each dog. The coordinates, along with additional attributed data for each dog (i.e. breed, age, sex, and seropositivity for different vector-borne diseases) were imported into the GIS and remote sensing software, TNTMips (Microimages Inc.). A map was generated to include the locations of all dogs from which serum was extracted, LAC virus antibody positive dogs, and dogs that were positive for antibody to *B. burgdorferi*.

Canine LAC virus seroprevalence between study areas were compared using either a Chi-square or Fisher's exact test in Graph Pad Prism (San Diego CA, 2007). Identical analyses were also used to compare canine seroprevalence for *B. burgdorferi* between the study areas. Fisher's

exact tests were used to compare ratios of male to female seropositive dogs for both LAC virus and *B. burgdorferi*. A P-value of ≤ 0.05 was deemed significant for all analyses.

3.3 Results

3.3.1 LAC Virus Antibody Assays

Of the 436 sera samples collected from dogs, 21 (4.8%) were positive for LAC virus antibodies. In addition, LAC virus antibodies were detected in dogs from each study area (Fig 3.1). Although the PRNTs showed no significant differences in seroprevalence among areas ($\chi^2 = 5.759$, df = 2, P = 0.0562), the highest percentage of LAC virus antibody positive dogs were detected from the NRV study area (57.1%), followed by Roanoke (28.6%), and then Wise (14.3%). Antibody titers of positive dogs ranged from 1:5 to 1:320 (Table 3.1).

Ages of dogs positive for LAC virus antibody varied from 1–16 years, with seroprevalence rates for each age class measuring approximately 5–8% (Table 3.2). All dogs ≤1 year old tested negative for LAC virus antibodies. Antibodies for LAC virus were found in all but one group (toy group) of American Kennel Club (AKC) breeds, which include sporting, hound, working, terrier, non-sporting and herding groups (Fig. 3.2). Several mixed breed dogs also tested positive for LAC virus antibodies. There were slightly more LAC virus antibody positive male dogs than female dogs, however the difference was not significant ($\chi^2 = 1.90$, df = 1, P = 0.1679). All positive dogs with the exception of 1 were housed indoors. Of these indoor dogs, 9 spent less than two hours outside each day, 8 spent less than 10, and 3 spent less than 24 hours outside each day. Also, 13 of the 21 dogs that tested positive for LAC virus did not have a previous travel history outside of the southwest Virginia area. The dogs with a travel history outside of southwest Virginia generally traveled to states bordering Virginia.

3.3.2 Idexx ® Snap 4Dx ® Tests

Seventeen (4.1%) of the dogs tested were found to be positive for *B. burgdorferi* antibodies. In the 2005/2006 study period, 8 out of 255 dogs (3.1%) tested positive for *B. burgdorferi* antibodies, while 9 out of 157 dogs (5.7%) tested positive in 2008 (Table 3.3). In both years, *B. burgdorferi* antibodies were detected from dogs in the NRV and Roanoke area, but not from the Wise area. Although canine seroprevalence was higher in the NRV than Roanoke, the difference between these areas was not significant (P = 0.1253).

The ages of *B. burgdorferi* antibody positive dogs were evenly distributed among all age classes, ranging between 3–5% for each class (Fig. 3.3). The ratio of positive males to positive females was 9:8, which was not significantly different ($\chi^2 = 0.230$, df = 1, P = 0.6315). Also, 13 of the 17 *B. burgdorferi* antibody positive dogs did not have a travel history prior to this study. The 4 positive dogs with a travel history outside of Virginia were all from the first study period in 2005/2006. The dogs with a travel history outside of southwest Virginia generally traveled to states bordering Virginia.

Ehrlichia canis antibodies were detected in 1 dog in Wise and 3 dogs in Roanoke in the 2005/2006 study period. There were no *E. canis* antibodies detected in any dog in 2008. In addition, 3 out of the 4 *E. canis* antibody positive dogs had no prior travel history.

Anaplasma phagocytophilum antibodies were detected in 1 dog in the NRV in 2005/2006, and 1 dog in Roanoke in 2008. The *A. phagocytophilum* antibody positive dog from Roanoke was also positive for *B. burgdorferi* antibodies. Also, this dog had no prior travel history outside of southwest Virginia. *Anaplasma phagocytophilum* was not detected in any dog from the Wise area during both study periods.

Overall, the majority of dogs that tested positive for antibodies to any vector-borne disease were housed indoors (Fig. 3.4). Also, no gender difference was found in the number of dogs with antibodies to the vector-borne diseases (Fig. 3.4). Lastly, all of the AKC breed groups were represented in this study, in addition to mixed breeds. Of these groups, the sporting and herding groups showed the highest proportion of dogs positive for vector-borne disease (Fig. 3.2).

3.4 Discussion

LAC virus seroprevalence among dogs was evident in each study region, including areas where LAC virus human cases and LAC virus positive mosquito isolates have not been reported. As a result, this study provided documentation of horizontal transmission of LAC virus throughout southwest Virginia, demonstrating that dogs make useful sentinels for LAC virus transmission in an area. A study in Indiana also demonstrated a widespread occurrence of LAC virus in an area with no reports of prior cases (Grimstad et al. 1984). The authors found a 2.3% LAC virus seroprevalence rate after testing 10,208 Indiana residents (0.2% of the state's population). Our finding of geographically widespread infection of LAC virus reiterates the fact that LAC virus infections are highly underreported.

Our LAC virus canine seroprevalence for southwest Virginia was 4.8%. Because dogs are closely associated with humans, we can make inferences about human risk for LAC virus based on this data. Previous seroprevalence studies in LAC virus endemic areas have shown human seroprevalence rates of 2.3% (Grimstad et al. 1984) to 9.6% (Szumlas et al. 1996b), and have noted that numerous inapparent infections occur for every apparent infection of LAC virus.

Therefore the LAC canine seroprevalence found in this study suggests a significant risk potential for human exposure to LAC virus in southwest Virginia.

The low prevalence of LAC positive dogs in the Wise study area may be due in part to the methodology used to sample dogs in this study. In fact, LAC virus antibody seroprevalence was likely underestimated in dogs from all of the study regions. Serum samples were convenience samples, because they were collected only from dogs brought to veterinary clinics for medical care, thus limiting this study to a subset of the true dog population. Relying on owners to bring their dogs in to veterinary clinics reduces the pool of possible dogs if owners are not seeking veterinary care for their animals. Increasing the pool of participating dogs could have been accomplished by including dogs from local animal shelters. However, this would have limited the collection of vital information, such as travel history and the animal's age, which could potentially skew our interpretation of the results.

The low LAC virus seroprevalence rates in Wise could also be due to its rural location, with many residences in remote areas. Travel to the veterinary clinic from remote residences can be inconvenient for owners, thereby limiting the amount of participating dogs. Also, rural areas are more permissive for keeping larger numbers of dogs, particularly in outdoor locations, thereby increasing the likelihood that LAC virus seroprevalence in Wise may actually be higher than we found in this study.

Antibody titers of LAC virus positive dogs ranged from 1:5 to 1:320. The majority of these antibody titers were low (i.e. 1:5 to 1:10), which is consistent with a prior study that measured canine LAC virus antibody titers following viral inoculation (Godsey et al. 1988). The authors concluded that dogs are not sufficient LAC virus amplifier hosts, however, they do develop detectable antibody responses when exposed to the pathogen. The detection of LAC

virus antibodies at low titers in the present study supports the conclusions of Godsey et al. (1988). However, one of the antibody titers in this study, 1:320, is higher than Godsey et al. (1988) reported for dogs. This may be due to the infection route, as host responses can vary depending on whether the infection was transmitted naturally by a mosquito vector, or artificially by inoculation. For example, Osorio et al. (1996) found that chipmunks exposed to LAC virus infected mosquitoes had significantly higher and longer viremias than the ones produced with artificial exposure by injection. In the present study, it is assumed that dogs were naturally infected from LAC virus mosquito vectors, in contrast to the previous study by Godsey et al. (1988) in which dogs were artificially infected with LAC virus. Also, LAC virus antibody titers have been shown to peak in chipmunks around 21 days post infection (Pantuwatana et al. 1972), but perhaps LAC virus antibody titers take longer to peak in dogs. Godsey et al. (1988) measured LAC virus antibody titers between days 21–32 post-infection. It is unknown how many days post LAC virus infection the dogs in our study were tested.

Because 20 of the 21 LAC virus antibody positive dogs were housed indoors, time spent outdoors was not predictive of exposure. However, since indoor dogs more closely parallel human lifestyle than outdoor dogs, indoor dogs provide a more realistic representation of human LAC virus exposure risk. Therefore, this finding in indoor dogs serves to reinforce the effectiveness of dogs as LAC virus sentinels.

A total of 17 dogs (4.1%) tested positive for *B. burgdorferi* antibodies in southwest Virginia. This high number of positives was unexpected, considering the infrequent reports of human Lyme cases in this region of the state. According to the Virginia Department of Health, human cases of Lyme disease occur at higher rates in the northern portion of the state, and in 2006, reported 255 human cases from northern Virginia and 17 cases from southwest Virginia at

rates of 12.6% and 1.3% (per 100,000), respectively (Va. Dept. of Health 2008). In addition, the Virginia Department of Health reports that the incidence of human Lyme disease is almost 7 times greater in the northern region than all other regions of the state combined. Based on prior studies, Virginia appears to represent a transitional zone between higher and lower levels of *B. burgdorferi* exposure (Duncan et al. 2004).

The reasons for the differences in levels of *B. burgdorferi* exposure are partially due to a greater availability of hosts to tick vectors. *Ixodes scapularis* commonly feed on the white-footed mouse (*Peromyscus leucopus*), the primary *B. burgdorferi* reservoir host, in northern portions of the U.S. where this host occurs in relatively higher abundance. In southern portions of the U.S., *I. scapularis* are also known to parasitize several species of lizards (Apperson et al. 1993), most of which are incompetent *B. burgdorferi* reservoirs. Feeding on incompetent *B. burgdorferi* reservoirs will disrupt enzootic cycles of Lyme transmission. Evidence for disruption of the enzootic Lyme cycle by lizards has been recorded as far north as southern Maryland (Swanson and Norris 2007), and it is likely that varying populations of lizards and white-footed mice between northern and southern portions of Virginia also has an effect on regional enzootic Lyme cycles. Additionally, Duncan et al. (2004) suggests that a lower percentage of infected ticks and hosts may exist in the southeastern U.S regions, relative to the northeast. Further investigation of *I. scapularis* host populations in Virginia is needed to understand regional differences in Lyme disease prevalence.

