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Title of Dissertation: Potential for *Aedes albopictus* and *Ochlerotatus j. japonicus* to change the field ecology of arboviruses of human health importance in the mid-Atlantic region of the United States

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ABSTRACT

Potential for *Aedes albopictus* and *Ochlerotatus j. japonicus* to change the field ecology of arboviruses of human health importance in the mid-Atlantic region of the United States

Michael R. Sardelis, Doctor of Philosophy, 2001

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Aedes albopictus and *Ochlerotatus j. japonicus* are mosquitoes that have been recently introduced into the United States. Since their introduction, they have been implicated in the transmission of one or more of the arboviruses of principal public health importance in the mid-Atlantic region. To more fully understand the potential of *Ae. albopictus* and *Oc. j. japonicus* to be vectors of endemic arboviruses, field and laboratory studies were conducted to assess their distribution in the region and to determine their vector competence for arboviruses for which data are lacking.

Aedes albopictus, a mosquito found in virtually all counties of states in the southeastern United States, was found to be established as far north as south-central Pennsylvania. *Ochlerotatus j. japonicus*, a mosquito that is generally found in more northern climates within its native range, was found to be established as far south as Maryland, and its relative abundance in Frederick County, Maryland, was found to be comparable to or greater than that of other container-inhabiting mosquito species.

Laboratory studies showed that *Oc. j. japonicus* is a competent vector of West Nile (WN), St. Louis encephalitis (SLE), eastern equine encephalitis (EEE), and La Crosse (LAC) viruses. Estimated transmission rates for these viruses by *Oc. j. japonicus*

and by known principal or suspected vector mosquito species were determined concurrently for comparison. Based on these comparisons, *Oc. j. japonicus* was a more efficient laboratory vector of WN virus than *Culex quinquefasciatus*, as efficient as *Culex pipiens* in transmitting SLE virus, a less efficient laboratory vector of EEE virus than *Ae. albopictus*, and as efficient as *Aedes triseriatus* in transmitting LAC virus. Additional studies indicated that the extrinsic incubation period (EIP) for WN and EEE virus in *Oc. j. japonicus* held at 26^{o}C was between 7-11 days and around 5 days for, respectively.

Experimental transmission studies showed that North American (NA) strains of *Ae. albopictus* were competent vectors of WN virus. Transmission rates varied among the NA strains tested, ranging from 36-92%. The EIP for WN virus in *Ae. albopictus* held at 26^{o}C was estimated to be 10 days. In a study to evaluate vertical transmission, no virus was recovered from >12,000 F_1 progeny.

This study provided key information for implicating *Ae. albopictus* and *Oc. j. japonicus* as vectors of arboviruses of public health importance. These data, combined with the behavioral and other biological characteristics of *Ae. albopictus* and *Oc. j japonicus*, indicate that these two introduced species could change the field ecology of arboviruses of human health importance in the mid-Atlantic region of the United States.

Potential for *Aedes albopictus* and *Ochlerotatus j. japonicus* to change the field ecology of arboviruses of human health importance in the mid-Atlantic region of the United States

by

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Table of Contents

List of Tables

List of Figures

Chapter 1

General introduction

INTRODUCTION

The introduction of nonnative mosquito species into the United States may result in severe, irreversible impacts on the environment, economy, and human quality of life and health. Biodiversity in an area may be affected, such as in the case in which the newly introduced mosquito species leads to the local elimination of indigenous mosquito species from shared breeding sites (Livdahl and Willey 1991, Hobbs et al. 1991). Additional funds may be needed to enforce legislation intended to stop future introductions and the spread of the invader species, and for mosquito abatement programs to control pestiferous species and potential disease vectors. When the distribution of a nonnative mosquito species, a pathogen, and susceptible hosts come together, there is the potential for future increased transmission of the pathogen in nature, to include possible transmission to humans. For example, two well-known mosquito species have had a significant impact on human health in areas where they were introduced. *Anopheles gambiae* Giles, a highly efficient malaria vector in Africa, was discovered in Brazil in the early 1930 s, having been transported there from Senegal, and subsequently deemed responsible for a large malaria epidemic in Northeast Brazil in 1938/39 (Soper and Wilson 1942). Likewise, the yellow fever mosquito, *Aedes aegypti* (L.), is thought to be native to Africa and at some unknown time brought to the Americas (Soper 1967, Strode 1951). This mosquito s impact on human health through the spread of yellow fever and dengue in the Americas has historical significance and is still a problem today (Pan American Health Organization 1997), despite extensive efforts to eradicate it (Camargo 1967).

Aedes albopictus (Skuse) and *Ochlerotatus j. japonicus* (Theobald) are nonnative mosquito species that are currently raising public health concern in the United States. Some news outlet headlines regarding these species include: Mosquito That Can Carry Dengue Fever Lands in L.A. (Surendran and Harvey 2001), Potentially Dangerous Mosquito Found In Portland (Channel 6000 2001), Asian Tigers Stalking Victims All Over County (Rein 1999), Asian Tiger Mosquito Spreads Trouble (Saulny 1999), New Mosquito Could Spread Virus, Experts Say (The New York Times 2000). Do these eye-catching headlines merit our concern? To assess the potential impact of *Ae. albopictus* and *Oc. j. japonicus* on human health, one must consider the distribution, behavior and other biological characteristics of these mosquitoes, what arboviruses of public health concern are in the region, and field and laboratory evidence that may incriminate these mosquitoes as vectors.

Aedes albopictus distribution and bionomics:

Established populations of *Ae. albopictus* were found in the United States in 1985, having been discovered in Houston, Texas [Centers for Disease Control and Prevention (CDC) 1986, Sprenger and Wuithiranyagool 1986]. Since that time, it has spread from Texas and up the eastern seaboard, where it now has become established as far north as Monmouth County, New Jersey (Crans et al. 1996). Moore (1999) reported that the *Ae. albopictus* has been found in 26 states, mainly in the eastern half of the United States (Figure 1). It has been reported from a few counties in each of the mid-Atlantic States of Virginia, Maryland, Delaware, Pennsylvania, and New Jersey. The northern limit of *Ae. albopictus* based on mean January temperatures has been estimated by Nawrocki and

Hawley (1987) to follow the 0°C isotherm, which puts its northern extent at about the northern half of New Jersey.

The bionomics of *Ae. albopictus* in the United States is well documented (see reviews Hawley 1988, Moore and Mitchell 1997). Its immature stages inhabit natural and artificial containers that are generally found in wooded areas. Populations of egg-laying *Ae. albopictus* in South Carolina increase steadily in June, peaking in the middle of July (Richardson et al. 1995), while the abundance of host-seeking *Ae. albopictus* in southwestern Louisiana is highest during July and August (Willis and Nasci 1994). Female *Ae. albopictus* are active during the daytime (Hawley 1988), and feed on a variety of mammals, to include man and birds (Tempelis et al. 1970, Sullivan et al. 1971). Relatively recent studies of host-seeking patterns of *Ae. albopictus* in the United States found that 3-16% of its blood meals are from birds (Niebylski et al 1994, Savage et al. 1993). The flight range of *Ae. albopictus* is relatively short, approximately a few hundred meters (Bonnet and Worcester 1946, Rosen et al. 1976).

***Ochlerotatus j. japonicus* distribution and bionomics:**

In the late summer of 1998, *Oc. j. japonicus* was found for the first time in the United States in New Jersey and New York (Peyton et al. 1999). This mosquito is native to Japan, Korea, Taiwan, and southern China (Tanaka et al. 1979). Little is known about the expanding distribution of *Oc. j. japonicus* in the United States. In 1999, *Oc. j. japonicus* was reported in Massachusetts, Connecticut, and Pennsylvania. In June 2000, it was discovered for the first time south of the Mason-Dixon Line, in Maryland, and then found a few months later in Virginia.

The bionomics of *Oc. j. japonicus* in the United States is currently unstudied. Within its native range, it is known to overwinter in the egg and larval stage and inhabit a wide range of natural and artificial containers (Tanaka et al. 1979). *Ochlerotatus j. japonicus* is generally found in association with wooded areas, and active primarily during the daytime (Tanaka et al. 1979). Little is known about the feeding preference of *Oc. j. japonicus* in the wild. It has been reported to bite humans as well as birds in Japan (Tanaka et al. 1979). In the laboratory, *Oc. j. japonicus* readily fed on birds and mice (Miyagi 1972). There have been no studies on the dispersal pattern or flight range of *Oc. j. japonicus*.

Arboviruses of human health importance in the mid-Atlantic region of the United States:

General. The principal arborviruses of human health importance in the mid-Atlantic region (defined as North Carolina, Virginia, Maryland, the District of Columbia, Delaware, Pennsylvania, New Jersey, and New York) of the United States are St. Louis encephalitis (SLE), West Nile (WN), eastern equine encephalitis (EEE), and La Crosse (LAC) viruses. Historically, SLE virus and eastern equine encephalitis (EEE) virus have been regarded as the arboviruses of greatest public health importance in the mid-Atlantic region. In this region during 1964-1997, the cumulative number of confirmed and probable cases of SLE was 205 (~6 cases per year) and of EEE was 40 (~1 case per year), with the majority of cases occurring in focal, widely intermittent outbreaks (CDC, unpublished data). La Crosse virus is endemic throughout the Midwest; however, the virus merits consideration when looking at the mid-Atlantic region due to the recent increase in LAC encephalitis in West Virginia (Nasci et al. 2000) and the presence of the mosquito vector, *Ochlerotatus triseriatus* (Say), throughout the mid-Atlantic region. In

1999, West Nile (WN) virus was reported for the first time in the Western hemisphere, specifically and primarily in New York City (CDC 1999a). This finding corresponded with reports of viral encephalitis in humans, including seven fatal cases (CDC 1999b), and of illness and die-off in wildlife (Anderson et al. 1999) and zoo animal (CDC 1999a) populations, which were attributed to WN virus. These four viruses represent three taxonomic families and are transmitted between various mosquito vector species and vertebrate hosts (Table 1). A further look at the distribution, ecology, and epidemiology of these viruses is needed to best understand what role *Ae. albopictus* and *Oc. j. japonicus* may play their transmission.

West Nile virus. West Nile virus is a member of the family Flaviviridae (genus *Flavivirus*). The geographic distribution of WN virus includes Africa, Asia, Europe and North America. It was first isolated in 1937 from a febrile woman in the West Nile province of Uganda (Smithburn et al. 1940). West Nile virus is assigned to the Japanese encephalitis antigentic complex, which, for example, includes the viruses responsible for Japanese encephalitis, Murray Valley encephalitis, and St. Louis encephalitis. West Nile virus particles are enveloped and 45 to 50 nm in diameter. Its genome consists of a single linear 10.9 kb molecule of ssRNA of positive polarity which is 5 capped but not 3 polyadenylated.

An excellent overview of West Nile virus in North America, with emphasis on its ecology and epidemiology, is provided by Petersen and Roehrig (2001). The ecology of WN virus in North America largely mimics that in the Old World: a wide range of birds (more than 70 species of birds) are susceptible to infection (Komar et al. 2001, Bernard et al. 2001); a wide variety of mosquitoes show evidence of natural infection with the

virus (primarily members of the *Culex* species) (Nasci et al. 2001, Bernard et al. 2001) and can transmit it by bite (Turell et al 2000, Turell et al. 2001, Sardelis et al. 2001); peak virus activity is during the hottest time of the year (CDC 2000); and humans and equines can develop encephalitis (CDC 1999b), but are not part of the natural transmission cycle of the virus (Figure 2). An interesting feature of 1999-2000 North American outbreak of WN virus was the high avian death rate, particularly in American Crows (Anderson et al. 1999). This was only the third time since the viruses discovery in 1937 that such an event has occurred, the two other instances involved pigeons in Egypt in 1953 (Work et al. 1953) and geese during 1997-2000 in Israel (Office of Internationale des Epizooties 1999). In regard to the recent North American outbreak of WN virus, it is not clear whether the high avian death rate was due to a more virulent strain of virus or higher susceptibility in the birds (Petersen and Roehrig 2001).

Infections of humans with WN virus are largely asymptomatic. The incubation period of WN virus in humans is usually 3 to 15 days. In persons with clinically apparent symptoms, mild symptoms include fever, headache, and body aches, occasionally with skin rash and swollen lymph glands; and severe symptoms include headache, high fever, neck stiffness, stupor, disorientation, coma, tremors, convulsions, muscle weakness, paralysis, and, rarely, death. Less than 1% of those infected with West Nile virus will develop severe illness. Among those with severe illness due to West Nile virus, case-fatality rates range from 3% to 15% and are highest among the elderly (those aged >65 years). There is no specific therapy for WN encephalitis; therapy consists of intensive supportive therapy. To date, there is no human vaccine for WN virus; however, there is a vaccine licensed for use in horses.

In 1999, some important concerns regarding WN virus were whether would it become established in the United States, and, assuming that WN virus became endemic, how far and rapid the virus may spread, based on the migration patterns of birds along the Eastern seaboard of the United States. West Nile virus was reported in four Atlantic states (New York, New Jersey, Connecticut and Maryland) in 1999 (CDC 1999b). In 2000, WN virus reappeared, and evidence of WN virus was found in 12 eastern states and the District of Columbia, having been detected as far south as North Carolina (CDC 2000). Interestingly in 2000, human cases of WN encephalitis were only reported from the vicinity of the 1999 outbreak (CDC 2000). As of September 2001, WN virus has been reported in 23 states including the District of Columbia, ranging from Maine to Florida and as far west as Iowa (CDC 2001). Additionally, human cases of WN encephalitis have been reported outside of the epicenter of the 1999-2000 cases, occurring in Maryland, Georgia, and Florida (CDC 20001).

St. Louis encephalitis virus. St. Louis encephalitis virus, as mentioned above, is closely related to WN virus; SLE virus a flavivirus in the Japanese encephalitis serogroup. The virus is named after St. Louis, MO, where, in 1933, it caused the largest ever outbreak of SLE (Webster and Fite 1933). St. Louis encephalitis virus is widely distributed throughout North, Central, and South America and the Caribbean region (Monath 1980), where it causes scattered human cases and occasional large outbreak in urban areas (Bleed et al. 1992, Brinker et al. 1979, Zweighaft et al. 1979, Levy et al. 1978, Powell and Blakey 1979). The virus s morphology and genome organization is similar to that of WN virus.

The transmission cycle of SLE virus is much like that of WN virus, cycling primarily between birds and mosquitoes (Figure 3). In the eastern United States, the principal avian hosts are common, urban and peri-urban Passiformes and Columbiformes (e.g., house sparrows, pigeon, blue jays, robins, cardinals, mourning doves, and mockingbirds) (McLean and Bowen 1980) and the mosquito vectors are members of the *Culex pipiens* complex and possibly *Culex restuans* Theobald (Mitchell et al. 1980). An important distinction between the ecology of SLE virus and WN virus is that natural infections of birds with SLE virus have not been found to be lethal (McLean and Bowen 1980). When conditions favor SLE virus amplification and transmission, human infections occasionally occur.

Most people who are infected with the SLE virus never show any outward symptoms. Mild cases may occur with flu-like symptoms, a slight fever and headache. Severe infections are marked by a rapid onset, headache, high fever, disorientation, coma, tremors, convulsions, paralysis or death. The elderly and the young are most at risk from SLE. As many as 30% of elderly patients infected with SLE die. There is neither a specific treatment for SLE, nor a vaccine to protect humans from SLE virus infection.

Eastern equine encephalitis virus. Eastern equine encephalitis virus was first isolated in 1933, during a major outbreak of illness in horses in the mid-Atlantic region of the United States (Giltner and Shahan 1933). The geographic distribution of EEE virus is now known to include North, Central, and South America and the Caribbean. In the United States, EEE virus is found mainly along the eastern seaboard and on the eastern Gulf coast. The virus is a member of the *Alphavirus* genus, family Togaviridae. Its particles are around 70 nm in diameter and enveloped, and the viral genome consists of a

single linear 11-12 kb molecule of ssRNA of positive polarity which is 5 capped and polyadenylated on its 3 end.

Transmission of EEE virus primarily takes place in marsh and swamp habitats, where it is maintained in an enzootic cycle involving passerine birds (e.g. sparrows, starlings, and blue jays) and the mosquito *Culiseta melanura* (Coquillett) (Morris 1988) (Figure 3). *Culiseta melanura* strictly feeds on birds, thus, human cases of EEE generally follow an increase in EEE viral activity in specific foci which leads to infection of bridge vector mosquito species, ones that feed on birds as well as mammals, such as *Ochlerotatus sollicitans* (Walker) (Crans et al. 1986), *Coquillettidia perturbans* (Walker) (Andreadis et al. 1998), and possibly *Ae. albopictus* (Mitchell et al. 1992).

Anyone can get eastern equine encephalitis, but symptomatic disease is more common in young children and in persons over the age of 55. Symptoms of infection usually occur 5 to 15 days after the bite of an infected mosquito. Infection with EEE virus can cause a range of illnesses. Most people infected with the virus have no symptoms, while others get only a mild flu-like illness with fever, headache, and a sore throat. In rare cases, infection of the central nervous system occurs, causing sudden fever and severe headache, and followed quickly by seizures and coma. About half of persons develop severe symptoms (encephalitis) die, and, of those who survive, many suffer varying degrees of permanent neurological damage. There is no specific treatment for eastern equine encephalitis. A vaccine is available for use in horses (Roehrig 1993) and for laboratory workers (Strizki and Repik 1995).

La Crosse virus. La Crosse virus was first isolated in 1960 from the brain of a girl from Minnesota in a La Crosse, WI, hospital (Thompson et al. 1965). The virus is distributed

throughout the upper Midwest of the United States (Ohio, Indiana, Iowa, Illinois, Wisconsin, and Minnesota) (Kappus et al. 1982). It is also endemic in a number of eastern states (Georgia, North Carolina, Tennessee, and West Virginia) (Sikes et al. 1984, Kappus et al. 1982, Jones et al. 1999, Woodruff et al. 1992).

La Crosse virus is a member of the California serogroup within the Genus *Bunyavirus* in the Family Bunyaviridae. La Crosse virus virions are spherical, around 90-100 nm in diameter, and have a lipid envelope with glycoprotein peplomers that enclose three circular nucleocapsids. The viral genome is segmented, consisting of three minus-sensed ssRNA molecules: a segment designated L (large) of approximately 7 kb, a segment designated M (medium) of approximately 4 kb, and a segment designated S (small) of approximately 1-2 kb. An interesting characteristics of the LAC virus genome is that genetic reassortment readily occurs when mosquitoes or cultured cells are coinfected with closely related bunyaviruses (Borucki et al. 1999). Like other ssRNA viruses, the genome of LAC virus evolves rather rapidly as a result of point mutations, deletions and duplications (Beaty and Bishop 1988).

La Crosse virus cycles between the mosquito *Oc. triseriatus*, the primary vector, and eastern chipmunks and gray squirrels during the warmer time of the year (Figure 4). Also, LAC virus is maintained indefinitely in *Oc. triseriatus* by transovarial transmission (Watts et al. 1974) and may be amplified by venereal transmission between infected males and uninfected female mosquitoes (Thompson and Beaty 1977). *Ochlerotatus triseriatus* are "tree hole mosquitoes that lay their eggs in water-holding containers like old tires and tree holes, and they will feed on larger mammals, including humans.

The groups at greatest risk of LAC virus infection are <16-year-old children due to their susceptibility and level of immunity. Due to their increased potential to be bitten by the vector, persons living in woodland environments or taking part in outdoor activities in woodland areas are at the greatest risk. The majority of LAC virus infections are subclinical or result in mild illness. Patients may have all or some of the following symptoms: fever, vomiting, stiff neck, headache, lethargy, seizure, and even coma. Encephalitis caused by LAC virus is most common in children and young adults (those under the age of 19). There is no specific therapy, however most patients recover (case-fatality ratio <1%). Persons that are severely afflicted may experience neurological sequelae that resolve within several years.

Association of *Ae. albopictus* and *Oc. j. japonicus* with discussed arboviruses of human health importance:

Aedes albopictus has been implicated as a dengue vector in rural areas of Southeast Asia (Smith 1956), found in association with a large dengue outbreak in Brazil in 1986 (Forattini 1986), and currently associated with a dengue outbreak in Hawaii (Promed 2001). In the United States, the greatest potential health impact of the introduction of *Ae. albopictus* is that of dengue transmission in urban areas. It has been suggested that *Ae. albopictus* may also play a role in the transmission of LAC virus in the Midwest (Tesh and Gubler 1975) and EEE viruses in the Eastern United States (Shroyer 1986). Eastern equine encephalitis virus was isolated from *Ae. albopictus* in Florida in 1992 (Mitchell et al. 1992). In the summer of 2000, LAC virus was isolated from a male *Ae. albopictus* reared from eggs collected in eastern Tennessee (Gerhardt et al. 2001).

Similarly, in 2000, a single pool of adult *Ae. albopictus* collected in southeastern Pennsylvania was found infected with WN virus (CDC 2000).

The vector competence of *Ae. albopictus* for many arboviruses has been studied and a comprehensive review of these studies was done by Mitchell (1991). The vector competence of North American strains of *Ae. albopictus* for the principal arboviruses of public health concern in the mid-Atlantic region of the United States is shown in Table 2. *Aedes albopictus* is an efficient experimental vector of EEE and LAC viruses (Scott et al. 1990, Turell et al. 1994, Grimstad et al 1989), but a poor laboratory vector of SLE virus (Savage at al. 1994). The vector competence of North American strains of *Ae. albopictus* for North American strains of WN virus has not been studied. In the 1940s, an Asian strain *Ae. albopictus* was shown to be a competent laboratory vector of an African strain of WN virus (Philip and Smadel 1943). Additionally, Turell and others (2001) have shown that *Ae. albopictus* from a long-standing colony that originated from mosquitoes collected in Hawaii over 30 years ago is a competent laboratory vector of a New York strain of WN virus.

No arbovirus has been isolated from wild *Oc. j. japonicus* collected within the mosquito s native range, though its abundance in Hokkaido, Japan, during an outbreak of Japanese encephalitis (JE) indicated that it may play some role in JE virus transmission (Takashima et al. 1989). In the United States during 2000, multiple pools of *Oc. j. japonicus* from New York and New Jersey showed evidence (i.e., isolation of virus and/or detection of viral genome) of WN virus infection (CDC 2000).

Little is known about the potential for *Oc. j. japonicus* to transmit arboviruses. Takashima and Rosen (1989) reported that *Oc. j. japonicus* was able to transmit JE virus

in the laboratory, and Turell and others (2001) found a New York strain of *Oc. j. japonicus* to be an efficient laboratory vector WN virus.

Vector incrimination in arbovirus transmission cycles:

The process of establishing that a particular arthropod may have a role in the transmission of a virus is referred to as vector incrimination. The criteria for incriminating a vector are fashioned along the same line as Koch s postulates for establishing the causal links between particular pathogens and specific diseases. The postulates have been adapted to incriminating arthropods in a disease transmission cycle (Barnett 1962, Reeves 1957). The criteria for incriminating a mosquito species in an arbovirus transmission cycle, modified from Barnett (1962), are shown in Table 3. Briefly, field and laboratory studies are done to 1) determine whether the mosquitoes are naturally infected with the virus, 2) assess the extent of human contact with the potential vector species, 3) evaluate the susceptibility of the mosquito to infection with the virus, and 4) determine the potential of transmission of the virus by the mosquito.

In general, an assortment of governmental and academic entities are involved in putting together the evidence to incriminate a vector species. State health or environmental personnel routinely collect mosquitoes for virus isolation as part of arbovirus surveillance programs. The goal of surveillance programs is to provide an "early warning system" for arbovirus outbreaks and to evaluate the need to intensify mosquito control efforts. When there is a paucity of information on the distribution and bionomics of a potential vector, such as in the case with newly introduced nonnative species, the urgency to conduct the necessary research will often be based on the species association with other arboviruses with epidemiologies similar to those in the new area.

This urgency may be enhanced should the particular species be an important biting pest or if a virus isolate is made. The assembly of the data to convincingly show an association between the suspected vector and the vertebrate reservoir, amplifying and/or dead-end host(s) of the virus takes years of effort by researchers in a number of specialties. Vector specific information includes daily survival and longevity, host preference and host feeding patterns, and density of vectors in relation to density of hosts (Eldridge and Edman 2000). This information then can be used to estimate the number of bites received daily by a single host. Lastly, research facilities, equipped with the appropriate biosafety level laboratories, conduct studies to determine the ability of the suspected vector to transmit the virus.

Vector competence studies:

Vector competence, in the context of mosquito-borne arbovirus, is the ability of a mosquito to transmit the virus. Vector competence information is required to incriminate a vector, as discussed above, even if an isolation of virus has been made from the vector. The reasons for this include misleading results from field isolates (Turell 1988) and the need to quantify the efficiency of transmission so vectorial capacity can be estimated. Also, in the absence of a field isolation of virus from a particular mosquito species, vector competence data may provide a basis for assessing the risk of virus transmission by a local problem mosquito species or a species in an area where a virus s distribution is projected to expand.

As mentioned above, vector competency is a parameter in determining vectorial capacity. Vectorial capacity, in the context of an arbovirus, is a term that attempts to

quantify virus transmission by mosquitoes. A formula for vectorial capacity, as modified from Reisen (1989), is as follows,

$$C = ma^2VP^n/-\log_e P,$$

Where C is the vectorial capacity, the number of infective bites received daily by a single host; m is the density of vectors in relation to density of hosts; a is the proportion of vectors feeding on a host divided by the length of the gonotrophic cycle in days; V is the vector competence; P is the daily survival of vector; and n is the extrinsic incubation period. Two factors in the above equation must be determined in controlled experimental conditions, vector competence and extrinsic incubation period (EIP).

Classically, vector competence is determined by allowing previously non-virus exposed mosquitoes to ingest a viremic blood meal, holding the engorged mosquitoes for an appropriate amount of time, and then refeeding these mosquitoes on a susceptible host. The percentage of refeeding mosquitoes that transmit virus by bite is the transmission rate. This rate is also the value for vector competence, V above.

Our knowledge of the interaction between a virus and mosquito vector has grown since the early days in the study of arboviruses. The sequential movement of an arborvirus through a vector mosquito has been described, the steps in arbovirus replication are known, barriers to infection of the mosquito have been elucidated, and terms, in addition to transmission rates or vector competence, have come into usage in experimental transmission trials.

There are six steps in the most common sequence for a competent female mosquito to transmit an arbovirus. Step 1 is ingestion of the infectious blood meal. Step 2 is infection and multiplication of virus in the midgut epithelial cells. Step 3 is release

of virus from the midgut epithelial cell into the hemolymph. Step 4 is infection of the salivary glands, with or without secondary amplification of virus in other tissues. Step 5 is release of virus from the salivary gland epithelial cells into the lumen of the salivary glands. Step 6 is transmission of virus in the saliva during blood feeding.

