La Crosse virus and *Dirofilaria immitis*: Abundance of Potential Vectors in Southwestern Virginia and the Effects of Dual Infection on *Aedes albopictus* and *Ochlerotatus triseriatus*

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Abstract

Microfilarial enhancement of viral transmission is well documented, however only one previously studied model used components that occur together in nature and therefore has realistic implications. La Crosse (LAC) virus encephalitis is the most common mosquito-borne illness affecting children in the United States. LAC virus is prevalent in the Great Lake and Mid-Atlantic states and coincidently this area overlaps the region of highest infection for *Dirofilaria immitis,* the nematode that cause canine heartworm disease. *Ae. albopictus* and *Oc. triseriatus* are important vectors of La Crosse virus and among the numerous species able to transmit *D. immitis*. In this study, *Aedes albopictus* and *Ochlerotatus triseriatus* were infected with La Crosse virus and *Dirofilaria immitis* to determine the effects of dual infection on the dissemination and transmission of the virus.

The effects of dual infection varied between the species tested. *Ae. albopictus* had significantly higher tolerance to *D. immitis* infection than *Oc. triseriatus*. Dissemination for dually infected *Ae. albopictus* were higher than the control group for all days tested, except one. Transmission rates for *D. immitis* infected *Ae. albopictus* were significantly higher than the control group on day 14 post infection. No microfilarial enhancement of viral dissemination or transmission was observed for *Oc. triseriatus*. The infection, dissemination, and tranmission rates were low for both species compared to rates of previous studies. Low rates could be a result of low susceptibility for the strains tested.

In a second study, mosquitoes were collected from two counties in Southwestern Virginia to determine the abundance of potential La Crosse virus and *D. immitis* vector species. The abundance and distribution of mosquito species were examined in 2003 and 2004 using gravid traps. An unexpected finding was the significant increase in the abundance of *Ochlerotatus japonicus*. In 2003, collections were made over 192 trap nights from June to August yielding 5,879 mosquitoes of which only 24 were *Oc. japonicus*. In 2004, 12,151 mosquitoes were trapped from June to September over 160 trap nights. *Oc. japonicus* was the second most abundant mosquito species and the dominant *Ochlerotatus* species collected in gravid traps. *Oc. japonicus* was collected in low numbers in June, but the abundance increased significantly in July and remained consistent throughout the rest of the season. Of the other major mosquito species collected in this study, only *Aedes albopictus* exhibited a similar seasonal pattern as *Oc. japonicus*. Other biological similarities of *Oc. japonicus* and *Ae. albopictus* are discussed.

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Introduction and Review of Literature

LA CROSSE VIRUS

Introduction

La Crosse (LAC) virus was first isolated in 1960 from the postmortem brain of a four-year old girl from La Crosse, Wisconsin (Thompson et al. 1965). LAC virus is a member of the California (CAL) serogroup of the family Bunyaviridae, the largest family of vertebrate viruses (Beaty et al. 2000). This virus, in the genus Bunyavirus, is a spherical, enveloped RNA virus. It causes La Crosse encephalitis (LACE) in humans, which is the most common mosquito-borne illness to affect children in the United States (McJunkin et al. 2001). After the initial isolation, LAC virus infections occurred primarily in the Great Lake states, but today the virus is also prevalent in all Mid-Atlantic States (Fig. 1.1) (CDC 2003).

La Crosse Virus in Humans

There are an estimated 300,000 new LAC virus infections per year (Rust et al. 1999), but most cases go unrecognized because they are subclinical (McJunkin et al. 2001). LAC virus affects mainly children 15 years old or younger; the probability of disease in children older than 15 is only 8% (Rust et al. 1999). A majority of children who become infected with LAC virus live on farms or near wooded areas (Rust et al. 1999) and children who spend a great amount time outside are at greater risk of becoming infected than children who spend most of their time indoors (Erwin et al. 2002).

Children with clinical infections develop illness similar to aseptic meningitis with symptoms including restlessness, irritability, depression, and fever. Fever occurs in nearly all cases and can range from 101-106°F. Approximately 42-60% of patients have seizures (Rust et al. 1999). Patients are often admitted to the hospital after the onset of seizures and the average duration of hospitalization is six days (McJunkin et al. 2001). The medical cost for a patient ranged from $13,967 to $19,320 in 2001 (Utz et al. 2003). Although the fatality rate for La Crosse encephalitis (LACE) is only 0.5% (Rust et al. 1999), patients who have contracted LACE may exhibit long-term effects of infection, or sequellae, such as decrease in overall IQ as well as an increased risk of attention-deficit-hyperactivity disorder (McJunkin et al. 2001).

La Crosse Virus in its Mammalian Hosts

La Crosse virus is maintained in nature by a cycle between mosquitoes and small wild mammals, with humans becoming infected occasionally (Thompson et al. 1972). Moulton and Thompson (1971) hypothesized that host species whose ecological habitat overlaps with that of *Ochlerotatus triseriatus*, the primary La Crosse virus vector, become infected more often than those hosts not interacting as closely with the mosquito. This hypothesis was confirmed with a serosurvey performed on a Wisconsin farm, which found that the Ohio chipmunk (*Tamias striatus*) and gray and fox squirrels (*Sciurius carolinensis* and *Sciurus niger*) tested positive for antibodies for La Crosse virus at rates of 53% and 39% respectively. Cottontail rabbits (*Sylvilagus floridanus*) and flying squirrels (*Glaucomys volans*) were also seropositive but at lower rates (15% and 5%). None of the white-footed mice tested were positive for antibodies (Moulton and Thompson 1971). Because squirrels and chipmunks are active during the day and spend

time in both the canopy and the forest floor, they are more likely to come into contact with *Oc. triseriatus* than nocturnal mammals such as white-footed mice (Moulton and Thompson 1971). Humans and white-tailed deer (*Odocoileus virginianus*) can also become infected with La Crosse virus, but are considered "dead-end" hosts because they do not produce adequate levels of viremia to pass on to uninfected mosquitoes (Osorio et al. 1996).

La Crosse Virus in Mosquitoes: Biology and Vector Competence of *Ochlerotatus triseriatus*

Ochlerotatus triseriatus (Say), the eastern treehole mosquito, is the primary vector of La Crosse virus in North America (Watts et al. 1972). In laboratory studies of LAC virus, the rate of transmission by *Oc. triseriatus* was between 70 and 77%, much greater than the other species studied (Watts et al. 1973a). This mosquito is distributed throughout the eastern United States and breeds in hardwood treeholes and artificial containers such as discarded automobile tires (Grimstad et al. 1989). A study performed in Wise County, Virginia, a LAC endemic area, revealed that *Oc. triseriatus* preferred wooded areas to urban or residential areas (Barker et al. 2003b). Wright and DeFoliart (1970) reported that *Oc. triseriatus* fed readily on humans, especially in wooded areas. The problem of *Oc. triseriatus* as a pest, therefore, is related to the availability of suitable habitats in an area (Horsfall 1972).

Mosquito species in Wisconsin were studied extensively to determine their host preferences. *Ochlerotatus triseriatus* was considered a general feeder, but fed on gray squirrels and chipmunks at higher rates than the other mosquito species studied (Wright and DeFoliart 1970). Gray squirrels were also the preferred host of *Oc. triseriatus* in

LAC endemic areas of Illinois, whereas as in non-endemic areas red squirrels, fox squirrels, and eastern chipmunks served as hosts (Nasci 1985). In contrast, *Oc. triseriatus* in a LAC virus endemic area of North Carolina preferred to feed on dogs (40.4%), rabbits (26.6%), or turtles (22.3%). Only 7.5% of mosquitoes fed on chipmunks and 2.1% fed on humans (Szumlas et al. 1996).

In most of its geographic range, *Oc. triseriatus* overwinters as diapausing eggs (Watts et al. 1974). The developing larvae inside the eggs are photosensitive (Shroyer and Craig 1980). Decreased temperatures and photoperiod are seasonal cues that signal the developing larvae that winter is approaching and that hatching should cease. The critical photoperiod varies for different geographic strains of *Oc. triseriatus* (Shroyer and Craig 1983). When days are long, the larvae develop normally and hatch, but exposure to short days induces diapause. Even at an optimal photoperiod, exposure to temperatures below 10°C can also induce diapause (Shroyer and Craig 1980). Termination of diapause can be caused by prolonged exposure to low temperature and short day or prolonged exposure to long days at 21°C (Shroyer and Craig 1983). It is in diapause eggs that La Crosse virus is able to survive cold winter months (Watts et al. 1973b).

Watts et al. (1973b) was the first to demonstrate transovarial transmission (TOT) of La Crosse virus in *Oc. triseriatus*. Before this research, it was unknown how LAC virus was able to survive the winter months when mosquitoes are inactive. Isolation of the virus from larvae and eggs laid by experimentally infected *Oc. triseriatus* adults suggested that the virus could overwinter in diapausing eggs laid by an infected female (Watts et al. 1973b). La Crosse virus infection negatively affects the overwintering eggs causing increased mortality compared to those eggs not infected with LAC virus (McGaw

et al. 1998). Miller et al. (1977) reported that over eight generations the TOT rate in *Oc. triseriatus* only decreased slightly (from 88% to 71%), but periodic horizontal amplification of the virus through vertebrate hosts ensures that the virus does not become too dilute. Transovarially transmitted virus may undergo genetic mutations and RNA exchange with other viruses within the egg. Because of this, there is a risk of more virulent strains of virus developing (Klimas et al. 1981).

Biology and Vector Competence of *Aedes albopictus*

In 1985, *Aedes albopictus* (Skuse), the Asian tiger mosquito, was introduced to North America and was identified breeding in artificial containers near Houston, Texas (Sprenger and Wuithiranyagool 1986). The species was most likely imported in used tires from Northern Asia (Hawley et al. 1987). *Ae. albopictus* spread rapidly through the United States and can now be found in 30 states (Gerhardt et al. 2001) in a wide variety of habitats, both man-made and natural (Hawley et al. 1987). In Southwestern Virginia, *Ae. albopictus* were collected in abundance in urban and residential areas and in relatively low numbers in wooded areas (Barker et al. 2003a).