The *B. burgdorferi* seroprevalence found among dogs in southwest Virginia was limited to the NRV and Roanoke areas, which are predominantly suburban and urban landscapes, interspersed with small fragments of forest. Risk of Lyme disease is dependent on contact with infected ticks, and previous studies have shown a connection between landscape and Lyme

disease risk (Frank et al. 1998, Ostfeld and Keesing 2000, Allan et al. 2003, Brownstein et al. 2005). Suburbanization results in fragmentation of forested habitat, which creates small patches of forest among residential areas. These small patches of forest provide ideal habitat for known host species of *I. scapularis*, including white-footed mice and white-tailed deer (Robinson et al. 1992, Nupp and Swihart 1998) and as such, are associated with increased densities of *I. scapularis* ticks. High densities of larval *I. scapularis* ticks are largely determined by distributions of white-tailed deer, as this is the primary host and mating location of adult *I. scapularis*. Larval *I. scapularis* prefer to feed on small mammals such as white–footed deer mice, where they are highly likely to become infected with *B. burgdorferi* (Frank et al. 1998, Ostfeld and Keesing 2000). Allan et al. (2003) found a high density of *I. scapularis* and an increased prevalence of *B. burgdorferi* among these ticks in small forest patches. This means there could be many ticks infected with *B. burgdorferi* that are concentrated within small forest patches. These small forest patches are surrounded by suburban residences, which put people in close contact with infected ticks. Therefore our findings of *B. burgdorferi* infection in the suburban and urban landscapes of the NRV and Roanoke, and absence of infection in Wise (a predominantly forested area) could be the result of landscape and subsequent tick exposure.

Canine *B. burgdorferi* seroprevalence increased in both the NRV and Roanoke from 2005/2006 to 2008. The Virginia Department of Health also noted an increase in the number of human Lyme cases, reporting that the number of cases in 2006 were 61% higher than the five year average. The reason for the increase in Lyme prevalence may be the increasing suburbanization of some parts of Virginia, which may create ideal environments for white-footed mice and white-tailed deer. Alternatively, the increasing awareness of Lyme disease by the

public and healthcare professionals may also contribute to the growing numbers of reported cases.

Based on the *B. burgdorferi* seroprevalence found in this study, we conclude that dogs make effective sentinels for *Borrelia* infection. Antibodies to *B. burgdorferi* were detected in two of our three study regions, both of which have low rates of reported human Lyme disease cases. Future studies are needed to assess distributions and seroprevalence of *I. scapularis*, as well as host populations in southwest Virginia.

Very few cases of *A. phagocytophilum* and *E. canis* were detected in the dogs that were sampled. Therefore, it is difficult to determine the effectiveness of canine sentinels for detection of these pathogens. A previous study determined that white-tailed deer are good sentinels for *A. phagocytophilum* (Dugan et al. 2006); however, ticks were collected from deer to determine vector distribution. Little has been published regarding the seroprevalence of *A. phagocytophilum* and *E. canis* in Virginia, and an evaluation of seroprevalence among vectors and reservoir hosts for these pathogens, as well as geographic distributions of vectors may be warranted in order to gain a better understanding of disease distribution.

Both *A. phagocytophilum* and *B. burgdorferi* were detected from the same dog in Roanoke in 2008. Dual infection with *A. phagocytophilum* and *B. burgdorferi* is likely because these pathogens share the same tick vector and reservoir hosts. Co-infections with *A. phagocytophilum* and *B. burgdorferi* in both tick vectors and in dogs have been reported in other studies (Levin and Fish 2000, Holden et al. 2003, Beall et al. 2008). Furthermore, *A. phagocytophilum* and *B. burgdorferi* appear to share the same regional distributions (Chapman et al. 2006).

Data for all participating dogs in this study included attributes such as sex and breed. Ratios of male to female dogs for all of the vector-borne diseases investigated in this study were almost equal. Based on this, we can conclude that sex has no effect on the likelihood of pathogen acquisition. Although vector-borne disease was found in a variety of AKC groups, it was most common among breeds in the sporting and herding groups (Fig. 3.2). A recent canine serosurvey for *Trypanosoma cruzi* in Texas also found the highest disease prevalence among the sporting breeds, and suggests that the high disease prevalence in this group is more likely due to lifestyle factors (i.e. time spent outdoors) or popularity of breed ownership rather than predilection for the pathogens (Kjos et al. 2008).

Because seroprevalence for LAC virus was apparent throughout southwest Virginia, using dogs to determine the distribution of LAC virus can be considered a success. Furthermore, LAC virus was detected in locations where there were no previous reports of human cases or mosquito isolates, indicating widespread horizontal transmission of LAC virus in southwest Virginia. Based on the *B. burgdorferi* serosurvey, dogs are also useful sentinels for Lyme disease. The reported number of human *B. burgdorferi* seroprevalence is low for southwest Virginia, yet we were able to detect *B. burgdorferi* in over 5% of our samples. Additionally, we observed an increase in *B. burgdorferi* seroprevalence between study periods. The Virginia Department of Health also reports an increase in human disease Lyme cases during this same time period. Further investigation of vectors and reservoir hosts associated with *A. phagocytophilum* and *E. canis* are warranted in order to conclude the effectiveness of dogs as sentinels.

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Tables and Figures

Table 3.1: Titers of LAC virus positive canine serum samples from plaque-reduction neutralization (PRNT) assays. Titers are expressed as the highest serum dilution that neutralized ≥80% of plaques.

Location of Veterinary Clinic	AGE of DOG (years)	LACV PRNT TITER	Location of Veterinary Clinic	AGE of DOG (years)	LACV PRNT TITER
NRV	2	5	Wise	12	5
	3	5		5	5
	7	5		1	320
	9	5			
	10	5			
	11	5			
	3	10	Roanoke	1	5
	5	10		6	5
	10	10		6	5
	10	20		11	5
	3	160		16	10
	6	160		9	40

Table 3:2: Prevalence of LAC virus antibodies among dogs of different age classes in the three study areas.

Age (years)	Number of Dogs and Seropositives (%)							
	NRV		Roanoke		Wise		Total	
	No.	Seropositive (%)	No.	Seropositive (%)	No.	Seropositive (%)	No.	Seropositive (%)
<1	15	0 (0.0)	10	0 (0.0)	1	0 (0.0)	26	0 (0.0)
1-3	54	4 (7.4)	42	1 (2.4)	28	1 (3.6)	124	6 (4.8)
4-6	33	2 (6.1)	51	2 (3.9)	16	1 (6.2)	100	5 (5.0)
7-10	23	5 (21.7)	55	1 (1.8)	43	0 (0.0)	121	6 (5.0)
>10	15	1 (6.7)	20	2(10.0)	16	1 (6.2)	51	4 (7.8)

Table 3.3: Serology results obtained from Idexx ® Snap 4Dx ® tests conducted on dog serum samples collected from three study areas in southwest Virginia.

	Borrelia burgdorferi		***Ehrlichia canis***		***Anaplasma phagocytophilum***	
	2005/2006	**2008**	**2005/2006**	**2008**	**2005/2006**	**2008**
Wise* No. of seroreactive samples / total no. of samples (%)	0 / 79 (0.00)	0 / 48 (0.00)	1 / 79 (1.26)	0 / 48 (0.00)	0 / 79 (0.00)	0 / 48 (0.00)
NRV* No. of seroreactive samples / total no. of samples (%)	7 / 115 (6.09)	7 / 65 (10.77)	0 / 115 (0.00)	0 / 65 (0.00)	1 / 115 (0.87)	0 / 65 (0.00)
Roanoke* No. of seroreactive samples / total no. of samples (%)	1 / 61 (1.64)	2 / 44 (4.54)	3 / 61 (4.92)	0 / 44 (0.00)	0 / 61 (0.00)	1 / 44 (2.27)
Overall No. of seroreactive samples / total no. of samples (%)	**8 / 255 (3.14)**	**9 / 157 (5.73)**	**4 / 255 (1.57)**	**0 / 157 (0.00)**	**1 / 255 (0.39)**	**1 / 157 (0.64)**
Total for combined sampling periods	**17 / 412 (4.13)**		**4 / 412 (0.97)**		**2 / 412 (0.48)**	