The sequence of events in arbovirus replication that lead to productive infections includes attachment, penetration, uncoating, replication of more viral genome and production of viral structural proteins, maturation, and budding. During attachment, the viral attachment proteins on the surface of the virion bind to receptors on the microvillar membrane of the mosquito s midgut. Penetration refers to the process through which the virions enter the midgut cells; arboviruses enter cell through receptor-mediated endocytosis. During uncoating, the virions at least partially uncoat so to make available the viral genome for transcription or translation, based on whether the viral genome positive or negative sense. Replication of more viral genome and production of viral structural proteins is done in manner determined by the organization of the viral genome and that often differs based on the family of virus. Viral proteins are further cleaved to form the final product during maturation. Lastly, through budding, the virions obtain their envelope and are released outside of the cell, into the hemolymph.

Based on events that attribute to reducing the efficiency of a mosquito to transmit virus under laboratory conditions (i.e., inability or reduced ability to complete the steps involved in the movement of an arborvirus through a vector mosquito and the breakdown in the arbovirus replication sequence), barriers to the biological transmission of arboviruses have been described. These barriers include the midgut infection barrier, midgut escaper barrier, and salivary gland barrier (Merrill and TenBroeck 1935, Kramer

et al. 1981). The salivary gland barrier has been further divided to include both a salivary gland infection barrier and salivary gland escape barrier (Hardy et al. 1983).

Terms now familiar in vector competence studies include the infection rate, dissemination rate, and estimated transmission rate. The infection rate is the number of mosquitoes infected divided by number that fed times 100. The dissemination rate, as a population parameter, is the number of mosquitoes with evidence of infection beyond the midgut, such as in the legs (Turell et al. 1984) or in the head, divided by the number that fed times 100. The estimated transmission rate is calculated by multiplying the percentage of individuals of a species that developed a disseminated infection (dissemination rate) times the proportion of mosquitoes with a disseminated infection, after either oral exposure to or intrathoracic inoculation with virus, that transmitted virus by bite (Turell et al. 1988). The advantage of determining the estimated transmission rate rather than the transmission rate is that this procedure allows for more efficient use of laboratory animals, an important issue in today s research environment. The number of animals needed to get a meaningful transmission rate can be quite high if the species being tested is one that is not likely to feed a second time in the laboratory or one in which the oral infection and/or subsequent dissemination rates are low.

When designing or evaluating laboratory studies of vector competence, one must consider factors that may affect the results. Variation in the transmission of viruses by different mosquito species is well documented (Grimstad 1983, Chamberlain and Sudia 1961). Moreover, intraspecific variation in vector competence has been described often (Reisen et al. 1996, Gubler et al. 1979, Tran et al. 1999, Boromisa et al 1987, Gubler and Rosen 1976, Hayes et al. 1984, Kay et al. 1984). As with mosquito strains, different

arbovirus strains might vary in their ability to infect and/or be transmitted by a mosquito vector (Saliba et al. 1973, Kramer and Scherer 1976). The viral dose of the infectious blood meal may affect vector competence, primarily through its affect on the infection rate. Numerous studies have shown that extrinsic incubation temperature affects the ability of a mosquito to transmit a virus (Bates and Roca-Garcia 1946, Chamberlain and Sudia 1955, Takahashi 1976, Turell et al. 1985, Watts et al. 1987, Reisen et al. 1993). These studies generally show that transmission rates are directly related to the extrinsic incubation temperature. Studies also show that female adult mosquitoes derived from nutritionally starved larvae or larvae reared in crowded conditions are more efficient transmitters of virus (Takahashi 1976, Grimstad and Haramis 1984). Ideally, vector competence studies should be done using sympatric mosquitoes and viruses, the reservoir host for the virus, exposure viral titers comparable to those observed in nature, and an extrinsic incubation temperature comparable to that experienced during the time of year when virus transmission in known to occur.

Though not a part of vector competence in a strict sense, the determination of EIP, the time from the ingestion of the infectious blood meal to the time the mosquito is capable of transmitting the virus, is occasionally integrated into vector competency studies. The EIP is determined by refeeding virus-exposed mosquitoes individually on susceptible hosts at various times after the infecting blood meal. The period of time until 50% of the mosquitoes transmit virus, EIP_{50}, is the epidemiologically relevant term. As in vector competence, viral dose and incubation temperature affect the EIP (Bates and Roca-Garcia 1946, Gresser et al. 1958, Turell et al. 1985, Watts et al. 1987, Reisen et al. 1993).

Research goals:

This research was designed to answer questions about the distribution of *Ae. albopictus* and *Oc. j. japonicus* in the mid-Atlantic region of the United States, and to evaluate the vector competence of these two species for the viruses of principal importance in the region for which studies are lacking. The goals were to 1) assess the distribution of *Ae. albopictus* and *Oc. j. japonicus* at selected sites in the mid-Atlantic region of the United States, 2) evaluate the distribution of *Oc. j. japonicus* and *Ae. albopictus* in an area in which both species had been recently discovered, 3) determine if North American strains of *Ae. albopictus* are competent experimental vectors of WN virus, and 4) determine if *Oc. j. japonicus* is a competent experimental vector of EEE, SLE, LAC and WN viruses.

This dissertation is presented in chapters that are largely aligned with the research goals. Each research chapter (2-7) was written and organized in a manner that would facilitate its publication in a peer-reviewed journal.

The laboratory studies were done at the US Army Medical Research Unit of Infectious Diseases (USAMRIID), Fort Detrick, Frederick, MD. The research was done in accordance with protocols V00-01 (dated 24 May 01) and V98-07 (10 Sep 98) (Appendix A). In conducting research using animals, I adhered to the "Guide for the Care and Use of Laboratory Animals," as prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (NIH Publication No. 86-23, revised 1996). The USAMRIID facilities are fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International.

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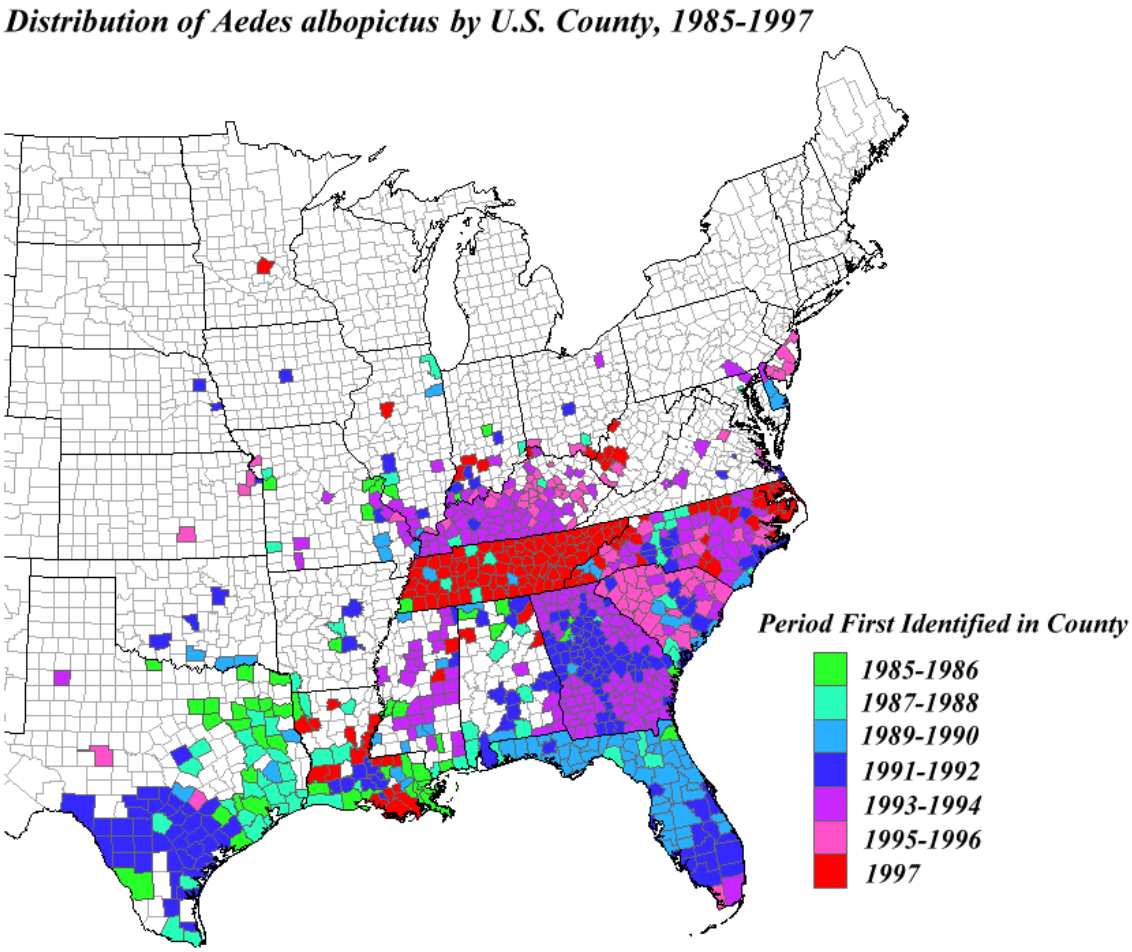

(DVBID, CDC, http://www.cdc.gov/ncidod/dvbid/arbor/albopic_97_sm.htm)

Figure 1. Distribution of *Aedes albopictus* by United States County, 1985-1997.

Table 1. Principal arboviruses of human health importance in the mid-Atlantic region of the United States

Virus	Family	Genus	Principle vector	Vertebrate reservoir	Human disease
St. Louis encephalitis	Flaviviridae	*Flavirivus*	*Culex pipiens*-complex	Birds	Encephalitis
West Nile	Flaviviridae	*Flavirivus*	*Culex (Cul)* spp.	Birds	Encephalitis
Eastern equine encephalitis	Togaviridae	*Alphavirus*	*Culiseta melanura*	Birds	Encephalitis
La Crosse	Bunyaviridae	*Bunyavirus*	*Ocherotatus triseriatus*	Small mammals	Encephalitis

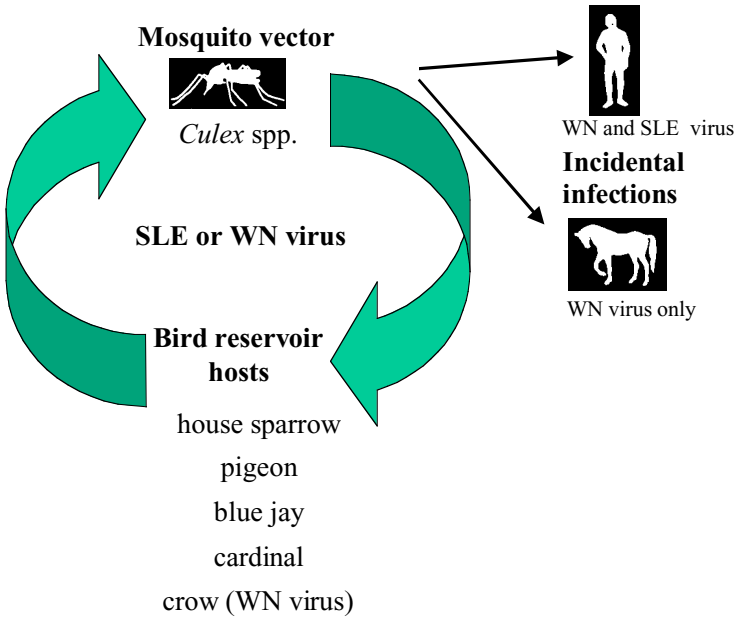

Figure 2. Transmission cycle of St. Louis encephalitis (SLE) and West Nile (WN) virus.

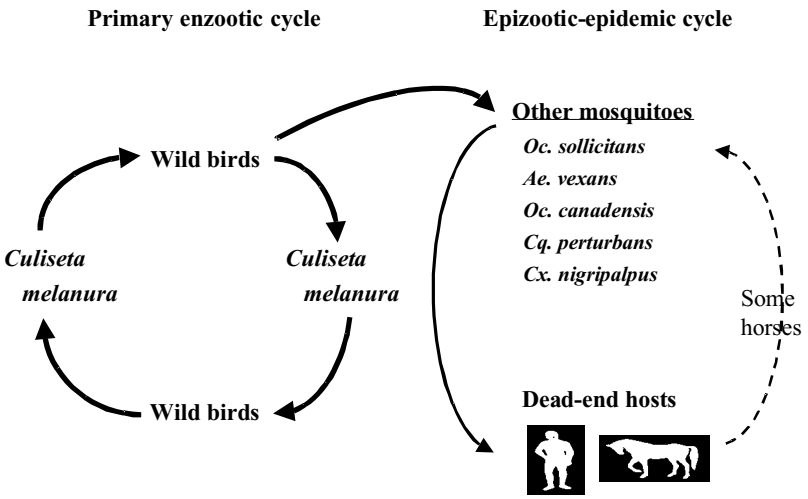

Figure 3. Transmission cycle of eastern equine encephalitis virus.

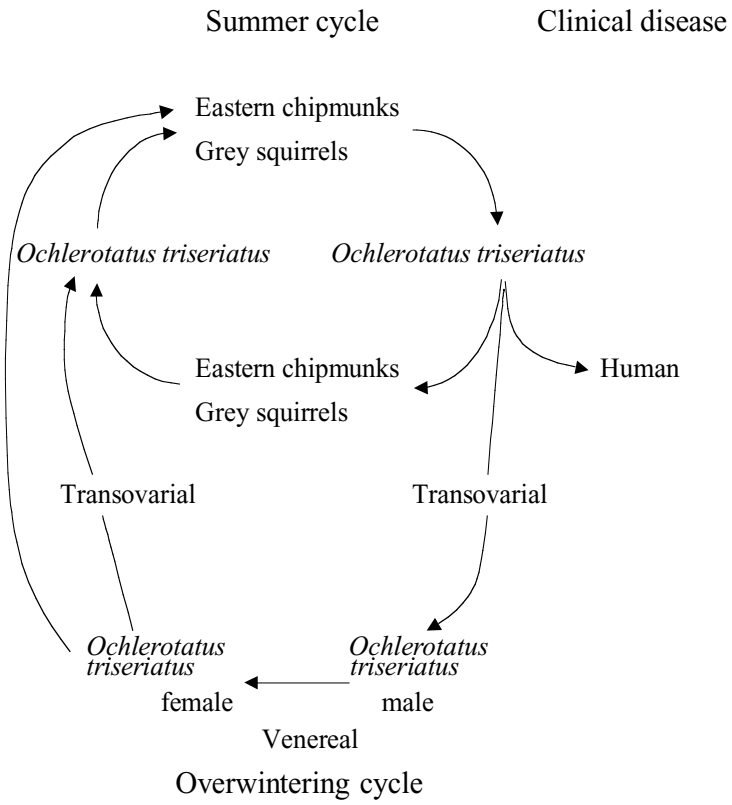

Figure 4. La Crosse virus cycle.

Table 2. Vector competence of North American strains of *Aedes albopictus* for principal viruses of public health importance in the mid-Atlantic region of the United States

Virus[1] strain	Mosquito strain	Infection[2] *n*	Infection[2] %	Transmission[3] *n*	Transmission[3] %	Reference
SLE						
AR91-2783	Pine Bluff	270	1	209	0.5	Savage et al. 1994
EEE						
ME 77132	Houston	10	100	20	25-57	Scott et al. 1990
FL91-4679	Polk II	15	100	12	42	Turell et al. 1994
FL91-4679	Polk X	45	100	25	52	Turell et al. 1994
FL91-4679	Gentilly	70	100	33	48	Turell et al. 1994
FL91-4679	Houston	33	100	11	45	Turell et al. 1994
LAC						
FL91-4679	Houston	80	93-98	75[4]	47	Grimstad et al. 1989
FL91-4679	Evansville	9	89	6[4]	33	Grimstad et al. 1989
FL91-4679	Indianapolis	10	80	6[4]	17	Grimstad et al. 1989

[1]Virus acronyms: SLE = St. Louis encephalitis, EEE = eastern equine encephalitis, LAC = La Crosse.
[2]*n* = number tested. % = percent infected.
[3]*n* = number tested, unless otherwise indicated. % = percent tranmitting.
[4]Mosquitoes with disseminated infections.

Table 3. Criteria for incrimination of mosquito species as a vector of a virus affecting human health

• Make multiple isolations of the virus from specimens of the suspected vector collected under natural conditions.
• Show that members of a suspected species population commonly feed upon vertebrate hosts of the virus.
• Show that there is convincing biological association in time and space between the suspected species and clinical or subclinical infections in vertebrate hosts.
• Demonstrate efficient transmission of the virus by the suspected vector under controlled laboratory conditions.

Modified from Barnett 1962.

Chapter 2

Survey of container-inhabiting *Aedes* and *Ochlerotatus* mosquitoes in the mid-Atlantic region of the United States, with emphasis on Frederick County, Maryland

ABSTRACT

Ochlerotatus j. japonicus is reported for the first time south of the Mason-Dixon Line, in Frederick County, Maryland. To assess the degree to which *Oc. j. japonicus* had established in the county, oviposition and larval surveys were conducted. Fifty-seven oviposition trap samples were collected throughout the county between June 30 and August 24, 2000. From 971 larvae reared from the oviposition traps, five species were identified: *Ochlerotatus triseriatus* (45%), *Oc. j. japonicus* (43%), *Aedes albopictus* (7%), *Culex pipiens* (4%), and *Toxorynchites ritulus septentrionalis* (<1%). During May 30 and June 29, 2001, mosquito larval samples were taken from containers at 39 separate collection sites in the ovitrapping survey area. *Ochlerotatus j. japonicus* was collected from 59% (23 of 39) of the containers, to include a variety of artificial and natural containers. In both surveys, *Oc. j. japonicus* was found widely distributed over the area sampled. This is the first record of *Ae. albopictus* in the Frederick County, Maryland, as well. Oviposition trapping done at sites from southern New York to Southern North Carolina show that the *Ae. albopictus* and *Oc. j. japonicus* are established in south-central Pennsylvania.

INTRODUCTION

Aedes albopictus (Skuse) is a container-inhabiting mosquito found in a variety of artificial and natural containers in forested, peri-urban, and urban areas (Hawley 1988). Established populations of *Ae. albopictus* were found in the United States in 1985, having been discovered in Houston, Texas [Centers for Disease Control and Prevention (CDC) 1986, Sprenger and Wuithiranyagool 1986]. Since that time, it has spread from Texas and up the eastern seaboard, where it now has become established as far north as Monmouth County, New Jersey (Crans et al. 1996). Moore (1999) reported that *Ae. albopictus* has been found in 26 states, mainly in the eastern half of the United States. It has been reported from a few counties in each of the Mid-Atlantic States of Virginia, Maryland, Delaware, Pennsylvania, and New Jersey.

Ochlerotatus j. japonicus (Theobald), like *Ae. albopictus*, inhabit a wide range of natural and artificial containers (Tanaka et al. 1979). Little is known about the geographic distribution of *Oc. j. japonicus* in the United States due to it being a 1998 discovery. The first published records of *Oc. j. japonicus* were from New York and New Jersey (Peyton et al. 1999). During 1999, *Oc. j. japonicus* was collected in Massachusetts, Connecticut, Ohio, and Pennsylvania.

On June 8, 2000, the author collected mosquito larvae from tires in an automobile salvage yard in Frederick, Frederick County, in western Maryland (39^{o}23 33 N, 77^{o}23 55 S). The tires (~70) were piled in a shaded area and the majority contained leaf litter. The entire contents of eight tires were collected and taken to the laboratory where the mosquito larvae were separated from the debris and reared to the adult stage. Of the 687 specimens collected, 508 (74%) were *Oc. j. japonicus*, 165 (24%) were *Ochlerotatus*

triseriatus (Say), and 14 (2%) were *Culex pipiens* L. The *Oc. j. japonicus* specimens were confirmed by taxonomists at the Walter Reed Biosystematics Unit (WRBU), Museum Support Center, Smithsonian Institution, Washington, DC, and voucher specimens were provided to WRBU. Specimens from this collection were subsequently used in a study of the population genetics of *Oc. j. japonicus* (Fonseca et al. 2001). When the operator of the salvage yard was asked about the origin of the tires, he indicated that all the tires were removed from rims of cars in his salvage yard.

Since 1989, the U.S. Army Center for Health Promotion and Preventive Medicine, Direct Support Activity-North (DSA-N), has conducted surveillance for container-inhabiting mosquitoes at military installations in the mid-Atlantic and northeastern United States. The primary objective of this surveillance was to assess the risk of arboviral disease transmission by *Ae. albopictus*. The results from this surveillance are summarized annually; however, none of the data has been published in the scientific literature.

Interest in *Ae. albopictus* and *Oc. j. japonicus* has been increasing due to the reports of West Nile infection in wild-caught specimens of both species in 2000 (CDC 2000). *Aedes albopictus* was previously implicated in eastern equine encephalitis virus transmission (Mitchell et al. 1992) and very recently implicated in La Crosse virus transmission (Gerhardt et al. 2001). In addition to evidence of natural infection and vector competence, basic information on the distribution and relative abundance of a particular mosquito species is needed to assess the role it may play in the circulation of viruses endemic in an area. The objectives of this study were to summarize and evaluate the distribution of *Ae. albopictus* and *Oc. j. japonicus* on a region-wide basis from the

DSA-N reports and to assess the distribution and relative abundance of container-inhabiting mosquitoes in an area in which *Oc. j. japonicus* was recently discovered; Frederick County, Maryland.

MATERIALS AND METHODS

Region-wide container breeding mosquito surveillance:

Data were analyzed from the U.S. Army Center for Health Promotion and Preventive Medicine, Direct Support Activity-N (DSA-N), mosquito oviposition trap surveillance program for 10 military installations (Table 1) located throughout the mid-Atlantic region of the United States (Figure 1) during 1996-2000. At these installations, the oviposition trapping was conducted by preventive medicine, natural resources, or engineering personnel. In 2000 at the United States Military Academy, West Point, NY, no ovitrapping was done; however, mosquito larvae were collected from containers on the installation in June and sent to DSA-N where they were reared to the adult stage.

The oviposition trapping was done using standard black cup traps as described by Zeichner and Perich (1999). Briefly, each trap consists of a black cup (473 ml capacity) filled with 250 ml of dechlorinated tap water. A velore strip (25 x 11 mm) (ovistrip) was affixed to the side of the cup by a paper clip to serve as the oviposition substrate. At each installation, 6 to 8 oviposition traps were placed in areas that may yield container-breeding mosquitoes, such as property disposal yards, tire storage areas, and residential areas. Samples were to be taken at 1-wk intervals from May through October. The ovitraps were left out for seven days after which the ovistrips are collected, placed in a sealable plastic bag and mailed to DSA-N.

At DSA-N, the ovistrips were visually examined for eggs under a dissecting microscope, and ovistrips with eggs were transferred to rearing containers (BioQuip Products Co., Gardena, CA) with dechlorinated tap water. Each rearing container was labeled with the date of collection, location, and site number. Emerging larvae were fed

ground rabbit chow and reared at 23-25^{o}C, 60-80% RH, and 16:8 h L:D photoperiod. The larvae were reared through adult, and the adults identified using Darsie and Ward (1981) with updates for identifying newly introduced mosquito species.

Surveys in Frederick County, Maryland:

Study site. Frederick County is located in western Maryland (Figure 2). The county has one major city, Frederick, population 50,411. Aside from Frederick and a few small towns, agricultural land dominates the landscape of the county. The northwestern portion of the county contains the Catoctin Mountains, ridges that are the leading edge of the Allegheny Mountains. The Catoctin Mountains and a small section of land in the southern part of the county are covered with eastern hardwood forest. Numerous small streams divide the elevated parts of the county. The Monocacy River divides the eastern third of the county from the western two-thirds, and flows south to the Potomac River.

Oviposition trapping survey. To assess the distribution of *Oc. j. japonicus* in Frederick County, Maryland, oviposition traps, as described above, were set throughout the county between June 30 and August 24, 2000. The traps were placed in sites that were at least partially shaded (e.g., the base of a tree) and just into the tree line of the road that was used to access the area. Seven days later, the traps were returned to the laboratory and checked for the presence of larvae and/or eggs. Larvae were transferred to pans containing dechlorinated tap water, provided ground catfish chow for nutrition, and reared at 26^{o}C, 80-85% RH, and 16:8 h L:D photoperiod. Eggs on ovistrips were flooded with dechlorinated tap water on the day they were collected and the larvae were reared as described above. Voucher specimens, 4th instar larvae, and adults were preserved in 80%

ethanol or pinned for later identification. A subsample of the larvae was allowed to pupate, and adults were identified after emergence.

Larval survey. Mosquito larval sampling was conducted throughout Frederick County between May 30 and June 29, 2001. Larval collection sites were identified by looking for containers while either driving along the road or walking in the vicinity of ovitrapping sites found positive *Ochlerotatus* spp. during the previous year s survey. Virtually all the collection sites had more than one container that could potentially contain mosquito larvae (e.g., a tire pile, an assortment of bird baths at a nursery, a grove of trees with tree holes, or a stream edge with rock pools). Containers at a site were first visually checked for mosquito larvae. The number of containers that were sampled at a particular collection site was limited to the number that were thought necessary to sample in order to ensure that all the common species at a site would be represented in the sample. Generally, larvae were sampled from less than five containers at each site. The containers sampled included artificial containers ranging in size from tin cans to 55-gallon barrels and natural containers such as tree holes and rock pools. From selected containers positive for larvae, a representative sample of larvae was collected using either a meat baster or larval dipper. The larvae were returned to the laboratory, reared to 4^{th} instar, and identified.

RESULTS

Region-wide container breeding mosquito surveillance:

Aedes albopictus was collected from Sunny Point, NC, to Fort Indian Town Gap, PA (Table 2). In the three southern most installations, *Ae. albopictus* was found as early as 1996. *Aedes albopictus* was collected in the central installations of Letterkenny Army Depot, PA, and Carlisle Barracks, PA, in 1998. *Ochlerotatus j. japonicus* was collected in 1999 at Letterkenny Army Depot, PA, and, in the subsequent year, it was found at all installations north of Fort Meade, MD (Table 2). Aside from instances in which either *Ae. albopictus* or *Oc. j. japonicus* were collected from an installation for the first time in 2000, collection of one of these species in a particular year was followed by a year in which the species was again collected.

Surveys in Frederick County, Maryland:

Oviposition trapping survey. Mosquito eggs or larvae were collected from 80% (44 of 57) of the oviposition traps. A total of 971 mosquitoes were collected and identified. *Ochlerotatus triseriatus* and *Oc. j. japonicus* accounted for 88%, and *Aedes albopictus* (Skuse), *Cx. pipiens*, and *Toxorynchites ritulus septentrionalis* (Dyar and Knab) accounted for the other 12% of these specimens (Table 3). The locations of oviposition traps in the study are shown in Figure 3. *Ochlerotatus j. japonicus* and *Oc. triseriatus* were collected across the entire sampling area. In contrast, *Ae. albopictus* were collected only in the vicinity of the city of Frederick and the southern border towns of Point of Rocks and Brunswick.