The biology and life cycle of *Aedes albopictus* are largely influenced by photoperiod. Biting activity varies by habitat, though usually peaks in the early morning and late afternoon (Hawley 1988). Adult *Ae. albopictus* are photoperiod sensitive. When exposed to long days, non-diapause eggs are produced; while exposure to short days results in the production of diapause eggs. Diapause eggs can overwinter and survive harsh winter weather (Hawley 1988).

Ae. albopictus was shown to be a competent vector of LAC virus in the laboratory (Cully et al. 1992); the virus was isolated from field collections of *Ae. albopictus*

collected as eggs from endemic areas in North Carolina and Tennessee (Gerhardt et al. 2001). LAC virus was also isolated from *Ae. albopictus* adults collected in southwestern Virginia in 2002 (Paulson, unpublished data). *Ae. albopictus* are able to transovarially transmit La Crosse virus, although the TOT rate was much less (2.7%) (Tesh and Gubler 1975) compared to that of *Oc. triseriatus* (34%) (Watts et al. 1973b).

Aedes albopictus feeds on a wide variety of mammalian hosts and will also feed on birds if no other host is available (Hawley 1988). In Thailand, *Ae. albopictus* was found to feed on humans, pigs, buffalo, chickens, and dogs, although humans were the preferred host (Sullivan et al. 1971). In the United States, a study was conducted to determine the blood host of *Aedes albopictus* collected in four states, Louisiana, Illinois, Florida, and Missouri. Ninety-two percent of the mosquitoes collected preferred to feed on mammals, whereas 1.9% fed on turtles, and 1.2% fed on birds (Niebyliski et al. 1994). Unlike the study in Thailand (Sullivan et al. 1971), the preferred hosts for *Ae. albopictus* in the U.S were rabbits (35.4%) and rats (26.8%). Although there were high landing and bite rates, only 3.9% of bloodmeals were taken from humans suggesting that humans are less susceptible and less tolerant of bites than other animals (Niebylski et al. 1994). A study performed in Potosi, MO confirmed that the preferred hosts of *Ae. albopictus* were mammals, with rabbits (24.5%), deer (14.5%), and dogs (13.6%) favored (Savage et al. 1993).

Biology and Vector Competence of *Ochlerotatus japonicus*

Ochlerotatus japonicus, a native of Asia (Tanaka et al. 1979), was first collected to the United States in 1998 when four adult females were collected in New Jersey and New York (Peyton et al. 1999). It is likely that this species, like *Aedes albopictus*, was

imported from Asia in used tires (Peyton et al. 1999). Since its introduction to the U.S., *Oc. japonicus* has been found in Washington (Washington State Health Department 2002), Connecticut (Andreadis et al. 2001), Maine (Foss and Dearborn 2001), Maryland (Scott et al. 2001), Virginia (Harrison et al. 2002), West Virginia (Joy 2004) and Georgia (Gray et al. 2005). Because the populations of *Oc. japonicus* in the United States differ genetically, the geographic spread of the species most likely resulted from multiple introductions (Fonseca et al. 2001).

In Japan, *Oc. japonicus* larvae occur in both natural and artificial habitats. Rockpools are a favored larval habitat in Asia (Tanaka et al. 1979). In the United States, immature *Oc. japonicus* have been collected from rock holes (Scott et al. 2002), as well as discarded tires and other artificial containers (Sardelis and Turell 2001, Oliver et al. 2003, Kutz et al. 2003, Joy 2004, and Gray et al. 2005). A common factor of preferred *Oc. japonicus* larval habitats in both Asia and the United States is the presence of decaying organic matter, a food source for the developing larvae (Tanaka et al. 1979, Gray et al. 2005).

Oc. japonicus can be found from early spring to early fall in Japan (Tanaka et al. 1979). *Oc. japonicus* larvae have been collected in New York as early as late March (Oliver et al. 2003), but this species is collected in highest abundance late in the trap season. In Connecticut, the peak collection time for *Oc. japonicus* was in September (Andreadis et al. 2001) and in Southern New York State collection of *Oc. japonicus* peaked in mid-August (Falco et al. 2002).

In Japan, adult *Oc. japonicus* typically reside in wooded areas and are diurnal biters (Tanaka et al. 1979). In the laboratory, *Oc. japonicus* are opportunistic feeders

(Sardelis et al. 2002a) and will feed on avian and mammalian hosts (Miyagi 1972 and Sardelis et al. 2002a). Although little is known about the host preference of *Oc. japonicus* in nature, Miyagi (1972) reported been bitten by *Oc. japonicus* females in a forested area in Japan. Likewise in the United States, *Oc. japonicus* have been observed aggressively biting humans for approximately one hour after dark (Gray et al. 2005).

Ochlerotatus japonicus has been examined for its potential as an arbovirus vector. In the laboratory, *Oc. japonicus* was found to be a competent vector of La Crosse virus (Sardelis et al. 2002b). In this research, the species was able to become infected, develop a disseminated infection, and transmit the virus to suckling mice at a rate similar to *Oc. triseriatus* (Sardelis et al. 2002b).

Mosquito Barriers Against Viral Infection

In order for a virus to be transmitted horizontally, the virus must penetrate the midgut cells of the mosquito, pass into the hemocoel, and infect the salivary glands (Turell et al. 1984). The extrinsic incubation (EI) period is the time from ingestion of an infectious bloodmeal by a mosquito to the oral transmission of the virus and is an important factor in disease transmission because it dictates how long a mosquito must survive after an infectious bloodmeal before it can transmit the virus (Hardy et al. 1983). A mosquito species may be susceptible to viral infection and yet not be able to transmit the virus once infected, because mosquitoes possess internal barriers against virus dissemination and infection (Hardy et al. 1983).

The mesenteronal infection (MI) barrier results in failure of a mosquito to develop a midgut infection after ingestion of an infectious bloodmeal (Hardy et al. 1983). The MI barrier may be the result of both chemical and physical factors, including midgut

epithelial cell surface charge, digestive enzyme degradation of virus, and presence and distribution of receptor sites for attachment of viruses (Hardy et al. 1983).

The mesenteronal escape (ME) barrier prevents the virus from entering into the hemocoel from infected midgut cells. Paulson and Grimstad (1989) found that nearly 100% of *Oc. triseriatus* that ingested blood-containing LAC developed a midgut infection, but in only 30-40% of those mosquitoes did the virus disseminate. Similar findings were reported for *Ae. albopictus* and LAC virus (Grimstad et al. 1989). The ME barrier is dose-dependent. In female mosquitoes with ME barriers, the virus does not replicate normally and therefore the viral titers are lower (Hardy et al. 1983).

The salivary gland infection (SGI) barrier prevents oral infection of the virus by a mosquito with a disseminated infection (Hardy et al. 1983). The SGI barrier occurs prior to the infection of the salivary glands but after the infection of the mesenteron (Hardy et al. 1983). This barrier is both dose- and time-dependent. In females with SGI barriers, the mean viral titer in the hemolymph is significantly lower than females lacking SGI barriers. Also the number of females with a SGI barrier decreases throughout extrinsic incubation period (Hardy et al. 1983).

Even after virus is detected in the salivary glands, mosquitoes may not be able to transmit the virus while feeding. The salivary gland escape barrier is not well defined but its function has been compared to that of the midgut escape barrier (Hardy et al. 1983).

DIROFILARIA IMMITIS

Introduction

In 1850 Joseph Leidy, a physician in Philadelphia, first described *Dirofilaria immitis* (Leidy), the nematode that causes canine and feline heartworm disease. Because

the nematode was isolated from the heart of a dog, Leidy first named the nematode *Filaria canis cordis* (Boreham and Atwell 1988). In 1901 Thomas Bancroft, an Australian physician, determined the complete life cycle of the parasite, including transmission by mosquito, and its development in the mosquito and vertebrate host (Bancroft 1901).

Dirofilaria immitis is widely distributed throughout the world in both tropical and subtropical climates, as well as northern and southern temperate zones (Otto 1969). Infections have been reported on six continents, all except Antarctica (Boreham and Atwell 1988). In the United States, the highest incidence of heartworm infection occurs in the southeastern states and states located along the Mississippi River, however localized infections occur in every state (Merial 2004).

A majority of dogs infected with heartworms live within 150 miles of either the Atlantic Ocean or the Gulf of Mexico (AHS 2005) and dogs traveling into these areas may be the cause of the spread of the parasite throughout the United States (Knight 1977). A study performed in Washington state noted that microfilaremic dogs traveling from endemic areas in Mississippi and Texas were responsible for the introduction of *D. immitis* to local mosquitoes in Washington and resulted in an infection in a native dog from Washington that lived in close proximity to both of these dogs. In western Washington, 0.8% of both male and female dogs that had traveled outside of the state tested positive for heartworm, compared to only 0.2% of non-traveling dogs (Theis et al. 2001).

One factor that appears to increase susceptibility of dogs to *D. immitis* infection is the housing that is provided for the dogs (Haddock 1987). Dogs that are housed outside

are at greater risk of exposure to mosquitoes and of *D. immitis* infection than dogs that are kept in the house (Haddock 1987).

In areas of high infection rate, dogs as young as one year of age can be diagnosed with heartworm infection (AHS 2005). In Northern Virginia, younger dogs, 1-3 years of age, were most at risk for heartworm infection and males and females were equally infected (Kimbell 1976). Although younger dogs can become infected, infections are most commonly diagnosed in dogs between three and eight years of age (AHS 2005). In Southeastern Michigan, the incidence of heartworm infection was 28% in dogs over five years of age, 24% in dogs ages 2-5 years and only 10% in dogs less than two years (Prouty 1972). A serosurvery performed in Maryland found that the peak of *D. immitis* infection was in 8-10 year old dogs (Wallenstein and Tibola 1960).

Along with age, other factors may affect a dog's risk of heartworm infection. In Michigan, more male dogs were infected (27%) compared to intact females (15%) and spayed females (17%) (Prouty 1972). Likewise in a serosurvey performed in Maryland, it was found that males were infected four times more often than females and Boxers were more commonly infected than any other species (Wallenstein and Tibola 1960).

Kimbell (1976) suggested that coat length might be a determining factor in the rate of infection and that a long coat provides the dog protection against mosquitoes. In this research, a greater number of short-haired dogs (25%) tested positive for heartworm, whereas only 14% of long-haired dogs were infected (Kimbell 1976). However, other research suggested that although a shaggy coat may provide some protection against mosquitoes, mosquitoes are still able to feed in the pelvic area, around the muzzle and by

penetrating the coat, therefore coat length is not a determining factor in infection rate (Otto 1969). Coat color does not seem to influence the rate of infection (Prouty 1972).