*** Indicates location of veterinary clinic where samples were collected**

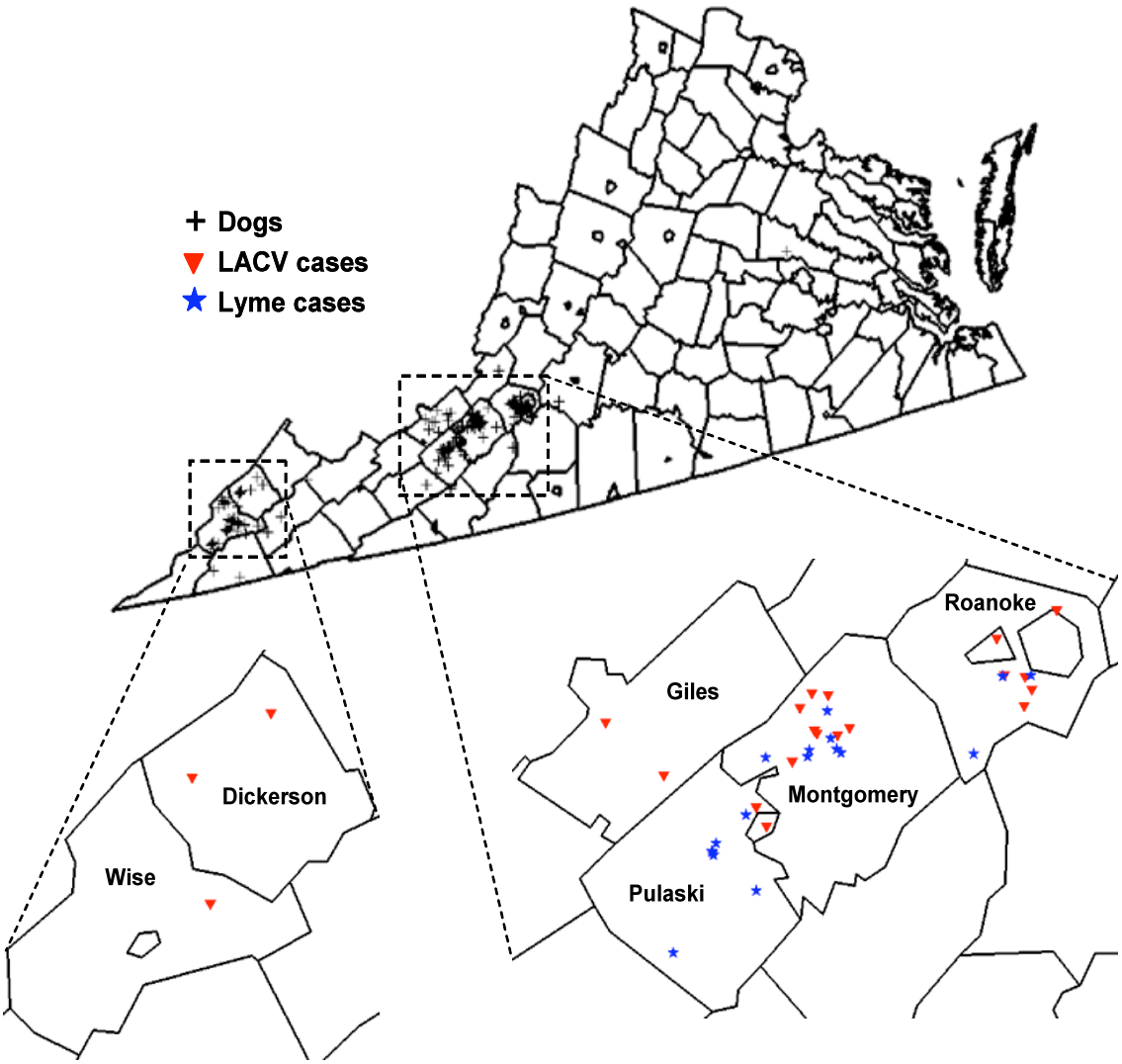

Fig. 3.1: Residential locations of dogs in Virginia from which sera samples were collected for testing for vector–borne infectious diseases. Areas and locations at which sera from dogs that tested positive for antibodies to La Crosse (LAC) virus or Lyme disease are shown in the enlarged area maps.

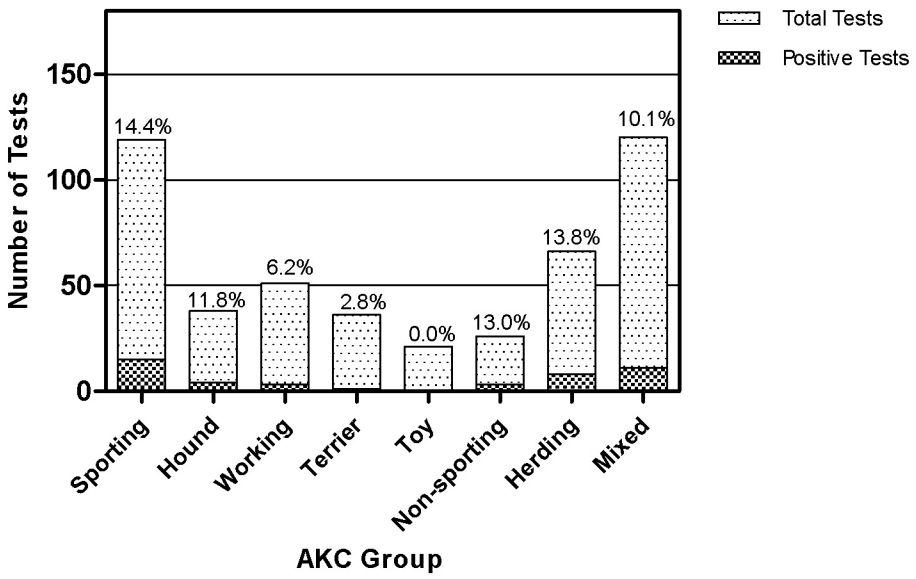

Fig. 3.2: AKC groups of dogs residing in southwestern Virginia that were tested for vector-borne diseases from 2005-2008. Percentage of positive tests is displayed for each breed.

Fig. 3.3: Percentage of dogs positive for *B. burgdorferi* antibodies by age class

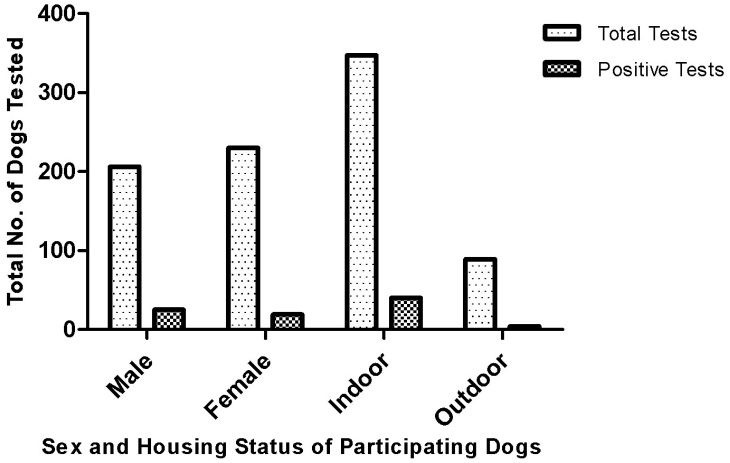

Fig. 3.4: Participating dogs grouped by sex and housing status. Numbers of positive tests are shown next to the total number of tests for that group.

4. Concurrent Infection of *Aedes* Mosquitoes by La Crosse Virus and Dog Heartworm (*Dirofilaria immitis*)

4.1 Introduction

When a mosquito ingests a bloodmeal dually infected with an arbovirus and filarial nematodes, enhanced transmission of the arbovirus can occur (Mellor and Boorman 1980, Turell et al. 1984, Turell et al. 1987, Zytoon et al. 1993a,b, Vaughan and Turell 1996, Vaughan et al. 1999). Dual infection of an arbovirus and larval filarial nematodes (microfilariae) can augment the infectivity of the virus to mosquito vectors, resulting in increased rates of arboviral infection, dissemination, and transmission. Enhanced transmission is accomplished through compromise of the mosquito's midgut barriers by microfilariae. The mosquito's midgut barriers consist of the mesenteronal infection (MI) and mesenteronal escape (ME) barriers. The MI barrier includes cell surface charge, digestive enzymes, and receptor-ligand incompatibility, while the ME is dose dependent, preventing virus from entering the hemocoel and disseminating (Chamberlain and Sudia 1961, Hardy 1983). The midgut barriers are normally overcome when a virus infects and replicates in the cells of the midgut epithelium. However when a mosquito ingests microfilariae, the microfilariae will puncture the mosquito's midgut epithelium to enter the hemocoel, creating holes through which a concurrently ingested virus could escape (Mellor and Boorman 1980, Turell et al. 1984, Zytoon et al. 1993a). Direct escape into the hemocoel by a virus bypasses the need for the virus to infect and replicate in the mosquito midgut epithelium, and is the basic mechanism of microfilaria enhancement.

Microfilaria enhanced transmission can affect arbovirus epidemiology in several ways, including shortened extrinsic incubation periods, lowered infection thresholds, and increasing vector competence. The extrinsic incubation (EI) period is the amount of time between the ingestion of an infective bloodmeal and the time when the virus can be transmitted orally while

refeeding. Shortened EI periods could significantly impact the epidemiology of the arbovirus because prolonged vector survival times or higher vector population densities would not be required to maintain the arbovirus in locations endemic to the reservoir hosts (Hardy et al. 1983, Vaughan et al. 1999). A lowered infection threshold, another potential impact of microfilaria enhanced transmission, could mean that less virus is required to establish an infection in the mosquito as a direct result of virus escaping into the hemocoel. Lowered infection thresholds create the potential for a mosquito to develop infections feeding on reservoir hosts with lower viremias. Lastly, microfilaria enhanced transmission can create vector competence from otherwise incompetent vectors. The existence of midgut barriers can prevent a virus from establishing a successful infection inside a mosquito (Chamberlain and Sudia 1961). The compromise of the midgut barrier therefore opens up the possibility for successful establishment of viral infections in a mosquito vector not normally capable of harboring that virus. As a result, vectorial capacity for that virus increases for the mosquito vector.

La Crosse (LAC) virus, an arbovirus in the California serogroup of Bunyaviruses, is the most prevalent arboviral infection in children in North America (McJunkin et al. 2001). LAC virus is transmitted by several mosquitoes, including the primary vector, *Aedes triseriatus*, and accessory vector *Ae. albopictus*. According to the CDC, LAC encephalitis has been reported from 27 states (CDC 2007), at a rate of about 70 cases per year. Historically, most cases have been reported from midwestern states, particularly Wisconsin, Ohio, and Minnesota (Calisher 1983). However, in the past decade, an increase in LAC encephalitis cases have been reported from mid-Atlantic and southeastern states, such as West Virginia, Virginia, North Carolina, and Tennessee (Nasci et al. 2000, Gerhardt et al. 2001, Barker et al. 2003a,b).

Dirofilaria immitis is a mosquito vectored-nematode that causes canine and feline heartworm disease. Dogs serve as primary reservoirs for *D. immitis*, and mosquitoes are the only known intermediate host (Knight 1977). *Dirofilaria immitis* has been reported in more than 60 species of mosquitoes, and has been found in all 50 states in North America, with the highest prevalence in the southeastern states and Gulf Coast regions (Otto 1969, Knight 1977, Haddock 1987, AHS 2005).

The geographic range of *D. immitis* overlaps with LAC virus throughout the eastern U.S., including several mid-Atlantic and southern states where there is a high prevalence of *D. immitis*. In addition, the LAC virus mosquito vectors *Ae. triseriatus*, and *Ae. albopictus* have been shown to be competent *D. immitis* vectors (Intermill 1973, Apperson et al. 1989, Nayar and Knight 1999). Furthermore, the competent *D. immitis* vector, *Ae. aegypti* can also be found within this geographic range, and is susceptible to LAC virus infection in laboratory settings (Hendrix et al. 1986, Apperson et al. 1989, Hughes et al. 2006, Tiawsirisup and Nithiuthai 2006). These mosquitoes feed on both humans and mammals, including dogs (Szumlas et al. 1996, Richards et al. 2006).