Larval survey. A total of 1,121 larvae, representing 4 genera and 6 species, were collected from 39 collection sites (Table 4). The primary container type from which the

samples were taken at these sites included tires (17), flower pots and bases (5), bird baths (4), large (55- to 6-gal capacity) barrels (3), small (≤ 5-gal capacity) buckets (2), assorted _-gal capacity plastic containers (2), vases (2), tin can (1), rock pool (1), tree hole (1), and tarp (1). *Ochlerotatus j. japonicus* were collected from 59% (23 of 39) of the sites (Table 4) and from every container type listed above except vase and tin can. *Ochlerotatus triseriatus* were collected from 38% (15 of 39) of the site (Table 4) and from every container type listed above except rock pool, tin can, tarp, and large barrel. The locations of the *Oc. j. japonicus*- and *Oc. triseriatus*-positive collection site are shown in Figure 4. Both species were broadly distributed in the county. No *Ae. albopictus* were collected.

DISCUSSION

The determination of the presence of a mosquito species in an area is one key element in assessing the risk of arboviral disease transmission by that species. In the mid-Atlantic region of the United States, established populations of *Ae. albopictus* have been reported from as far north as Keyport, Monmouth County, New Jersey (40°26 N lat) (Crans et al. 1996). Similarly, this study found established populations of *Ae. albopictus* as far north as 40°23 N lat, at Fort Indian Town Gap, Dauphin County, PA. In 1993, *Ae. albopictus* had been previously collected at a military base near Harrisburg in south-central Pennsylvania; however, none was collected the following year (B. Pagac, unpublished data). The significance of the finding at Fort Indian Town Gap is two-fold: 1) it indicates that *Ae. albopictus* is now established in the area and 2) the northern extent of its range now encompasses some large population centers (e.g. Harrisburg, PA) where it is likely to become an important biting pest.

This study showed that *Oc. j. japonicus* is firmly established in south-central Pennsylvania and western Maryland. Previous published reports and recent informal reports indicate that it is also found in New York, New Jersey, Connecticut, Pennsylvania, Ohio, and Virginia. Although *Oc. j. japonicus* is generally described as a northern climate species within its native range in Japan, it has been reported as far south as 33°N, in Chejudo, Republic of Korea (Tanaka et al. 1979). Thus, based solely on climatic information, *Oc. j. japonicus* may expand its range as far south as Jacksonville, FL, in the US.

Given its distribution within Frederick County and its abundance relative to *Oc. triseriatus*, the introduction of *Oc. j. japonicus* apparently occurred before 2000. Surveys

during 1999 in Connecticut looking specifically for *Oc. j. japonicus*, found it to be widespread and breeding in areas away from tire dumps (Andreadis T, unpublished data). *Ochlerotatus j. japonicus* has been present in Connecticut for between 2 and 11 years, based on the reevaluation of adult collections from recent years and from a 1989 survey of tire-breeding mosquitoes (Andreadis 1989). The data from the region-wide ovitrapping survey seem to indicate that the introduction of *Oc. j. japonicus* into Frederick County may have occurred prior to 2000. In 1999, it was collected at Letterkenny Army Depot, PA, a location just 30 km northwest of the Frederick County. Thus, based on the data presented here and on the Connecticut studies, *Oc. j. japonicus* either has been in Frederick County for at least a few years or may be an extremely fast invader of new territory.

The data from the Frederick County study also seem to suggest that local expansion of the range of *Oc. j. japonicus* is not driven only by the movement of infested, used automobile tires. Oviposition traps placed in remote areas were often positive for *Oc. j. japonicus*. Also, the larval survey revealed that *Oc. j. japonicus* was taking advantage of natural breeding sites such as rock pools and tree holes.

The distribution of *Oc. j. japonicus* appeared to be associated with *Oc. triseriatus*. Seventeen percent of the ovitraps contained both species and the distribution of positive ovitraps for either species were interwoven. Likewise, larvae of both species were found breeding in the same container. This overlap of the two species would be likely, given that they both breed in containers. In the United States, *Oc. j. japonicus* has been collected in a broad variety of container types which can be found around virtually all residential areas, such as the containers described in this study (e.g., tires, bird baths, and

tarps), as well as tin cans, concrete rain-water drainage forms, and 3.8-liter milk jugs (MRS, unpublished data). Thus, the likelihood for it to come in contact with humans may be similar to that of *Oc. triseriatus*. To date, there have been no studies published on biting preference of *Oc. j. japonicus* in the United States. It also remains to be shown whether there will be some type of competition between these two species for breeding sites and survival.

Though the focus of the Frederick County oviposition survey was not on *Ae. albopictus*, there are some items of information regarding this species that are noteworthy. This is the first record of *Ae. albopictus* in Frederick County, Maryland. The collection of *Ae. albopictus* in the city of Frederick and in two other small towns in the county indicates that there is potential for this species to become a nuisance to residents.

Given the widening distribution and apparent relative abundance of *Oc. j. japonicus* in the United States, the vector competence of this mosquito for arbovirus of human health importance in the region (see Chapters 3-6), and evidence of natural infection with WN virus (CDC 2000), it is important to evaluate the other factors that influence the potential for *Oc. j. japonicus* to become involved in arbovirus transmission. Examples of some of the factors that require study include host preference, host-feeding pattern, daily survival and longevity, seasonality, and flight range. Unlike *Oc. j. japonicus*, the behavioral and biological characteristic of *Ae. albopictus* are well documented (Hawley 1988) and it already has a reputation as an important biting pest. West Nile and La Crosse virus have been detected in field-collected *Ae. albopictus* (CDC 2000, Gerhardt et al. 2001), and it is a competent laboratory vector of these viruses as

well as St. Louis encephalitis and eastern equine encephalitis viruses (see Chapter 6 and review Mitchell 1991). This information regarding the potential vector status of *Ae. albopictus* and the recent evidence that it continues to expand or fill-in its distribution around its northern range makes this mosquito worthy of attention when and wherever it is found.

The findings in this chapter on the oviposition trapping in Frederick County, Maryland have been recently published and are included in Appendix B.

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Table 1. Installations that participated in oviposition trapping program

Installation, state	County
Military Ocean Terminal—Sunny Point, NC	New Hanover
Fort Bragg, NC	Cumberland
Fort A.P. Hill, VA	Caroline
Fort Lee, VA	Petersburg
Fort Meade, MD	Anne Arundel
Aberdeen Proving Ground, MD	Harford
Letterkenny Army Depot, PA	Franklin
Carlisle Barracks, PA	Cumberland
Fort Indian Town Gap, PA	Dauphin
U.S. Military Academy, NY	Orange

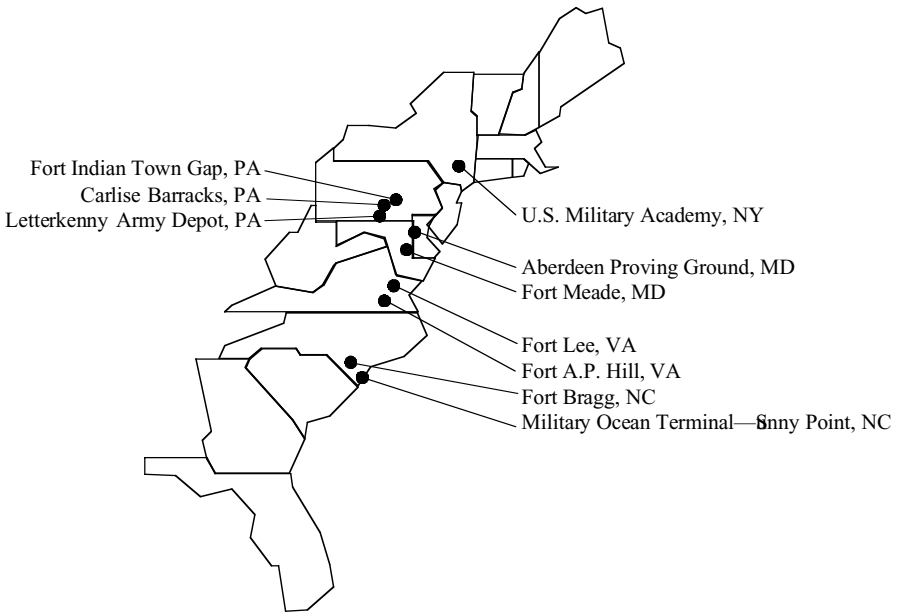

Figure 1. Location of military installations that participated in the oviposition trap survey

Table 2. Summary of the oviposition trap surveys conducted at military installations in the mid-Atlantic region of the United States, 1996-2000.

Installation, state	1996		1997		1998		1999		2000	
	Ae. *albo.*	*Oc. j. japo.*	*Ae. albo.*	*Oc. j. japo*	*Ae. albo.*	*Oc. j. japo*	*Ae. albo.*	*Oc. j. japo*	*Ae. albo.*	*Oc. j. japo*
Military Ocean Terminal—Sunny Point, NC	+	–	+	–	+	–	+	–	+	–
Fort Bragg, NC	+	–	+	–	+	–	+	–	+	–
Fort A.P. Hill, VA	+	–	+	–	nd	nd	nd	nd	nd	nd
Fort Lee, VA	nd	nd	+	–	+	–	nd	nd	+	–
Fort Meade, MD	–	–	–	–	–	–	–	–	+	–
Aberdeen Proving Ground, MD	–	–	–	–	–	–	+	–	+	+
Letterkenny Army Depot, PA	–	–	–	–	+	–	+	+	+	+
Carlisle Barracks, PA	–	–	–	–	+	–	+	–	+	+
Fort Indian Town Gap, PA	–	–	–	–	–	–	+	–	+	+
U.S. Military Academy, NY[1]	nd	nd	–	–	–	–	nd	nd	-L	+L

+ = positive, – = negative, nd = not determined.

[1]No ovitrapping done in 2000; however, larvae collected from containers. -L = negative for larvae, +L = positive for larvae.

Figure 2. Topographic map of Frederick County, Maryland

Table 3. Summary of the oviposition trap survey done in Frederick County, Maryland, between June 30 and August 24, 2000.

			Ovitraps[1]	
				Percent
Species or species combination	No. *n*	Percent of total	positive	positive
Ochlerotatus triseriatus	*437*	*45*	*25*	*45*
Ochlerotatus j. japonicus	*422*	*43*	*25*	*45*
Aedes albopictus	68	7	6	11
Culex pipiens	*43*	*4*	*1*	*2*
Toxorhynchites rutilus septentrionalis	1	<1	1	2
Total	971	100	44	80
Oc. triseriatus and *Oc. j. japonicus*	38 (54)[2]	39[3]	10	18
Ae. albopictus and *Oc. triseriatus*	28 (54)	3	2	4
Oc. j. japonicus and *Ae. albopictus*	13 (92)	1	1	2
Oc. j. japonicus, *Oc. triseriatus*, and *Ae. albopictus*	0	0	0	0

[1]Fifty-seven oviposition traps were set at 57 different sites.
[2]Percentage of the first species listed in the combination.
[3]Percent of total for species combinations calculated using the total for the entire collection, 971.

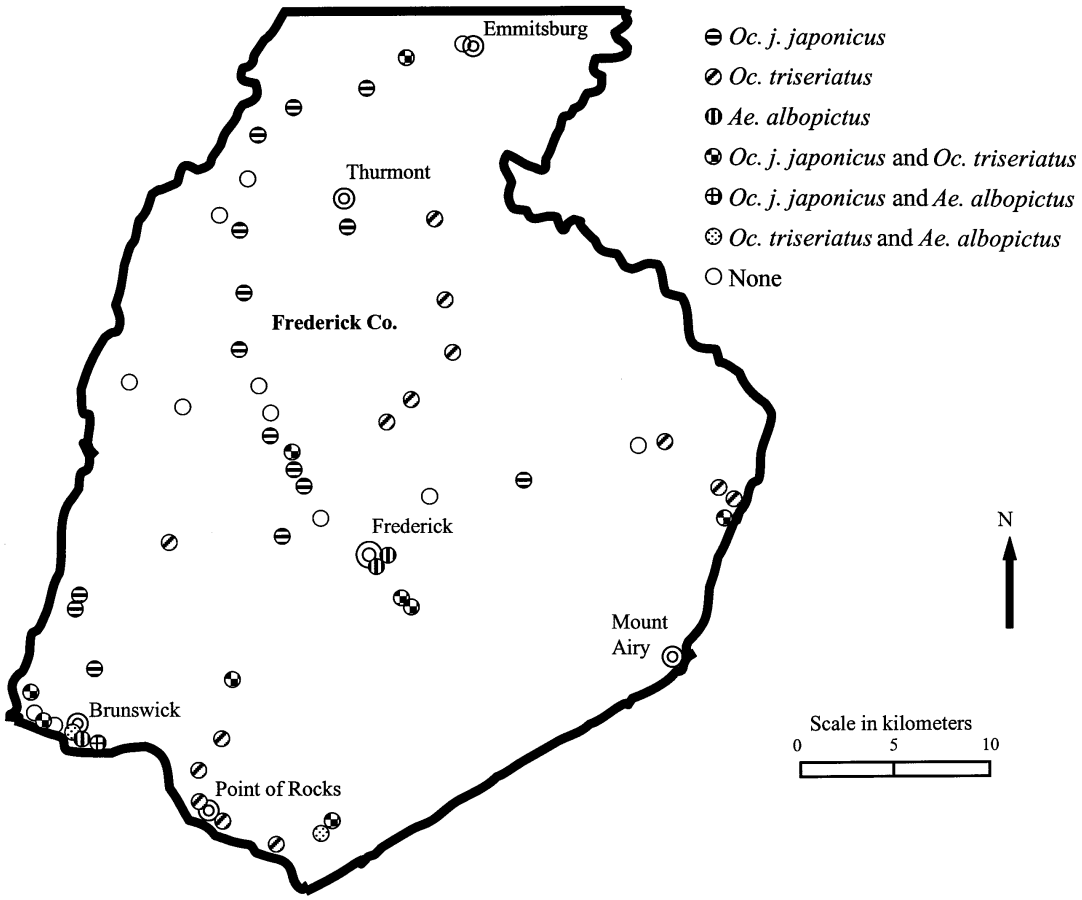

Figure 3. Map of Frederick County, Maryland, showing locations of oviposition traps and *Ochlerotatus* and *Aedes* mosquitoes captured at each site. Based on a single survey using 57 oviposition traps between June 30 and August 26, 2000.

Table 3. Summary of the container survey done in Frederick County, Maryland, between May 30 and June 29, 2001.

Species	*n* [1]	Percent of total	No. of positive containers[2]	Percent positive
Culex restuans	*592*	*53*	*24*	*62*
Ochlerotatus j. japonicus	*408*	*36*	*23*	*59*
Ochlerotatus triseriatus	*108*	*10*	*15*	*38*
Orthopodomyia signifera	*10*	*<1*	*2*	*5*
Anopheles punctipennis	2	<1	2	5
Toxorhynchites rutilus septentrionalis	1	<1	1	3

[1]Total number of larvae collected was 1,121.
[2]Total number of containers surveyed was 39.

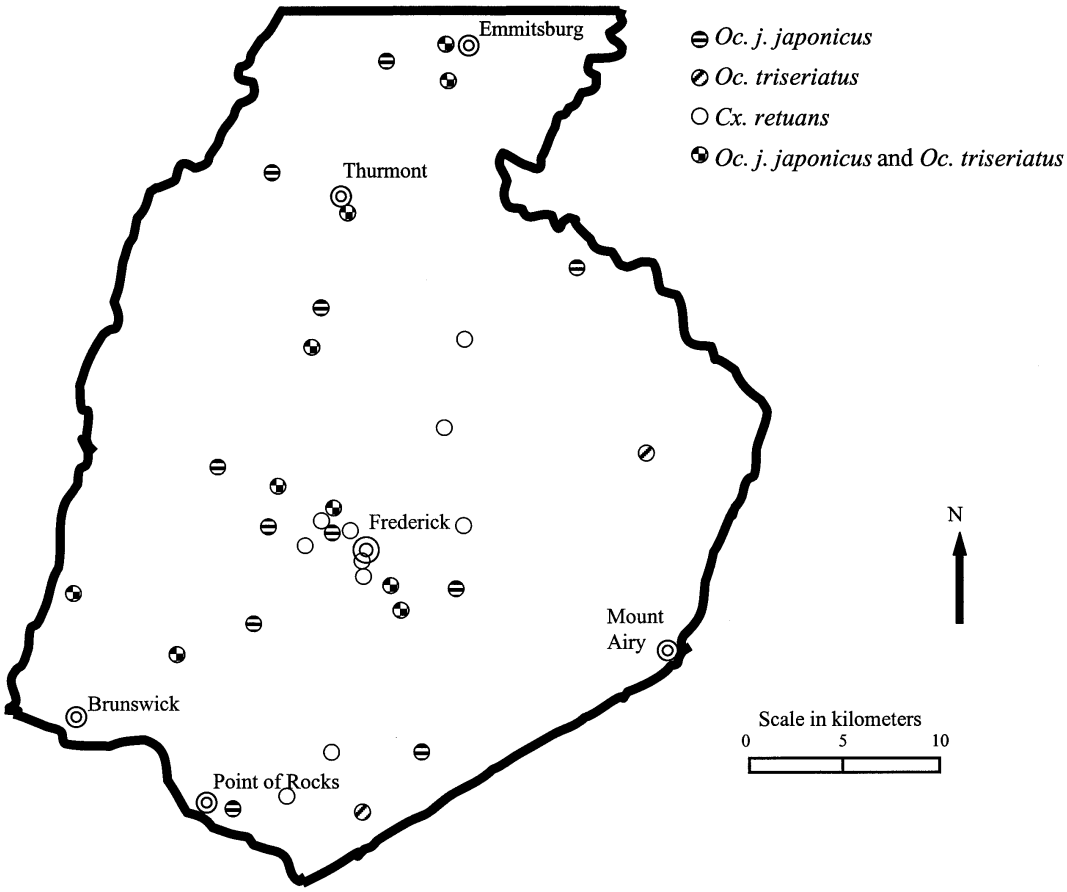

Figure 4. Map of Frederick County, Maryland, showing locations of larval collection sites and *Ochlerotatus j. japonicus, Ochlerotatus triseriatus,* and *Culex restuans* mosquitoes captured at each site. Collections were made at 39 locations between May 30 and June 29, 2001.

Chapter 3

Ochlerotatus j. japonicus and West Nile virus: vector competence and viral replication and dissemination

ABSTRACT

This study evaluated the potential for a Maryland strain of *Ochlerotatus j. japonicus* to transmit a North American isolate of West Nile (WN) virus and examined WN virus replication and dissemination in this mosquito. Vector competence studies indicated that *Oc. j. japonicus* is an efficient laboratory vector of WN virus. Depending on the WN viral titer at time of feeding, infection rates were 57-80% and estimated transmission rates were 53-75% for *Oc. j. japonicus*. Studies of the viral titers in mosquitoes showed that titers in the bodies of infected *Oc. j. japonicus* reached their peak (~$10^{6.5}$ plaque-forming units/mosquito) between 7 and 11 days after taking an infectious blood meal, and that virus became detectable in the legs (an indicator of disseminated infection) as early as 3 days after taking an infectious blood meal. In addition to laboratory vector competence, host-feeding preference, survivorship, and abundance also determine the role that *Oc. j. japonicus* could play in transmitting WN virus.

INTRODUCTION

The subspecies *Ochlerotatus j. japonicus* (Theobald) was reported for the first time in the USA in New Jersey and New York in the late summer of 1998 (Peyton et al. 1999). This mosquito has since been found in Connecticut (Andreadis T., Connecticut Agricultural Experiment Station, New Haven, CT, unpublished data), Ohio (Restifo R., Ohio Vector Borne Disease Unit, Columbus, OH, unpublished data), and Pennsylvania (Pagac B., US Army Centers for Health Promotion and Preventive Medicine-North, Fort Meade, MD, unpublished data). *Ochlerotatus japonicus sensu lato* (*s.l.*) is native to Japan, Korea, Taiwan, and southern China (Tanaka et al. 1979). Its distribution and bionomics in the USA is still largely unknown. This species breeds in natural and artificial containers and is generally found associated with wooded areas. Within its native range, *Oc. japonicus s.l.* is active primarily during the daytime (Tanaka et al. 1979). Little is known about the feeding preference of *Oc. japonicus s.l.* In Japan, it was reported to bite humans as well as birds (Tanaka et al. 1979). In the laboratory, *Oc. japonicus s.l.* readily fed on birds and mice (Miyagi 1971).

The public health importance of *Oc. j. japonicus* in the USA has not been studied in detail. Takashima and Rosen (1989) reported that this species was able to transmit Japanese encephalitis virus in the laboratory, and Turell et al. (2001) found a New Jersey strain of *Oc. j. japonicus* to be an efficient laboratory vector of WN virus. West Nile virus was detected in *Oc. j. japonicus* captured in New York in 2000 (Centers for Disease Control and Prevention (CDC) 2000a).

Because of recent interest in WN virus (CDC 1999) and the need to elucidate the role newly invasive mosquito species may play in the epidemiology of WN virus in the

eastern US, laboratory studies of the vector competence of Maryland-collected *Oc. j. japonicus* for WN virus were conducted. Additionally, a study was done to evaluate viral replication and dissemination in these mosquitoes.

MATERIALS AND METHODS

Mosquitoes:

The *Oc. j. japonicus* mosquitoes were from larvae found in discarded tires in Frederick, MD, and from eggs collected in oviposition traps set throughout Frederick County, MD. These specimens were collected June through July 2000. The larvae were transferred to pans (31 × 19 × 6 cm) containing dechlorinated tap water, provided ground catfish chow (AquaMax Pond Plus 3000, Purina Mills, Inc., St. Louis, MO) for nutrition, and reared at 26^{o}C, 80-85% RH, and 16:8 hr L:D photoperiod in an environmental chamber. Eggs on ovistrips were flooded with dechlorinated tap water on the day they were collected and the resulting larvae were reared as described above. Adults were maintained in 3.8-liter cardboard cartons with netting covering one end in the same environmental chamber that the immature stages were held and provided water soaked gauze pads and apple slices for sustenance.

Culex quinquefasciatus Say from a colony established in 1988 from specimens collected in Sebring County, Florida, was used as the comparison species in the virus studies. This species was tested because it is a vector of St. Louis encephalitis virus in the southern USA (Chamberlain et al. 1959), a virus with an epidemiology similar to WN virus, and preliminary studies (Sardelis et al. 2001) indicate that it is a competent laboratory vector, with transmission efficiency comparable to *Cx. pipiens* (Turell et al. 2001), the presumed primary vector of WN virus (CDC 2000a). The *Cx. quinquefasciatus* mosquitoes were reared and maintained under similar conditions as the *Oc. j. japonicus*.

Four- to 10-day-old adult mosquitoes were used in the susceptibility, transmission, or viral replication studies. For feeding on chickens, apple slices were removed one day prior to feeding and mosquitoes were transferred to 0.9-liter cartons with netting over one end.

Virus and viral assay:

The WN virus strain (Crow 397-99) used in these studies was isolated from a dead crow found in the Bronx, New York, during an epizootic in 1999 (Turell et al. 2000) and had been passaged once in Vero cell culture. Viral stock suspensions, triturated mosquito suspensions, and chicken blood samples were tested for infectious virus by plaque assay on Vero cells as described by Gargan et al. (1983), except that the second overlay, containing neutral red stain, was added 2 days after the first overlay.

Viremia profile studies:

Preliminary studies were done to determine WN virus viremia profiles in young white leghorn chickens (*Gallus gallus*). Less than 1-day-old chickens were inoculated subcutaneously with 0.1 ml of a suspension containing $10^{4.2}$ plaque-forming units (PFU) of WN virus. These chickens were bled daily from the jugular vein (0.1 ml of blood in heparinized diluent) 24, 48, and 72 hr after inoculation. The blood was diluted in 0.9 ml of diluent (10% heat-inactivated fetal bovine serum in Medium 199 with Earle s salts, $NaHCO_3$, and antibiotics) plus 10 units of heparin per ml and frozen at —70°C until tested for virus.

Vector competence:

Mosquitoes were allowed to feed on 1- to 2-day-old chickens that had been inoculated with approximately 10^3 PFU of WN virus 24 or 48 hr earlier. To best

compare oral susceptibility to WN virus of *Oc. j. japonicus* and *Cx. quinquefasciatus*, mosquitoes were placed in the single carton and allowed to feed on the same chicken. Immediately after mosquito feeding, a 0.1-ml blood sample was obtained from the jugular vein of each chicken to determine the viremia at the time of mosquito feeding. Engorged mosquitoes were separated by species and transferred to 3.8-liter cardboard cartons with netting over the open end and maintained at 26°C with a 16:8 (L:D) photoperiod. Four days after the infectious blood meal, an oviposition substrate was added to each carton. After incubation for 12-14 days, the mosquitoes were allowed to refeed on <1- to 2-day-old chickens either individually or in small groups to determine if they could transmit virus by bite. Immediately after the feeding attempt, the mosquitoes were killed by freezing (5 min at —20°C), feeding status determined, and their legs and bodies triturated separately in 1 ml of diluent and frozen at —70°C until assayed for virus. Presence of virus in a mosquito s body indicated infection, while virus in the legs indicated the mosquito had a disseminated infection (Turell et al. 1984).

To more efficiently examine virus transmission, some of the unfed mosquitoes were inoculated intrathoracically (Rosen and Gubler 1974) with 0.3 µl of a suspension containing $10^{4.2}$ PFU of WN virus/ml ($10^{0.7}$ PFU/mosquito) and allowed to feed on 1- to 3-day-old chickens 7-10 days later. Mosquito and blood specimens from these chickens were processed as described for the orally exposed mosquitoes, except that the mosquitoes were ground whole. This exception was made because these mosquitoes had been inoculated with virus, so it was not necessary to determine if they had a disseminated infection.

Viral replication and dissemination over time:

To evaluate viral replication and dissemination, mosquitoes were fed on a viremic chicken and held at 26°C. The *Oc. japonicus* and the *Cx. quinquefasciatus*, one species followed immediately by the other, fed upon the same chicken inoculated 48 hr earlier with WN virus as described above. Samples of three to five mosquitoes were assayed, leg and bodies separately, for virus immediately after the infectious blood meal and on days 1, 3, 5, 7, 11-12, and 14 after blood feeding.

Data analysis:

The infection rate was calculated as: (the number of infected mosquitoes/total tested) × 100. The dissemination rate was calculated as: (the number of mosquitoes with positive legs/total tested) × 100. The estimated transmission rate was calculated by multiplying the percentage of mosquitoes with a disseminated infection (after either oral or by intrathoracic inoculation) that transmitted virus by bite times the percentage of mosquitoes that developed a disseminated infection after feeding on a host with a particular viremia. The various percentages (e.g., infection and dissemination) were compared by Chi-square or Fisher exact tests as appropriate, and significant differences were determined at the 95% confidence level (SAS Institute Inc. 1999). Log transformed mean titers in blood of the chickens at one sample time to the next in the viremia profile study were compared using paired *t*-tests.