Dirofilaria immitis in Mosquitoes

Mosquitoes are the intermediate host of *D. immitis.* Larvae of *D. immitis* molt twice inside the mosquito without replicating. During a bloodmeal, a mosquito ingests the microfilariae circulating in the blood of the host. The microfilariae penetrate to midgut of the mosquito and migrate to the indirect flight muscles (Knight 1977). Using electron micrographs, the microfilariae were seen migrating toward the outer wall of the midgut within minutes after ingestion of infected blood and completely penetrated the midgut wall by four to six hours post-ingestion (Zytoon et al. 1993). Within 48 hours of ingestion the larvae molt into the second instar, or the sausage stage. These larvae migrate to the Malpighian tubules, and approximately two weeks after ingestion, a second molt occurs. The L3 or 3rd stage larvae move out of the Malpighian tubules into the hemocoel, eventually reaching the head and infecting the mouthparts. Approximately 15-17 days after ingestion, the infected mosquito can transmit the infective L3 larvae to the host while blood feeding (Knight 1977).

The complete larval development of *D. immitis* has been reported in more than 60 species of mosquitoes; however a majority of these are considered experimental vectors (Knight 1977). Because *Oc. triseriatus* typically reside in forests or wooded areas and therefore have less contact with dogs than other species of mosquitoes, they were not originally considered to an important heartworm vector (Intermill 1973). However, field-collected *Oc. triseriatus* from Mississippi were found to be a competent host and vector for development and transmission of *D. immitis* (Intermill 1973). A laboratory study

suggested that because of their preference for ground level activity and feeding on a variety of hosts, *Oc. triseriatus* were a likely vector of *D. immitis* especially in areas where dogs are present in the natural habitat of the mosquito (Rogers and Newson 1979).

Soon after *Ae. albopictus* was discovered in the United States, research was conducted to test the ability of the mosquito as a competent vector of *D. immitis*. *Aedes albopictus* in North Carolina were studied, and it was determined that they were unsuitable vectors because mortality after infection was high and the presence of L3 microfilariae was low (Apperson et al. 1989). Seven strains of *Ae. albopictus* from New Orleans, Louisiana were tested for their susceptibility to *D. immitis* infection; susceptibility of strains to infection ranged from 22-74% (Scoles and Dickson 1995). A second study tested the survival and susceptibility to heartworm infection of U.S. strains of *Ae. albopictus* compared to the South China strain of *Ae. albopictus* and laboratory strains of *Oc. triseriatus* and *Ae. aegypti* (Scoles and Craig 1993). The survival after feeding on infected dogs was similar for all the mosquito strains, ranging from 68% survival of the South China *Ae. albopictus* strain to 61% survival of *Oc. triseriatus*. Conversely, the susceptibility of the mosquitoes to infection varied significantly over the mosquito strains. Nearly 100% of *Oc. triseriatus* were susceptible to infection. The South China strain and *Ae. aegypti* were slightly less susceptible at 73% and 79%, respectively. All U.S strains of *Ae albopictus* had low susceptibility to infection; the greatest susceptibility rate was 36% (Scoles and Craig 1993). The variation in vector competence across populations may suggest that strains of *Ae. albopictus* have adapted to become heartworm vectors, and that susceptibility to infection may have a genetic basis (Nayar and Knight 1999).

Dirofilaria immitis in Humans

Human dirofilariasis occurs when a canine heartworm develops in or near the heart or within a branch of the pulmonary artery (Beaver and Orihel 1965). The latter case is known more specifically as pulmonary dirofilariasis (Faust 1957). The first case of pulmonary dirofilariasis was reported in 1939, when a male worm was recovered from the inferior vena cava of a 73-year old man. The worm was first identified as a new species, *Dirofilaria louisianensis*, but after reviewing of the case the parasite was re-identified as *D. immitis* (Faust 1957). A second case of human infection by *D. immitis* was reported in 1960 when the parasite was isolated from the pulmonary artery of a 40-year woman during an autopsy. The parasite was not the cause of death (Abadie et al. 1965). Approximately 150 cases of human infection of *D. immitis* have been reported worldwide (Echeverri et al. 1999) and the distribution of the disease throughout the United State closely follows that of canine heartworm disease (Ciferri 1982). Patients ranged in age from 28 to 77 years old and males were infected twice as often as females. Of the patients, 59% were asymptomatic and were diagnosed when a nodule, known as a coin lesion, was discovered in their lung during a routine chest radiograph. Patients experiencing symptoms of infection reported coughing, chest pain, fever, and malaise (Ciferri 1982).

Dirofilaria immitis in its Mammalian Hosts

Dirofilaria immitis is horizontally transmitted between mosquitoes and a vertebrate host. The parasite is not transmitted directly between vertebrates or between mosquitoes (Otto and Jachowshi 1980). Although canine and feline hosts are most commonly infected, over 30 species of wild and domestic animals can become infected

and serve as abnormal or accidental hosts for the parasite, including California sea lions, ferrets, otters, bears, horses, rabbits, and humans (Otto and Jachowshi 1980, Boreham and Atwell 1988). Domestic dogs are the primary reservoir for infection, but because red fox and coyotes have high infection rates in some areas, there is concern that they may also serve as reservoirs (Otto and Jachowshi 1980). In one experimental study performed in the heartworm endemic areas of the southeastern United States, 93% of heartworm-negative dogs became infected by natural exposure within one year (McTier et al. 1992a). In laboratory experiments, 60-80% of cats inoculated with *D. immitis* developed infections (McTier et al. 1992b).

Vertebrate hosts become infected during mosquito feeding. As an infected mosquito feeds on a dog, the L3 larvae are released inside a drop of hemolymph and are maintained within the droplet until the mosquito is finished feeding. The larvae, approximately 1 mm in length, enter the host by way of the puncture wound, migrate to the subcutaneous tissue surrounding the wound, and molt. Approximately three weeks after transmission a majority of the larvae can be found within the abdominal cavity of the host. The larvae molt to the 5^{th} instar and enter to the right side of the heart by penetrating the wall of a systemic vein. Within three to four months of transmission, all worms reach the right ventricle and the pulmonary blood vessel where they become sexually mature.

Within one year of reaching the heart, adults will be fully grown (AHS 2005). At maturity, the female worms reach an average length of 25 cm (10 inches), but can be as long as 30 cm (12 inches). Male worms are usually not longer than 15 cm (6 inches) (Otto 1969). Sex differentiation can be made in the third-stage larvae, and adults can

easily be distinguished because of the females' larger size and the corkscrew shaped tail of the male (Knight 1977). The lifespan of adult worms in dogs is at least five to seven years (AHS 2005).

Microfilaremia is the presence of heartworm offspring, or microfilariae, in the circulating blood of the host (AHS 2005). Adult female of heartworms produce eggs which are fertilized and develop in the uterus. Each embryo is contained with the vitelline membrane, which acts as a sheath and is shed before the microfilariae are discharged into the blood of the host (Otto 1969). These first-stage larvae survive in the bloodstream for as long as two years (Knight 1977).

Although the microfilariae are present in the circulating blood throughout the day, they display a daily periodicity (Knight 1977). The microfilariae can be five to ten times more prevalent and up to 50 times more prevalent in the blood during the early evening and night than in the morning (Otto 1969). Because heartworm infection is temperature dependent, transmission is seasonally limited in most part of the United States (Knight 2002). Mosquitoes require an average daily temperature of 64°F (18C) for ingested microfilariae to mature (HSP 1995). Heartworm transmission peaks during the months of July and August (HSP 1995), although the length of transmission depends on latitude (Knight 2002). A study performed on field-collected mosquitoes in Florida and Louisiana found that heartworms are not transmitted year-around, and that even in mild climates mosquitoes were only infected from April to November (Watts et al. 2001).

Microfilaremia occurs in a majority of infected dogs, however circulating microfilariae are not found in all cases (AHS 2005). An occult infection, when circulating microfilariae are not present, can result from single sex infection,

administration of heartworm preventives, or host immune responses against the microfilariae (AHS 2005).

Prevention, Diagnosis, and Treatment of *Dirofilaria immitis* infection

The symptoms of *D. immitis* infection are dependent on the number of filaria present, duration of infection, and the interaction between parasite and host (Calvert 1985). Heartworm infections are classified as early, mild, moderate or severe. In early infection, there are no abnormal clinical symptoms (AHS 2005). In mild cases, dogs show few physical symptoms and the disease is diagnosed by way of laboratory tests (Boreham and Atwell 1988). Moderate infections can be diagnosed clinically by symptoms including coughing, intolerance to exercise, weight loss, poor coat quality, and distended abdomen. These symptoms may not be a direct result of infection but a result of stress caused by the disease (Calvert 1985). In severe cases, infection can lead to right-sided congestive heart failure and respiratory disease (Boreham and Atwell 1988).

Infection of canine heartworms can be detected using several diagnostic procedures including: x-ray, ultrasound, blood testing, microfilarial detection, and clinical laboratory tests (AHS 2005). Detection of heartworm antigen, produced by female heartworms, in the blood has now replaced microfilariae testing as the primary test for diagnosis of infection (AHS 2005). Most antigen tests can accurately detect the presence of one or more mature female heartworm (AHS 2005). The earliest that heartworm antigen is detectable in the blood is approximately five months after infection; therefore testing in the late spring is likely to confirm infection from the preceding summer (Knight 2002).

Detection of microfilariae in the blood indicates infection of adult heartworms (AHS 2005). The modified Knott technique is sensitive to low concentrations of microfilariae and discriminates between *D. immitis* and other *Dirofilaria* species (Haddock 1987). Practitioners may also look for the presence of microfilariae in a blood smear. This method is not extremely accurate and therefore if larvae are not found in the sample, the possibility of infection cannot be completely ruled out. However, detection of microfilariae in the sample does confirm infection of adult worms (AHS 2005).

Heartworm infections may also be diagnosed by way of thoracic radiographs, where infection is typically indicated by enlarged peripheral branches of the pulmonary arteries (HSP 1995) or the right side of the heart (AHS 2005). Inflammation of the lung tissue, a sign of heartworm infection, can also be detected using radiographs (AHS 2005). Ultrasonography can be utilized to evaluate the heart and to detect the presence of heartworms in the right ventricle or pulmonary artery (AHS 2005).