Because dogs are the primary reservoir for *D. immitis*, coinfections with both LAC virus and *D. immitis* are possible in mosquito vectors, particularly in LAC virus and *D. immitis* endemic areas where mosquitoes can feed on LAC virus reservoir hosts (small mammals) and dogs. Furthermore, *Aedes triseriatus* and *Ae. albopictus*, have shown an affinity for canine hosts. In fact, blood meal analyses studies in North Carolina demonstrated that *Aedes triseriatus* fed predominantly on dogs, and that dogs are a common mammalian host for *Aedes albopictus* (Szumlas et al. 1996, Richards et al. 2006). While dogs do not develop LAC virus infections (unless they are less than 12 weeks of age), multiple feedings by a mosquito could result in

acquisition of *D. immitis* from dogs, and LAC virus from reservoir hosts. Therefore, the coexistence of LAC virus, *D. immitis*, and mosquito vectors in the mid-Atlantic and southern U.S. states suggests a potential for the occurrence of microfilaria enhanced transmission.

Microfilaria enhanced transmission of an arbovirus by *D. immitis* has been shown in prior studies (Zytoon et al. 1993a,b). Zytoon et al. (1993a,b) demonstrated enhanced dissemination and transovarial transmission of Chikungunya (CHIK) virus in *Ae. albopictus* after ingestion of a bloodmeal dually infected with CHIK virus and *D. immitis*. Furthermore, penetration of the mosquito midgut by *D. immitis* was shown using histopathology and electron microscopy, with micrographs exhibiting complete midgut penetration by *D. immitis* within 4 – 6 hours post ingestion (Zytoon et al. 1993a).

The evidence of midgut penetration by *D. immitis* shown by Zytoon et al. (1993a) provides justification for the investigation of mosquito vectors with known midgut escape barriers to LAC virus. Paulson et al. (1989) reported a distinct ME barrier in *Ae. triseriatus* after finding a significant proportion of mosquitoes with LAC virus infected midguts that did not develop disseminated infections. Similarly, Hughes et al. (2006) showed LAC virus dissemination rates of *Ae. albopictus* and *Ae. aegypti* to be approximately half the value of the midgut infection rates, also indicating ME barriers for these species.

In this study, we assess the effects of coinfection with *D. immitis* and LAC virus in *Ae. triseriatus*, *Ae. albopictus and Ae. aegypti.* We evaluate mosquitoes for microfilaria enhanced LAC virus transmission by measuring percentages of infection, dissemination, and transmission, as well as extrinsic incubation periods and lowered virus infection thresholds.

4.2 Materials and Methods

4.2.1 Mosquitoes

The mosquitoes were reared from eggs collected in Montgomery Co. Virginia, with the exception of *Ae. aegypti*, which was obtained from the Department of Biological Sciences at the University of Notre Dame (KHW strain). Eggs were hatched and reared according to the methods described by Munstermann and Wasmuth (1985) under the following conditions: 24°C, 75% RH, and 16:8 (L:D) photoperiod. Adult mosquitoes were maintained on 10% sucrose solution on moistened cotton balls and apple slices.

4.2.2 Microfilariae

Canine blood infected with *D. immitis* microfilariae was provided by the NIH / NIAID Filariasis Research Reagent Repository Center at the University of Georgia, College of Veterinary Medicine (Athens, GA). Prior to each experiment, microfilariae concentrations were determined by counting five wet smears of 1 µl of blood each at 100X magnification. The numbers of microfilariae were averaged to determine the final concentration of microfilariae per ml (MF / ml).

4.2.3 Virus and Virus Assays

The VA0921075 strain of LAC virus was isolated from *Aedes triseriatus* collected from Wise County, Virginia in 1999. This isolate was passaged twice in Vero cells before its use in these studies. Mosquito abdomens were tested for virus infection, wings and legs for virus dissemination, and salivary expectorate was collected in capillary tubes to test for oral virus transmission. All abdomens, wings and legs, and portions of capillary tubes containing salivary expectorate were placed in Eppendorf tubes containing 1ml of M-199 cell culture medium, and prepared for assay according to the methods described by Nasci et al. (2000). Prepared

specimens were tested for virus by plaque assay on Vero Cells according to the methods described by Barker et al. (2003b). Salivary expectorate was inoculated onto Vero cells in 12-well plates and incubated for 4 days at 34°C to allow for virus amplification. A sample of the liquid culture medium was then tested for virus according to the methods described by Barker et al. (2003b).

4.2.4 Mosquito Co-infection

Five to six day old mosquitoes were starved for three days prior to feeding. Infectious blood meals were delivered in a water-jacketed artificial membrane feeder (Rutledge et al. 1964) with stretched parafilm used as a membrane (Failloux et al. 1991). Suspensions of microfilariae and LAC virus were prepared in canine blood treated with EDTA (Bioreclamation, Liverpool, NY). Mosquitoes were allowed to feed for ~ 1 hour. During this time, three freshly engorged mosquitoes from the experimental group were removed and their midguts were examined in order to confirm ingestion of microfilaria. After the feeding, engorged mosquitoes were removed and placed into a separate cage. These mosquitoes were maintained on a diet of 10% sucrose, and housed in the insectary at 24°C, 75% RH, and a 16:8 (L:D) photoperiod.

Mosquitoes were evaluated for virus infection, dissemination, and transmission at designated days post infection (P.I.). Infection is confirmed by the detection of LAC virus in the mosquito midgut, while dissemination is presumed when LAC virus is detected in the mosquito's wings and legs. The presence of LAC virus in the salivary expectorate confirms the mosquito's ability for virus transmission. The mosquitoes were anesthetized in the 20°C freezer for 4 minutes, and then placed on ice. Wings and legs were removed and placed in Eppendorf tubes for virus dissemination assays. Each mosquito was then suspended from a capillary tube by its

proboscis, and saliva was collected using the methods of Boromisa and Grayson (1991). Upon completion of saliva collection, mosquito abdomens, and capillary tubes were placed in Eppendorf tubes for respective virus infection and transmission assays, respectively.

4.2.5 Microfilariae in Mosquito Midguts

Aedes triseriatus, *Ae. albopictus* and *Ae. aegypti* were each divided into three groups and fed three *D. immitis* microfilaremic bloodmeals containing doses of 2000, 4000, and 8000 MF / ml. During the feeding, at least 15 mosquitoes were removed from each group, aspirated into 70% ethanol, and transferred to chilled saline. Of these mosquitoes, half were used to determine the numbers of ingested microfilariae, and the remaining mosquitoes were used to quantify midgut penetration by microfilariae. To determine numbers of ingested microfilariae, replete midguts were removed, squashed on a glass slide, and examined at 100X magnification with a binocular compound microscope. To measure the penetration by microfilariae, replete midguts were carefully excised intact, placed individually into wells of a 12-well plastic tissue culture plate containing Medium 199 (with Earle's balanced salt solution, 5% fetal bovine serum, and antibiotics) (Vaughan and Turell 1996, Vaughan et al. 1999), and stored in the insectary (at 24°C, 75% RH). After 8 – 10 hours, the penetrating microfilariae in each well were visualized and counted at 100X magnification using an inverted phase contrast microscope (Bausch and Lomb Photozoom, Buffalo, NY).

4.2.6 Mosquito Mortality

All engorged mosquitoes remaining from the latter experiment were grouped by species and microfilaria dose, and maintained in the insectary for 14 days on a 10% sucrose diet. After

this time, numbers of live and dead mosquitoes were counted to determine the percentages of mortality for each group.

4.2.7 Data Analyses

Percentages of viral infection, dissemination, and transmission were compared between groups of mosquitoes using either a Chi-square or Fisher's exact test analyses in Graph Pad Prism (San Diego CA, 2007). All microfilaria data were transformed to $\log_{10}$ before analysis in order to equalize their variance-to-mean ratios. Numbers of ingested microfilariae and penetrated microfilariae were compared using a one-way analysis of variance (ANOVA), and significant results were further analyzed by comparing individual groups using t-tests with Welsh's correction. Identical analyses were also used to compare mean numbers of ingested microfilariae and penetrated microfilariae between species at each dose (i.e. 2000, 4000 and 8000 MF /ml). Log transformed numbers of microfilariae were back transformed in order to report means and confidence intervals.

Mosquito mortality was compared between species for each dose using a Fisher's exact analysis. Mortality rates were computed as percentages and the results were graphed using Graph Pad Prism. A P-value of ≤ 0.05 was indicated significance throughout the study.

4.3 Results

4.3.1 Effect of concurrent infection on dissemination and transmission of LAC virus

No significant differences in the percentages of viral infection, dissemination, and transmission were found between the co-infected and LAC virus alone groups for any species. The percent dissemination and transmission for *Ae. triseriatus* on day 15 P.I. (Table 4.1) are

slightly higher in the co-infected group, although not significantly different from the LAC virus alone group. *Aedes albopictus* also had a higher percentage of LAC virus transmission (Table 4.1) in the co-infected group, although again, not significantly different from the group infected with LAC alone.

Although slightly higher dissemination and transmission percentages were detected on day 9 in the co-infected group for *Ae. triseriatus*, the differences were not significant from the LAC virus alone group (Table 4.2). Also LAC virus was only detected in the saliva of *Ae. triseriatus* in the co-infected group on day 9 in contrast to the LAC virus alone group where LAC virus was not detected in the saliva of any mosquito. However, overall differences between the co-infected and LAC virus alone groups were not significant. *Aedes aegypti* demonstrated no significant differences in percentages of viral infection, dissemination, and transmission between the co-infected and LAC virus alone groups (Table 4.2). The co-infected group shows only a very slight increase in percentages of infection, dissemination, and transmission compared to the LAC alone group (Table 4.2).

No significant differences in percentages of viral infection, dissemination, and transmission were found between the groups of *Aedes albopictus* with ten-fold decreased LAC virus doses and microfilariae, and decreased LAC virus doses alone (Table 4.1). Percentages of infection and dissemination were slightly higher in the co-infected group, though these were low in general for both groups. LAC virus transmission was not detected in either group.

4.3.2 Microfilariae in Mosquito Midguts

When comparing amounts of ingested microfilariae between the species, *Ae. triseriatus* ingested the highest numbers of microfilaria while *Ae. aegypti* ingested the least amount of

microfilariae for every dose tested (Table 4.3). At the 2000 MF / ml dose, *Ae. aegypti* ingested significantly fewer microfilariae than *Ae. triseriatus* and *Ae. albopictus* ($F = 13.05$ df = 2, 19 $P = < 0.01$) . At the 4000 MF /ml dose, *Ae. aegypti* also ingested significantly less microfilariae than *Ae. triseriatus* or *Ae. albopictus* ($F = 7.110$ df = 2, 19 $P = < 0.05$). At the 8000 MF / ml dose, there were no significant differences in numbers of ingested microfilariae among any groups ($F = 2.882$ df = 2, 19 $P = 0.0836$).