RESULTS

The viremia profile for WN virus in chickens is shown in Figure 1. Mean (95% CI) viral titers were 5.9 (5.6 — 6.1), 6.8 (6.5 — 7.0), and 6.9 (6.7 — 7.1) at 24, 48, and 72 hr after inoculation, respectively. Mean viral titer at 48 hr after inoculation was significantly ($P < 0.001$) higher than the mean viral titer 24 hr after inoculation. However, there was no significant difference ($P = 0.277$) between viral titer in chickens 48 hr after inoculation and 72 hr after inoculation.

Ochlerotatus j. japonicus was susceptible to infection with WN virus at the viral titers tested (Table 1). The proportion of *Oc. j. japonicus* and *Cx. quinquefasciatus* infected with WN virus significantly increased with the viral titer of the blood meal ($\chi^2 \geq 9.27$, df = 1, $P < 0.003$). At the low viral titer, infection rates for *Oc. j. japonicus* (57%) and *Cx. quinquefasciatus* (69%) were not significantly different ($\chi^2 = 1.80$, df = 1, $P = 0.18$). By contrast, at the higher viral titer, infection rate for *Cx. quinquefasciatus* (93%) was significantly higher ($\chi^2 = 4.97$, df = 1, $P = 0.026$) than infection rate for *Oc. j. japonicus* (80%). As with infection rates, dissemination rates significantly increased ($\chi^2 \geq 6.96$, df = 1, $P < 0.008$) with the viral titer of the blood meal. Dissemination rates for *Oc. j. japonicus* at both viral doses were 2-3% lower than the corresponding infection rate, while dissemination rates for *Cx. quinquefasciatus* at both viral doses were 63-65% lower than the corresponding infection rate. Depending on the viral titer at time of feeding, the estimated transmission rates for *Oc. j. japonicus* for WN virus were three to nine times higher than that for *Cx. quinquefasciatus.*

Both *Oc. j. japonicus* and *Cx. quinquefasciatus* with a disseminated infection transmitted WN virus by bite (Table 2). Ninety-seven percent (29/30) of *Oc. j. japonicus* and 94% (17/18) of *Cx. quinquefasciatus* with a disseminated infection transmitted WN virus by bite. Route of infection did not significantly affect transmission of virus by mosquitoes with disseminated infections of either species (Fisher s exact test, $P > 0.22$).

For mosquitoes that fed on a chicken with a viremia of $10^{6.8}$ PFU/ml of blood, viral titers in both species generally increased from day 3-11, with titers reaching nearly 10^{7} PFU per body for *Oc. j. japonicus* and approximately $10^{4.9}$ PFU per body for *Cx. quinquefasciatus* (Table 3). Disseminated infections were detected 3 days after the infectious blood meal in *Oc. j. japonicus*, but not until days 11-12 in *Cx. quinquefasciatus* (Table 3).

DISCUSSION

The study of viremias in chicken inoculated with WN virus showed that viremias increased during the first two days after inoculation. This resulted in two distinct viremia levels, one at approximately $10^{5.9}$ PFU/ml of blood (24 hr post-inoculation) and one at approximately $10^{7.0}$ PFU/ml of blood (48-72 hr post-inoculation). These viremias are similar to those found in wild birds, such as crows and house sparrows, in Egypt (Work et al. 1955). Thus, the viremias at the time of mosquito feeding in this study should be representative of that which mosquitoes would be exposed to in nature. Additionally, the viremias in this study produced by inoculation with once-passaged WN virus (Crow 397-99) were comparable to the viremias produced from the parent (unpassed) virus (Turell et al. 2001). In the Turell study, viremias were around $10^{5.5}$, $10^{6.8}$, $10^{7.5}$ PFU/ml of blood at 24, 48, and 72 hr after inoculation, respectively.

This study showed that the Maryland strain of *Oc. j. japonicus* was highly susceptible to infection with WN virus, and depending on viral titer in the blood meal, an estimated 75% of orally exposed *Oc. j. japonicus* may transmit virus 12-14 days later. These results are similar to those of Turell et al. (2001), who reported that the estimated transmission rate for a New Jersey strain of *Oc. j. japonicus* was 64% percent. Both this study and the Turell study indicated that transmission efficiency of WN virus by *Oc. j. japonicus* is primarily determined by the susceptibility of the midgut to infection. In other words, should enough virus be ingested in the blood meal to infect the midgut, the virus will then readily escape the midgut epithelial cells, enter the hemocoel, infect the salivary gland, pass into the lumen of the salivary glands and be passed to a host during feeding via the saliva. The absence of significant midgut escape and salivary gland

barriers in the Maryland strain of *Oc. j. japonicus* was indicated by the close agreement of the infection and dissemination rates (a difference of ≤3%) and the highly efficient transmission of virus by mosquitoes with a disseminated infection (97%). In comparison, for a New Jersey strain of *Oc. j. japonicus* tested under comparable conditions, the difference between infection and dissemination rate was 5% and transmission of virus by mosquitoes with a disseminated infection was 100% (Turell et al. 2001).

Previous studies have shown that the vector competence of a mosquito species for flaviviruses may vary between geographic strains (Gubler et al. 1979, Tran et al. 1999, Boromisa et al 1987, Gubler and Rosen 1976, Hayes et al. 1984, Kay et al. 1984). A recent study of the population genetics of *Oc. j. japonicus* that included the Maryland strain used in this study and the New Jersey strain used by Turell et al. (2001) indicated that the two stains are genetically distinct (Fonseca et al. 2001). Still, the vector competence of the two stains for WN virus was similar.

In the biological transmission of arboviruses, the extrinsic incubation period (EIP), the period from ingestion of an infectious blood meal by a vector to when the vector can transmit the virus by bite, is an important component in determining vectorial capacity. The EIP for flaviviruses in mosquitoes incubated at 26-28^{o}C is 9-12 days (Miller et al. 1989, Watts et al. 1987, Reisen et al. 1993). This study showed that WN virus is transmitted by nearly all *Oc. j. japonicus* 12-14 days after oral exposure and incubation at 26^{o}C. Additional evidence from this study indicated that the EIP might be shorter than 12 days. The studies of viral dissemination over time indicated that virus was present in the hemocoel as early as 3 days after ingestion of the viremic blood and that nearly all *Oc. j. japonicus* had disseminated infections 7 days after ingestion of the

viremic blood. Furthermore, the study of viral replication over time indicated that the amount of virus circulating in the hemolymph (as estimated by the titer in a mosquito s legs) peaked between 7 and 11 days after the infectious blood meal. These additional findings appear to indicate that the EIP may be around 9 days. Because the EIP_{50}, the time period required for 50% of a population to be capable to transmitting virus, is commonly used in modeling arbovirus transmission, the 9 day EIP estimated may be a bit conservative (i.e., 1-3 days shorter). Additional transmission studies are needed to more precisely determine the EIP and to evaluate the effect of temperature on EIP for WN virus in *Oc. j. japonicus*.

The high vector efficiency of *Oc. j. japonicus* compared to *Cx. quinquefasciatus* or *Cx. pipiens* may have an important bearing on the epidemiology of WN virus. *Culex* mosquitoes are considered the primary enzootic vector of WN virus in the eastern USA, and their status as the primary vector is supported by the >350 field isolates of WN virus from these mosquitoes during 2000 in the eastern USA (CDC 2000b). During 2000, nine pools of *Oc. j. japonicus* showed evidence of WN virus infection (CDC 2000b). However, in the absence of information on the survivorship, host preference, and abundance of *Oc. j. japonicus* in the USA, it is difficult to make an accurate prediction on the possible impact of this newly invasive species. Findings reported in Chapter 2 of this dissertation indicated that the distribution of *Oc. j. japonicus* in the eastern USA is expanding and that in Frederick County, Maryland, it is widely distributed and abundant. The expansion of the geographic range of *Oc. j. japonicus* lends impetus to the need to determine the bionomics of this species in the USA.

The findings in this chapter on the vector competence of *Oc. j. japonicus* for WN virus have been recently published and are included in Appendix B.

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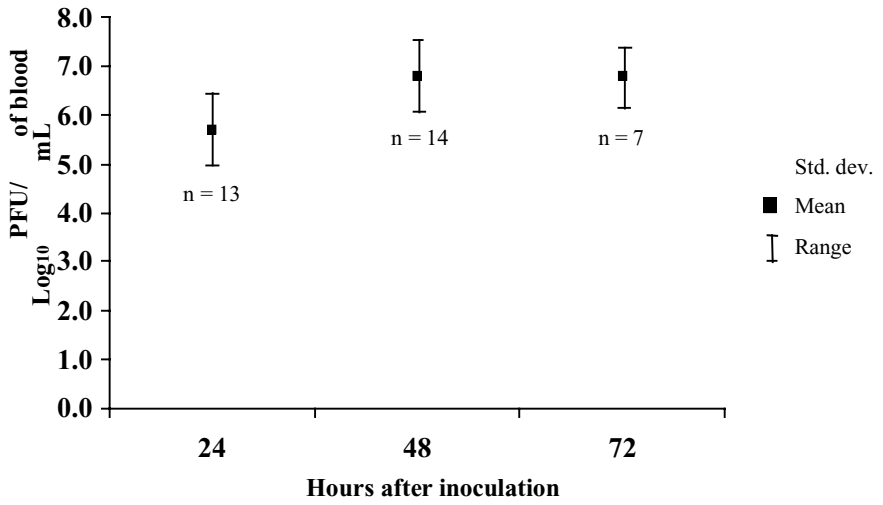

Figure 1. Viremias in chickens (*Gallus gallus*) 24, 48, and 72 hr after inoculation with 0.1 ml of a suspension containing $10^{4.2}$ plaque-forming units (PFU) of West Nile virus (Crow NY397-99, Vero-1 passage). Chickens were less than 1-day-old at time of inoculation.

Table 1. Infection, dissemination, and estimated transmission rates of *Ochlerotatus j. japonicus* and *Culex quinquefasciatus* for West Nile virus.

Species	Viral titer at time of feeding[1]	Infection rate[2]	Dissemination rate[3]	Estimated transmission rate[4]
Oc. j. japonicus	6.0 ± 0.5	52/92 (57)	51/92 (55)	53
	7.0 ± 0.4	66/83 (80)	64/83 (77)	75
Cx. quinquefasciatus	6.0 ± 0.5	25/36 (69)	2/36 (6)	6
	7.0 ± 0.4	54/58 (93)	16/58 (28)	26

[1]Titer in chickens (*Gallus gallus*) inoculated 24 or 48 h previously with a West Nile virus (Crow 397-99).
[2]Number of mosquitoes containing virus in their bodies/number tested (% infected).
[3]Number of mosquitoes containing virus in their legs/number tested (% disseminated).
[4]The estimate transmission rate = the percentage of mosquitoes that developed a disseminated infection 12-14 days after ingesting WN virus multiplied by the percentage of individuals with a disseminated infection that transmitted virus by bite (see Table 2). The percentage of individuals with a disseminated infection that transmitted virus by bite was previously determined to be 97% for *Oc. j. japonicus* and 94% for *Cx. quinquefasciatus*.

Table 2. Transmission of virus by bite for mosquitoes with a disseminated infection after either oral exposure to or intrathoracic inoculation with WN virus

Species	Route of exposure		
	Oral[1]	Intrathoracic inoculation[2]	Combined[3]
Ochlerotatus j. japonicus	10/11 (91)	19/19 (100)	29/30 (97)
Culex quinquefasciatus	3/4 (75)	14/14 (100)	17/18 (94)

[1]Number transmitting virus by bite/number refeeding (% transmitting). Mosquitoes orally exposed to chickens (*Gallus gallus*) inoculated 24 or 48 hr previously with a WN virus (Crow 397-99) and held at 26°C for 12-14 days prior to refeeding.
[2]Number transmitting virus by bite/number feeding (% transmitting). Mosquitoes intrathoracically inoculated with 0.3 µl of a suspension containing $10^{4.2}$ PFU of WN virus/ml ($10^{0.7}$ PFU/mosquito), held at 26°C, and then allowed to feed on individual on 1- to 3-day-old chickens 7-10 d later.
[3]Number transmitting virus by bite/number refeeding or feeding (% transmitting).

Table 3. Viral titers[1] over time in the bodies and legs of *Ochlerotatus j. japonicus* and *Culex quinquefasciatus* after oral exposure to a West Nile virus-infected chicken with a viremia of $10^{6.8}$ plaque-forming units/ml of blood and incubation at 26°C.

	Part	**Days after oral exposure**						
Species	assayed	0	1	3	5	7	11-12	14
Oc. j. japonicus	Body	3.1 - 4.0 (1/3)	0[2] - 3.1 (3/3)	3.8 - 4.7 (3/3)	4.2 - 4.9 (3/3)	4.2 - 6.9 (5/5)	6.0 - 7.0 (3/3)	6.0 - 6.8
	Leg	0 (0/3)	0 (2/3)	0 - 2.7 (3/3)	2.1 - 2.9 (2/3)	0 - 5.7 (5/5)	3.7 - 5.4 (3/3)	4.1 - 5.3
Cx. quinquefasciatus	Body	3.9 - 4.0 (3/3)	0 - 2.6 (2/3)	2.4 - 3.8 (3/3)	2.9 - 4.2 (3/3)	2.9 - 4.9 (3/3)	4.4 - 4.7 (5/5)	4.5 - 5.3 (5/5)
	Leg	0 (0/3)	0 (0/3)	0 (0/3)	0 (0/3)	0 (1/5)	0 - 2.5 (1/5)	0 - 3.0

[1]Log_{10} PFU/ml of body or leg suspension. Range (number of mosquitoes with virus in the respective part assayed/number assayed).
[2]A viral titer of zero (0) indicates that virus was not present or that the viral titer was below the detection limit of the assay (a titer <1.7 Log_{10} PFU/ml).

Chapter 4

Transmission of St. Louis encephalitis virus by *Ochlerotatus j. japonicus*

ABSTRACT

Ochlerotatus j. japonicus, a newly discovered non-indigenous mosquito species, and a colonized strain of *Culex pipiens* were compared for their vector competence for St. Louis encephalitis (SLE) virus. After feeding on chickens with viremias between $10^{4.1}$ and $10^{4.7}$ plaque-forming units/ml of blood, *Oc. j. japonicus* infection rates were 0-33%. In comparison, infection rates were 12-94% for *Cx. pipiens* at the same range of viral doses. When the viral titer in a donor chicken was increased to between $10^{5.3}$ and $10^{5.6}$ plaque-forming units/ml of blood, infection rates for *Oc. j. japonicus* and *Cx. pipiens* were similar, at 94% and 100%, respectively. After 12-14 days of extrinsic incubation at 26°C, nearly all (98%, 57/58) infected *Oc. j. japonicus* had a disseminated infection. In contrast, only 43% (23/54) of infected *Cx. pipiens* had a disseminated infection. Transmission of virus by mosquitoes (both species) with disseminated infection was efficient, at ≥83%. Estimated transmission rates at viral doses sufficient to infect both of the tested species were 31-90% for *Oc. j. japonicus* and 30-50% for *Cx. pipiens*. Because of its continued geographic expansion, field and laboratory evidence incriminating it as a vector of the closely related West Nile virus, and its ability to transmit of SLE virus in the laboratory, *Oc. j. japonicus* should be considered as a potential enzootic or epizootic vector of SLE virus.

INTRODUCTION

The recent introduction of *Ochlerotatus j. japonicus* (Theobald) into the northeastern and mid-Atlantic regions of the USA (Peyton et al. 1999, Sardelis and Turell 2001, Scott et al. 2001) has heightened concerns about the possibility of enhanced future transmission of arboviruses of human health importance in the eastern USA. A primary reason for this concern stems from repeated evidence of West Nile (WN) virus infection, including both virus isolates and detection of WN virus genome in *Oc. j. japonicus* collected in the New York and New Jersey in 2000 (Centers for Disease Control and Prevention 2000). The importance of these findings have been substantiated by laboratory studies that show *Oc. j. japonicus* is a highly efficient laboratory vector of WN virus (Turell et al. 2001, Sardelis and Turell 2001).

St. Louis encephalitis (SLE) virus, a member of the Genus *Flavivirus*, Family Flaviviridae, is enzootic throughout mainland USA and is closely related serologically and epidemiologically to WN virus. In the eastern USA and north of Florida, the virus primarily cycles between birds and *Culex pipiens*-complex mosquitoes and has been responsible for urban and widespread epidemics (Bleed et al. 1992, Brinker et al. 1979, Zweighaft et al. 1979, Levy et al. 1978, Powell and Blakey 1979).

The objective of this study was to assess the vector competence of *Oc. j. japonicus* for two strain of SLE virus. For comparison, *Culex pipiens* (L.) was tested in conjunction with *Oc. j. japonicus*.

MATERIALS AND METHODS

Mosquitoes:

The *Oc. j. japonicus* were from eggs collected in oviposition traps (Zeichner and Perich 1999) set throughout Frederick County, Maryland, during May through June of 2001. On the day the oviposition traps were returned to the laboratory, the eggs on ovistrips were placed in 31 × 19 × 6 cm plastic pans and flooded with dechlorinated tap water. The resulting larvae were provided ground catfish chow (AquaMax Pond Plus 3000, Purina Mills, Inc., St. Louis, MO) for nutrition, and reared at 26^{o}C, 80-85% RH, and 16:8 hr L:D photoperiod in an environmental chamber. Adults were maintained in 3.8-liter cardboard cartons with netting covering one end in the same environmental chamber that the immature stages were held and provided water-soaked gauze pads and apple slices for sustenance.

For comparison, *Culex pipiens* from a colony established in 1999 from specimens collected in Westchester County, New York (Turell et al. 2000), was used. These mosquitoes were reared and maintained under similar conditions as the *Oc. j. japonicus*.

To minimize age related effects, only 4- to 10-day-old adult mosquitoes were used. For feeding on chickens, the apple slices were removed one day prior to feeding and mosquitoes were transferred to 0.9-liter cartons with netting over one end.

Virus and viral assay:

The Fort Washington (FTWASH) strain of SLE virus was isolated from overwintering *Cx. pipiens* mosquitoes collected in Fort Washington, MD, in 1977 (Bailey et al. 1978) and had been passaged ≤ 3 times in Vero cell culture. The TBH-28 strain was isolated from a fatal case of SLE in Tampa Bay, FL, in 1962 (Coleman et al. 1968)

and had been passaged 12 times in mouse brains and once in Vero cell culture. Stocks of virus at a concentration of $10^{4.6}$ plaque-forming units (PFU)/ml for the FTWASH strain and $10^{4.3}$ PFU/ml for the TBH-28 strain were prepared in a standard diluent (10% heat-inactivated fetal bovine serum in Medium 199 with Earle's salts, $NaHCO_3$ and antibiotics). Viral stocks, triturated mosquito suspensions, and chicken blood samples were tested for infectious virus by plaque assay on Vero cells as previously described (Gargan et al. 1983), except that the second overlay, containing neutral red stain, was added 5 days instead of 4 days after the first overlay.

Viremia profile studies:

Preliminary studies were done to determined SLE virus viremia profiles in young white leghorn chickens (*Gallus gallus*). Less than 1-day-old chickens were inoculated subcutaneously with 0.1 ml of a suspension containing 10^4 plaque-forming units (PFU) of one of the SLE viruses. These chickens were bled daily from the jugular vein (0.1 ml of blood) 24, 48, 72, and 96 hr after inoculation. The blood was diluted in 0.9 ml of the standard diluent plus 10 units of heparin per ml and frozen at —70°C until tested for virus.

Vector competence studies:

Mosquitoes were allowed to feed on 1- to 2-day-old leghorn chickens (*Gallus gallus*) that had been inoculated with 10^4 PFU of one of the SLE viruses 1-2 days earlier. Immediately after mosquito feeding, blood was obtained from the jugular vein of each chicken (0.1 ml of blood into 0.9 ml of heparinized diluent) and the blood suspensions were frozen at —70°C until assayed for virus to determine the viremias at the time of mosquito feeding. After feeding on viremic chickens, engorged mosquitoes were transferred to 3.8-liter screen-topped cardboard cages and held at 26°C with a 16:8 (L:D)

hr photoperiod. After an incubation period of 12 to 14 days, the mosquitoes were allowed to refeed on <1- to 2-day-old chickens either individually or in groups of 5 to determine if they could transmit virus by bite. Immediately after the transmission attempt, the mosquitoes were killed by freezing, the feeding status determined, and their legs and bodies triturated separately in 1 ml of diluent. Infection was determined by recovery of virus from the mosquito tissue suspension. If virus was recovered from its body, but not its legs, the mosquito was considered to have a nondisseminated infection limited to its midgut. In contrast, if virus was recovered from both the body and leg suspensions, the mosquito was considered to have a disseminated infection (Turell et al. 1984). Infection and dissemination rates were defined as the percentages of mosquitoes tested that contained virus in their body or legs, respectively. Chickens used in the transmission attempts were bled from the jugular vein 1 or 2 days after mosquito feeding and the blood handled as described above. Recovery of virus from this blood indicated transmission.

To more efficiently examine viral transmission, some of the original, unfed mosquitoes were inoculated intrathoracically (Rosen and Gubler 1974) with 0.3 μl of a viral suspension containing $10^{4.3-4.6}$ PFU of SLE virus/ml ($10^{0.8-1.1}$ PFU/mosquito), held 7-9 days, and allowed to feed on <1- to 2-d-old chickens. Mosquitoes and blood specimens from these chicks were processed as described for the orally exposed mosquitoes.

In order to estimate transmission rates for the two species, the percentage of mosquitoes with a disseminated infection (after either oral exposure or by intrathoracic inoculation) that transmitted virus by bite was determined. This value was then

multiplied times the percentage of mosquitoes that developed a disseminated infection after feeding on a host with a particular viremia. This resulted in an estimated transmission rate for those mosquitoes.

Data analysis:

Infection and dissemination rates were compared by Chi-square or Fisher exact tests as appropriate and significant differences were determined at the 95% confidence level (SAS Institute Inc. 1999).

RESULTS

The viremia profile for the two strains of SLE virus in chickens is shown in Figure 1. For the TBH-28 strain, mean viral titers were highest ($10^{3.8}$-$10^{4.3}$ PFU/ml of blood) 24 hr after inoculation and fell to undetectable levels by 72 hr after inoculation. In comparison, the peak viral titer in the FTWASH inoculated chickens occurred 48 hr after inoculation and ranged from $10^{4.8}$-$10^{5.9}$ PFU/ml of blood.

Ochlerotatus j. japonicus was susceptible to infection with both of the SLE viral strains. For TBH-28, the infection rate for *Cx. pipiens* after ingesting a blood meal with a titer of $10^{4.1}$ PFU/ml of blood was 12%, while the infection rate for *Oc. j. japonicus* at the same titer was 0% (Table 1). There was no significant difference between these two rates (Fisher s exact test, $P = 0.057$). When the viral titer in the infectious blood meal was $10^{4.4}$ PFU/ml of blood, nearly doubled from $10^{4.1}$ PFU/ml of blood, 15% of *Oc. j. japonicus* became infected. Three out of 4 of these infected mosquitoes developed a disseminated infection. Transmission of TBH-28 by *Oc. j. japonicus* and *Cx. pipiens* with a disseminated infection as a result of intrathoracic inoculation with virus was 83% (15 of 18) and 88% (7 of 8), respectively. Because dissemination rates for mosquitoes orally exposed to TBH-28 were low (0-12%), no data on the transmission of this virus by orally exposed mosquitoes was obtained. The estimated transmission rate for *Oc. j. japonicus* at the highest viral titer blood meal was 10% (Table 1).

Infection, dissemination, and estimated transmission rates for the FTWASH strain of SLE virus by *Ochlerotatus j. japonicus* and *Cx. pipiens* are shown in Table 2. The proportion of *Oc. j. japonicus* infected with the Fort Washington strain significantly increased with the viral titer of the blood meal ($\chi^2 = 22.55$, df = 1, $P < 0.001$), while there

was no significant difference (Fisher s exact test, $P = 0.53$) in infection rates for *Cx. pipiens* associated with the concentration of virus in the blood meal. At low viral titers, the lower infection rates for *Oc. j. japonicus* (33%) compared to the rate *Cx. pipiens* (94%) were statistically significant ($\chi^2 = 26.91$, df = 1, $P < 0.001$). In comparison, at the higher viral titer range, there was no significant difference (Fisher s exact test, $P = 0.56$) between the infection rates for *Oc. j. japonicus* (96%) and *Cx. pipiens* (100%). As with infection rates, dissemination rates for *Oc. j. japonicus* increased significantly ($\chi^2 = 6.96$, df = 1, $P = 0.008$) with the viral titer of the blood meal. However, dissemination rates for *Cx. pipiens* did not significantly increase ($\chi^2 = 2.53$, df = 1, $P = 0.11$) with increase in viral titer in the blood meal. Dissemination rates for *Oc. j. japonicus* at both viral doses were identical to the corresponding infection rate, while dissemination rates for *Cx. pipiens* at both viral doses were 59-42% lower than the corresponding infection rate. Depending on the viral titer at time of feeding, the estimated transmission rates for *Oc. j. japonicus* for WN virus were comparable to or nearly 2 times higher than that for *Cx. pipiens.*

Both *Oc. j. japonicus* and *Cx. pipiens* with a disseminated infection transmitted the FTWASH strain of SLE virus by bite (Table 3). Ninety four percent (15/16) of *Oc. j. japonicus* and 86% (6/7) of *Cx. pipiens* with a disseminated infection transmitted virus by bite. Route of infection did not significantly affect transmission of virus by mosquitoes with disseminated infections of either species (Fisher s exact test, $P > 0.29$).

DISCUSSION

The study of viremias in chicken inoculated with SLE virus showed that duration and levels of viremias varied with virus strain. This finding was consistent with previous studies that indicate that there can be a great deal of variability in viremic response in wild and domestic bird species based on the particular strain of SLE virus (Bowen et al. 1980, Reisen et al. 2000). Most importantly, the viremias at the time of mosquito feeding in this study, between $10^{3.5}$ and $10^{5.6}$ PFU/ml of blood, are similar to those produced in an number of epidemiologically important avian hosts, such as robins, cardinals and house sparrows, experimentally infected with SLE virus (McLean et al. 1985, Savage et al. 1994). Thus, the rates observed in this study should reflect those that would occur in nature.

This study showed that *Oc. j. japonicus* was moderately susceptible to infection with SLE virus, and, depending on viral titer in the blood meal, an estimated 10-90% of orally exposed *Oc. j. japonicus* may transmit virus when allowed to refeed 12-14 days later. Compared to *Cx. pipiens*, *Oc. j. japonicus* needed to be exposed to a slightly higher concentration of virus to become infected, and virus more readily escaped the midgut of *Oc. j. japonicus*. These two differences resulted in similar estimated transmission rates for *Oc. j. japonicus* and *Cx. pipiens* under the conditions in this study.

This is the first evidence of laboratory transmission of SLE virus by *Oc. j. japonicus*. Because *Oc. j. japonicus* is just now expanding into areas of SLE activity, there are no data on its potential to serve as a natural vector of SLE virus. However, *Oc. j. japonicus* is known to be an efficient experimental vector of two other flaviviruses:

Japanese encephalitis virus (Takashima and Rosen 1989) and West Nile virus (Turell et al 2001, Sardelis and Turell 2001).