Most dogs infected with heartworm can be successfully treated. Treatment is a two step process: use of an adulticide to kill mature heartworms and use of a larvicide to kill circulating microfiliariae (AHS 2005). Currently there is only one adulticide available for treating heartworm infection (Knight 2002). Melarsomine dihydrochloride, known commercially as Immiticide®, is an organoarsenic that is administered via intramuscular injection in two doses (2.5 mg/kg) given 24 hours apart (HSP 1995). Thromboembolism, blockage of a blood vessel by dead worms, is a common complication of adulticide treatment (Tanner et al. 1997) and in severe cases can cause fever, coughing, and even right-sided heart failure (HSP 1995). The complications due to

pulmonary thromboembolism can be reduced by administering anti-inflammatory steroids and limiting exercise (HSP 1995).

Currently there are no drugs approved for use as microfilaricides (AHS 2005). Although their use as microfilaricides has not been approved by the FDA, macrocyclic lactone anthelmintics are commonly used for that purpose (AHS 2005). The active ingredient in these drugs is the same used in heartworm preventives. Because macrocyclic lactone anthelmintics cause rapid death of the microfilariae, it is recommended that after treatment dogs are hospitalized for at least eight hours for observation for any adverse reactions (AHS 2005).

Prevention of heartworm infection has been transformed within the last decade by the introduction of monthly chemoprophylactic formulations (McTier et al. 2000). Preventive should be administered at least one month before the start of the transmission season and the last dose should be given one month after the transmission season has ended (HSP 1995). All dogs at least seven months of age should be tested for presence both of microfilariae and antigen before starting preventive (HSP 1995).

The major difference between preventive treatments are the method and frequency with which they are administered. Diethylcarbamazine citrate (DEC) is administered orally every day throughout the transmission season (Knight 2002). Drawbacks of DEC are that dogs must be microfilariae-free before use and clients must comply with the daily administration for the drug to be effective (AHS 2005). Macrocyclic lactones, such as ivermectin and milbemycin, are administered orally every month and may provide additional control for intestinal and external parasite activity (AHS 2005). Oral macrolide preventives administered during the transmission season are

effective in preventing adult heartworm infections, but studies have also shown that chronic administration of these products also affects larvae older than the age susceptible to a single treatment (Rawlings 2002).

In addition to oral chemoprophylaxis, topical formulations have proven effective in preventing heartworm development when applied 30 or 60 days after infection (McTier et al. 2000). Selamectin, also administered once a month, is applied topically (Knight 2002) and has intestinal and external parasiticide activity similar to other macrocyclic lactones (AHS 2005). A study was performed to test the efficiency of selamectin, sold commercially as Revolution®, when it was applied topically 30 and 60 days post-infection to experimentally infected dogs and cats (McTier et al. 2000). It was concluded that when administered at the recommended dosage (6 mg/kg) 30 days post-infection, selamectin was 100% effective in preventing heartworm infection in all animals infected. At 60 days post-infection, simulating a missed monthly treatment, selamectin remained completely effective (McTier et al. 2000).

A slow release injectable formulation of moxidectin provides six months of continuous protection against heartworm infection (Knight 2002). However in 2004, the U.S Food and Drug Administration requested a recall of moxidectin as an injectable heartworm preventive due to its adverse effects (FDA 2004).

No treatments have been approved for feline heartworm disease (AHS 2005).

CONCURRENT INFECTION OF FILARID NEMATODES AND VIRUS IN INSECT VECTORS

Introduction

In nature, vertebrates (including humans) infected with virus are often also infected with filarid worms (Vaughan and Turell 1996). In tropical regions where both

viral and filarial infections readily occur, the probability of a vertebrate to develop dual infection can be greater than 70% (Vaughan et al. 1999). It has been suggested that when mosquitoes are concurrently infected with a microfilariae and a virus, the microfilariae penetrate the midgut and allow the virus to bypass the midgut barrier, therefore enhancing the infectivity of the vector to the virus (Vaughan and Turell 1996, Vaughan et al. 1999, Turell et al. 1984, and Zytoon et al. 1993).

Co-infection of microfilariae and virus could have significant medical and veterinary effects by increasing vector competence of normally incompetent mosquitoes, reducing the amount of virus needed for a mosquito to become infectious, and decreasing the normal extrinsic incubation period (Vaughan and Turell 1996).

Culcioides nubeculosus - *Onchocerca cervicalis* – Bluetongue Virus

The first research regarding dual infection of microfilariae and virus was performed with *Culcicoides nubeculosus,* a biting midge (Mellor and Boorman 1980). *C. nubeculosus* do not develop a disseminated infection after oral ingestion of bluetongue virus (BTV), most likely due to the presence of a midgut infection barrier. However when the midges were intrathoracically inoculated with the virus, the barrier is bypassed and a disseminated infection did develop (Mellor and Boorman 1980).

Culcioides nubeculosus is a natural vector of the filarial parasite, *Onchocerca cervicalis*, and after the parasite is ingested, it penetrates the midgut and enters the hemocoel within two hours. It was hypothesized that dual infection of the *Onchocerca cervicalis* and BTV may affect the transmission of the virus because virus would be able to enter the hemocoel with penetrating microfilariae (Mellor and Boorman 1980). Midges were fed an infectious bloodmeal containing *O. cervicalis* and BTV via an

artificial membrane feeder. For comparison, a second group of midges were fed an infectious bloodmeal containing only BTV. A third group of midges were intrathoracically inoculated with virus (Mellor and Boorman 1980).

Mellor and Boorman (1980) found that within two hours of ingestion, the microfilariae had penetrated the midgut wall and entered the hemocoel. Even though the midges initially became infected with BTV, viral replication was not observed in either of the orally infected groups of midges. Because the volume of blood taken by *C. nubeculosus* was only 10^{-4} ml each female ingested an estimated one microfilaria during the bloodmeal, an amount probably insufficient to enhance viral infection (Mellor and Boorman 1980). However, this original research opened the door for other vector, virus, and parasite combinations to be studied.

Aedes taeniorhynchus – Brugia malayi – Rift Valley virus

Aedes taeniorhynchus were concurrently infected with *Brugia malayi* and Rift Valley virus (Turell et al. 1984). When mosquitoes ingested virus alone, 59% of them became infected. After 4-5 days 9% of these mosquitoes developed a disseminated infection and after 39 days of infection, 78% of the mosquitoes had a disseminated infection. However, of mosquitoes that ingested *B. malayi* and Rift Valley virus, 89% became infected with virus and after 4-5 days more than 50% of the mosquitoes developed a disseminated infection. It was concluded that mosquitoes infected with both microfilariae and virus have increased infectivity and also increased in viral dissemination and transmission rates (Turell et al. 1984).

Aedes aegypti – Brugia malayi – Dengue-2 virus

In Thailand *Ae. aegypti* is a competent vector of dengue-2 virus and *B. malayi* (Turell et al. 1987). In this study mosquitoes were infected with *B. malayi* and dengue-2 virus to test the dissemination and transmission rate of the virus. Although dual infection did not change the rate of viral transmission, the rate of dissemination did increase (Turell et al. 1987). Turell et al. (1987) also found that the dissemination rate of mosquitoes that were dually infected with microfilariae and virus was four times higher than those infected with virus alone, and the time it took the virus to disseminate was decreased by four days in coinfected mosquitoes. Turell et al. (1987) concluded that infection of both *B. malayi* and Dengue-2 virus increased the vector potential of *Ae. aegypti* for the virus.

Aedes species – *Brugia* species – Eastern Equine Encephalitis virus

Research was conducted to determine whether concurrent infection of two species of *Brugia* microfilaria and the virus that causes eastern equine encephalitis (EEE) would affect the transmission of virus in 3 species of *Aedes* mosquitoes (Vaughan and Turell 1996). Vaughan and Turell (1996) found a significant increase in viral dissemination in two of the species (*Ae. aegypti* and *Ae. taeniorhunchus*) that were dually infected compared to those infected only with virus. No significant difference in viral infection or dissemination was observed for *Ae. triseriatus*.

Dissemination of virus may depend on the concentration of microfilaria that penetrates the midgut. Vaughan and Turell (1996) showed that at high concentration of microfilaria, 6% of *Ae. triseriatus* developed a disseminated infection. At low microfilarial concentration, 8% of *Ae. triseriatus* became infected with virus, but none had a disseminated infection. Similarly, at high microfilarial concentration, 71%

mosquitoes were infected and 55% of *Aedes taeniorhynchus* developed a disseminated infection. At low microfilarial concentration, 35% became infected and only 17% developed disseminated infection (Vaughan and Turell 1996). Because increased dissemination rates were observed in 2 of the species tested but not in the third species, it is possible that the minimum infectious dose varies among mosquito species. They hypothesize that the amount of virus entering the hemocoel after the microfilariae have penetrated the midgut may vary among species and result in differences in the infectious dose. *Ae. taeniorhynchus* takes a larger bloodmeal than *Ae. triseriatus* so the midgut wall may become more stretched and tight. The penetrating microfilariae may cause larger holes or rips the midgut, allowing more virus to enter the hemocoel.

Aedes aegypti and *Ochlerotatus triseriatus – Brugia malayi* – Venezuelan Equine Encephalitis virus

Further experimentation using *Aedes* mosquitoes also showed differences of microfilarial enhancement of viral dissemination across the species. *Aedes aegypti, Oc. triseriatus*, and *Oc. taeniorhynchus* were concurrently infected with Venezuelan Equine Encephalitis virus (VEE) and *B. malayi* (Vaughan et al. 1999). Vaughan et al. (1999) found that in *Ae. aegypti* there was no significant effect on viral dissemination between dually infected mosquitoes and mosquitoes infected with virus alone. However in *Oc. triseriatus* between 11-22% of mosquitoes infected with VEE and *B. malayi* developed a disseminated infection compared to only 2% of the mosquitoes infected with VEE only. Also, in *Oc. taeniorhynchus* greater dissemination rates were seen in dually infected mosquitoes (between 50-61%) than in those infected with only VEE (0%).