Numbers of microfilariae that penetrated the midgut increased with increasing doses of microfilariae in all 3 mosquito species. However, there were no significant differences found in the mean numbers of penetrating microfilariae between any species at any dose ($F = 1.095$ df = 2, 24 $P = 0.3506$; Table 4.3).

There was a significant difference between the numbers of ingested microfilariae and the numbers of penetrated microfilariae (Figs 4.1, 4.2, & 4.3) for all groups of mosquitoes with the exception of *Ae. albopictus* and *Ae. aegypti* at a dose of 2000 MF / ml. Table 4.3 shows mean numbers of both ingested microfilariae and penetrated microfilariae for all 3 species tested at doses ranging from 2000 – 8000 MF / ml.

4.3.3 Mosquito Mortality

Percent mosquito mortality (Fig 4.4) increased for all 3 species with increasing doses of microfilariae. When comparing mortality between the species, *Aedes triseriatus* had the greatest mortality while *Ae. albopictus* had the lowest mortality, at each microfilaria dose. Table 4.4 shows the percent mortality for all 3 species at doses ranging from 2000 – 8000 MF / ml. At a dose of 2000 MF / ml, the percent mortality among these species were significantly different ($F = 17.27$ df = 2 $P = <0.001$) with *Ae. triseriatus* experiencing significantly greater mortality than

Ae. albopictus and *Ae. aegypti*. At doses of 4000 MF / ml and 8000 MF ml, both *Ae. triseriatus* and *Ae. aegypti* have significantly higher mortality than *Ae. albopictus* ((4000 MF /ml: $F = 8.878$ $df = 2$ $P = <0.05$) (8000 MF / ml: $F = 14.55$ $df = 2$ $P = <0.001$)).

4.4 Discussion

Overall, microfilaria enhanced transmission of LAC virus using *D. immitis* microfilaria was not observed in *Ae. triseriatus*, *Ae. albopictus*, and *Ae. aegypti*. There were no significant differences in the percentages of infection, dissemination or transmission of mosquitoes fed dually infected bloodmeals (i.e. *D. immitis* microfilaria and LAC virus) compared with those fed bloodmeals containing LAC virus only. In many cases, there was a slight increase in dissemination and transmission in the co-infected groups, however these increases were not significant. The failure to demonstrate enhancement of LAC virus is likely due to poor midgut penetration rates by *D. immitis*, despite using biologically significant doses of microfilariae.

Previous studies have shown that microfilarial enhancement is dose dependent, which means there must be sufficient midgut penetration by microfilariae in order for a minimum infectious dose of virus to pass into the hemocoel (Vaughan and Turell 1996, Vaughan et al. 1999). Our study quantified the *D. immitis* microfilariae that penetrated the midgut and found that the numbers were significantly lower than the numbers of ingested microfilariae. Low percentages of midgut penetration by *D. immitis* is an important finding that suggests that microfilarial enhancement of LAC virus may not occur. In other words, there may have been a lack of midgut lesions because *D. immitis* did not damage the midgut, which resulted in the subsequent failure of a minimum infectious dose of LAC virus to pass into the hemocoel.

While microfilarial enhancement of arboviral transmission has been demonstrated on a variety of virus-vector-microfilaria species combinations, few studies have used *D. immitis* in their dual infection models. Varying lifecycles of different filarid parasite species are known to have an effect on numbers of microfilariae that penetrate the midgut. For example, shortly after ingestion, the microfilariae of *Brugia pahangi* and *B. malayi* must penetrate the mosquito midgut in order to reach the indirect flight muscles in the thorax. As a result, penetration of *B. pahangi* and *B. malayi* microfilariae occur in relatively higher numbers in efficient mosquito hosts (Ewert 1965). Conversely, *D. immitis* migrate to the Malpighian tubules after ingestion, which occurs by traveling along the digestive tract after ingestion, presumably without the need for midgut penetration. However, midgut penetration has been demonstrated in *D. immitis* with the use of electron microscopy and histopathology, although penetration rates have not been quantified (Zytoon et al. 1993a).

In addition to the numbers of midgut lesions caused by microfilariae, researchers have suggested that the size of the blood meal leakage from the lesions also determines the outcome of microfilarial enhancement (Vaughan and Turell 1996, Vaughan et al. 1999). Furthermore, blood meal leakage from microfilaria-induced midgut lesions varies between mosquito species. For example, after feeding 3 *Aedes* species a bloodmeal with *B. pahangi*, Vaughan et al. (1999) describes visible blood in the hemocoel of only one of the species, despite having the same or lower microfilarial penetration rates than the other two species. Although the reasons for differences in microfilaria-induced midgut pathology among mosquito species are unknown, Vaughan and Turell (1996) speculate that differences may be due to varying blood meal sizes between mosquito species and resulting tonicity forces placed on a stretched midgut epithelium. Penetrating microfilariae may create larger rips or tears in mosquitoes with a highly stretched

midgut epithelium. Because we did not see microfilaria enhanced transmission in any of our species, but instead, observed low midgut penetration rates, we can conclude that *D. immitis* microfilariae failed to reach a "penetration threshold" for enhancement to occur. A penetration threshold can be defined as a minimum number of microfilariae needed to cause sufficient blood meal leakage or numbers of midgut lesions to allow passage of a minimum infectious virus dose.

Previous studies successfully demonstrating microfilarial enhancement have recorded higher numbers of penetrating microfilariae than we found in our study. For example, in our study, mean numbers of microfilariae penetrating the midgut of *Ae. triseriatus* ranged from 1.8 to 5.8 microfilariae (Table 4.3). Vaughan et al. (1996) documented microfilarial enhancement of Eastern Equine Encephalitis in *Ae. triseriatus* with a mean number of 34 penetrating microfilariae. Additionally, Vaughan et al. (1996) showed no microfilarial enhancement with a mean number of 12 penetrating microfilariae for these same species.

It is well established that ingesting high numbers of *D. immitis* microfilariae will have an adverse effect on the longevity of mosquitoes (Kershaw et al. 1953), presumably because of injury caused by high numbers of microfilariae in the Malpighian tubules (Webber and Hawking 1955, Apperson et al. 1989, Nayar and Knight 1999). Therefore, it is imperative to use a microfilaria dose that minimizes mosquito mortality, yet is representative of a dose obtained by a naturally infected mosquito. Walters and Lavopierre (1984) showed that low *D. immitis* microfilarial densities (< 10,000 MF / ml) are common in naturally infected dog populations. Thus, in our study we used dosages between 2000 and 4000 MF / ml. These doses represent a likely number of circulating microfilariae that a mosquito would naturally ingest and be able survive ingestion (long enough to transmit the parasite). Also, previous studies have documented high mortality rates in *Ae. triseriatus*, *Ae. albopictus* and *Ae. aegypti* exposed to

doses of *D. immitis* greater than 4000 MF / ml. For example, after documenting high mortality in several *Aedes* species, Weiner and Bradley (1970) used a microfilaria dose ranging from 1300 – 5300 MF / ml.

Because the tolerance for microfilarial burdens among mosquito species is variable, the differences in mortality rates we observed in this study were not unexpected. We experienced the highest mortality rates with *Ae. triseriatus*, regardless of the microfilaria dose. Mortality of 65% at a dose of 4000 MF / ml, prompted us to decrease the microfilaria dose to 2000 MF / ml for a second experiment, which reduced mortality by about 5%. Rodgers and Newson (1979) also experienced high mortality in *Ae. triseriatus* after feeding the mosquitoes a bloodmeal containing *D. immitis*. Mortality for *Ae. aegypti* were high as well, especially at doses ≥ 4000 MF /ml. Apperson et al. (1989) experienced similar mortality for this species, although different strains of *Ae. aegypti* vary in refractoriness to the parasite and subsequent mortality (Buxton and Mullen 1981, Apperson 1989). The KHW strain of *Ae. aegypti* used in this study appears to have a low tolerance to microfilaria burdens.

Aedes albopictus had the highest tolerance to microfilaria burdens throughout our study. *Dirofilaria immitis*–induced mortality rates for *Ae. albopictus* were not as high at doses < 4000 MF / ml relative to *Ae. triseriatus* and *Ae. aegypti*, however at 8000 MF / ml, mortality rates reached nearly 40%. Tiawsirisup and Kaewthamason (2007) report similar findings, noting that *D. immitis*–induced *Ae. albopictus* mortality becomes an issue at doses higher than 5000 MF /ml. However like *Ae. aegypti*, different strains of *Ae. albopictus* show varied susceptibility to *D. immitis* microfilaria burdens (Apperson et al. 1989, Scoles and Craig 1993, Nayar and Knight 1999, Tiawsirisup and Kaewthamason 2007).

An additional finding in this study was the variability of microfilariae ingestion among the mosquito species. Because numbers of ingested microfilariae can potentially determine the outcome of enhanced transmission, this is an important finding with respect to initial dosing in the infective bloodmeals. In our study, we found that *Ae. triseriatus* ingested significantly more microfilariae than *Ae. aegypti* regardless of being fed the same dose of microfilariae as the other species.

There have been several studies in the past which have addressed the issue of microfilariae intake (Kartman 1953, Kershaw et al.1953). Kartman (1953) describes variable microfilariae uptake between mosquito species commonly occurring due to feeding preferences shown by the mosquito. This includes feeding locations on their host (i.e. feeding directly from a capillary which contains more microfilariae versus extravasated blood from a previously lacerated capillary that contains fewer microfilariae), or the time of day in which the mosquito feeds because the time of day affects the numbers of circulating microfilariae in the host's peripheral blood vessels. Kartman (1953) noted that the effects of location on the host and time of day can be minimized by using an in vitro feeding device.