At the present time, it is difficult to predict the extent to which *Oc. j. japonicus* will become involved in the transmission cycle of SLE virus. As noted in the previous chapter, little is known about the behavioral and other biological characteristics of *Oc. j. japonicus* in the USA. In 2000, evidence of WN virus infection was reported in wild-caught *Oc. j. japonicus* (CDC 2000). This is important because the enzootic cycles of WN and SLE viruses in urban settings in the eastern USA are similar. As overlap in distribution of *Oc. j. japonicus* and SLE virus increases, contact between the two will increase. Based on the above points and on the vector competence *Oc. j. japonicus*, it should be considered a potential secondary vector in the enzootic cycle of SLE virus and a possible bridge vector of this virus to dead-end hosts.

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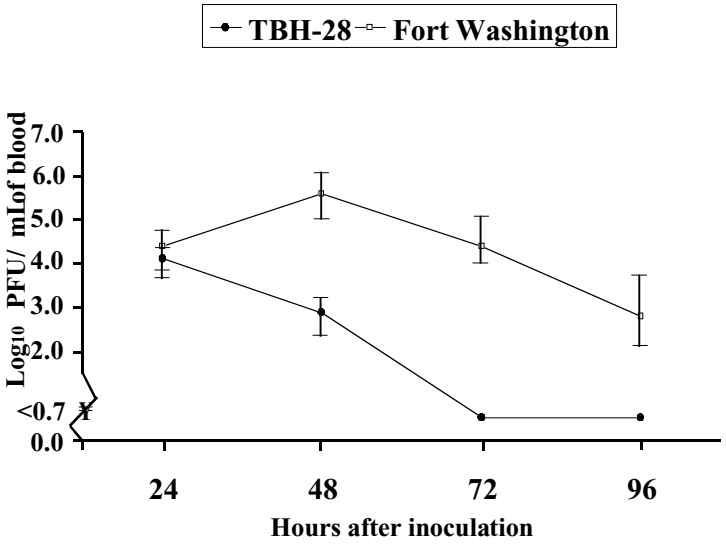

Figure 1. Viremias in chickens (*Gallus gallus*) 24, 48, 72, and 96 hr after inoculation with 0.1 ml of a suspension containing approximately 10^4 plaque-forming units (PFU) of either the TBH-28 or Fort Washington strain of St. Louis encephalitis virus. Chickens were less than 1-day-old at time of inoculation. Bars indicate range of viremias.

Table 1. Infection, dissemination, and estimated transmission rates of the TBH-28 strain of St. Louis encephalitis virus by *Ochlerotatus j. japonicus* and *Culex pipiens*

Species	Viral titer at time of feeding[1]	Infection rate[2]	Dissemination rate[3]	Estimated transmission rate[4]
Oc. j. japonicus	3.5	0/14 (0)	0/14 (0)	0
	4.1	0/40 (0)	0/40 (0)	0
	4.4	4/26 (15)	3/26 (12)	10
Cx. pipiens	3.5	0/13 (0)	0/13 (0)	0
	4.1	3/26 (12)	0/26 (0)	0

[1]Titer in chickens (*Gallus gallus*) inoculated 24 hr previously with virus.
[2]Number of mosquitoes containing virus in their bodies/number tested (% infected).
[3]Number of mosquitoes containing virus in their legs/number tested (% disseminated).
[4]The estimate transmission rate = the percentage of mosquitoes that developed a disseminated infection 12-14 days after ingesting SLE virus multiplied by the percentage of individuals with a disseminated infection that transmitted virus by bite. The percentage of individuals with a disseminated infection that transmitted virus by bite was previously determined to be 83% for *Oc. j. japonicus* and 88% for *Cx. pipiens*.

Table 2. Infection, dissemination, and estimated transmission rates of the Fort Washington strain of St. Louis encephalitis virus by *Ochlerotatus j. japonicus* and *Culex pipiens*

Species	Viral titer at time of feeding[1]	Infection rate[2]	Dissemination rate[3]	Estimated transmission rate[4]
Oc. j. japonicus	4.4-4.7	11/33 (33)	11/33 (33)	31
	5.3-5.6	23/24 (96)	23/24 (96)	90
Cx. pipiens	4.4-4.7	32/34 (94)	12/34 (35)	30
	5.3-5.6	19/19 (100)	11/19 (58)	50

[1]Titer in chickens (*Gallus gallus*) inoculated 24 or 48 hr previously with virus.
[2]Number of mosquitoes containing virus in their bodies/number tested (% infected).
[3]Number of mosquitoes containing virus in their legs/number tested (% disseminated).
[4]The estimate transmission rate = the percentage of mosquitoes that developed a disseminated infection 12-14 days after ingesting SLE virus multiplied by the percentage of individuals with a disseminated infection that transmitted virus by bite (see Table 3). The percentage of individuals with a disseminated infection that transmitted virus by bite was previously determined to be 94% for *Oc. j. japonicus* and 86% for *Cx. pipiens*.

Table 3. Transmission of virus by bite for mosquitoes with a disseminated infection after either oral exposure to or intrathoracic inoculation with the Fort Washington strain of St. Louis encephalitis virus

Species	Route of exposure		
	Oral[1]	Intrathoracic inoculation[2]	Combined[3]
Oc. j. japonicus	7/8 (88)	8/8 (100)	15/16 (94)
Culex pipiens	1/2 (50)	5/5 (100)	6/7 (86)

[1]Number transmitting virus by bite/number refeeding (% transmitting). Mosquitoes orally exposed to chickens (*Gallus gallus*) inoculated 48 hr previously with a SLE virus and held at 26°C for 12-14 days prior to refeeding.
[2]Number transmitting virus by bite/number feeding (% transmitting). Mosquitoes intrathoracically inoculated with 0.3 μl of a suspension containing $10^{4.6}$ PFU of WN virus/ml ($10^{1.1}$ PFU/mosquito), held at 26°C, and then allowed to feed on individual on 1- to 2-day-old chickens 7-9 d later.
[3]Number transmitting virus by bite/number refeeding or feeding (% transmitting).

Chapter 5

Experimental transmission of eastern equine encephalitis virus by *Ochlerotatus j. japonicus*

ABSTRACT

The potential for *Ochlerotatus j. japonicus* (Theobald), a newly recognized invasive mosquito species in the USA, to transmit eastern equine encephalitis (EEE) virus was evaluated. *Aedes albopictus* and *Culex pipiens* were tested for comparison. *Ochlerotatus j. japonicus* and *Ae. albopictus* became infected and transmitted EEE virus by bite after feeding on young chickens 1 day after they had been inoculated with EEE virus (viremias ranging from $10^{7.0\text{-}8.7}$ plaque-forming units [PFU]/ml of blood). No *Cx. pipiens* (n = 20) had detectable levels of virus 14 days after feeding on an EEE-virus infected chicken with a viremia of $10^{8.1}$ PFU per ml of blood. Depending on the viral titer in the donor chicken, infection rates ranged from 55-100% for *Oc. j. japonicus* and 93-100% for *Ae. albopictus*. In these two species, dissemination rates were identical to or nearly identical to infection rates. Depending on the viral titer in the blood meal, estimated transmission rates ranged from 15-25% for *Oc. j. japonicus* and 59-63% for *Ae. albopictus*. Studies of replication of EEE virus in *Oc. j. japonicus* showed that there was an eclipse phase in the first 4 days after an infectious blood meal, that viral titers peak by day 7 at around $10^{5.7}$ per mosquito, and that virus escaped the mid-gut as soon as 3 days after the infectious blood meal. These data, combined with the opportunistic feeding behavior of *Oc. j. japonicus* in Asia and the reported expansion of its range in the eastern USA, indicate that it could function as a bridge vector for EEE virus between the enzootic *Culiseta melanura*-avian cycle and susceptible mammalian hosts.

INTRODUCTION

Since the initial report of *Ochlerotatus japonicus japonicus* (Theobald) in the USA in New Jersey and New York in the late summer of 1998 (Peyton et al. 1999), this mosquito has been found in Connecticut (Munstermann and Andreadis 1999), Maryland (See Chapter 2), Ohio (Restifo R., Connecticut Agricultural Experiment Station, New Haven, CT, unpublished data), Pennsylvania (Pagac B., US Army Centers for Health Promotion and Preventive Medicine-North, Fort Meade, MD, unpublished data), and Virginia (Harrison B., North Carolina Department of Environment, Health & Natural Resources, Winston-Salem, NC, unpublished data). The apparent rapid spread of *Oc. j. japonicus* has raised concern whether it will become an important pest species or be involved in the transmission of North American arboviruses. *Ochlerotatus j. japonicus* from New York have been found infected with West Nile (WN) virus (CDC 2000), and vector competence studies indicate that this species can become infected with and transmit WN and St. Louis encephalitis virus (Turell et al. 2001, See Chapters 3 and 4).

Eastern equine encephalitis (EEE) virus is a member of the Genus *Alphavirus*, in the Family Togaviridae. In the USA, EEE virus is found in marsh and swamp habitats along the eastern seaboard and is maintained in an enzootic cycle involving birds and the mosquito *Culiseta melanura* (Coquillett) (Morris 1988). Between 0 and 14 human cases per year of EEE were reported from 1983 to 1997 (CDC 1998). These cases generally follow an increase in EEE viral activity in specific foci which lead to infection of bridge vector mosquito species, ones that feed on birds as well as mammals, such as *Ochlerotatus sollicitans* (Walker) (Crans et al. 1986), *Coquillettidia perturbans* (Walker) (Andreadis et al. 1998), and possibly *Aedes albopictus* (Skuse) (Mitchell et al. 1992).

To assist public health personnel assess the risk that *Oc. j. japonicus* represents for transmission of EEE virus, laboratory studies are needed to evaluate its vector competence. In addition, studies were done to evaluate viral replication and dissemination over time. For comparison, *Ae. albopictus*, an efficient laboratory vector (Turell et al. 1994), and *Culex pipiens* (L.), were similarly studied.

MATERIALS AND METHODS

Mosquitoes:

The *Oc. j. japonicus* used in this study were reared from eggs collected at Letterkenny Army Depot (LEAD), near Chambersburg, PA, during 2000, or obtained from a recently established colony (LEAD stain) derived from progeny of these eggs and maintained at the United States Army Medical Research Institute of Infectious Diseases. The *Ae. albopictus* were from a colony of mosquitoes that were originally collected in Oahu, HI, in 1971, and the *Cx. pipiens* were from a colony established in 1999 with specimens collected in Westchester County, New York. Mosquitoes were reared in containers containing dechlorinated tap water, provided ground catfish chow for nutrition, and maintained in an environmental chamber at 26^{o}C, 80-85% RH and 16:8 h L:D photoperiod. Four- to 10-d-old adult mosquitoes were used in the susceptibility, transmission, and viral replication studies.

Virus and viral assays:

The EEE virus strain (MA92-1406) used was isolated from a pool of *Cs. melanura* collected in Massachusetts and had been passaged once in Vero cell culture. Viral stock suspensions, triturated mosquito suspensions, and chicken blood samples were tested for infectious virus by plaque assay on Vero cells as described by Gargan et al. (1983), except that the second overlay, containing neutral red, was added 2 rather than 4 days after the first overlay.

Viremia profile studies:

Preliminary studies were done to determine EEE virus viremia profiles in young white leghorn chickens (*Gallus gallus*). Three- to 9-day-old chickens were inoculated subcutaneously with 0.1 ml of a suspension containing $10^{4.2}$ plaque-forming units (PFU)

of EEE virus. These chickens were bled daily from the jugular vein (0.1 ml of blood in heparinized diluent) 24 hr after inoculation. The blood was diluted in 0.9 ml of diluent (10% heat-inactivated fetal bovine serum in Medium 199 with Earle s salts, $NaHCO_3$, and antibiotics) plus 10 units of heparin per ml and frozen at —70°C until tested for virus. Five to 10 chickens were tested at each age.

Vector competence studies:

Three- to 9-day-old chickens (*Gallus gallus* L.) were inoculated subcutaneously with 0.1 ml of a suspension containing $10^{4.2}$ plaque-forming units (PFU) ($10^{5.2}$ PFU/ml) of EEE virus, and mosquitoes were allowed to feed on them 24 h later. Immediately after mosquito feeding, a 0.1-ml blood sample was obtained from the jugular vein of each chicken and treated as describe above to determine the viremia at the time of mosquito feeding. Engorged mosquitoes were transferred to 3.8-liter cardboard cartons with netting over the open end and maintained in an environmental chamber as described above. Four days after the infectious blood meal, an oviposition substrate was added to each cage. After 14 days, the mosquitoes were killed by freezing at —20°C and their legs and bodies triturated separately in 1 ml of diluent and frozen at —70°C until assayed for virus. Presence of virus in a mosquito s body indicated infection, while virus in the legs indicated the mosquito had a disseminated infection (Turell et al. 1984). The infection and dissemination rates were defined as the percentages of mosquitoes tested that contained virus in their body or legs, respectively.

To estimate the transmission rate for each species, we multiplied the percentage of individuals of that species that developed a disseminated infection times the proportion of mosquitoes with a disseminated infection that transmitted EEE virus by bite. To

determine transmission rates, some of the *Oc. j. japonicus* that had taken an infectious blood meal were allowed to refeed individually on 1- to 7-day-old chickens 7-21 days after the initial infectious blood meal. Chickens were bled 24 h after mosquito feeding, and the blood tested for EEE virus by plaque assay. Presence of virus in this blood indicated viral transmission. In addition to the orally exposed mosquitoes, *Oc. j. japonicus* intrathoracically inoculated (Rosen and Gubler 1974) 7-21 days previously with 0.3 µl of a suspension containing $10^{5.2}$ PFU of EEE virus/ml ($10^{1.7}$ PFU/inoculum) were allowed to feed on individual chickens that were 1- to 7-days-old. The transmission rate for *Ae. albopictus,* 14 days after oral exposure to EEE virus, was determined as described above. Transmission rates were not determined for *Culex pipiens* because this species was not susceptible to oral infection with EEE virus. Immediately after the transmission attempt, the mosquitoes were killed by freezing, their feeding status was determined, and their legs and bodies were triturated separately as describe above.

Viral replication and dissemination studies:

To evaluate viral replication and dissemination over time, mosquitoes were fed on a viremic chicken and held in an environmental chamber as described above. Samples of ≥ 5 mosquitoes were assayed, leg and bodies separately, for virus immediately after the infectious blood meal and at selected time intervals after blood feeding.

Data analysis:

Infection, dissemination, and transmission rates for mosquitoes with disseminated infections were compared by Chi-square or Fisher exact tests as appropriate and

significant differences were determined at the 95% confidence level (SAS Institute Inc. 1999).

RESULTS

Viremias in chickens generally decreased with increasing age of the chickens at time of inoculation (Figure 1). The highest viremias, approximately $10^{9.7}$ PFU/ml of blood, were observed in chickens that were 3 days old at time of inoculation. In contrast, viremias were lowest in chickens that were 9 days old at the time of inoculation, with viremias of approximately $10^{7.5}$ PFU/ml of blood.

When fed on chickens with viremias ranging from $10^{7.0\text{-}8.1}$ PFU/ml of blood, *Oc. j. japonicus* and *Ae. albopictus* were susceptible to infection with EEE virus, while *Cx. pipiens* was not (Table 1). The proportion of *Oc. j. japonicus* infected with EEE virus increased significantly ($\chi^2 = 29.99$, df = 2, $P < 0.001$) as the viral titer in the blood meal increased. Likewise, the viral titer of the blood meal significantly affected infection rates in *Ae. albopictus* (Fisher exact, $P = 0.035$). Although highly susceptible to infection with EEE virus at the two ranges of viral titers in blood meals that were directly comparable, *Oc. j. japonicus* was significantly less susceptible than *Ae. albopictus* when mosquitoes ingested $10^{7.0\text{-}7.5}$ PFU/ml of blood ($\chi^2 = 17.76$, df = 1, $P < 0.001$) or $10^{8.0\text{-}8.7}$ PFU/ml of blood (Fisher exact, $P = 0.002$). Dissemination rates were identical to or nearly identical to infection rates for any of the species tested and at all viral titers to which they were exposed.

Transmission rates for *Oc. j. japonicus* with a disseminated infection were similar regardless of route of EEE virus exposure (orally or by intrathoracic inoculation) or day after virus exposure (7, 10, 14 or 21 day) ($\chi^2 \leq 2.3$, df = 1, $P \geq 0.129$) (data not shown). Thus, the transmission data were combined for further analysis. Overall, 27% (16 of 60) of *Oc. j. japonicus* with a disseminated infection transmitted EEE virus. Likewise, 63%

(7 of 11) of *Ae. albopictus* with a disseminated infection after oral exposure to EEE virus transmitted virus by bite 14 days after ingesting an infectious blood meal. Thus, depending on the viral titer in the blood meal, estimated transmission rates ranged from 15-25% for *Oc. j. japonicus* and 59-63% for *Ae. albopictus* orally exposed to EEE virus (Table 1).

Viral replication over time in the bodies of *Oc. j. japonicus, Ae. albopictus,* and *Cx. pipiens* that fed on a chicken with a viremia of $10^{8.1}$ PFU/ml of blood is shown in Figure 2. The amount of virus ingested per mosquito was $10^{5.8 \pm 0.3}$ PFU. In *Oc. j. japonicus*, the amount of virus in the bodies of mosquitoes decreased during the 2 days after ingestion of the infectious blood meal, increased during days 3 and 4, and remained about $10^{5.7}$ PFU during days 7-21. Viral titers in *Ae. albopictus* from days 4-21 after the infectious blood meal paralleled those of *Oc. j. japonicus*; however, viral titers were consistently 10-fold higher than those for *Oc. j. japonicus*. Virus was not detected in *Cx. pipiens* ≥ 7 days after the infectious blood meal.

At ≥ 4 days after an infectious blood meal, virus was detected in the legs of all *Oc. j. japonicus* and *Ae. albopictus* that had evidence of virus in their bodies. Viral titers in legs tested ≥ 4 days after the infectious blood meal were approximately $10^{4.8}$ and $10^{5.7}$ PFU/set of legs in *Oc. j. japonicus* and *Ae. albopictus*, respectively. Additionally, virus was detected 3 days after oral exposure to virus in the legs of two of five *Oc. j. japonicus* found to have infected bodies. Regardless of day after oral exposure, no virus was detected in the legs of *Cx. pipiens*.

DISCUSSION

Results from this study show that, although not a highly efficient vector under laboratory conditions, *Oc. j. japonicus* can become infected with EEE virus and transmit it by bite. Susceptibility to infection was moderate to high (55-100%) depending on the viral titer of the blood meal. The titers used in this study, $10^{7.0}$—$10^{8.9}$ are consistent with those observed in North American birds inoculated with EEE virus (Komar et al. 1999). EEE virus escaped the midgut readily and quickly (as soon as 3 days after exposure) after mosquitoes fed on a chicken with a viremia of $10^{8.1}$ PFU/ml of blood. However, there was evidence of a salivary gland barrier to EEE virus in *Oc. j. japonicus*, and to a lesser extent in *Ae. albopictus*. These estimated transmission rates for *Ae. albopictus*, 59-63%, are similar to those reported by other investigators for this virus using various strains of *Ae. albopictus* (Scott et al. 1990, Turell et al. 1994). In other laboratory studies, *Cs. melanura*, the enzootic vector of EEE virus, had a transmission rate of 94% (Vaidyanathan et al. 1997). While estimated transmission rates were found to be 15-25% for *Oc. j. japonicus,* other potential epizootic vectors [*Aedes canadensis* (Theobald), *Aedes vexans* (Meigan), *Cq. perturbans*, and *Culex salinarius* Coquillett] transmit EEE virus at rates ranging from 0-13% after feeding on chickens with comparable viremias (Vaidyanathan et al. 1997).

The decrease in detectable titers of EEE virus in *Oc. j. japonicus* during the first 4 days after an infectious blood meal indicated a eclipse phase in virus replication. This phase was previously described in *Aedes aegypti* (L.), *Ochlerotatus triseriatus* (Say), and *Ae. albopictus* with EEE virus (Chamberlain et al. 1954; Scott et al. 1990). By comparison, EEE virus in *Cs. melanura* replicates and disseminates so rapidly that no

detectable drop in virus titer is observed during the first 2 days after oral exposure (Scott et al. 1984). Dissemination of EEE virus in *Oc. j. japonicus* in our study was detected as early as 3 days after oral exposure. Although transmission trials were not done until 7 days after an infectious blood meal, the rapid dissemination of virus and evidence that transmission rates did not change over time (day 7-21) indicate that the extrinsic incubation period at 26^{o}C for EEE virus in *Oc. j. japonicus* may be around 5 days.

Culex pipiens was found to be an incompetent vector of EEE virus. This was due to the presence of a midgut infection barrier. Two likely hypotheses to explain why the virus was not able to infect the midgut are inactivation of the virus by digestive enzymes in the lumen of the midgut, and the absence or reduced number of cellular receptor sites for virus attachment (Murphy 1975, McLintock 1978). To date, there has never been a report of natural EEE virus infection of *Culex pipiens*-complex mosquitoes in North America. The vector competence results reported here provide a possible reason for the lack of field isolates in this species complex. Interestingly, EEE virus was detected four days after ingestion of the blood meal. This could complicate the interpretation of evidence of natural infection of *Cules pipiens* with EEE virus, if it is ever reported to occur.

Experimental transmission studies only provide a piece of the puzzle in estimating the role *Oc. j. japonicus* may play in the epidemiology of EEE virus. Distribution and key bionomic characteristics (e.g., host preference, activity time, flight range, seasonality, breeding habitat) must be considered. Because *Oc. j. japonicus* is a newly recognized invasive species, there is a lack of distribution and bionomics data on this species in the USA. In its native range, *Oc. j. japonicus* is an opportunistic feeder that will take a blood

meal from avian and mammalian hosts (Miyagi 1972). It reportedly will bite humans that enter its forest habitat (Knight 1969). In Frederick County, Maryland, female *Oc. j. japonicus* were not observed during daytime hours while we collected larvae during June - August of 2000; however, two to three females were collected while landing on humans ~30 min after sunset on consecutive evenings in late September. This was in a backyard where no breeding sites were found within a 200-m radius from the collection point (Sardelis M., unpublished data). The distribution of *Oc. j. japonicus* in the USA remains largely unknown. In Frederick County, Maryland, *Oc. j. japonicus* is found widely distributed throughout the county and its relative abundance, as determined by oviposition trapping, equaled that of *Ochlerotatus triseriatus* (Say) in the same area (Sardelis and Turell 2001). The fact that *Oc. j. japonicus* is a northern climate mosquito (Tanaka et al. 1979) may have an impact on arbovirus transmission during the typical, early season viral amplification period. In the USA, *Oc. j. japonicus* breeds in natural containers such as rock holes (Andreadis, personal communication) and numerous human artifacts including discarded tires, tin cans, water dishes for potted plants, concrete rain-water drainage forms, buckets, pans, and plastic milk jugs (Munstermann and Andreadis 1999; M.R.S, unpublished data).

It is necessary for there to be repeated isolations of EEE virus from feral *Oc. j. japonicus* to establish that this species plays a role in virus transmission in natural settings. Transmission rates for EEE virus by *Oc. j. japonicus* in the laboratory are comparable to other suspected bridge vectors, and there is evidence that *Oc. j. japonicus* is expanding geographically into many known foci of EEE virus transmission in the northeast and eastern USA. Therefore, persons involved in arbovirus surveillance and

control programs should consider this species among those that require heightened concern when rainfall, temperature, and other environmental conditions favor viral amplification and transmission of virus from birds to tangential hosts.

The results of this study combined with our limited knowledge of the distribution and bionomics of this species in the USA suggest that *Oc. j. japonicus* could function as a bridge vector for EEE virus between the enzootic *Cs. melanura*—avian cycle and susceptible mammalian hosts. To clarify the significance of our vector competence tests, additional studies of the behavioral and ecological components of vectorial capacity of this newly invasive species and the evaluation of field-collected adults for evidence of EEE infection are needed. Furthermore, vertical transmission of EEE virus by *Oc. j. japonicus* remains unstudied.

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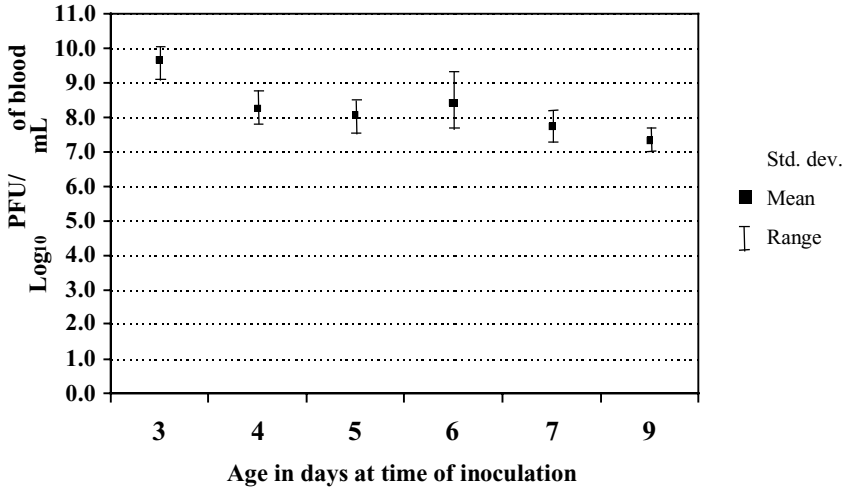

Figure 1. Viremias at 24 h in chickens (*Gallus gallus*) inoculated at various ages with 0.1 ml of a suspension containing $10^{5.2}$ plaque-forming units (PFU) of eastern equine encephalitis virus (Mosquito MA92-1406, Vero-1 passage). Number tested was between 5-10 chickens at each age at time of inoculation category.

Table 1. Infection, dissemination, and estimated transmission rates for EEE virus by *Ochlerotatus j. japonicus*, *Aedes albopictus*, and *Culex pipiens*

Species	Viral titer at time of feeding[1]	No. tested	Infection rate, %[2]	Dissemination rate, %[3]	Estimated transmission rate, %[4]
Oc. j. japonicus	7.0 - 7.5	33	55	55	15
	8.0 - 8.7	77	88	86	23
	9.1 - 9.9	44	100	93	25
Ae. albopictus	7.0 - 7.5	55	93	93	59
	8.0 - 8.7	70	100	100	63
Cx. pipiens	8.1	20	0	0	nd

[1]Log $_{10}$ PFU/ml of blood in donor chickens (*Gallus gallus*) inoculated 24 previously with a Massachusetts isolate of EEE (Mosquito MA92-1406).
[2]Percentage of mosquitoes with virus in their body 14 d after blood feeding.
[3]Percentage of mosquitoes with virus in their legs 14 d after blood feeding.
[4]An estimate of the percentage of mosquitoes that will transmit virus by bite 14 d after an infectious blood meal and storage at 26^{o}C, calculated by multiplying percent dissemination times the percentage of disseminated mosquitoes that transmitted virus. These percentages were previously determined to be 27% for *Oc. j. japonicus* and 63% for *Ae. albopictus*. nd = not determined.