Aedes albopictus – Dirofilaria immitis – Chickungunya virus

Ae. albopictus is a natural vector of Chickungunya virus and *Dirofilaria immitis* (Zytoon et al. 1993). Zytoon et al. (1993) determined that mosquitoes dually infected with Chickungunya virus and *D. immitis* have a significantly higher dissemination and transmission rate than those infected with virus only. In mosquitoes infected with both virus and *D. immitis*, infection and dissemination rates (39% and 18%, respectively) were higher than rates in mosquitoes mosquitoes infected with virus alone (6% and 0.6%).

Because high mortality rates have been reported in previous research with dually infected mosquitoes, the midgut of the mosquitoes was punctured artificially shortly after ingestion of virus to determine the amount of midgut injury the mosquito could withstand (Zytoon et al. 1993). Viral transmission rate increased for those mosquitoes with midgut punctures as compared with those without, and mortality rates increased proportionally to the number of punctures made. All mosquitoes with midgut punctures died by day 12, whereas the control mosquitoes survived beyond day 12.

RESEARCH OBJECTIVES

- To determine the effects of dual infection of La Crosse virus and *Dirofilaria immitis* on the dissemination and transmission of La Crosse virus by *Aedes albopictus* and *Ochlerotatus triseriatus*
- To determine the distribution and seasonal abundance of potential La Crosse virus and *Dirofilaria immitis* vectors in Southwestern Virginia

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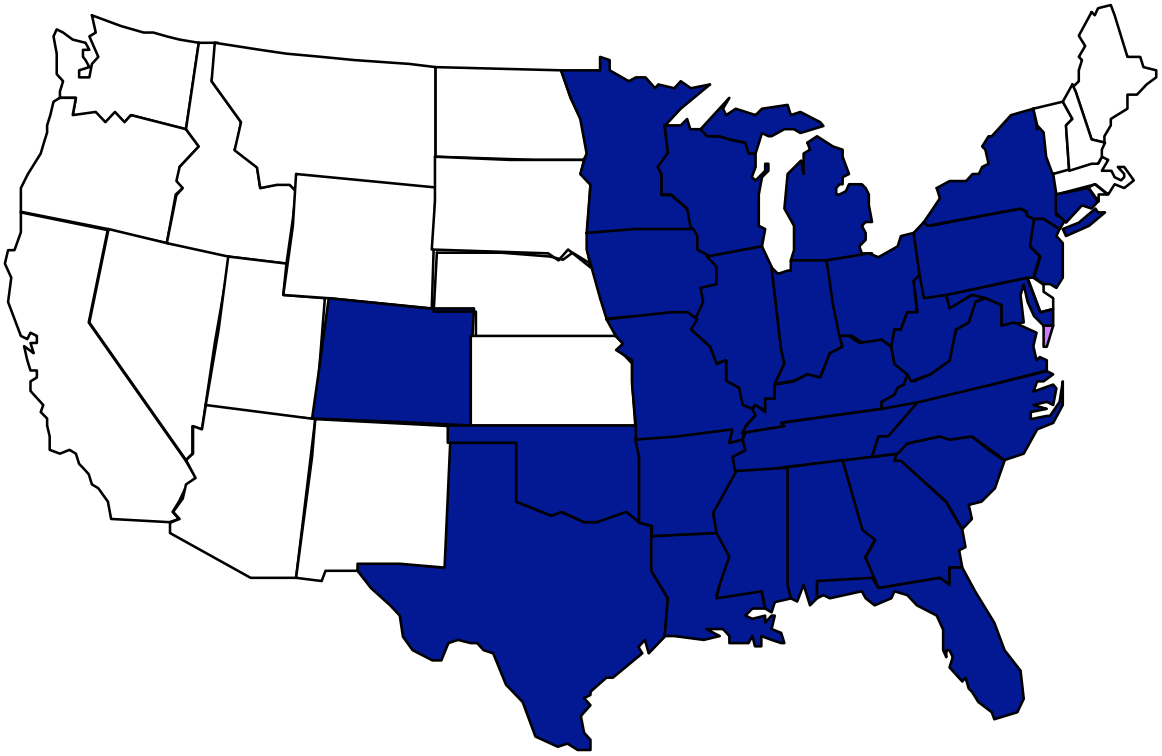
Fig. 1.1. Distribution of Confirmed LAC Virus Cases, 2003. As of 2003, LAC virus cases have been reported in 29 states.



Chapter 2

La Crosse virus and *Dirofiliaria immitis*: The Effects of Dual Infection on the Dissemination and Transmission of La Crosse virus by *Aedes albopictus* and *Ochlerotatus triseriatus*

Dual infection of an arbovirus and microfilariae enhances the infectivity of the virus to the insect vector (Mellor and Boorman 1980, Turell et al. 1984, Turell et al. 1987, Zytoon et al. 1993, Vaughan and Turell 1996, and Vaughan et al. 1999). Increased infectivity is a result of the virus particles escaping into the hemocoel through the punctures in the midgut created by the microfilariae (Zytoon et al. 1993, Vaughan and Turell 1996, and Vaughan et al. 1999), thus bypassing the need to infect and replicate in the midgut epithelium cells (Chamberlain and Sudia 1961). In *Aedes albopictus* experimentally infected with Chickungunya virus and *Dirofilaria immitis,* electron micrographs showed microfilariae migrating toward the outer wall of the midgut within minutes after being ingested and completely penetrating the midgut wall by four to six hours post-ingestion (Zytoon et al. 1993).

Because virus particles can pass into the hemocoel without first infecting the midgut cells, this can have a significant impact on vector potential. Less virus may be needed for a mosquito to become infected (Vaughan and Turell 1996), the extrinsic incubation period may be reduced (Turell et al. 1987), and vector competence of normally incompetent mosquitoes may be increased (Mellor and Boorman 1980, Turell et al. 1984, Zytoon et al. 1993).

Although a variety of microfiliaria, virus, and vector models have been used to study dual infection infection in the laboratory, only one of those models had realistic

implications. Turell et al. (1987) used dengue 2 virus, *Aedes aegypti*, a natural vector of the virus, and *Brugia malayi*, to determine the effects of dual infection. All of the components of this study occur together in Thailand. When a realistic model is chosen for experimentation, results can help to estimate the impact that dual infection might have in the field. Mosquitoes can take blood from multiple hosts in a single meal (Richards et al. 2006); therefore it is possible for a mosquito to become infected with multiple pathogens. The primary requirement for dual infection in nature is the presence of vertebrates that are both viremic and microfilaremic within the same area.

La Crosse (LAC) virus encephalitis is the most common mosquito-borne illness affecting children in the United States (McJunkin et al. 2001). LAC virus is prevalent in the Great Lake and Mid-Atlantic states (CDC 2003), and coincidently *Dirofilaria immitis,* the nematode that causes canine heartworm disease (Merial 2004), has also been reported in this region (Fig. 2.1). Exposure to mosquito vectors is most important factor influencing the rate of infection for both LAC virus and *D. immitis*. A majority of children who become infected with LAC virus live on farms or near wooded areas (Rust et al. 1999) and children who spend a great amount time outside are at greater risk of becoming infected than children who spend most of their time indoors (Erwin et al. 2002). Likewise, outdoor dogs are at greater risk of exposure to mosquitoes and of *D. immitis* infection than dogs that are kept inside (Haddock 1987).

The primary vector of LAC virus is the eastern treehole mosquito, *Ochlerotatus triseriatus* (Say) (Watts et al. 1972). *Aedes albopictus* (Skuse), the Asian tiger mosquito, is an important accessory vector for the virus (Beaty et al. 2000). Both of these mosquitoes are competent vectors of *D. immitis* in the lab and in the field (Intermill 1973,

Rogers and Newson 1979, Scoles and Craig 1993, Scoles and Dickson 1995). The purpose of these experiments was to determine the effects of dual infection of La Crosse virus and *Dirofilaria immitis* on the dissemination and transmission of La Crosse virus by *Ae. albopictus* and *Oc. triseriatus.*

Materials and Methods:

Mosquito strains and rearing: A laboratory colony of *Ae. albopictus* Sri Lanka strain was used. Eggs from this colony were provided by Notre Dame University, where a colony is maintained. *Ochlerotatus triseriatus* were derived from eggs collected in Montgomery County, VA in 2005.

The rearing of larvae and the maintenance of the adult mosquitoes was done at a controlled temperature of 75°F and 80% relative humidity with a photoperiod (16L:8D). Newly emerged adult mosquitoes were maintained on a diet of 10% sugar solution on moistened cotton balls.

Virus and Viral Assays: The La Crosse virus strain, isolated from *Ochlerotatus triseriatus* collected in Duncan Gap, VA in 1999, was used in this experiment after three laboratory passages on green African monkey kidney (VERO) cells (American Type Culture Collection, Manassas, Virginia). The titer of virus was 1.0×10^8 PFU/ml (plaque-forming units)

All specimens (mosquito bodies, legs and wings, and capillary tubes) were tested for viral infection using plaque assay on VERO cells as described by Gargan et al. (1983). The mosquito bodies were assayed to test for viral infection. The legs and wings were used to determine viral dissemination rate. The capillary tubes were assayed to test for viral transmission.

Microfilariae: Canine blood infected with *Dirofilariae immitis* was provided by Dr. John McCall at the University of Georgia School of Veterinary Medicine. Before each feeding, the microfilarial concentration of the blood was determined. The microfilariae in a wet smear of 1µl of blood were counted at 100x magnification. The average microfilarial density was 77,500 mf/ml (± 24,200 mf/ml) of blood.

Laboratory infection of mosquitoes: Five- to six-day-old mosquitoes were starved for three days prior to feeding. Mosquitoes were fed using a membrane feeding apparatus covered with a Baundruche membrane (Joseph Long, Inc. Belleview, NJ) according to the methods of Rutledge et al. (1964). Canine blood treated with EDTA was mixed with *D. immitis* infected canine blood and/or La Crosse virus for a final concentration of approximately 27,000 mf/ml of blood and 1.0×10^7 PFU/ml, respectively. Each mosquito was expected to acquire a bloodmeal of 2.0×10^4 PFU of virus and 54 microfilariae, assuming that the mean bloodmeal was 2 µl of blood (Zytoon et al. 1993).

Mosquitoes were divided into two groups that were fed via membrane feeding apparatus until fully engorged. The experimental group of mosquitoes ingested an infectious blood meal containing both *D. immitis* and La Crosse virus. The control group of mosquitoes was composed of mosquitoes fed an infectious blood meal containing only La Crosse virus.