Since we used an *in vitro* feeding device for all mosquito bloodmeals, and because we still had great variability of microfilariae ingestion among species, other variables should be considered such as the size of the mosquitoes. For example, *Ae. triseriatus* is a relatively larger mosquito than *Ae. albopictus* and *Ae. aegypti*, therefore we may expect that it ingests a larger bloodmeal. One study reported average bloodmeal sizes of *Ae. triseriatus* and *Ae. albopictus* to be 5.7µl and 3.3µl respectively (Bryan Jackson, unpublished data). A study on *Ae. aegypti* reported that their bloodmeal sizes ranged from 3.5 to 5µl, depending on their size (Klowden and Lea 1978). Despite the range of bloodmeal sizes between these species, *Ae. triseriatus* and *Ae.*

albopictus ingested similar numbers of microfilariae, while *Ae. aegypti* ingested a significantly lower amount of microfilariae. Prior research by Kershaw et al. (1953), quantified *D. immitis* microfilaria intake by *Ae. aegypti* using computed bloodmeal sizes and microfilariae from a dog's peripheral blood samples. Kershaw et al. (1953) concluded that while most of these mosquitoes took in far fewer microfilariae than might be expected, a few took in more microfilaria than expected, suggesting that microfilariae uptake has an unpredictable nature. Because initial dosing of microfilariae may significantly impact the enhanced transmission outcome, future studies regarding microfilariae intake by in vitro feeding devices are essential, especially when using multiple mosquito species of varying sizes.

While the percentages of LAC virus infection, dissemination, and transmission in *Ae. albopictus* and *Ae. aegypti* were comparable to previous reports (Grimstad et al. 1989, Hughes et al. 2006), percentages in *Ae. triseriatus* were relatively low (Grimstad et al. 1977, Paulson et al. 1989, Hughes et al. 2006). Because endemic strains of *Ae. triseriatus* and LAC virus were used, perhaps host resistance to virus by the mosquito has evolved as a result of virus adaptation. Grimstad et al. (1977) showed that different strains of *Ae. triseriatus* differ widely in response to LAC virus, and revealed that non-endemic strains were more susceptible to infection and more likely to transmit the virus. Also, our low LAC virus transmission rates may have been a result of our laboratory techniques for sample collection. We measured LAC virus transmission by assaying mosquito salivary expectorate. Collection of salivary expectorate was done via capillary tube feeding (Cornell and Jupp 1989, Colton et al. 2005), a method not previously reported for measuring LAC virus transmission. Mosquito expectorate was difficult to visualize and therefore confirmation of its presence was not always feasible. Therefore, all capillary tubes

were tested with the assumption that the mosquitoes had in fact salivated into them. Also, it is possible some individuals shed virus particles at levels not detectable by this testing method.

Aedes albopictus that were fed a virus dose with a ten-fold reduction had very low percentages of infection and dissemination. Also, LAC virus was not detected in the saliva of any mosquito in either group. The low infection and dissemination percentages are most likely due to the ten-fold dose reduction of LAC virus. Other studies have observed high oral infection and transmission rates of LAC virus only when relatively high virus titers were used (Pantuwatana et al. 1972, Watts et al. 1973, Patrican et al. 1985). Patrican et al. (1985) also observed significantly reduced rates of infection and transmission after feeding *Ae. triseriatus* on LAC viruses doses with a ten-fold or more reduction. Significantly reduced rates of infection and transmission could also be due in part to the use of an artificial feeding apparatus. Blood must be treated with an anticoagulant when it is outside of the body to ensure clotting does not occur in the artificial feeding device. Anticoagulants could induce physiologic changes in the blood, and Patrican et al. (1985) speculated that changes in blood pH and electrostatic forces could affect viral attachment to midgut epithelial cells. Lastly, even when adequate infectious doses are used, infection thresholds can vary between mosquito species. For example, Watts et al. (1973) infected several species of mosquitoes with LAC virus at similar doses and found that many were infected but at rates much lower than the primary LAC vector, *Ae. triseriatus*. Therefore, the reduced virus dose used in this study may have been well below the LAC infection threshold for the accessory vector, *Ae. albopictus*.

Failure to demonstrate enhancement of LAC virus infection, dissemination and transmission in the primary vector *Ae. triseriatus*, accessory vector *Ae. albopictus*, and incompetent vector *Ae. aegypti* led us to conclude that *D. immitis* does not have a significant

impact on LAC virus epidemiology in areas where these organisms co-exist. Microfilaria enhanced transmission is dose dependent, and mosquitoes ingesting bloodmeals with high numbers of microfilaria are not likely to survive long enough to transmit arboviruses ingested simultaneously. Midgut penetration rates of *D. immitis* are low, and in the case of LAC virus, fail to meet the penetration threshold that makes arboviral enhancement successful.

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Tables and Figures

Table 4.1: Percentages of LAC virus infection, dissemination and transmission in *Aedes triseriatus* and *Ae. albopictus* 14-15 days after concurrent ingestion of *D. immitis* and LAC virus.

MF/ml*	Virus dose**	n	% Infection	% Dissemination	% Transmission
Aedes triseriatus					
None	7	80	67.5	45	21.3
2000	7	80	65	48.8	23.8
Aedes albopictus					
None	7	25	96	96	16
4000	7	25	84	84	28
None	6	40	7.5	7.5	0
4000	6	40	12.5	12.5	0

*MF, microfilariae.

**Logarithm$_{10}$ plaque-forming units per milliliter of blood.

Table 4.2: Percentages of LAC virus infection, dissemination and transmission in *Aedes triseriatus* and *Ae. aegypti* on specific days P.I. after concurrent ingestion of *D. immitis* and LAC virus

		***D. immitis* Microfilariae (2000 – 4000 MF / ml) + LAC Virus**			**LAC Virus Alone**		
Species	**Days P.I.**	**Infection Rate (%)**	**Dissemination Rate (%)**	**Transmission Rate (%)**	**Infection Rate (%)**	**Dissemination Rate (%)**	**Transmission Rate (%)**
Ae. triseriatus	9	7/10 (70)	7/10 (70)	2/10 (20)	5/10 (50)	5/10 (50)	0/10 (0)
	13	6/11 (55)	6/11 (55)	2/11 (18)	7/11 (64)	6/11 (55)	2/11 (18)
	Total	13/21 (62)	13/21 (62)	4/21 (19)	12/21 (57)	11/21 (52)	2/21 (10)
Ae. aegypti	7	5/25 (20)	4/25 (16)	0/25 (0)	2/25 (8)	2/25 (8)	0/25 (0)
	10	5/25 (20)	5/25 (20)	2/25 (8)	3/25 (12)	3/25 (12)	2/25 (8)
	14	2/25 (8)	2/25 (8)	1/25 (4)	1/25 (4)	1/25 (4)	0/25 (0)
	21	2/14 (14)	1/14 (7)	0/14 (0)	1/14 (7)	1/14 (7)	0/14 (0)
	Total	14/89 (16)	12/89 (13)	3/89 (3)	7/89 (8)	7/89 (8)	2/89 (2)

Table 4.3: Mean numbers of ingested and midgut penetrating *D. immitis* microfilariae (MF) in *Ae. triseriatus*, *Ae. albopictus*, and *Ae. aegypti**

	Mean number of ingested MF (95% C.I.)			**Mean number of penetrated MF (95% C.I.)**		
MF / ml	2000	4000	8000	2000	4000	8000
Ae. triseriatus	8.75a (6.41 – 11.93)	19.54a (17.99 – 21.23)	38.11a (30.90 – 46.99)	1.82a (0.46 – 7.22)	2.99a (0.68 – 13.14)	5.85a (1.62 – 21.06)
Ae. albopictus	6.67a (4.63 – 9.60)	17.14a 12.42 – 23.66)	36.39a (29.31 – 45.18)	2.0a (0.36 – 11.19)	5.83a (2.44 – 13.94)	10.3a (5.18 – 20.49)
Ae. aegypti	3.59b (2.87 – 4.50)	11.27b (7.48 – 16.97)	29.17a (24.72 – 34.43)	3.21a (0.16 – 63.27)	3.3a (2.18 – 4.99)	8.37a (4.89 – 14.33)

*Means within a column followed by the same letter are not significantly different ($P < 0.05$) when analyzed by ANOVA and unpaired T- tests

Table 4.4: Mortality of *Ae. triseriatus*, *Ae. albopictus*, and *Ae. aegypti* 14 days P.I. after ingestion of *D. immitis* microfilariae*

	2000 MF / ml		**4000 MF / ml**		**8000 MF / ml**	
Mosquito species	**No. surviving /No. fed**	**% Mortality**	**No. surviving /No. fed**	**% Surviving**	**No. surviving /No. fed**	**% Surviving**
Ae. triseriatus	12/29	58.6%a	9/26	65.3%a	5/29	82.7%a
Ae. albopictus	36/41	12.2%b	16/21	23.8%b	22/36	38.9%b
Ae. aegypti	22/31	29.0%b	10/24	58.3%a	5/19	73.7%a

* Means within a column followed by different letters are significantly different ($P < 0.05$) when analyzed by Chi Square or Fisher's Exact analyses.

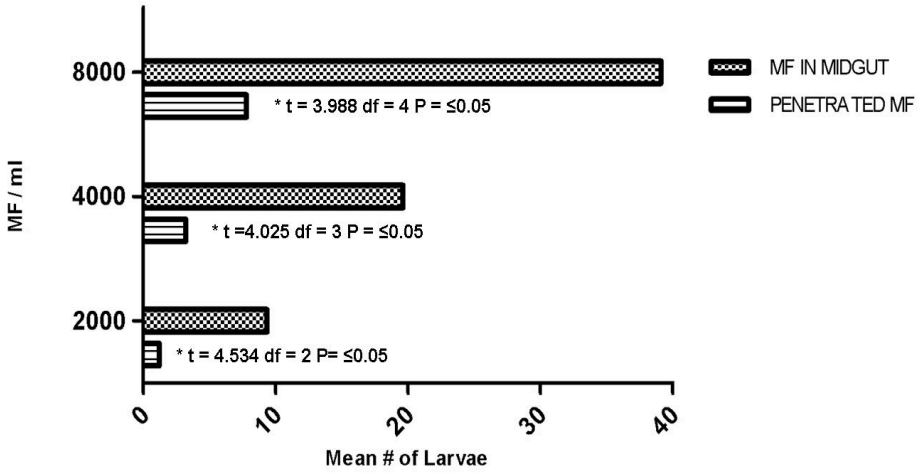

Figure 4.1: Comparison of mean midgut numbers of microfilariae after bloodmeal vs. numbers of penetrating microfilariae 10 hours after bloodmeal in *Ae. triseriatus*
* Denotes significant difference between mean numbers of ingested and penetrated microfilariae