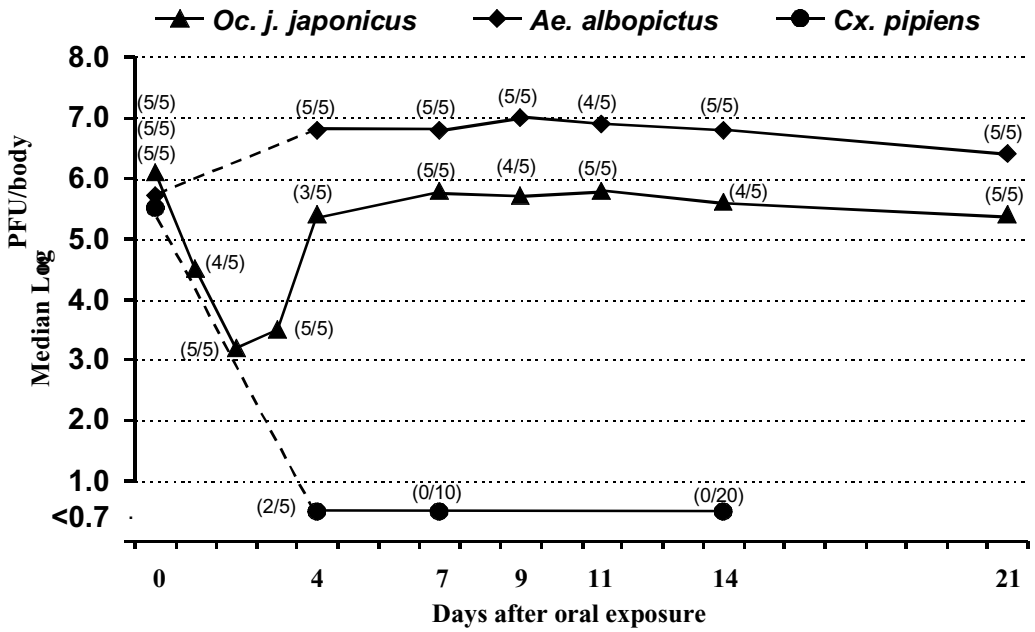

Figure 2. Replication of EEE virus over time in the bodies of *Ochlerotatus j. japonicus*, *Aedes albopictus*, *Culex pipiens*, after oral exposure to a chicken with a viremia of $10^{8.1}$ plaque-forming units (PFU)/ml of blood and held at 26°C. Number of mosquitoes positive/number tested in parentheses above or next to median values. *Aedes albopictus* and *Cx. pipiens* were not tested on days 1-3, thus, a dashed line connects values in this part of the growth curves.

Chapter 6

Laboratory transmission of La Crosse virus by *Ochlerotatus j. japonicus*

ABSTRACT

Ochlerotatus j. japonicus, a recent introduction to the United States, was studied to determine its capability to serve as a vector of La Crosse (LAC) virus. A field-collected population of *Ochlerotatus triseriatus*, the primary vector of LAC virus, was similarly tested for comparison. After ingesting virus from hamsters with viremias of $10^{3.6-5.4}$ plaque-forming units (PFU)/ml of blood, estimated transmission rates for *Oc. j. japonicus* were 35-88%. These rates were slightly lower than, though similar to, those for *Oc. triseriatus*, 75-100%. Viral titers in *Oc. j. japonicus* peaked approximately$10^{5.5}$ PFU/mosquito about 7 days after ingesting a blood meal in which the concentration of LAC virus was $10^{5.4}$ PFU/ml of blood, and virus had disseminated from the midgut in 100% (8/8) of these specimens. These data, combined with the close association between the habitats of *Oc. j. japonicus* and *Oc. triseriatus* and the reported expansion of the range of this newly discovered species in the eastern USA, indicate that *Oc. j. japonicus* could function as an additional vector of LAC virus.

INTRODUCTION

The mosquito *Ochlerotatus japonicus japonicus* (Theobald), known previously only from Japan, Taiwan, southern China, and the Republic of Korea (Tanaka et al. 1979), has been recently discovered in the USA (Peyton et al. 1999) and has an expanding distribution (See Chapter 2). This mosquito uses natural and artificial containers as larval habitats in the USA (See Chapter 2, Scott et al 2001a). In its native range, *Oc. j. japonicus* is categorized as a woodland species and is regarded as an opportunistic feeder, feeding on bird and mammals, including humans (Tanaka et al. 1979).

La Crosse (LAC) virus is a member of the California serogroup within the Genus *Bunyavirus* in the Family Bunyaviridae. The virus s distribution is well documented in the upper Midwest of the USA (Ohio, Indiana, Iowa, Illinois, Wisconsin, and Minnesota) (Kappus et al. 1982). It is also endemic in a number of eastern states (Georgia, North Carolina, Tennessee, and West Virginia) (Sikes et al. 1984, Kappus et al. 1982, Jones et al. 1999, Woodruff et al. 1992). La Crosse virus cycles between the mosquito *Ochlerotatus triseriatus* (Say), the primary vector, and eastern chipmunks and gray squirrels during the months of mosquito activity. The virus is additionally maintained indefinitely in *Oc. triseriatus* by transovarial transmission (Watts et al. 1974) and its prevalence in mosquitoes may be amplified by venereal transmission between infected males and uninfected female mosquitoes (Thompson and Beaty 1977). Between 30 and 180 cases of La Crosse encephalitis are reported each year (Centers for Disease Control and Prevention 1998), primarily in children, making it the most common and important endemic mosquito-borne illness in the United States.

Because *Oc. j. japonicus* now inhabits the fringe of LAC virus s distribution and its spread into highly endemic areas, such as West Virginia, seems imminent, it would be prudent to evaluate the potential influence of this newly introduced mosquito on the ecology of LAC virus. The objective of this study was to determine the oral susceptibility and to estimate the transmission efficiency of *Oc. j. japonicus* for LAC virus under laboratory conditions. For comparison, *Oc. triseriatus*, the primary vector of this virus, was similarly evaluated.

MATERIALS AND METHODS

Mosquitoes:

The *Oc. j. japonicus* used in this study were reared from eggs and larvae collected in Frederick County, Maryland, in 2001. The *Oc. triseriatus* were from larvae collected from tree holes in the vicinity of Sugarloaf Mountain in the southern part of Frederick County, Maryland. Mosquitoes were reared in pans containing dechlorinated tap water, provided ground catfish chow for nutrition, and maintained in an environmental chamber at 26^{o}C, 80-85% RH and 16:8 h L:D photoperiod. Four- to 8- day-old adult mosquitoes were used in these studies.

Virus and viral assays:

Two stains of LAC virus were used in the study: strain 2-3-95, isolated from *Oc. triseriatus* mosquitoes collected in West Virginia in 1995, and strain 97WV-131, isolated from a human from West Virginia who was stricken with LAC encephalitis in 1997. Both strains were used in their third Vero cell culture passage. Viral stock suspensions, triturated mosquito suspensions, and hamster blood samples were tested for infectious virus by plaque assay on Vero cells as described by Gargan et al. (1983).

Determination of vector competence:

Young (7-8-wk-old) Syrian hamsters (*Mesocricetus auratus*) were inoculated intraperitoneally with 0.1 ml of a suspension containing approximately $10^{4.2}$ plaque-forming units (PFU) of one of the strains of LAC virus. The hamsters were anesthetized 24, 48, or 96 hr after inoculation and placed on top of a cage containing 10-60 *Oc. j. japonicus* or *Oc. triseriatus*. The feeding of one mosquito species was immediately followed by the feeding of the other, with each species provided ≈30 min in which to take

a blood meal. Immediately after mosquito feeding, a 0.1-ml blood sample was obtained from the hamster by cardiac puncture and added to 0.9 ml of diluent (10% heat-inactivated fetal bovine serum in Medium 199 with Earl s salts, $NaHCO_3$, and antibiotics) plus 10 units of heparin per ml to determine the hamster viremia at the time of mosquito feeding. After exposure to the viremic hamster, engorged mosquitoes were transferred to 3.8-liter cardboard cartons with netting over the open end and maintained in an environmental chamber as described above. Four days after the infectious blood meal oviposition substrates were provided.

To evaluate viral replication over time, samples of 5-10 mosquitoes that fed on a hamster inoculated with 2-3-95 virus were killed by freezing for 10 min at —20°C on 7, 14, and 21 days after the infectious blood meal. Their legs and bodies were triturated separately in 1 ml of diluent and frozen at —70°C until assayed for virus. Most of the mosquitoes that fed on the other LAC virus-inoculated hamsters were ground, legs and bodies separately, after 14 days of extrinsic incubation. However, some of the mosquitoes were held for 21-22 days and then allowed to feed individually on suckling mice to determine if they could transmit virus by bite. Immediately after the transmission attempts, these mosquitoes were killed by freezing, the feeding status determined, and their legs and bodies triturated separately as describe above. For all mosquitoes, infection was determined by recovery of virus from the mosquito tissue suspension. If virus was recovered from its body, but not its legs, the mosquito was considered to have a nondisseminated infection limited to its midgut. In contrast, if virus was recovered from both the body and leg suspensions, the mosquito was considered to have a disseminated infection (Turell et al. 1984). The infection and dissemination rates were defined as the

percentages of mosquitoes tested that contained virus in their body or legs, respectively. The mice used in the transmission attempts were observed for 7 days. The brains of moribund or dead mice were removed and suspended in 3 ml of diluent. Because LAC virus infection is consistently fatal to suckling mice, death of these animals was used to indicate virus transmission. Transmission was verified by isolating virus from brain tissue.

In addition, some of the original, unfed mosquitoes were inoculated intrathoracically (Rosen and Gubler 1974) with 0.3 μl of a viral suspension containing $\approx 10^{4.4}$ PFU of LAC virus/ml ($10^{0.9}$ PFU/mosquito). These were held 9-16 days and allowed to feed on suckling mice. Mosquitoes from these transmission attempts were triturated whole and to confirm infection status. The mice were observed and treated as described above.

In order to estimate transmission rates for both species, the percentage of mosquitoes with a disseminated infection (after either oral exposure or by intrathoracic inoculation) that transmitted virus by bite for each species of mosquito was determined. This value was then multiplied by the percentage of mosquitoes that developed a disseminated infection after feeding on a hamster with each viremia. This resulted in the estimated transmission rate for those mosquitoes.

To assess if the vector competence determination for *Oc. triseriatus* may have been biased by the presence of naturally (vertically) infected specimens, males (n = 183) reared from the field-collected populations of *Oc. triseriatus* were tested in pools (≤25 per pool) for LAC virus as described above.

Data analysis:

Infection and dissemination rates were compared by Chi-square or Fisher exact tests as appropriate and significant differences were determined at the 95% confidence level (SAS Institute Inc. 1999).

RESULTS

Hamster viremias at the time of mosquito feeding ranged from $10^{2.0}$ to $10^{5.4}$ PFU/ml of blood (Table 1). In general, both infection and dissemination rates increased as hamster viremia titers increased, and both species were highly susceptible to infection with LAC virus when virus titers were $\geq 10^{4.8}$ PFU/ml of blood. However, *Oc. triseriatus* was significantly more susceptible to infection with LAC virus than *Oc. j. japonicus* when fed on a hamster with a viremia of $10^{3.6}$ PFU/ml of blood ($\chi^2 > 11.0$, df = 1, $P < 0.001$). All ($n = 80$) *Oc. j. japonicus* and virtually all (27/28, 96%) *Oc. triseriatus* that became infected developed disseminated infections (Table 1).

Viral titers were similar in *Oc. j. japonicus* on days 7, 14, and 21 after ingesting a viremic blood meal (Table 2). At every time period, virtually all infected mosquitoes were found to have virus in their legs.

All mosquitoes with disseminated infections, either after oral exposure or intrathoracic inoculation, transmitted LAC virus by bite (Table 3). Thus, the rate of transmission of virus by *Oc. j. japonicus* and *Oc. triseriatus* with a disseminated infection was determined to be 100%. This value was then multiplied by the dissemination rates for a mosquito species after ingesting an infectious blood to determine the estimated transmission rate (Table 1). At viral titer levels sufficient to infect *Oc. j. japonicus*, estimated transmission rates for *Oc. j. japonicus* (35-88%) were comparable to those for *Oc. triseriatus* (75-100%).

None of the pools of male specimens tested to screen the *Oc. triseriatus* populations for natural infection with LAC virus was positive.

DISCUSSION

Results from this study show that *Oc. j. japonicus* can become infected with and transmit LAC virus by bite. Oral infection, dissemination, and estimated transmission rates for this species at viral titer levels sufficient to produce infections were moderate (35%) to high (>75%). Because eastern chipmunks and gray squirrels can develop viremias up to 10^6 PFU/ml of blood (Pantuwatana et al. 1972), the viremias used in this study, $10^{2.0}$—$10^{5.4}$ PFU/ml of blood, should be representative of those that mosquitoes would be exposed to in nature.

The efficiency of *Oc. j. japonicus* as a laboratory vector of LAC virus was found to be similar to that of the native vector, *Oc. triseriatus*, when viral titer in the blood meal was ≥$10^{4.8}$ PFU/ml of blood. However, at lower viral titers, *Oc. triseriatus* appeared to be more susceptible to infection than *Oc. j. japonicus*. The vector competence of the *Oc. triseriatus* strain evaluated in this study was consistent with other laboratory studies using a variety of colonized and field-collected strains of *Oc. triseriatus* (Watts et al. 1972 , Pantuwatana et al. 1972, Paulson et al. 1989, Grimstad et al 1989).

The possibility that *Oc. j. japonicus* may be an efficient vector of and suitable host for LAC virus is supported by the data on the propagation and persistence of this virus over time. Although the amount of virus ingested by a single mosquito in the blood meal was not determined, the amount of virus ingested can be estimated based on the volume of blood ingested when a mosquito feeds to repletion. Given that the volume blood an average-sized mosquito ingests is approximately 5 µl (Klowden and Lea 1979), the amount of virus that would be ingested while taking of blood meal from a host with a viremia of $10^{5.4}$ PFU/ml of blood would be $10^{3.1}$/mosquito. Using this value as the day 0

amount of virus ingested per mosquito, multiplication of virus was evident by 7 days after taking the blood meal, reaching on average $10^{5.5}$ PFU/mosquito. Additionally, at day 7, all *Oc. j. japonicus* had disseminated infections and the viral titer per mosquito had peaked. Although transmission attempts were not done on day 7, it appears likely that a high proportion of *Oc. j. japonicus* would be capable of transmitting virus on by this day. Watts et al. (1972) found that 60% of *Oc. triseriatus* transmitted LAC virus by bite 7 days after exposure oral exposure to virus and incubation at 27°C. Further studies need to be done to establish the extrinsic incubation period for LAC virus in this mosquito species. The average viral titer per mosquito was still above 10^5 PFU at 21 days after ingestion of the infectious blood meal, indicating that *Oc. j. japonicus* can support substantial quantities of LAC virus for an epidemiologically important period of time.

In addition to this study with *Oc. j. japonicus*, other introduced mosquito species [e.g., *Aedes albopictus* (Skuse) and *Ochelrotatus atropalpus* (Coquillett)] have the potential to transmit LAC virus (Grimstad et al. 1989, Freier and Beier 1984). However, until recently, no field evidence existed that they are involved in the natural transmission of LAC virus. La Crosse virus was recently isolated from *Ae. albopictus* capture in eastern Tennessee (Gerhardt et al. 2001), indicating that it may take several years after a virus and a mosquito (i.e., *Oc. j. japonicus*) become sympatric before a newly introduced mosquito may become involved in the transmission cycle.

There are many gaps in the current knowledge of bionomics and distribution of *Oc. j. japonicus* in the United States that make it difficult to predict what role it may play in the ecology of LAC virus. The flight range, natural host preference, and host seeking time of *Oc. j. japonicus* in the United States have not been studied. On a local scale, the

distribution of *Oc. j. japonicus* and *Oc. triseriatus* in a county in western Maryland overlaps to a large degree (See Chapter 2), and these same two species share several of the same breeding sites (See Chapter 2). Regionally, the reported distribution of *Oc. j. japonicus* stretches from Massachusetts to Virginia and from New Jersey to Ohio. Because *Oc. j. japonicus* has been found just across the Potomac River from West Virginia (See Chapter 2), it seems likely that this mosquito will soon invade areas that are highly endemic for LAC virus.

Because of the demonstrated ability of *Oc. j. japonicus* to efficiently transmit LAC virus, it would be prudent for persons involved in LAC virus surveillance and control programs to begin monitoring for this species and testing specimens for virus. Ovipostion traps and gravid traps are known to be effective in collecting *Oc. j. japonicus* (See Chapter 2, Scott et al. 2001b). Additionally, laboratory studies need to be done to determine the ability of this mosquito to transmit LAC virus vertically.

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Table 1. Infection, dissemination, and estimated transmission rates for La Crosse encephalitis virus by *Ochlerotatus j. japonicus* and *Ochlerotatus triseriatus*

Mosquito species	Viral titer at time of feeding[1]	No. tested	Infection rate, %[2]	Dissemination rate, %[3]	Estimated transmission rate, %[4]
Oc. j. japonicus	2.0	17	0	0	0
Oc. triseriatus	2.0	12	17	17	17
Oc. j. japonicus	3.6[5]	34	35	35	35
Oc. triseriatus	3.6[5]	15	93	87	87
Oc. j. japonicus	4.8	32	75	75	75
Oc. triseriatus	4.8	4	75	75	75
Oc. j. japonicus	5.4	50	88	88	88
Oc. triseriatus	5.4	9	100	100	100

[1]Log $_{10}$ PFU/ml of blood in donor hamster (*Mesocricetus auratus*) inoculated with the 2-3-95 strain of LAC virus, unless otherwise noted.
[2]Percentage of mosquitoes with virus in their body 14-22 d after blood feeding.
[3]Percentage of mosquitoes with virus in their legs 14-22 d after blood feeding.
[4]An estimate of the percentage of mosquitoes that will transmit virus by bite 14-22 d after an infectious blood meal and storage at 26^{o}C, calculated by multiplying percent dissemination times the percentage of disseminated mosquitoes that transmitted virus. These percentages were previously determined to be 100% for both *Oc. j. japonicus* and *Oc. triseriatus* (Table 3).
[5]Hamster inoculated with the 97WV-131 strain of LAC virus.

Table 2. Recovery of virus from *Oc. j. japonicus* and *Oc. triseriatus* at 7, 14, and 21 days after ingesting blood meal from a hamster with a viremia of $10^{5.4}$ PFU/ml of blood after inoculation with La Crosse virus (strain 2-3-95)

		Day after oral exposure[1]		
Species		7	14	21
Oc. j. japonicus	No. tested	8	9	9
	Mean	5.5	5.4	5.2
	Range	5.3-5.6	5.1-5.6	5.0-5.5
Oc. triseriatus	No. tested	ND[2]	5	5
	Mean		5.8	4.9
	Range		5.6-6.1	4.7-5.1

[1]Log_{10} PFU/mosquito.
[2]ND = not determined.

Table 3. Transmission of La Crosse virus strains 2-3-95 and 97WV-131 by *Oc. j. japonicus* and *Oc. triseriatus* with a disseminated infection either after oral exposure to or intrathoracic inoculation with virus

		Route of exposure		
Mosquito species	LAC strain	Oral[1]	Inoculation	Combined
Oc. j. japonicus	2-3-95	100 (2)	100 (5)	100 (7)
	97WV-131	100 (1)	100 (3)	100 (4)
	Total	100 (3)	100 (8)	100 (11)
Oc. triseriatus	2-3-95	100 (4)	100 (5)	100 (9)
	97WV-131	100 (5)	100 (1)	100 (6)
	Total	100 (9)	100 (6)	100 (15)

[1]Percent transmitting (no. feeding)

Chapter 7

Vector competence of North American strains of *Aedes albopictus* for West Nile virus

ABSTRACT

To evaluate the potential for North American (NA) *Aedes albopictus* to transmit West Nile (WN) virus, we tested strains derived from three NA sources (Frederick Co., Maryland, FRED strain; Cheverly, MD, CHEV strain; Chambers Co. and Liberty Co., Texas, TAMU strain) and a Hawaiian source (Honolulu, HI, OAHU strain). Mosquitoes were fed upon 2- to 3-day old chickens previously inoculated with a New York strain (Crow 397-99) of WN virus. All of the NA strains were competent laboratory vectors of WN virus, with transmission rates of 36, 50, 83, and 92% for the FRED, CHEV, OAHU, and TAMU strains, respectively. The extrinsic incubation period for WN virus in *Ae. albopictus* held at 26^{o}C was estimated to be 10 days. In the study to evaluate vertical transmission, no virus was recovered from 12,183 F_1 progeny (5,936 females, 6,007 males, 240 larvae) of the WN virus-inoculated mosquitoes. Based on efficiency of virus transmission, evidence of natural infection, bionomics, and distribution, *Ae. albopictus* could be an important bridge vector of WN virus in the southeastern USA.

INTRODUCTION

West Nile (WN) virus was reported for the first time in the Western Hemisphere in 1999 when it caused encephalitis in humans in New York City and an epizootic in native and exotic avian species [Centers for Disease Control and Prevention (CDC) 1999a, CDC 1999b, Lanciotti et al. 2000]. Testing of field-collected mosquitoes for evidence of WN virus infection during 1999 and 2000 indicates that *Culex pipiens* L. is the primary vector of this virus and that a number of additional species may be secondary vectors or bridge vectors (CDC 1999b, 2000). Potential secondary or bridge vector species from which there were multiple instances of WN virus infection reported include *Culex restuans* Theobald, *Culex salinarius* Coquillett, *Aedes vexans* (Meigan), *Ochlerotatus j. japonicus* (Theobald), *Ochlerotatus triseriatus* (Say), *Ochlerotatus trivittatus* (Coquillett), and *Culiseta melanura* (Coquillett). In September 2000, a single pool of *Aedes albopictus* (Skuse) collected in southeast Pennsylvania showed evidence of WN virus infection (CDC 2000).

Since discovery of *Ae. albopictus* in Memphis, TN, (Reiter and Darsie 1984) and Houston , TX (Sprenger and Wuithiranyagool 1986) in the 1980s, this species has become established throughout most of the southeastern USA, extending as far north as New Jersey (Moore and Mitchell 1997). Throughout its range in the USA, *Ae. albopictus* is an important human pest, aggressively biting during daylight hours close to its breeding sites. Experimental transmission studies with a strain of WN virus from the outbreak of WN in New York in 1999 indicate that *Ae. albopictus* is a highly efficient laboratory vector of WN virus (Turell et al. 2001). However, this study used a long-

colonized strain of *Ae. albopictus* from Hawaii, and therefore may not represent what occurs in nature in regions of the USA where WN virus is now enzootic.

Previous studies have shown that vector competence for arboviruses is widely variable among populations of vectors. Some examples of this variation are reported in *Culex tarsalis* Coquillett for western equine encephalomyelitis virus (Reisen et al. 1996), *Ae. aegypti* (L.) for dengue viruses (Gubler et al. 1979, Tran et al. 1999), *Ae. albopictus* for dengue viruses (Boromisa et al 1987, Gubler and Rosen 1976), *Culex tritaeniorhynchus* Giles for WN virus (Hayes et al. 1984), and *Culex annulirostris* Skuse for Murray Valley encephalitis and Kunjin viruses (Kay et al. 1984).

To elucidate the role *Ae. albopictus* may play in the epidemiology of WN virus in the eastern USA, we conducted laboratory studies of the vector competence of four strains: two newly colonized ones from Maryland where WN is considered enzootic, a long-colonized Texan strain, and the Hawaiian strain (OAHU) used by Turell et al. (2001). Additionally, studies were done to evaluate viral replication and dissemination in these mosquitoes over time.

MATERIALS AND METHODS

Mosquitoes:

Three North American strains of *Ae. albopictus* were evaluated for their ability to transmit WN virus. These included the Frederick (FRED) strain, derived from eggs collected in Frederick County, Maryland, in July 1999 (See Chapter 2); the Cheverly (CHEV) strain, derived larvae collected from discarded tires in Cheverly, Prince George s County, Maryland, in June 1999; and the Texas A&M University (TAMU) strain, derived from specimens collected in Chambers and Liberty Counties, Texas, in 1987. Additionally, the OAHU strain of *Ae. albopictus*, derived from specimens collected in Honolulu, HI, in 1971, was evaluated. The generation tested was F_3 for FRED and CHEV, and $>F_{30}$ for TAMU and OAHU.

To ensure random sampling and consistent age of specimens, we submerged egg papers containing approximately 2,500-3,000 eggs of each strain in separate rearing pans (31 × 19 × 6 cm) containing dechlorinated water on the same day. Two days later, the larvae, by strain, were culled to the number required for the study and divided into rearing pans, about 120 larvae per pan to avoid overcrowding. The larvae were reared in an incubator at 26 ± 1^{o}C with a relative humidity of 80-85% and 16-hr photoperiod. The larval diet consisted was ground catfish chow (AquaMax Pond Plus 3000, Purina Mills, Inc., St. Louis, MO). Adult mosquitoes were kept in 3.8-liter cartons with netting over one end and given apple slices and water-soaked gauze pads as food. For exposure to virus, 60-90 females were transferred to 0.9-liter cartons with netting over one end, and they were deprived of carbohydrates for one day prior to blood feeding. To minimize age-related differences we used only 4-5-day-old mosquitoes.

Virus and viral assay:

The WN virus strain (Crow 397-99) used was isolated from a dead crow found in the Bronx, New York, during an epizootic in 1999 (Turell et al. 2000) and had been passaged once in Vero cell culture. Viral stock suspensions, triturated mosquito suspensions, and chicken blood samples were tested for infectious virus by plaque assay on African green monkey kidney (Vero) cells as described by Gargan et al. (1983), except that the second overlay, containing neutral red, was added 2 days after the first overlay.

Vector competence studies:

Mosquitoes were allowed to feed on a chicken (*Gallus gallus* L.) that had been inoculated subcutaneously 24 or 48 hr earlier with 0.1 ml of a suspension containing $10^{4.2}$ plaque-forming units (PFU) of WN virus. As soon as most of the mosquitoes in a carton had fed (approximately 15 min), the chicken was transferred to second carton containing a different strain of mosquitoes. This was repeated until all four strains of mosquitoes had fed upon the same chicken. Immediately after mosquito feeding, a 0.1-ml blood sample was obtained from the jugular vein of each chicken and diluted in 0.9 ml of diluent (10% heat-inactivated fetal bovine serum in Medium 199 with Earle s salts, $NaHCO_3$, and antibiotics) plus 10 units of heparin per ml to determine the viremia at the time of mosquito feeding. Engorged mosquitoes were transferred to 3.8-liter cartons with netting over the open end and maintained in an incubator as described above. Four days after the infectious blood meal, an oviposition substrate was added to each cage.