Fully engorged mosquitoes were transferred to other cages and maintained on a diet of 10% sucrose solution.

Percent Survival: Mosquitoes were fed blood with a microfilarial density of approximately 13,500, 27,000, 37,800, 45,900, or 54,000 mf/ml. All densities were made by diluting microfilaremic blood with uninfected canine blood. Control groups were fed

uninfected canine blood. Fully engorged females were quantified and moved to a separate cage. The number of mosquitoes surviving at 14 d post infection (p.i.) were counted.

Detection of *D. immits* Infection: On 7 d p.i., five mosquitoes were removed and dissected to examine for the presence of *D. immitis*.

Detection of Viral Transmission: Approximately 20 *Ae. albopictus* were removed from each group on day 10, 14, and 21 p.i. For *Oc. triseriatus*, transmission was only tested on day 10 and 14 p.i. The legs and wings of each mosquito were removed and frozen at -80°C. Viral transmission was tested by way of capillary tube feeding according to the methods of Boromisa et al. (1987). The mosquitoes were allowed to feed for 45 minutes and before being removed from the capillary tube, the tube was examined for signs of salivation. Then the body of the mosquito and the tube were frozen at -80°C.

Statistical analysis: Rates of viral infection, dissemination, and transmission were compared among groups using chi-square or Fisher's exact analyses depending on the size of the cells (SAS Institute, 2004). Probit analyses from computing LD_{50} values were performed using the software package, PoloPlus. A significant value of 0.05 was used throughout.

Results:

Survival rates of *Aedes albopictus* and *Ochlerotatus triseriatus* 14 d after the ingestion of *D. immtis* infectious-blood containing microfilariae levels from 13,500 to 54,000 mf/ml ranged from 0-84% and 0-69%, respectively (Table 2.1). Both species had 100% mortality at 14 d after ingesting a bloodmeal containing 54,000 mf/ml. The

survival rates of *Ae. albopictus* and *Oc. triseriatus* varied significantly. The LD_{50} values (95% confidence intervals) for *Ae. albopictus* and *Oc. triseriatus* were 43,572 mf/ml (40,376 – 47,790 mf/mf) and 34,020 ml/mf (22,734 – 38,556 mf/ml), respectively. A majority of the mortality occur by day 5 post ingestion of the infection bloodmeal.

At a rate of 27,000 mf/ml, 80% of *Ae. albopictus* and 70% of *Oc. triseriatus* became infected, however only 50% of *Ae. albopictus* and 20% of *Oc. triseriatus* became infected after ingesting a bloodmeal containing 13,500 mf/ml. A microfiliarial concentration of 27,000 mf/ml was used throughout the rest of the experiment for maximal infection and survival.

Viral infection, dissemination, and transmission rates of *Ae. albopictus* were compared (Table 2.2). Infection rates of the experimental and control groups were not significantly different. Disseminated infection was detected on 2 d p.i. in both the dually infected mosquitoes and control group. Although there were no significant differences in the viral dissemination rates between the dually infected mosquitoes when compared with the dissemination rates of the control group, the rates of the dually infected were greater for every day tested, except day 7.

On all days tested, transmission by experimental group was greater than that of the mosquitoes that ingested virus alone. The transmission rate of the dually infected mosquitoes continued to increase and was significantly higher on 14 d p.i. (χ-value = 5.62, df = 1, p-value = 0.018) on 14 d p.i. (28%) than the control group (4%). The total transmission by the dually infected mosquitoes was also significantly higher (χ-value = 7.06, df = 1, p-value = 0.008) compared to the control group.

Viral infection, dissemination, and transmission rates of *Oc. triseriatus* were compared (Table 2.3). Dissemination rate of the dually infected mosquitoes was not significantly different than that of the control group. Interestingly, dissemination was greater for the control group on 10 d p.i. (30%) compared to the dually infected mosquitoes (25%). On day 14 the dissemination rate of the dually infected mosquitoes increased (40%), while the rate of the control group stayed constant (30%). The viral transmission rates were not significantly different, however on both day 10 and 14, the transmission rate was greater for the dually infected group (10% and 5%) than the control mosquitoes (0% and 5%).

Discussion:

Microfilarial enhancement of viral transmission is dose dependent (Vaughan et al. 1999) meaning the greater concentration of microfilariae ingested the greater potential for enhancement of viral transmission. Microfilarial induced mortality is also correlated to a mosquito's filarial burden (Ibrahim and Trpis 1987). A naturally infected mosquito generally ingests less than 10 microfilariae, but infections of 50 or more larvae are not uncommon (Otto 1969), but tolerance to infection can vary among species.

In this experiment, both tolerance to microfilarial infection and microfilarial enhancement of viral transmission varied between the species tested. *Oc. triseriatus* had low tolerance to microfilarial infection and no enhancement of dissemination or transmission for dually infected mosquitoes. *Ae. albopictus* had high tolerance to microfilarial infection and significant increase of transmission for dually infected mosquitoes.

Infection, dissemination, and transmission rates for even the control group of mosquitoes were relatively low compared to previous studies. Paulson and Grimstad (1989) found that 100% of La Crosse virus-infected *Oc. triseriatus* became infected and 60-70% of those mosquitoes developed a disseminated infection compared to only 30% in this study. On day 21 p.i. 71% of La Crosse virus-fed *Oc. triseriatus* were able to transmit the virus to sucking mice (Paulson and Grimstad 1989) compared to only 5% of control mosquitoes on day 14 p.i. in our experiment. Likewise, 45.0% and 42.5% of *Ae. albopictus* could transmit the virus 14 d and 21 d after ingesting a bloodmeal containing La Crosse virus (Grimstad et al. 1989), compared to 4% on day 14 p.i. and 20% on day 21 p.i in this study.

Susceptibility to viral infection can vary among strains of the same species. Grimstad et al. (1977) reported significant variation in susceptibility to La Crosse virus infection by geographic strains of *Oc. triseriatus*. Horizontal and transovarial transmission rates of La Crosse virus also differ among strains of *Oc. triseriatus* (Grimstad et al. 1977, Miller et al. 1982). Watts et al. (1973) found that La Crosse virus transmission rate by field collected *Oc. triseriatus* were lower than those observed with laboratory strains. Similarly, differences have been observed in the susceptibility of strains of *Ae. albopictus* to viral infection (Tesh et al. 1976, Boromisa et al. 1987).

Vaughan et al. (1999) point out that an important epidemiological consequence of dual infections is the infectious dose of virus required for a mosquito to become infected may be reduced. In nature if less virus is needed for a mosquito to become infected, a host that would normally be considered incompetent may become a competent reservoir of the virus when dually infected with microfilariae. In many areas where LAC virus is

endemic, rate of *D. immitis* infection of dogs is also high (Fig. 1). Given the chronic nature of *D. immitis* infections, we would expect that dually infected dogs would not be uncommon. Mosquitoes readily feed on dogs. In field studies in North Carolina, of the blood fed mosquitoes collected, approximately 40% of *Oc. triseriatus* and 14% of *Ae. albopictus* had fed on dogs (Szumlas et al. 1996, Richards et al. 2006). However, Godsey et al. (1988) had concluded, based on laboratory testing of virus transmission, that dogs are not an efficient amplifier species of LAC virus. But if coinfection with microfilariae can lower the minimum infectious dose of virus needed to infect mosquitoes, then dually infected dogs may become more competent reservoir hosts. We suggest further experimentation to determine if the infectious dose of La Crosse virus required for a mosquito to become infected is reduced for dually infected mosquitoes.

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Figure 2.1. Distribution of La Crosse virus and *Dirofilaria immits* cases reported in the United States. La Crosse virus cases are all those reported through 2006. *Dirofilaria immitis* cases have been reported in all 50 states.

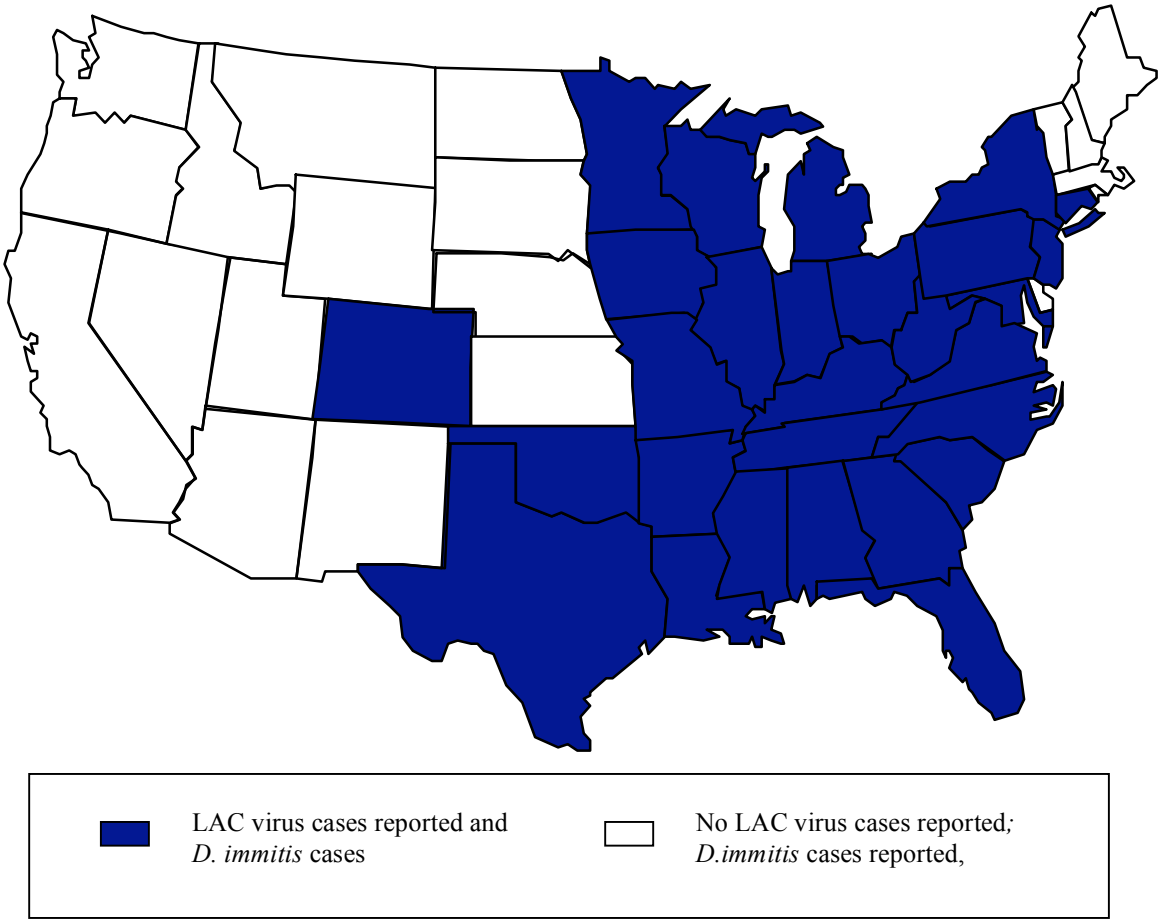

Table 2.1. Survival and infection rates of *Aedes albopictus* and *Ochlerotatus triseriatus* fed *Dirofilaria immitis* infectious-blood containing different densities of microfilariae (mf)