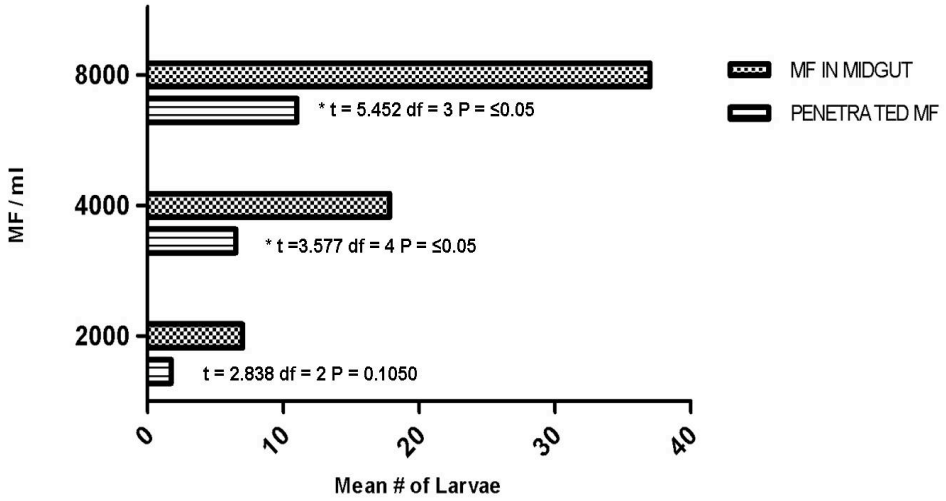

Figure 4.2: Comparison of mean midgut numbers of microfilariae after bloodmeal vs. numbers of penetrating microfilariae 10 hours after bloodmeal in *Ae. albopictus*
* Denotes significant difference between mean numbers of ingested and penetrated microfilariae

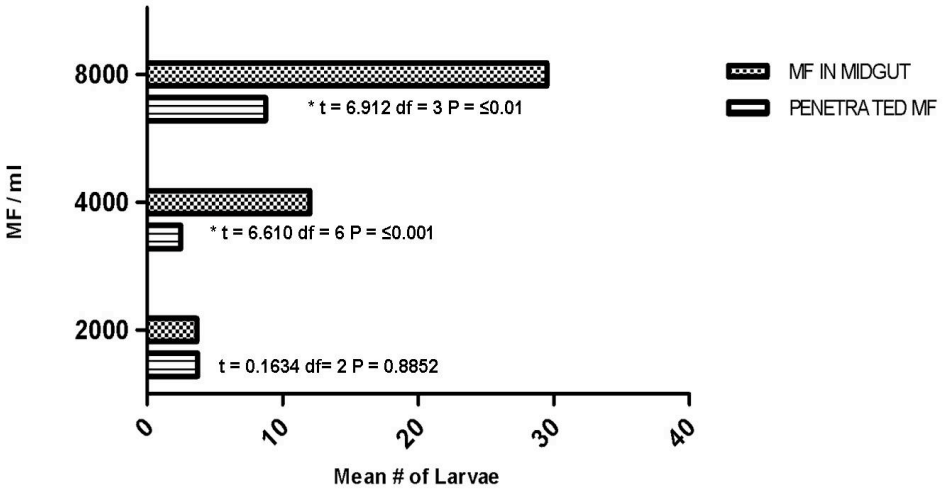

Figure 4.3: Comparison of mean midgut numbers of microfilariae after bloodmeal vs. numbers of penetrating microfilariae 10 hours after bloodmeal in *Ae. aegypti*
* Denotes significant difference between mean numbers of ingested and penetrated microfilariae

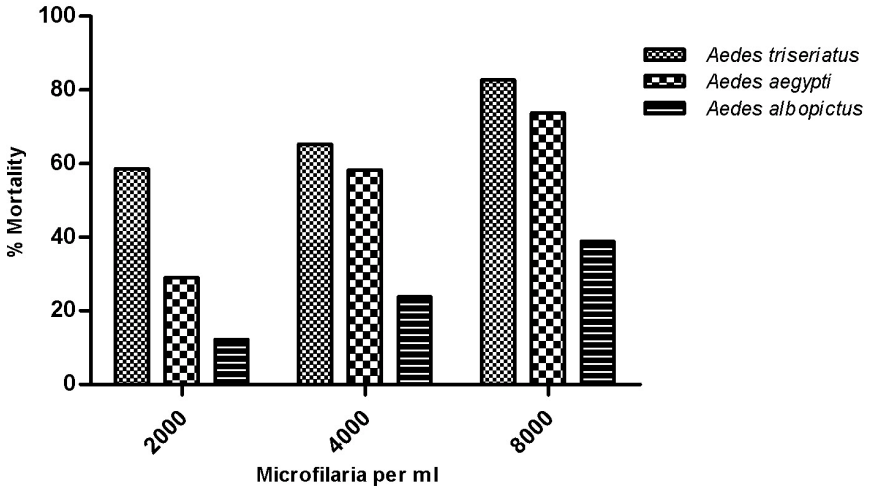

Figure 4.4: Mortality of *Aedes triseriatus*, *Ae. albopictus*, and *Ae. aegypti* after ingestion of *D. immitis* microfilariae 14 days P.I.

5. Summary

Southwest Virginia has become an emerging focus of LAC virus activity (Barker et al. 2003a,b, CDC 2007, VA Dept. of Health 2008). I had the unique opportunity to study the regional occurrences of LAC virus vectors and LAC virus seroprevalence rates of sentinel animals in southwest Virginia, in three areas that differ in the number of human cases of LAC encephalitis and LAC virus positive mosquito isolates. In addition, I investigated the potential impacts of microfilaria enhanced arbovirus transmission on LAC virus epidemiology.

Reasons for the rise in human cases of LAC encephalitis in southwest Virginia may include the widespread occurrence of the primary LAC virus vector, *Aedes triseriatus* and the relatively recent invasions of mosquito species such as *Ae. albopictus* and *Ae. japonicus*. An important first step for improving our understanding and ability to predict the risk of LAC encephalitis in the southwestern region of Virginia is to develop a knowledge-base of information on the spatiotemporal dynamics of the vector-species. Therefore, my first objective (Objective I) was to investigate the spatiotemporal activity of vectors. In 2005 and 2006, I conducted ovitrap surveys across a wide region of southwest Virginia, in order to assess oviposition abundance and dispersion patterns, and species co-occurrence and displacement. Analyses of data included comparing egg abundance among study areas, determining habitat preferences for each vector species, and examination of spatial patterns of oviposition.

My study found that the egg abundance and oviposition patterns of *Ae. triseriatus*, *Ae. albopictus*, and *Ae. japonicus* varied across the three study areas, which demonstrated that these mosquito vectors are not evenly distributed throughout southwest Virginia. There is a connection between the risk of LAC virus infection in endemic areas and the density of breeding sites available to the vector (Defoliart et al. 1986), which suggests that LAC virus endemic areas

are sporadic and levels of risk probably vary throughout southwest Virginia. In addition, the primary LAC virus vector, *Ae. triseriatus*, was collected in the greatest abundance from all three study areas. Previous ovitrap studies in southwest Virginia and North Carolina have also shown *Ae. triseriatus* as the most commonly collected mosquito (Szumlas et al. 1996a,b, Barker et al. 2003a,b).

Using a habitat selection index, oviposition preferences were determined for each of the mosquito species based on their tendency to oviposit in urban or forested areas. My results show that *Ae. triseriatus* has a preference for forested habitat. The preference for forested habitat has been well documented for *Aedes triseriatus* in previous studies (Walker 1992, Joy and Hildreth-Whitehair 2000, Nasci et al. 2000). As such, the risk for LAC virus is often associated with the proximity of residences to forest edges or children playing in wooded areas, and the subsequent probability of coming into contact with this predominantly forest–dwelling mosquito.

Knowledge of habitat preference is especially crucial for the recently invasive mosquito species *Ae. albopictus* and *Ae. japonicus*, because laboratory studies have shown that they are capable LAC virus vectors (Tesh and Gubler 1975, Sardelis et al. 2002). Furthermore, LAC virus has been isolated in natural populations in *Ae. albopictus* (Gerhardt et al. 2001). The habitat selection index showed that *Ae. albopictus* had an affinity for urban habitats, which has also been shown in prior habitat selection studies for this mosquito species (Francy et al. 1990, Rai 1991, Barker et al. 2003a,b). Perhaps the role of *Ae. albopictus* in this new southeastern expansion of focus of LAC virus is to serve as a bridge vector, carrying LAC virus from the forests into urban areas where the primary vector is usually found in fewer numbers.

The habitat preferences of the recently introduced species, *Ae. japonicus,* are difficult to define, however my analysis suggested that this species has a preference for urban habitats

similar to that of *Ae. albopictus*. I also found that *Ae. japonicus* is actively expanding its range throughout southwest Virginia, as eggs from this species were collected at many more trap sites in 2006 relative to 2005. A recent study in southwest Virginia also indicated that *Ae. japonicus* is actively expanding its range (Grim et al. 2007). Grim et al. (2007) reported that *Ae. japonicus* were rarely found during an adult mosquito collection in 2002, but found *Ae. japonicus* to be the second most commonly collected mosquito in a similar collection in 2004.

My assessment of dispersion and spatial distribution patterns of egg-laying found that the spatial dispersion of oviposition among trap sites by *Ae. triseriatus* and *Ae. albopictus* were aggregated. Similar results on the spatial distribution of oviposition for these vectors have been found in other studies (Kitron et al. 1989, Focks et al. 1999, Richards et al. 2006a). While I did not investigate reasons for aggregated oviposition patterns, Kitron et al. (1989) suggested these patterns may be due to the deposition of a large number of eggs per gonotrophic cycle by females. Regardless, information on the spatial distribution of egg-laying for a species when combined with information that the pattern across years are associated can prove useful for tracking the spread of the species and for developing management programs.

Because the majority of LAC virus infections are undiagnosed, little is actually known about the distribution of LAC virus in endemic areas such as southwest Virginia. Using dogs as sentinel animals for the detection of LAC virus may help to determine locations of LAC virus foci. Dogs are particularly good LAC virus sentinels because they develop detectable serum antibody levels in response to LAC virus (Godsey et al. 1988), and studies have shown that *Ae. triseriatus* and *Ae. albopictus* commonly feed on dogs (Szumlas et al. 1996b, Richards et al. 2006b). Therefore my second objective (Objective II) measured the efficacy of canine sentinels to determine the distribution of LAC virus in southwest Virginia.