To determine transmission rates, some of the mosquitoes that had taken an infectious blood meal were individually allowed to refeed on a 2-day-old chicken 13 days

after the initial infectious blood meal. Immediately after the transmission attempt, the mosquitoes were killed by freezing at —20°C for approximately 5 min and their legs and bodies triturated separately in 1 ml of diluent and frozen at —70°C until assayed for virus. Presence of virus in a mosquito s body indicated infection, while virus in the legs indicated the mosquito had a disseminated infection (Turell et al. 1984). The remaining mosquitoes, the ones not used in the transmission attempts, were killed and ground as described above on day 14 after the infectious blood meal.

Viral replication and dissemination studies:

To evaluate viral replication and dissemination over time, each strain of mosquito was fed on a single chicken that had been inoculated with WN virus 48 hr earlier. Immediately after taking the infectious blood meal, five mosquitoes of each strain were killed, triturated, frozen, and assayed as described above. Handling and maintenance after blood feeding for the remaining mosquitoes was as described above, except that samples of 10 mosquitoes of each strain were killed, ground, and frozen for later assay on days 1, 4, 7, 10, 14, 21 and 28 after blood feeding.

Vertical transmission evaluation:

To test for vertical transmission, female *Ae. albopictus* (FRED strain, n = 140) were inoculated intrathoracically with WN virus as described above and allowed to feed on a hamster 7, 14, 21, 28, 35, and 42 days later. Eggs resulting from these blood meals were allowed to hatch and mature, in the vast majority of instances, to the adult stage. In cases in when >98% of the larvae in a rearing pan had pupated and the remaining larvae began to show signs of languishing, the specimens were harvested at the larval stage. The adult mosquitoes were separated according to sex and placed in pools of ≤ 25

mosquitoes each and triturated in 2 ml of diluent. Larvae were placed in pools of ≤ 12 mosquitoes each and triturated in 1 ml of diluent. The triturated pools were frozen at —70℃ until assayed for virus on Vero cells as described above. After the day 7 and the day 21 blood meals, 10 WN virus-inoculated mosquitoes were individually triturated in 1 ml of diluent and tested for infection.

Statistics:

The infection rate was calculated as (the number of infected mosquitoes/total tested) X 100. The dissemination rate was calculated as (the number of mosquitoes with positive legs/total tested) X 100. The transmission rate was calculated as (the number of mosquitoes transmitting virus by bite/the number of mosquitoes that took a blood meal) X 100. Infection, dissemination, and transmission rates were compared by Chi-square or Fisher exact tests as appropriate and significant differences were determined at the 95% confidence level (SAS Institute Inc. 1999). A one-way analysis of variance (ANOVA) was used to test for differences in the mean titer (log transformed) of WN virus ingested among the strains. The means (log transformed) from the study of viral replication in the bodies of infected mosquitoes were subjected to two-way ANOVA to evaluate the main effects for strain and day after oral exposure and the interaction term. For terms found significant (alpha = 0.05) by ANOVA, the means were compared by Duncan s multiple range test (SAS Institute Inc. 1999).

RESULTS

All strains of *Ae. albopictus* were susceptible to infection with WN virus at both viral titers tested (Table 1). Susceptibility to infection for each strain significantly increased with viral titer ($\chi^2 > 5.4$, df = 1, $P < 0.02$). Additionally, within viral titer levels, susceptibility to infection was associated with strain tested ($\chi^2 \geq 11.6$, df = 3, $P \leq 0.009$). At the higher viral titer, infection rates of the TAMU (96%) and OAHU (93%) strains were significantly higher ($\chi^2 \geq 10.0$, df = 1, $P \leq 0.002$) than infection rates of the FRED (56%) and CHEV (67%) stains. Dissemination rates for all strains and at both viral doses were 0-11% lower than the corresponding infection rate. Transmission rates for mosquitoes exposed to a chicken with a viremia of $10^{6.8}$ were significantly affected by strain ($\chi^2 = 10.80$, df = 3, $P = 0.013$), ranging from 36% for the FRED strain to 92% for the TAMU strain.

From a chicken with a viremia of $10^{6.8}$ PFU/ml of blood, approximately $10^{4.2}$ infectious virions were taken up in the blood meal of a single mosquito (Figure 1. A). The strain of mosquito had no significant effect on the amount of virus ingested (ANOVA; $F = 0.04$; df = 3,16; $P = 0.99$). Two-way ANOVA revealed that the main effect for strain proved to be nonsignificant ($F = 2.65$; df = 3,140; $P = 0.052$) and that the main effect for day after oral exposure was significant ($F = 141.5$; df = 5,140; $P < 0.001$). The mean titers in the bodies by strain and day after oral exposure are displayed in Figure 1.A. Multiple comparison tests showed that viral titers significantly increased between days 4, 7, and 10 and did not significantly increase from day 14-28. The interaction between strain and day after oral exposure proved to be nonsignificant ($F =$

0.40; df = 15,140; $P = 0.967$). During this particular study, infection rates for the FRED, CHEV, TAMU, and OAHU were 42, 59, 88, 86%, respectively.

There was evidence of virus escaping the midgut of the FRED and OAHU strains 4 days after oral exposure (Figure 1, B). By 7 days after oral exposure, virus was detected in the legs of all strains. Viral titers in the legs of all strains peaked between 10 and 14 days after the infectious blood meal. For all the strains combined, the percentages of infected mosquitoes that showed evidence of having a disseminated infection (virus in the legs) on days 4, 7, 10, 14, 21, and 28 were 14, 85, 100, 96, 100, and 96%, respectively.

In the vertical transmission study, all ($n = 20$) WN virus-inoculated *Ae. albopictus* tested to confirm infection status were infected. No virus was recovered from 12,183 F_1 progeny (5,936 females, 6,007 males, 240 larvae) of the WN virus-inoculated mosquitoes (Table 2).

DISCUSSION

This study showed that the North American strains of *Ae. albopictus* have the potential to serve as WN viral vectors based on their susceptibility to infection and their ability to transmit WN virus efficiently. This finding is consistent with previously laboratory transmission studies that have shown that WN virus is transmitted by a broad range of North American mosquito species, to include a number of *Culex*, *Aedes* and *Ochlerotatus* species (Turell et al. 2000, 2001; See Chapter 3; Sardelis et al. 2001). Additionally, the transmission rate for the OAHU strain in this study (83%) closely agree with the rate reported by Turell et al. (2001), who estimated the transmission rate for the same strain to be 73% when tested under nearly identical conditions. Because WN virus-infected wild birds (e.g., crows and house sparrows) can develop viremias $>10^8$ PFU/ml of blood (Work et al. 1955), the viral titers of $10^{5.7\text{-}6.8}$ PFU/ml used in our study should be representative of what the mosquitoes would be exposed in nature.

Of the barriers to the biological transmission of arboviruses by bite (midgut infection barrier, MIB; midgut escaper barrier, MEB; and salivary gland barrier, SGB), the MIB appeared to be the most important determinant of vector competence of the strains tested in this study. However, by increasing the titer of the infecting blood meal the MIB could largely be overcome. Infection rates were identical or nearly identical to dissemination rates, thus indicating that a MEB was not important. Lastly, because transmission rates were comparable with dissemination rates, there appeared to be only a slight, if any, SGB. Of the North American mosquito species tested for West Nile virus transmission thus far (Turell et al. 2000, 2001; See Chapter 3; Sardelis et al. 2001), laboratory vector efficiency is primarily modulated by a MIB. Additionally, these earlier

studies indicate that there is a wide range in vector competence level, from inefficient (<5% transmission rate) through highly efficient (>60% transmission rate), and that there is a pronounced MEB in a number of common *Culex* species. The *Ae. albopictus* strains tested here were efficient to highly efficient laboratory vectors of WN virus.

Intraspecific variation was found in the vector competence of *Ae. albopictus* for WN virus. Because the FRED and CHEV strains were collected within a month of one another and in a relatively close proximity of one another (60 km), it is likely that these two strains are genetically similar. This study showed that FRED and CHEV strains were very similar in vector competence, thus they could be considered as representing one population. Variation in vector competence was found between the distinct North American strains [i.e., the two Maryland strains and the TAMU (Texas) strain] and between the Maryland strains and the Hawaiian strain. Other studies have found intraspecific variation in vector competence for arboviruses, including a number of studies involving *Aedes* species and flaviviruses (Tran et al 1999, Boromisa et al 1987, Kay et al. 1984, Gubler et al. 1979, Gubler and Rosen 1976). The finding of such variation in the current study highlights the need to evaluate the vector competence of local mosquitoes strains when trying to determine the impact that species on arbovirus transmission in a particular area.

Knowledge of the extrinsic incubation period (EIP), along with other factors, is vital for estimating the vectorial capacity. The transmission studies showed that *Ae. albopictus* transmitted WN virus 13 days after taking an infectious blood meal. Additionally, the studies of viral replication and dissemination over time indicated that virus had escaped the midgut by day 7 in >80% of all infected mosquitoes and that the

amount of virus circulating in the hemolymph (as estimated by the titer in a mosquitoes legs) peaked between 10 and 14 days after the infectious blood meal. Although we realize that the presence of virus in the hemolymph does not indicate that the salivary glands are infected and that there are infectious virions in the saliva, it is probable that, based on the time studies, the EIP may be around 10 days. Additional transmission studies are needed to more precisely determine the EIP and to evaluate the effect of temperature on EIP for WN virus in *Ae. albopictus*. The duration of the EIP in this study is typical of the EIP reported for other flavivirus-mosquito combinations at comparable incubation temperatures, which is generally 9-12 days (Miller et al. 1989, Watts et al. 1987, Reisen et al. 1993).

The rate of vertical transmission observed in this study indicate that the chances of vertical transmission of WN virus by *Ae. albopictus* may be low (<1 in 12,000). This finding supports the current notion that vertical transmission of flaviviruses in *Aedes* mosquitoes as a means of maintaining the transmission cycle of these viruses is generally not considered important. This is thought to be due to the relative inefficiency of vertical transmission of flaviviruses relative to bunyaviruses as shown in laboratory studies and to the rarity of documented instances of vertical transmission of flavivirues by *Aedes* mosquitoes in nature. The differences in efficiency in vertical transmission between flaviviruses and bunyaviruses in *Aedes* mosquitoes (generally around 0.1% for flaviviruses and up to 80% for bunyaviruses) is apparently due to the mechanism by which the parent passes the virus to progeny. Vertical transmission of flaviviruses supposedly occurs through transovum infection, in which virus enters the egg during

oviposition. By contrast, vertical transmission of bunyaviruses involves transovarial transmission, in which virus infects the developing ova.

Despite the lack of extensive evidence of natural vertical transmission of flaviviruses compared to bunyaviruses, there are few recent reports. Natural infection of *Ae. aegypti* with yellow fever virus was reported for the first time in 1995 in Senegal (Fontenille et al 1997). In 1993, Broom et al. (1995) isolated Murray Valley encephalitis virus, a virus in the same serogroup as West Nile virus, from a pool of male *Ae. tremulus* collected in western Australia. West Nile virus was isolated from male *Culex univittatus* complex mosquitoes collected in the Rift Valley province, Kenya, in 1998 (Miller et al. 2000). These studies seem to suggest that the role of vertical transmission in flavivirus transmission may be underestimated.

The absence of evidence of vertical transmission of WN virus by *Ae. albopictus* in this study could possibly be related to factors such as the geographic strain of mosquito (Bossio et al. 1992) and passage level of the virus (Baqar et al. 1993). Given that the horizontal vector competence varied significantly among the strains of *Ae. albopictus* tested, it is not unreasonable to suspect that the strains may vary in their ability to transmit WN virus vertically. Thus, this issue warrants further study.

Although *Ae. albopictus* appears to be among the most efficient laboratory vectors of WN virus, one needs to consider a number of important aspects of the mosquito s bionomics to properly evaluate whether or not this species will become important in the transmission of WN virus in nature. *Aedes albopictus* is considered an opportunistic feeder, taking blood meal from birds and mammals, including humans (Tempelis et al. 1970, Sullivan et al. 1971). Relatively recent studies of host-seeking patterns of *Ae.*

albopictus in North American found that 3-16% of its blood meals are from birds (Niebylski et al 1994, Savage et al. 1993). The flight range of *Ae. albopictus* is relatively short, approximately a few hundred meters (Bonnet and Worcester 1946, Rosen et al. 1976), which may limit its role in WN virus transmission. However, the mosquito s container breeding sites are often found in association with human habitation and the avian reservoir hosts of WN virus (e.g., crows, blue jays, and house sparrows) are found everywhere, particularly in peridomestic situations.

Aedes albopictus is established throughout the southeastern USA (Moore and Mitchell 1997) and is a commonly reported to be an important biting pest. *Aedes albopictus* has been found infected with EEE virus (Mitchell et al. 1992), a virus with a similar epidemiology to WN virus. In 2000, WN virus was detected in a single pool of *Ae. albopictus* captured in southeastern Pennsylvania (CDC 2000), close to the northernmost established range of this species in the mid-Atlantic region. West Nile virus was detected in four Atlantic states (Connecticut, Maryland, New Jersey, and New York) in 1999, and in 12 states and the District of Columbia, extending from most of the New England states to North Carolina (CDC 2000), in 2000. If WN virus continues its apparent southward spread, the probability of contact between this pathogen and *Ae. albopictus* will be greater. As such, personnel involved in the entomological arm of WN virus surveillance programs should ensure that *Ae. albopictus* are collected and tested. Also, further evidence of WN virus infection in wild-caught *Ae. albopictus* is needed to more firmly incriminate this mosquito as a vector of WN virus.

The results of this study combined with evidence of natural infection in and knowledge of the distribution and bionomics of *Ae. albopictus* suggest that it could

function as a bridge vector for WN-virus between the enzootic *Cx.* (*Cux.*) spp.—avian cycle and susceptible mammalian hosts, including man. Due to intraspecific variation in vector competence, testing of local strains of *Ae. albopictus* to determine their transmission efficiency is warranted to best estimate the role this species may play in the epidemiology of WN virus in a particular area.

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Table 1. Infection, dissemination, and transmission rates for four strains of *Ae. albopictus* orally exposed to West Nile virus

Strain (Collection location)	Number tested	Infection rate[a], %	Dissemination rate[b], %	Transmission rate[c] , %
Infectious dose = $10^{5.7}$ PFU/ml of blood				
FRED (Frederick Co., MD)	45	27a	24a	nt
CHEV (Cheverly, MD)	45	42ab	38ab	nt
TAMU (Chambers Co. and Liberty Co., TX)	45	53ab	49ab	nt
OAHU (Honolulu, HI)	45	60b	58b	nt
Infectious dose = $10^{6.8}$ PFU/ml of blood				
FRED (Frederick Co., MD)	45	56a	49a	36 (11)a
CHEV (Cheverly, MD)	45	67a	64ab	50 (12)ab
TAMU (Chambers Co. and Liberty Co., TX)	45	96b	87b	92 (12)b
OAHU (Honolulu, HI)	45	93b	93b	83 (12)b

[a] Percentage of mosquitoes containing virus in their bodies. By infectious dose, infection rates followed by the same lower case letter are not significantly different at _ = 0.05 after adjusting for multiple comparisons.

[b] Percentage of mosquitoes containing virus in their legs. By infectious dose, dissemination rates followed by the same letter are not significantly different at _ = 0.05 after adjusting for multiple comparisons.

[c] Percentage of mosquitoes which that transmitted virus by bite 13 days after ingesting a WN virus-infected blood meal (number fed). Transmission rates followed by the same letter are not significantly different at _ = 0.05 after adjusting for multiple comparisons. nt = not tested.

Table 2. Number of negative specimens of *Ae. albopictus*[a] tested for vertical transmission of West Nile virus following rearing at one of two temperatures

	Rearing temperature		
Sex or stage	26°C	20°C	Combined
Female	4,683	1,253	5,936
Male	4,824	1,183	6,007
Larval	190	50	240
Total	9,697	2,486	12,183

[a]Frederick Co., Maryland, strain *Ae. albopictus* (n = 140) were intrathoracically inoculated with Crow 397-99 strain of WN virus. Mosquitoes were held for 7 days then allowed to feed on a hamster. Following oviposition, mosquitoes were provided another opportunity to take a blood meal. Specimens tested were from the eggs resulting from a total of 6 ovipositon cycles.

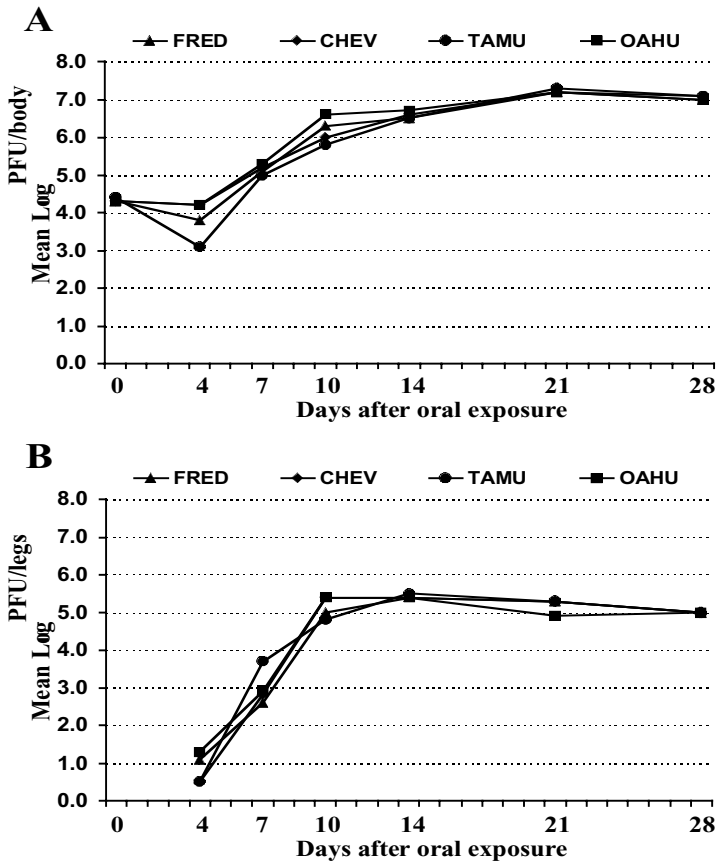

Figure 1. Mean viral titers over time in the bodies (A) and legs (B) of infected *Aedes albopictus* after oral exposure to a West Nile virus-infected chicken with a viremia of $10^{6.8}$ plaque-forming units (PFU)/ml of blood and held at 26°C. FRED, Frederick Co., Maryland strain; CHEV, Cheverly, MD strain; TAMU, Texas strain; OAHU, Hawaii strain. N = 5 on day 0 and 3-9 on days 4-28. Standard error of the mean was less than ± 0.4 at each data point.

Chapter 8

Conclusion

CONCLUSION

The results from this dissertation indicate that *Aedes albopictus* (Skuse) and *Ochlerotatus j. japonicus* (Theobald) could change the ecology of arboviruses of public health importance in the mid-Atlantic region of the Unites States. Both species are competent laboratory vectors of WN, SLE, EEE, and LAC viruses (Table 1). Additionally, *Ae. albopictus* is well established in the southeastern United States (Moore 1999, Chapter 2), while *Oc. j. japonicus*, though only detected a few years ago, has a distribution that appears to be rapidly expanding (Chapter 2). How important *Ae. albopictus* and *Oc. j. japonicus* are or may become in the natural transmission of endemic arboviruses will depend on the factors reported in this dissertation as well as other factors related to the vectors, virus, and reservoirs that affect arbovirus ecology.

There is a growing body of evidence that indicates that *Ae. albopictus* and *Oc. j. japonicus* may become important vectors of WN virus. Both species were found to be highly efficient laboratory vectors of WN virus (Chapters 3 and 7). Evidence of WN infection in wild-caught *Ae. albopictus* and *Oc. j. japonicus* has been found. There has only been one reported natural infection of *Ae. albopictus* with WN virus (CDC 2000). However, there have been numerous isolates of WN virus from *Oc. j. japonicus* over the years 1999-2001 (CDC 1999, 2000, 2001), and the minimum infection rates for WN virus in *Oc. j. japonicus* are among the highest for any species tested during 2000 in New York (White et al. 2001). An interesting aspect concerning the evidence of natural infection of *Oc. j. japonicus* from the New York study was that infections with WN virus were detected early in the transmission season (the first week of July) and late in the transmission season (the middle of September). This could indicate that *Oc. j. japonicus*

may have a role in virus amplification early in the transmission season and in transmission of virus to tangential hosts late in the transmission season when the number of avian blood meal sources decrease due to migration. The range of WN virus activity is increasing, pushing both southward and westward (CDC 2001). The southward expansion of WN virus may increase the probability that *Ae. albopictus* will transmit the virus, provided that hosts that develop viremias sufficient to infect the mosquitoes are present. Indeed, avian hosts of WN virus throughout the virus s range continue to show high susceptibility to infection, as evidenced by the continued reports of deaths in the crows (CDC 2001).

Based on vector competence, lack of evidence of natural infection, and viral distribution and ecology, *Ae. albopictus* and *Oc. j. japonicus* may not be important in the natural transmission of SLE virus. In the laboratory, infection rates for SLE virus in *Ae. albopictus* are low (<5%) (Savage et al. 1994), and *Oc. j. japonicus* needed to ingest a blood meal that contained a higher concentration of virus to become infected than did *Cx. pipiens*, a primary vector species (Chapter 4). To date, SLE virus has not been isolated from *Ae. albopictus* or *Oc. j. japonicus*. This could be due to one or more factors, such as the routine arbovirus surveillance methods do not efficiently sample *Ae. albopictus* or *Oc. j. japonicus*, the low to moderate susceptibility to SLE virus infection of these mosquitoes, the distribution of *Oc. j. japonicus* does not yet overlap the historically highly endemic areas of SLE virus transmission in the United States (mainly the lower Mississippi and Ohio River Valleys, the Gulf States and Florida), and little current evidence of SLE virus activity in nature. The viremic response (both level and duration) in avian hosts varies widely based on the geographic strain of SLE virus (Bowen et al.

1980, Reisen et al. 2000). This finding appears to be further substantiated in the laboratory studies done as part of this dissertation; a Maryland strain of SLE virus produced viremia in young chickens that were >10-fold higher than that produced by a Florida strain (Chapter 4). Variability of viral titers in vertebrate hosts may directly affect infection rates in the mosquito vectors, and subsequently reduce virus transmission in nature. Lastly, incidence of SLE in the United States fluctuates widely from year to year (Monath 1980), and major outbreaks of SLE have around an 8-yr periodicity (Creech 1975). These findings are probably related to the variability in the strains of SLE virus, and seem to indicate that highly virulent strains of SLE virus do not rapidly arise. If highly virulent strains of SLE virus are slow to arise, there would be a reduction in the probability of transmission of SLE virus by all vectors, except maybe the most highly susceptible ones, and the role that low to moderately efficient laboratory vectors (e.g., *Ae. albopictus* and *Oc. j. japonicus*) may play in viral transmission in nature may be severely reduced.

Aedes albopictus and *Oc. j. japonicus* may function as bridge vectors for EEE virus between the enzootic *Cs. melanura*-avian cycle and susceptible mammalian hosts. Both species are highly susceptible to infection with EEE virus and transmit the virus by bite. Yet, the two species are not highly efficient vectors of EEE virus due to the presence of a salivary gland barrier (Chapter 5). The transmission rates for *Ae. albopictus* and *Oc. j. japonicus* are similar to or higher than the transmission rates for native bridge vector species that are commonly found to be infected with EEE virus during epizootics [e.g., *Aedes canadensis* (Theobald), *Aedes vexans* (Meigan), *Coquillettidia perturbans* (Walker), and *Culex salinarius* Coquillett] (Vaidyanathan et al.

1997). A key factor that may limit the ability of *Ae. albopictus* and *Oc. j. japonicus* to transmit EEE virus to tangential hosts (e.g., man and horses) is flight range. The flight range for *Ae. albopictus* is around 200 m (Bonnet and Worcester 1946, Rosen et al. 1976). The flight range of *Oc. j. japonicus* is unstudied; however, it would be reasonable to suspect that its flight range would be short (a few hundred meters), like most container-inhabiting, woodland mosquito species. By comparison, native bridge vector species mentioned above have flight ranges of many kilometers (Moore et al. 1993). Thus, they would be more likely to bring EEE virus from the usually distant endemic foci to the tangential hosts. As people increasingly encroach (either by building homes or participating in outdoor activities) on the swamp foci of EEE virus transmission, the probability of *Ae. albopictus* and *Oc. j. japonicus* to serve as epidemic vectors may increase.

In comparison to the potential of WN, SLE, or EEE virus transmission by *Ae. albopictus* and *Oc. j. japonicus*, the risk of increased transmission of LAC virus by these two mosquitoes in nature, to include transmission to humans, may be very high. A number of factors, in addition to vector competence, favor transmission of LAC virus by *Ae. albopictus* and *Oc. j. japonicus*. The bionomics of these two mosquitoes are similar to that of the primary vector, *Ochlerotatus triseriatus* (Say) All three species prefer woodland habitats, and breed in artificial and natural containers. *Ochlerotatus triseriatus* and *Ae. albopictus* are mammalophagic and bite during the day. The host preference of *Oc. j. japonicus* in the United States is unstudied; however, it is likely to be mammalophagic based on reports from within its native range (Tanaka et al. 1997), and anecdotal reports of it coming to human bait in the United States (MRS, unpublished

data). The ecology and behavior of *Ae. albopictus* and *Oc. j. japonicus* place them in locations of LAC virus transmission and indicate that they are likely to feed on the mammalian hosts of the virus, eastern chipmunks and gray squirrels. In 2000, the first report was made of LAC virus infection in natural populations of *Ae. albopictus* (Gerhardt et al. 2001). As the distribution of *Oc. j. japonicus* expands into LAC virus-endemic areas, it is likely to become involved in the natural transmission of the virus.

To clarify the significance of the results presented in this dissertation, additional studies are needed. Much remains to be studied about the behavioral and ecological components of vectorial capacity of *Oc. j. japonicus*. Specifically, studies are needed to assess host preference, host-feeding pattern, daily survival and longevity, seasonality, and flight range. Lastly, more *Ae. albopictus* and *Oc. j japonicus* need to be collected and tested for evidence of arbovirus infection to prove that there is natural association with arboviruses or to further determine the extent to which they are involved in the transmission of a particular virus.