Species	Bloodmeal (mf/ml)	No. bloodfed	Survival rate[#]	Transmission rate[#]
Ae. albopictus	0	34	91	0
	13,500	51	84	50
	27,000	45	82	80
	37,800	52	63	ND
	45,900	29	38	ND
	54,000	33	0	ND
Oc. triseriatus	0	45	69	0
	13,500	22	64	20
	27,000	26	50	70
	37,800	28	25	ND
	45,900	25	20	ND
	54,000	32	0	ND

# = determined 14 d post infection
ND = not determined

Table 2.2. Infection, dissemination, and transmission rates of La Crosse virus in *Aedes albopictus* after ingestion of infected blood

	Virus plus microfilaria			Virus alone		
Days after infection	Infection rate (%)	Dissemination rate (%)	Transmission rate (%)	Infection rate (%)	Dissemination rate (%)	Transmission rate (%)
2	10/15 (67)	4/15 (27)	ND	8/14 (57)	1/14 (7)	ND
5	13/15 (87)	7/15 (47)	ND	9/15 (60)	5/15 (33)	ND
7	10/15 (67)	7/15 (47)	ND	11/15 (73)	8/15 (53)	ND
10	17/20 (85)	12/20 (60)	1/20 (5)	14/20 (70)	10/20 (50)	0/20 (0)
14	19/25 (76)	15/25 (60)	7/25 (28)*	13/26 (50)	12/26 (46)	1/26 (4)
21	12/20 (60)	11/20 (55)	8/20 (40)	12/20 (60)	9/20 (45)	4/20 (20)
Total	81/110 (74)	56/110 (51)	16/65 (25)*	67/110 (61)	45/110 (41)	5/66 (8)

* denotes significant difference
ND = not determined

Table 2.3. Infection, dissemination, and transmission rates of La Crosse virus by *Ochlerotatus triseriatus* after ingestion of infectious blood

	Virus plus microfilaria			Virus alone		
Days after infection	Infection rate (percent)	Dissemination rate (percent)	Transmission rate (percent)	Infection rate (percent)	Dissemination rate (percent)	Transmission rate (percent)
10	10/20 (50)	5/20 (25)	2/20 (10)	12/20 (60)	6/20 (30)	0/20 (0)
14	15/20 (75)	8/20 (40)	1/20 (5)	12/20 (60)	6/20 (30)	1/20 (5)
Total	25/40 (63)	13/40 (33)	3/40 (8)	24/40 (60)	12/40 (30)	1/40 (3)

Chapter 3

Abundance and Bionomics of *Ochlerotatus japonicus* (Diptera: Culicidae) in Two Counties in Southwestern Virginia

The first report of *Ochlerotatus japonicus* in the United States was the collection of four adult females in New York and New Jersey in 1998 (Peyton et al. 1999). The species has since been found in Connecticut (Andreadis et al. 2001), Maryland (Scott et al. 2001), Maine (Foss and Dearborn 2001), Ohio (Fonseca et al. 2001), Virginia (Harrison et al. 2002), Washington (Roppo et al. 2004), West Virginia (Joy 2004), North Carolina and Georgia (Gray et al. 2005), Tennessee (Caldwell et al. 2005) and Missouri (Gallitano et al. 2005). Because the populations of *Oc. japonicus* in the United States differ genetically, the geographic spread of the species most likely resulted from multiple introductions (Fonseca et al. 2001).

Expansion of *Oc. japonicus* into virus endemic areas has raised serious concern about its vector potential. In laboratory studies, *Oc. japonicus* was shown to be competent vector of West Nile virus and transmitted the virus more efficiently than the primary vector, *Culex pipens* (Sardelis and Turell 2001). West Nile Virus has been isolated from field-collected *Oc. japonicus* every year since 2000 (CDC 2005). Also, *Oc. japonicus* was shown to be susceptible to oral infection by La Crosse virus and to subsequently transmit virus to suckling mice at a rate similar to *Oc. triseriatus* (Sardelis et al. 2002).

Ochlerotatus japonicus was first collected in Virginia in 2000, when three male and two female larvae were collected in Prince William County, Virginia (Harrison et al. 2002). During the summer of 2002, surveillance for West Nile virus vectors was done in

the Montgomery, Pulaski, Roanoke, and Giles counties in southwestern Virginia using CO_2-baited light traps and hay-infused gravid traps. *Oc. japonicus* was found at only one site; one adult female was caught in a gravid trap in Roanoke, Virginia on 9 August 2002 and a second female was trapped at the same location the following week. The purpose of this study was to demonstrate the increasing abundance and seasonal distribution of *Oc. japonicus* in southwest Virginia.

Materials and Methods:

Location: Mosquito collections were done in Montgomery and Pulaski counties in the New River Valley (NRV) of southwestern Virginia. The valley, between the Appalachian and Blue Ridge Mountains, has an elevation ranging from 535 m to 750 m, and 50-60% forest cover of oak-hickory (Johnson 1992). Average summer (June, July, and August) temperature and rainfall are 20.8ºC and 29 cm (SRCC 2005).

Sampling techniques: In 2003, mosquito trapping was conducted from June through August. In 2004, trapping began in June and continued through September. In both years, sampling was done at a total of six sites in Montgomery and Pulaski Counties (Fig. 3.1) that included private residences, recreational parks, private educational facilities, and university-managed farmland.

Adult mosquitoes were collected in gravid traps (Hausherr's Machine Works, Toms River, NJ). In 2003, two different infusions were used to bait the traps: a hay infusion prepared according to the methods of Reiter (1986) and a cow manure infusion made by mixing 550 ml (2.35 cups) of cow manure, from a dairy cow manure retention pond, with 3.24 liter (0.85 gal) of warm tap water. In 2004, all traps were baited with the hay infusion. The traps were placed at the sites in the afternoon and checked the following

morning. Adult mosquitoes were collected from the traps with a hand held aspirator (Hausherr's Machine Works, Toms River, NJ), and placed in vials labeled with date, site, and infusion. All vials were placed on ice in an ice chest to keep the mosquitoes inactive and cool. In the lab, the adults were stored in a –20 °C freezer for 24 hours. Male mosquitoes were discarded and all females were sorted and identified using the keys and descriptions of Slaff and Apperson (1989) and Darsie (2002). *Culex pipens* and *Culex restuans* were pooled together into a group called *Culex pipiens-restuans*.

Statistical Analysis: Fisher's LSD was calculated to determine significant differences in abundance by month of the major mosquito species in 2004 (SAS Institute).

Results:

In 2003, 5,879 mosquitoes were collected over the course of 192 trap nights from June through August. The most abundant species were *Cx. pipiens-restuans* (93.5%), *Ochlerotatus* triseriatus (4.1%), and *Aedes albopictus* (2%) (Fig. 3.2). A total of 24 *Oc. japonicus* were collected, all but one individual in August (Table 3.1). *Oc. japonicus* were collected at five of six trap sites.

In 2004, a total of 12,151 mosquitoes were trapped over 160 trap nights. *Oc. japonicus* was collected at all six sites and comprised 16.8% of the total catch. Other major species trapped were *Cx. pipiens-restuans* (70.7%), *Oc. triseriatus* (8.7%), and *Ae. albopictus* (3.3%).

The seasonal pattern of abundance for each of the major mosquito species differed significantly by month (Table 3.2); *Cx. pipiens-restuans* (F-value = 4.68, df = 3, p-value = 0.0360), *Oc. japonicus* (F-value = 4.78, df = 3, p-value = 0.0342), *Oc. triseriatus* (F-

value = 4.52, df = 3, p-value = 0.0391), *Ae. albopictus* (F-value = 4.52, df = 3, p-value = 0.0391).

Both *Cx. pipiens-restuans* and *Oc. triseriatus* were collected in significantly higher numbers in July when compared to the rest of the season. *Ae. albopictus* abundance peaked in August. After June, the abundance of *Oc. japonicus* increased significantly and stayed consistent throughout the rest of the season.

Discussion:

Oc. japonicus has rapidly become established in southwest Virginia, going from rare in 2002 to the second most abundant mosquito and the dominant *Ochlerotatus* species collected in gravid traps in 2004. Choice of trap was fortuitous because gravid traps had originally been employed to survey *Culex* species as part of West Nile virus surveillance. However, recent studies have shown that gravid traps are an effective method for monitoring *Oc. japonicus* (Andreadis et al. 2001, Falco et al. 2002, Scott et al. 2001). Other traps vary in their selectivity; CDC light traps collect fewer *Oc. japonicus* females than gravid traps (Andreadis et al. 2001, Scott et al. 2001) and *Oc. triseriatus* is more abundant than *Oc. japonicus* in oviposition cups (Paulson et al., unpublished data).