Canine serum samples were tested for LAC virus antibodies using plaque reduction neutralization (PRNT) assays. Of the 436 collected canine serum samples, 21 (4.8%) were positive for LAC virus antibodies. LAC virus seroprevalence was evident in dogs from each study region, with no significant differences in seroprevalence among the three areas. LAC virus antibodies were found in dogs residing in areas where LAC virus human cases and LAC virus positive mosquito isolates have not been reported. As a result, this study provided documentation of horizontal transmission of LAC virus throughout southwest Virginia, demonstrating that dogs make useful sentinels for assessing the distribution of LAC virus in an area. Also, our finding of widespread infection of LAC virus reiterates the fact that this virus is highly underreported.

Additionally, this seroprevalence study was expanded to examine the usefulness of dogs as indicators of other vector-borne diseases in the region including *Borrelia burgdorferi* (the causative agent of Lyme Disease), *Ehrlichia canis*, and *Anaplasma phagocytophilum*. The detection of *B. burgdorferi* was of particular interest, as Lyme disease has also been reported as an emerging infectious disease in southwest Virginia (Virginia Dept of Health 2008). Surveillance for Lyme disease in southwest Virginia is passive, and as a result may be ineffective (Johnson et al. 2004), as cases oftentimes go unreported and tick submission rates depend on public awareness of Lyme disease and local concerns of individuals.

The ELISA-based Idexx ® Snap 4Dx ® test (Idexx Laboratories, Westbrook, ME, USA) was used to screen 412 canine serum samples for antibodies to *B. burgdorferi*, *Ehrlichia canis*, and *Anaplasma phagocytophilum*. While very little antibodies were detected for *E. canis* and *A. phagocytophilum*, seventeen (4.1%) of the 412 dogs tested were found to be positive for *B. burgdorferi* antibodies. Antibodies to *B. burgdorferi* were detected in two of the three study

areas, both of which have low rates of reported human Lyme cases. The widespread seroprevalence of *B. burgdorferi* found in this study suggests that dogs also make effective sentinels for *Borrelia* infection in southwest Virginia.

The final objective of this project investigated the potential occurrence of microfilarial enhanced arbovirus transmission, which can occur when a mosquito ingests a bloodmeal dually infected with an arbovirus and filarial nematodes (microfilariae) (Turell et al. 1987, Zytoon et al. 1993, Vaughan and Turell 1996, Vaughan et al 1999). The basic mechanism of arbovirus enhancement occurs when ingested microfilariae penetrate the mosquito's midgut epithelium, leaving holes that a concurrently ingested virus could escape through to directly enter the hemocoel. Direct entrance into the hemocoel bypasses the need for a virus to infect and replicate in the mosquito midgut, and can augment the infectivity of that virus in the mosquito.

In the U.S., the potential exists for the occurrence of microfilaria enhanced arbovirus transmission because of the overlapping ranges of the filarial nematode *Dirofilaria immitis* (canine heartworm), and LAC virus. In addition, the LAC virus mosquito vectors *Ae. triseriatus*, and *Ae. albopictus* have been shown to be competent *D. immitis* vectors (Intermill 1973, Apperson et al. 1989, Nayar and Knight 1999). I investigated the occurrence of microfilaria enhanced transmission for LAC virus in mosquito vectors by examining the effects of coinfection with *D. immitis* microfilaria and LAC virus in *Ae. triseriatus*, *Ae. albopictus,* and the incompetent LAC vector, *Ae. aegypti.*

Overall, microfilaria enhanced transmission of LAC virus using *D. immitis* microfilariae was not observed in *Ae. triseriatus*, *Ae. albopictus*, and *Ae. aegypti*. There were no significant differences in percentages of LAC virus infection, dissemination or transmission in mosquitoes

fed dually infected bloodmeals (i.e. *D. immitis* microfilariae and LAC virus) compared to those fed bloodmeals containing LAC virus only.

Because previous studies have shown that microfilarial enhancement is dose dependent, I hypothesized that the failure to demonstrate enhancement of LAC virus was likely due to poor midgut penetration rates by *D. immitis*. Poor midgut penetration would not allow the passage of the minimum infectious dose of virus needed enhance arbovirus transmission. Since very little is known about the midgut penetration behavior of *D. immitis*, a follow-up study was conducted in order to examine *D. immitis* microfilariae in the midguts of the three mosquito species previously used in this experiment. Specifically, numbers of ingested and midgut–penetrating microfilaria were quantified, and mortality rates for different doses of microfilariae were assessed for *Ae. triseriatus*, *Ae. albopictus*, and *Ae. aegypti*.

The results of this follow-up *D. immitis* investigation showed significant differences between the numbers of ingested and midgut–penetrating *D. immitis* microfilariae for each mosquito species. In general, midgut penetration by *D. immitis* was very low, and I concluded that the low numbers of penetrating microfilariae were not sufficient enough to allow for the passage of the minimum amount of virus required for enhancement to occur.

Microfilaria-induced mosquito mortality prevented the use of higher doses of microfilariae throughout the study, however biologically significant doses were used in each experiment. Mortality assessment of the mosquito species showed that *Aedes triseriatus* had the lowest tolerance to microfilaria burdens, while *Ae. albopictus* had the highest tolerance. Failure to demonstrate enhancement of LAC virus infection, dissemination and transmission in the primary vector *Ae. triseriatus*, accessory vector *Ae. albopictus*, and incompetent vector *Ae.*

aegypti, suggest that *D. immitis* does not have a significant impact on LAC virus epidemiology in areas where these organisms co-exist.

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APPENDIX A: Vector-Borne Disease Client Handout

Vector Borne Disease Survey

What is Vector Borne Disease?

A vector borne disease is a disease that is transmitted to a human or animal by an insect or other arthropod. Vectors that commonly transmit disease in Virginia are mostly mosquitoes and ticks. Mosquitoes are responsible for transmitting diseases such as La Crosse encephalitis virus, West Nile virus, and canine heartworms, while diseases such as Lyme disease are transmitted by ticks.

Dogs that have been exposed to some of the vector borne organisms will make antibodies that circulate in the bloodstream. Finding these antibodies can provide valuable information on the distribution of vector borne diseases that may be occurring in this area.

Researchers in the Department of Entomology and the College of Veterinary Medicine at Virginia Tech are conducting a survey using blood samples from dogs to monitor levels of exposure to vector borne diseases. Dogs can be tested more easily than people and can be a very effective monitor of exposure because many dogs spend more time outside than their owners and have a greater chance of being bitten by an infected mosquito or tick.

How can you help?

Please allow your vet to take a blood sample from your dog(s) during your office visit and complete this short questionnaire about your dog's activities. We will test the blood for antibodies to La Crosse virus / other vector borne diseases. The testing will be done by Virginia Tech and there will be **NO CHARGE** to you. A positive test result does not mean that your dog can infect you or your family. Your participation will help us determine where La Crosse encephalitis virus and other vector borne diseases are occurring in southwestern Virginia.

Thank you very much for your help. If you have questions you can contact:

Dr. Anne Zajac (Email: azajac@vt.edu, Phone (540) 231-7017)
Virginia/ Maryland Regional College of Veterinary Medicine
Virginia Tech, Blacksburg VA 24061

_____
Sample number

Pet Information

1. Age of your dog:_____ Sex: Male:_____Female:_____

2. Breed of dog (if unsure, just put "mixed")_____

3. **County** of residence:_____

4. Address (if P.O. box, please include street name of residence)_____

_____

5. How long has your dog lived in this area? _____

6. Has your dog ever traveled outside western Virginia?

Yes_____ No_____ Don't know_____

7. If you answered yes to the above question, what parts of the U.S. or the world has the dog traveled to? _____

8. About how much time does the dog spend outdoors each day?
   _____less than 2 hours
   _____more than 2 hours but less than 10 hours
   _____more than 10 hours but less than 24 hours
   _____lives outdoors

9. My veterinarian has my permission to draw a blood sample from my dog for use in the Virginia Tech Vector Borne Disease Survey.

_____ _____
Signature Date

10. May we contact you for further information? yes / no (circle one)

11. If you answered yes, please fill in your name, and phone number and/or e-mail address.

_____
Name

_____
Phone number and/or e-mail address

APPENDIX B: IACUC Approval Letter

VirginiaTech

Institutional Animal Care and Use Committee
Stephen A. Smith, Chair
2000 Kraft Drive, Suite 2000 (0497)
Blacksburg, Virginia 24061
540/231-5131
Email: stsmith7@vt.edu
www.acc.vt.edu

June 22, 2007

MEMORANDUM

TO: Sally L. Paulson

FROM: Stephen A. Smith

SUBJECT: Review of Protocols Involving Animals

The purpose of this memo is to verify that the Virginia Tech Institutional Animal Care and Use Committee has reviewed and granted approval of protocol **#07-074-ENT**, entitled **"Emerging Foci of La Crosse Encephalitis Virus in Southwestern Virginia"**, submitted by **Sally L. Paulson**. The funding source for this protocol is (OSP# 431448).

Period of Approval

This research protocol is approved for a period of three years, from **June 22, 2007 to June 21, 2010**. If the research experiments offered under this protocol will be conducted on a continuing basis throughout the three-year approval period, the protocol must undergo continuing review on an annual basis. In such cases, the principal investigator must submit an annual continuing review form prior to the one-and two-year anniversaries of the approval date. If the reseaarch conducted under this protocol will continue to be conducted after the end of the three-year approval period, a new protocol must be submitted and approved prior to the three-year anniversary of the original approval date. The principal investigator is responsible for submitting all paperwork required to maintain IACUC approval.

Changes to Approved Protocols

Any changes in animal numbers, species, procedures/treatments, or pain category must be submitted to the IACUC for review and approval **before** those changes are implemented. Failure to seek IACUC approval for amending approved protocol procedures may result in withdrawal of permission to conduct the research.

Federal Compliance Assurance

All proposals involving the use of living vertebrates are reviewed by the Virginia Tech Institutional Animal Care and Use Committee to assure humane care and treatment of the animals involved. Approved proposals comply with:

1. "U.S. Government Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research, and Training"
2. The Animal Welfare Act, As Amended
3. The Public Health Service (PHS) Policy on Humane Care and Use of Laboratory Animals
4. "Virginia Tech Policies Governing the Use of Animals in Research and Teaching"

Virginia Tech has a written, approved Animal Welfare Assurance on file with the PHS Office of Laboratory Animal Welfare (OLAW). The university's Animal Welfare Assurance number is A-3208-01, expiration date 3 -31-2010.