So, what action should be taken now to reduce the potential of *Ae. albopictus* and *Oc. j. japonicus* to become involved in arbovirus transmission? Possible responses range from conducting an elimination campaign to doing nothing. Although elimination campaigns targeting nonnative mosquitoes have been successful against *Anopheles gambiae* Giles in Brazil (Soper and Wilson 1942) and *Aedes aegypti* in South America (at least during the period of active control) (Camargo 1967), it is unlikely that either *Ae. albopictus* or *Oc. j. japonicus* could now be eliminated from the United States. Elimination of these mosquitoes would be improbable because their distribution now covers many states and they breed in small, natural containers away from human

habitation. Realistically, doing nothing is the probable response because evidence is lacking to incriminate either *Ae. albopictus* or *Oc. j. japonicus* as the vector of even a single case of human disease in the United States. However, it may just be a matter of time before such evidence is produced. For example, consider the circumstances in eastern Tennessee. By the mid- to late-1990s, *Ae. albopictus* had infested every county in Tennessee (Moore 1998), and this finding overlapped with the finding of a new foci of LAC virus transmission in the state (Jones et al. 1999). The first report of LAC virus infection in field collected *Ae. albopictus* was made in 2000 (Gerhardt et al. 2001). Although one could argue that more evidence is needed (i.e., multiple field isolates, vector density determination, etc.), the risk of human disease eventually reaches the point where the do nothing approach is unacceptable.

An appropriate response to reduce the potential of *Ae. albopictus* and *Oc. j. japonicus* to become involved in arbovirus transmission would be one that includes mosquito and arbovirus surveillance, localized mosquito population suppression measures, and an active public education campaign. The mosquito and arbovirus surveillance should employ trapping methods that will ensure adequate sampling of *Ae. albopictus* and *Oc. j. japonicus*. Ovitrapping is a sensitive tool for identifying the presence of these species in an area and it has been effective in monitoring for vertical transmission of LAC virus in *Oc. triseriatus* and *Ae. albopictus* (Moore et al. 1993, Gerhardt et al. 2001). Gravid traps are effective for collecting adult *Oc. j. japonicus* (Scott et al. 2001), and carbon dioxide-baited traps set out in the daytime in the vicinity of *Ae. albopictus* breeding sites are effective for collecting adults of this species (Freier and Francy 1991). While state, county, or city governmental organizations will be

responsible for mosquito and arbovirus surveillance, the enactment of localized mosquito population suppression measures will have to be a shared responsibility between governmental organization and individual residents. The most effective way to control *Ae. albopictus* and *Oc. j. japonicus* is to find and eliminate their breeding sites. Homeowners need to dispose of items such as tin cans, old tires, buckets, unused plastic swimming pools or other containers that collect and hold water; change water in bird baths at least once a week; and not allow water to accumulate at the base of flower pots or in pet dishes for more than 2 days. Governmental organizations must actively search out and eliminate large breeding sites (e.g., tire dumps). The goal of the mosquito suppression measures would be to establish a breeding-container free buffer zone around human habitations. Because *Ae. albopictus* and *Oc. j japonicus* presumably have short flight ranges, this zone may limit mosquito-human contact. Lastly, an innovative and sustainable public outreach program is an essential tool in preventing arbovirus transmission. These programs need to clearly describe the modes of mosquito-borne disease transmission, present information on the biology and risk of disease transmission by *Ae. albopictus* and *Oc. j. japonicus*, define the responsibilities of each level of the overall mosquito control effort, and educate the public on the means of preventing or reducing risk for exposure.

As evidence incriminating *Ae. albopictus* and *Oc. j. japonicus* as vectors of viruses affecting human accumulates, it is becoming increasingly likely that these two nonnative mosquitoes will change or have already changed the ecology of endemic arboviruses in the United States. It is clear that *Ae. albopictus* and *Oc. j. japonicus* are in

the United States to stay. Preventing or reducing the risk of virus transmission by *Ae. albopictus* and *Oc. j. japonicus* will be tasks that require diligence by all.

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Table 1. Relative vector competence [a] (reference) of North American strains of *Aedes albopictus* and *Ochlerotatus j. japonicus* for principal arboviruses of public health importance in the mid-Atlantic region of the United States

	Virus			
	SLE	WN	EEE	LAC
Ae. albopictus	+ (Savage et al. 1994)	+++ (Chapter 7)	++ (Scott et al. 1990, Turell et al. 1994)	+++ (Grimstad et al. 1989)
Oc. j. japonicus	++ (Chapter 4)	+++ (Chapter 3, Turell et al. 2001)	+ (Chapter 5)	++ (Chapter 6)

[a]+, transmission or estimated transmission rate 1-25% or inefficient; ++, transmission or estimated transmission rate 26-60% or moderately efficient; and +++, transmission or estimated transmission rate >60% or highly efficient.

Appendix A

Animal use approvals

USAMRIID RESEARCH PROTOCOL

Proposal No. 00-26 Protocol No. V00-10

Date Approved: 29 May 00

APC:

Title: DETERMINATION OF VECTOR COMPETENCE AND INVESTIGATION OF FACTORS AFFECTING THE ABILITY OF ARTHROPODS TO TRANSMIT SELECTED VIRUSES TO CHICKENS

Principal Investigator: Michael J. Turell/Virology/x4921
Monica O'Guinn/Virology/x4689

Scientific/Division Review:

Recommend Approval: Monica L. O'Guinn date 27Apr00
(Department Chief Signature)

date 27 APR 00
(Division Chief Signature)

Attending/Consulting Veterinarian:

date 27APR00
(Signature)

Statistical Review:

date 25 APR 2000
(Biostatistician Signature)

LACUC:

Recommend Approval:
~~Disapproved~~

MAJ, VC date 23MAY00
(Committee chairman)

Commander:

Approved:

date 29 May 00
(Signature)

USAMRIID RESEARCH PROTOCOL

Proposal No. 98-32 Protocol No. V98-07

Date Approved: 9 September 1998

APC:

Title: USE OF MICE, HAMSTERS, AND GUINEA PIGS IN THE RISK ASSESSMENT AND EVALUATION OF VIRAL AGENTS AND THEIR VECTORS THAT POSE A THREAT TO MILITARY PERSONNEL

Principal Investigators: Michael J. Turell/Virology/x4921
Terry A. Klein/Virology/x4724
James W. Jones/Virology/x4920

Scientific/Division Review:

Recommend Approval: _____ date 2 July 98
(Department Chief Signature)

_____ date 2 July 98
(Division Chief Signature)

Attending/Consulting Veterinarian:

_____ date 7 July 98
(Signature)

Statistical Review:

_____ date 2 July 98
(Biostatistician Signature)

LACUC:

Recommend Approval:
~~Disapproved~~:

_____ date 30 Jul 98
(committee chairman)

Commander:

Approved:

_____ date 8 Sep 98
(signature)

Appendix B

Published manuscript

Journal of the American Mosquito Control Association, 17(2):137–141, 2001

SCIENTIFIC NOTE

OCHLEROTATUS J. JAPONICUS IN FREDERICK COUNTY, MARYLAND: DISCOVERY, DISTRIBUTION, AND VECTOR COMPETENCE FOR WEST NILE VIRUS[1]

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ABSTRACT. *Ochlerotatus japonicus japonicus* is reported for the 1st time south of the Mason–Dixon Line, in Frederick County, Maryland. Fifty-seven oviposition trap samples were collected throughout the county between June 30 and August 24, 2000. From 971 larvae reared from the oviposition traps, 5 species were identified: *Ochlerotatus triseriatus* (45%), *Oc. j. japonicus* (43%), *Aedes albopictus* (7%), *Culex pipiens* (4%), and *Toxorynchites ritulus septentrionalis* (<1%). *Ochlerotatus j. japonicus* was found widely distributed over the area sampled. This is the 1st record of *Ae. albopictus* in the county as well. Vector competence studies indicated that *Oc. j. japonicus* is an efficient laboratory vector of West Nile (WN) virus. Depending on the viral titer at time of feeding, the estimated transmission rates for *Oc. j. japonicus* for WN virus were 2–4 times higher than that for *Cx. pipiens.* Studies of the viral titer in mosquitoes over time showed that titers in the bodies of infected *Oc. j. japonicus* reached their peak ($\sim 10^{6.5}$ plaque-forming units/mosquito) between 7 and 11 days after taking an infectious blood meal, and that virus became detectable in the legs (an indicator of disseminated infection) as early as 3 days after taking an infectious blood meal.

KEY WORDS *Ochlerotatus j. japonicus,* Maryland, distribution, West Nile virus, vector competence

The subspecies *Ochlerotatus japonicus japonicus* (Theobald) was reported for the 1st time in the USA in New Jersey and New York in the late summer of 1998 (Peyton et al. 1999). This mosquito has since been found in Connecticut (Andreadis, unpublished data), Ohio (Restifo, unpublished data), and Pennsylvania (Pagac, unpublished data). *Ochlerotatus japonicus* sensu lato (s.l.) is native to Japan, Korea, Taiwan, and southern China (Tanaka et al. 1979). Its distribution and bionomics in the USA are still largely unknown. This species breeds in natural and artificial containers and is generally found associated with wooded areas. Within its native range, *Oc. japonicus* s.l. is active primarily during the daytime (Tanaka et al. 1979). Little is known about the feeding preference of *Oc. japonicus* s.l. In Japan, it was reported to bite humans as well as birds (Tanaka et al. 1979). In the laboratory, *Oc. japonicus* s.l. readily fed on birds and mice (Miyagi 1971).

The public health importance of *Oc. j. japonicus* in the USA has not been studied in detail. Takashima and Rosen (1989) reported that this species was able to transmit Japanese encephalitis virus in the laboratory, and Turell et al. (2001) found a New Jersey strain of *Oc. j. japonicus* to be an efficient laboratory vector of West Nile (WN) virus. West Nile virus was detected in *Oc. j. japonicus* captured in New York in 2000 (Centers for Disease Control and Prevention 2000).

On June 8, 2000, mosquito larvae were collected from tires in an automobile salvage yard in Frederick, Frederick County, in western Maryland (39°23′33″N, 77°23′55″S). The tires (~70) were piled in a shaded area and the majority contained leaf litter. The entire contents of 8 tires were collected and taken to the laboratory, where the mosquito larvae were separated from the debris and reared to adults. Of the 687 specimens collected, 508 (74%) were *Oc. j. japonicus,* 165 (24%) were *Ochlerotatus triseriatus* (Say), and 14 (2%) were *Culex pipiens* L. The *Oc. j. japonicus* specimens were confirmed by taxonomists at the Walter Reed Biosystematics Unit (WRBU), Museum Support Center, Smithsonian Institution, Washington, DC, and voucher specimens were provided to WRBU. Specimens from this collection were subsequently used in a study of the population genetics of *Oc. j.*

[1] The views of the authors do not necessarily reflect the position of the Department of Defense or the Department of the Army. In conducting research using animals, the investigators adhered to the *Guide for the Care and Use of Laboratory Animals,* as prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (NIH Publication 86-23, revised 1996). The facilities are fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International.

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Table 1. Summary of the oviposition trap survey done in Frederick County, Maryland, between June 30 and August 24, 2000.

Species or species combination	*n*	% of total	Ovitraps[1]	
			No. positive	% positive
Ochlerotatus triseriatus	437	45	25	45
Ochlerotatus j. japonicus	422	43	25	45
Aedes albopictus	68	7	6	11
Culex pipiens	43	4	1	2
Toxorhychites rutilus septentrionalis	1	<1	1	2
Total	971	100	44	80
Oc. triseriatus and *Oc. j. japonicus*	38 (54)[2]	39[3]	10	18
Ae. albopictus and *Oc. triseriatus*	28 (54)	3	2	4
Oc. j. japonicus and *Ae. albopictus*	13 (92)	1	1	2
Oc. j. japonicus, Oc. triseriatus, and *Ae. albopictus*	0	0	0	0

[1] Fifty-seven oviposition traps were set at 57 different sites.
[2] Percentage of the 1st species listed in the combination.
[3] Percent of total of species combinations calculated using the total for the entire collection (971).

japonicus (Fonseca et al. 2001). When the operator of the salvage yard was asked about the origin of the tires, he indicated that all the tires were removed from rims of cars in his salvage yard.

To survey the distribution of *Oc. j. japonicus* in Frederick County, Maryland, oviposition traps (Zeichner and Perich 1999) were set throughout the county between June 30 and August 24, 2000. Each ovitrap consisted of a black cup (473-ml capacity) filled with 250 ml of dechlorinated tap water. A velore ovistrip (25 × 11 mm) was affixed to the side of the cup by a paper clip to serve as the oviposition substrate. The traps were placed in sites that were at least partially shaded (e.g., the base of a tree) and just into the tree line of the road that was used to access the area. Seven days later, the traps were returned to the laboratory and checked for the presence of larvae or eggs. Larvae were transferred to containers containing dechlorinated tap water, provided ground catfish chow for nutrition, and reared at 26°C, 80–85% relative humidity, and 16:8 h light:dark photoperiod. Eggs on ovistrips were flooded with dechlorinated tap water on the day they were collected and the larvae were reared as described above. Voucher specimens, 4th-stage larvae, and adults were preserved in 80% ethanol or pinned for later identification. A subsample of the larvae was allowed to pupate, and adults were identified after emergence.

Mosquito eggs or larvae were collected from 80% (44 of 57) of the oviposition traps. A total of 971 mosquitoes was collected and identified. *Ochlerotatus triseriatus* and *Oc. j. japonicus* accounted for 88%, and *Aedes albopictus* (Skuse), *Cx. pipiens,* and *Toxorynchites rutulus septentrionalis* (Dyar and Knab) accounted for the other 12% of these specimens (Table 1). The location of oviposition traps in our study is shown in Fig. 1. *Ochlerotatus j. japonicus* and *Oc. triseriatus* were collected across the entire sampling area. In contrast, *Ae. albopictus* was collected in the vicinity of the city of Frederick and the southern border towns of Point of Rocks and Brunswick.

Because of recent interest in WN virus and the need to elucidate the role newly invasive mosquito species may play in the epidemiology of WN virus in the eastern USA, we conducted laboratory studies of the vector competence of Maryland-collected *Oc. j. japonicus* for WN virus. Additionally, a study was done to evaluate viral replication and dissemination in these mosquitoes over time.

The *Oc. j. japonicus* used in the vector studies were reared from larvae collected at the original discovery site and from eggs collected during the countywide ovitrapping. The immature stages of mosquitoes were handled and reared as described above. Four- to 10-day-old adult mosquitoes were used in the susceptibility, transmission, or viral growth studies.

The WN virus strain (Crow 397–99) used was isolated from a dead crow found in Bronx, NY, during an epizootic in 1999 (Turell et al. 2000) and had been passaged once in Vero cell culture. Viral stock suspensions, triturated mosquito suspensions, and chicken blood samples were tested for infectious virus by plaque assay on Vero cells as described by Gargan et al. (1983), except that the 2nd overlay, containing neutral red stain, was added 2 days after the 1st overlay.

One-day-old chickens (*Gallus gallus*) were inoculated subcutaneously with 0.1 ml of a suspension containing $10^{4.2}$ plaque-forming units (PFU) of WN virus and mosquitoes were allowed to feed on them 1 or 2 days later. Immediately after mosquito feeding, a 0.1-ml blood sample was obtained from the jugular vein of each chicken and diluted in 0.9 ml of diluent (10% heat-inactivated fetal bovine serum in medium 199 with Earl's salts, $NaHCO_3$, and antibiotics) plus 10 units of heparin per milliliter to determine the viremia at the time of mosquito feeding. Engorged mosquitoes were transferred to 3.8-liter cardboard cartons with netting over the open

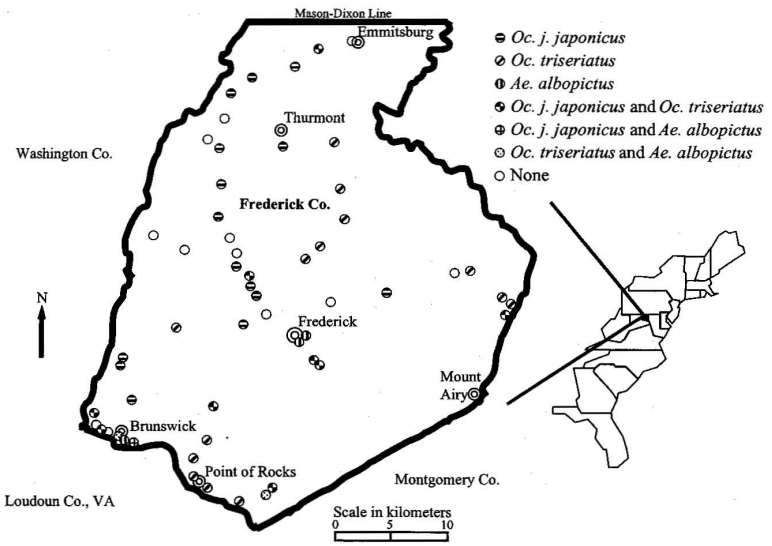

Fig. 1. Map of Frederick County, Maryland, showing locations of oviposition traps and *Ochlerotatus* and *Aedes* mosquitoes captured at each site; based on a single survey using 57 oviposition traps between June 30 and August 26, 2000.

end and maintained at 26°C. Four days after the infectious blood meal, an oviposition substrate was added to each cage. After 12–14 days, the mosquitoes were killed and their legs and bodies were triturated separately in 1 ml of diluent and frozen at −70°C until assayed for virus. Presence of virus in a mosquito's body indicated infection, whereas virus in the legs indicated that the mosquito had a disseminated infection (Turell et al. 1984).

To determine transmission rates, some of the mosquitoes that had taken an infectious blood meal were individually allowed to refeed on a 1- to 2-day-old chicken 12 or 13 days after the initial infectious blood meal. In addition to the orally exposed mosquitoes, mosquitoes intrathoracically inoculated (Rosen and Gubler 1974) 6–8 days previously with 0.3 μl of a suspension containing $10^{4.2}$ PFU of WN virus/ml were allowed to feed on individual chickens. The presence of virus in the blood of a chicken 24–48 h after mosquito feeding was used to indicate viral transmission. The proportion of mosquitoes with a disseminated infection that transmitted virus by bite (T(d)) was determined for orally exposed and inoculated mosquitoes. These percentages were used to calculate an overall T(d) percentage, which was then multiplied by the dissemination rate to obtain an estimated transmission rate.

To evaluate viral growth and dissemination over time, mosquitoes were fed on a viremic chicken and held at 26°C. Samples of 3–5 mosquitoes were assayed, leg and bodies separately, for virus immediately after the infectious blood meal and on days 1, 3, 5, 7, 11–12, and 14 after blood feeding.

Ochlerotatus j. japonicus was susceptible to infection with WN virus at both of the viral titers tested (Table 2). Data for *Cx. pipiens,* the suspected vector in New York (Centers for Disease Control and Prevention 1999), is included for comparison. The proportion of *Oc. j. japonicus* infected with WN virus significantly increased with the viral titer of the blood meal ($\chi^2 = 10.5$, df = 1, $P = 0.001$), whereas for *Cx. pipiens,* the titer of the blood meal did not significantly affect the rate of infection (Fisher's exact test, $P = 0.544$). *Ochlerotatus j. japonicus* was significantly less susceptible to oral infection than was *Cx. pipiens* at the low titer range (Fisher's exact test, $P = 0.038$); however, at the high titer range, no significant difference was found in oral susceptibility to infection between the 2 species ($\chi^2 = 0.0$, df = 1, $P = 0.996$). The proportion of *Oc. j. japonicus* developing a disseminated infection was significantly higher than that of *Cx. pipiens* at each of the viral titers tested ($\chi^2 = 6.3$, df = 1, $P = 0.012$ and $\chi^2 = 44.8$, df = 1, $P < 0.001$ at the low and high titer ranges, respectively).

Nearly all (97% or 29 of 30) *Oc. j. japonicus* with a disseminated infection transmitted WN virus by bite. This included 10 of 11 orally exposed and 19 of 19 inoculated individuals. Thus, route of infection did not significantly affect transmission rates (Fisher's exact test, $P = 0.367$), and the overall T(d) percentage for *Oc. j. japonicus* in our study, 97%, was virtually identical to the 100% (6

Table 2. Oral susceptibility to and transmission of West Nile virus by a Maryland strain of *Ochlerotatus j. japonicus.*

Species	Virus titer at time of feeding[1] (log_{10} PFU/ml)	No. tested	Infection rate[2]	Dissemination rate[3]	Estimated transmission rate[4]
Oc. j. japonicus	6.0 ± 0.5	92	57	56	54
	7.0 ± 0.4	83	80	77	75
Culex pipiens[5]	6.0 ± 0.5	17	82	23	20
	7.0 ± 0.4	78	79	24	21

[1] Titer in chickens (*Gallus gallus*) inoculated 24 or 48 h previously with a New York stain of West Nile virus (Crow 397-99) (PFU, plaque-forming units).
[2] Percentage of mosquitos with virus in their body.
[3] Percentage of mosquitos with virus in their legs.
[4] An estimate of the percentage of mosquitos that will transmit virus by bite 12–13 days after an infectious blood meal and storage at 26°C, calculated by multiplying percent dissemination times the percentage of mosquitoes with disseminated virus that transmitted virus [T(d)]. Overall T(d) percentages were previously determined to be 97% for *Oc. j. japonicus* and 88% for *Cx. pipiens.*
[5] *Culex pipiens* data at the lower titer range from D. Dohm (unpublished data) and at the higher titer range from Turell et al. (2001).

of 6) rate reported for New Jersey *Oc. j. japonicus* by Turell et al. (in press).

Based on the proportion of mosquitoes that developed a disseminated infection and the overall T(d) percentage, *Oc. j. japonicus* was more efficient at transmitting WN virus than was *Cx. pipiens*: more than 2 times more efficient at the lower viral dose ($10^{6.0\pm0.5}$), and nearly 4 times more efficient at transmitting virus at the higher viral dose ($10^{7.0\pm0.4}$; Table 2).

For mosquitoes that fed on chickens with a mean viremia level of $10^{6.5}$ PFU/ml of blood, viral titers in both species generally increased from day 3 to 11, with titers reaching nearly 10^7 PFU per body for *Oc. j. japonicus* and approximately $10^{6.5}$ PFU per body for *Cx. pipiens* (Table 3). Disseminated infections were detected 3 days after the infectious blood meal in *Oc. j. japonicus,* but not until days 11–12 in *Cx. pipiens* (Table 3).

This is the 1st report of *Oc. j. japonicus* south of the Mason–Dixon Line and it indicates that this species' range in the USA is expanding. Previous published reports and recent informal reports indicate that it is also found in New York, New Jersey, Connecticut, Pennsylvania, and Ohio. Although *Oc. j. japonicus* is generally described as a northern-climate species within its native range in Japan, it has been reported as far south as 33°N, in Chejudo Island, Republic of Korea (Tanaka et al. 1979). Thus, based solely on climatic information, *Oc. j. japonicus* may expand its range as far south as Jacksonville, FL, in the USA.

Given its distribution within Frederick County and its abundance relative to *Oc. triseriatus,* the introduction of *Oc. j. japonicus* apparently occurred before this year's discovery. Surveys in Connecticut looking specifically for *Oc. j. japonicus* found it to be widespread and breeding in areas away from tire dumps (Andreadis, unpublished data). *Ochlerotatus j. japonicus* has been present in Connecticut for between 2 and 11 years, based on the reevaluation of adult collections from recent years and from a 1989 survey of tire-breeding mosquitoes (Andreadis 1989). Thus, based on the data from this and the Connecticut studies, *Oc. j. japonicus* has been in Frederick County, Maryland, for at least a few years. Analysis of the data from our study also seems to suggest that local expansion of the range of *Oc. j. japonicus* is not driven only by the movement of infested, used automobile tires.

Table 3. Viral titers[1] over time in the bodies and legs of a Maryland strain of *Ochlerotatus j. japonicus* after oral exposure to a West Nile virus–infected chicken with a viremia of $10^{6.5}$ plaque-forming units (PFU)/ml of blood.

Species	Part assayed	Days after oral exposure: 0	1	3	5	7	11–12	14
Oc. j. japonicus	Body	3.1–4.0 (3/3)	0[2]–3.1 (1/3)	3.8–4.7 (3/3)	4.2–4.9 (3/3)	4.2–6.9 (3/3)	6.0–7.0 (5/5)	6.0–6.8 (3/3)
	Leg	0 (0/3)	0 (0/3)	0–2.7 (2/3)	2.1–2.9 (3/3)	0–5.7 (2/3)	3.7–5.4 (5/5)	4.1–5.3 (3/3)
Cx. pipiens[3]	Body	3.9–4.0 (3/3)	NT	3.0–4.0 (3/3)	3.6–4.2 (3/3)	3.9–4.3 (3/3)	0–6.6 (5/6)	2.6–6.4 (6/6)
	Leg	0 (0/3)	NT	0 (0/3)	0 (0/3)	0 (0/3)	0–4.2 (2/6)	0–4.5 (3/6)

[1] Log_{10} PFU/ml of body or leg suspension. Range (number of mosquitoes with virus in the respective part assayed/number assayed) NT, not test.
[2] A viral titer of zero (0) indicates that virus was not present or that the viral titer was below the detection limit of the assay.
[3] *Culex pipiens* data from D. Dohm (unpublished data).

The distribution of *Oc. j. japonicus* seemed to be associated with *Oc. triseriatus.* Seventeen percent of the ovitraps contained both species (Table 1) and the distribution of positive ovitraps for either species were interwoven (Fig. 1). This overlap of the 2 species would be likely, given that they both breed in containers. In the USA, *Oc. j. japonicus* has been collected in a broad variety of container types, such as tin cans, water dishes for potted plants, concrete rainwater drainage forms, buckets, pans, and 3.8-liter milk jugs (Sardelis, unpublished data). Thus, the likelihood for this species to come in contact with humans may be similar to that of *Oc. triseriatus.* To date, no studies have been published on biting preference of *Oc. j. japonicus* in the USA. Whether some type of competition will occur between these 2 species for breeding sites and survival also remains to be shown.

Although the focus of this study was not on *Ae. albopictus,* some items of information regarding this species are noteworthy. This is the 1st record of *Ae. albopictus* in Frederick County, Maryland. The collection of *Ae. albopictus* in the city of Frederick and in 2 other small towns in the county indicates that potential exists for this species to become a nuisance to residents.

This study showed that the Maryland strain of *Oc. j. japonicus* has the potential to serve as a WN viral vector, based on its susceptibility to infection, and its ability to transmit WN virus efficiently. These data are in agreement with a similar study by Turell et al. (2000), who studied a New Jersey strain of *Oc. j. japonicus.* Additionally, the high relative efficiency of transmission and the shorter extrinsic incubation period for *Oc. j. japonicus* compared to *Cx. pipiens* may have an important bearing on the epidemiology of WN virus. However, in the absence of information on the survivorship, host preference, and abundance of *Oc. j. japonicus* in the USA, making an accurate prediction on the possible impact of this newly invasive species is difficult.

Given the widening distribution and apparent relative abundance of *Oc. j. japonicus* in the USA, it is important to evaluate its potential to become involved in transmission of other arboviruses, such as eastern equine encephalitis, St. Louis encephalitis, and La Crosse encephalitis viruses.

We thank D. Dohm for the use of his unpublished data; R. Leon, M. O'Guinn, L. Wasieloski, and K. Kenyon for their critical reading of the manuscript; and M. Delgado and D. Schachner for their assistance with the mosquito rearing. This research is part of the doctoral dissertation of Michael R. Sardelis.

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