Oc. japonicus shares many biological similarities with another invasive mosquito, *Ae. albopictus*, both of which were imported to the U.S. from Asia, presumably a result of the used tire trade (Hawley et al. 1987, Peyton et al. 1999). Both species utilize a wide range of natural and artificial container habitats, lay desiccation-resistant eggs, and overwinter as diapausing eggs in the more northern parts of their distributions (Hawley 1988, Lounibos 2002). Also, both are daytime feeders and feed on a variety of hosts including humans (Hawley 1988, Andreadis et al. 2001). Since their original

introductions into the United States, both species have increased their geographic range rapidly. *Ae. albopictus* is most abundant is the southeastern US (Moore 1999). Its range is limited by climatic factors, with winter temperature determining the northern overwintering boundary (Nawrocki and Hawley 1987) and rainfall likely restricting western expansion (Washburn and Hartmann 1992). The geographic range of *Oc. japonicus* in North America continues to expand and is being closely monitored, however, it is likely to colonize more northern locations than *Ae. albopictus*. In Japan, the northern limit for *Ae albopictus* is central Honshu Island, which corresponds with the 0^{o}C isotherm (Nawrocki and Hawley 1987), but *Oc. japonicus* is found on northern Honshu Island and Hokkaido Island (Tanaka et al. 1979). Therefore, *Oc. japonicus* is probably more cold hardy than *Ae. albopictus*; reports of *Oc. japonicus* from Maine (Foss and Dearborn 2001), Vermont (Graham and Turmel, personal communication), and Ontario (Theilman and Hunter 2006) support this hypothesis At present, Georgia represents the southern-most record for *Oc. japonicus* in the U.S. (Gray et al. 2005).

Oc. japonicus and *Ae. albopictus* share a similar seasonal pattern. In this study, *Oc. japonicus* adults were collected at significantly lower rates during June and the abundance increased in July and stayed constant throughout September. Other researchers also reported that *Oc. japonicus* adults were most abundant late in the trap season. In Connecticut, the population peaked in September (Andreadis et al. 2001) and in New York, *Oc. japonicus* abundance peaked in mid-August (Falco et al. 2002). Of the other major species collected in this study, only *Ae. albopictus* exhibited a similar seasonal pattern as *Oc. japonicus* (Fig. 2). *Ae. albopictus* were trapped in the highest numbers in August, whereas *Oc. triseriatus* and *Cx. pipiens-restuans* were significantly

more abundant earlier in the trap season. Similar results were reported in previous studies. Late season abundance was also seen in *Ae. albopictus* when larvae (Joy 2004) or eggs (Barker et al. 2003) were collected.

Because the native vector of the La Crosse virus, *Oc. triseriatus*, must compete with these exotic species for food and habitats, they could have a significant impact on the transmission cycle of La Crosse (LAC) virus. *Ae. albopictus* is considered an accessory LAC vector and has been shown to be a competent vector of the virus in laboratory (Grimstad et al. 1989; Cully et al. 1992). LAC virus has been isolated from *Ae. albopictu*s eggs in Tennessee and North Carolina (Gerhardt et al. 2001), indicating transovarial transmission, and from adults in Virginia (Paulson, unpublished data).

Although LAC virus has not been isolated from *Oc. japonicus* in the field, the species also has been shown to be a competent vector of the virus in the laboratory (Sardelis et al. 2002). Both its high abundance and its ability to adapt to a variety of habitat types increase the likelihood of this species to be in contact with virus reservoir species. LAC virus-infected *Ae. albopictus* were first collected in the field approximately 15 years after the initial introduction of the species to the U.S. Thus, it may take years for an introduced species to become established in the natural cycle of the virus. We should continue to monitor the spread of *Oc. japonicus* and evaluate its potential as a vector of LAC virus and other arboviruses.

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Figure 3.1. Map of 2002 and 2003 trap sites

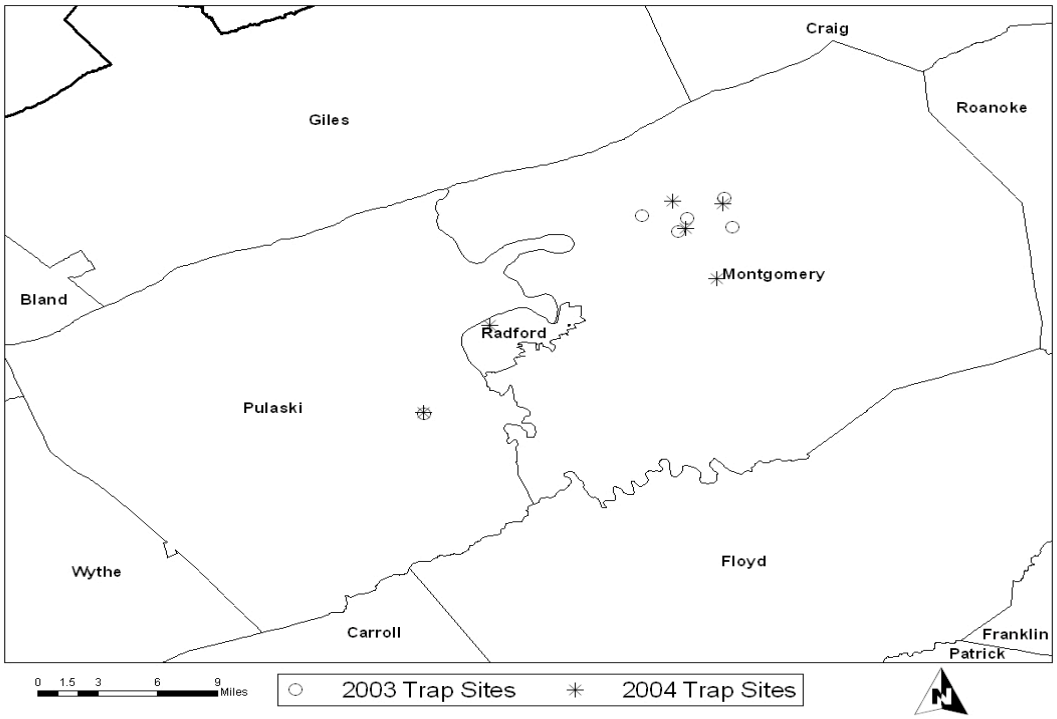

Fig. 3.2. Comparative abundance of mosquito species collected in Montgomery and Pulaski Counties, 2003 and 2004

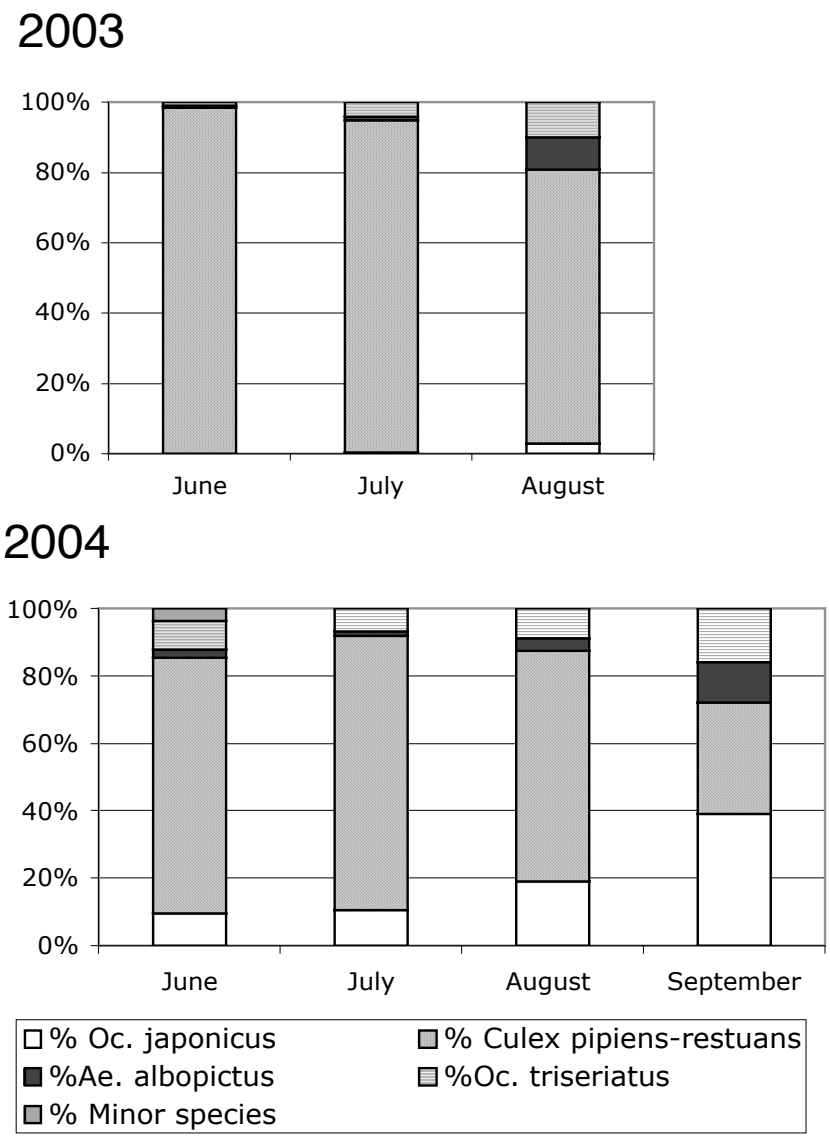

Table 3.1. Monthly abundance of *Oc. japonicus* in Montgomery and Pulaski Counties, 2003

Week	Number of trap nights[1]	Number of sites positive (percent)	Total collected
June 11 - June 13	32	0/6 (0)	0
June 17 - June 19	32	0/6 (0)	0
July 8 - July 10	32	1/6 (17)	1
July 15 - July 17	32	0/6 (0)	0
August 5 - August 7	32	3/6 (50)	17
August 12 - August14	32	3/6 (50)	6

[1]Two gravid traps were placed at each of six locations.

Table 3.2. Mean (± standard deviation) monthly abundance of major mosquito species caught per trap in Montgomery and Pulaski Counties, 2004.

Species	June (40 trap nights)	July (40 trap nights)	August (50 trap nights)	September (30 trap nights)
Cx. pipiens-restuans	34.8 ± 29.5	104.9 ± 41.6*	51.6 ± 28.7	13.9 ± 6.1
Oc. japonicus	4.2 ± 3.0*	13.9 ± 5.9	15.9 ± 6.4	17.7 ± 5.2
Oc. triseriatus	5.3 ± 1.7	11.0 ± 4.9*	2.9 ± 0.5	1.3 ± 0.1
Ae. albopictus	0.8 ± 0.4	1.3 ± 0.5	2.9 ± 1.0*	1.3 ± 1.3

***** Indicates value significantly different from others within a row

Vitae

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Devin attended Virginia Polytechnic Institute and State University and earned a Bachelor of Science in biology, with a minor in chemistry in 2003. She remained at Virginia Tech to pursue her Master's degree in entomology.