Vector competence of *Ochlerotatus trivittatus* (Coquillett), *Aedes albopictus* (Skuse), and *Culex pipiens* (L.) (Diptera: Culicidae) for West Nile virus

by

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ABSTRACT

West Nile virus (WNV) was recognized in North America in 1999. Avian species are the primary reservoir of the virus and can develop viremias with titers higher than $10^{10.0}$ CID_{50s} / ml. *Culex* species of mosquitoes are the primary amplifying vectors. Many species of mammals are also susceptible to WNV but develop viremias that seldom exceed $10^{5.0}$ CID_{50s} / ml. Mammals may play a significant role in WNV ecology if mosquitoes that feed on them during periods of viremia can be infected and transmit the virus. The primary objective of this study was to determine vector competency of *Ochlerotatus trivittatus* (Coq.) a potential bridge vector that feeds primarily on mammals including humans and occasionally on birds. Susceptibility of *Oc. trivittatus* to WNV was compared to that of *Aedes albopictus* (Skuse) a known bridge vector, and *Culex pipiens* (L.), a primary amplifying vector by determining infection rates after feeding mosquitoes on chicks with blood meal titers (BMTs) of $10^{2.5}$ to $10^{10.0}$ CID_{50s} / ml. The ability of *Oc. trivittatus* to transmit WNV was determined by comparing transmission rates (%) of *Oc. trivittatus, Ae. albopictus* and *Cx. pipiens* by the capillary tube method following infection by blood meals with titers ranging from $10^{2.5}$ to $10^{9.5}$ CID_{50s} / ml. Susceptibility of *Oc. trivittatus* and *Cx. pipiens* to WNV was essentially the same but greater than *Ae. albopictus.* The lowest infective BMTs for *Oc. trivittatus, Ae. albopictus* and *Cx. pipiens* were $10^{4.5}$, $10^{5.5}$, and$10^{4.5}$ CID_{50s} / ml. The 50 % infective BMTs for the 3 species were $10^{6.0}$, $10^{6.6}$, and $10^{6.2}$ CID_{50s} / ml. Transmission rates of *Oc. trivittatus, Ae. albopictus,* and *Cx. pipiens* after blood meals with titers higher than $10^{7.0}$ CID_{50s} / ml were 41.7, 72.4 and 46.8 %. The lowest BMTs that resulted in transmission by the 3 species

were $10^{5.5}$, $10^{7.0}$, and $10^{5.5}$ CID_{50s} / ml. These observations suggest that *Oc. trivittatus* might play a more significant role than *Ae. albopictus* in maintaining WNV in populations of mammals which typically develop low levels of viremia.

CHAPTER 1. GENERAL INTRODUCTION

Introduction

West Nile virus (WNV) is a linear positive, single-stranded RNA virus in the Japanese encephalitis serogroup. It belongs to the genus *Flavivirus* of the family *Flaviviridae* (Burke and Monath, 2001). This virus was first isolated in 1937 from the blood of a woman in the West Nile province of Uganda (Smithburn et al. 1940). The virus is widely distributed in Africa, the Middle East, parts of Europe, the former Soviet Union, and Asia (Burke and Monath, 2001).

The first outbreak of the WNV in the United States was in the New York City metropolitan area in August 1999 (Briese et al. 1999, Lanciotti et al. 1999, van der Poel 1999). Sixty-two human cases of WNV infection were reported in the USA during the first year of the outbreak. The number of human cases has increased every year. There were more than 8,000 WNV human cases reported in 2003 (CDC, 2003b).

The transmission cycle of WNV in nature involves mosquito vectors and WNV amplifying hosts. The principle amplifying hosts are avian species in which viremias can exceed $> 10^{10.0}$ PFU / ml. Mammals are also susceptible to WNV but generally do not develop high levels of viremia and therefore may not play a significant role in the ecology of WNV. Not all species of mosquitoes are competent vectors for WNV.

Mosquitoes generally pick up the virus with their blood meal but the virus must overcome several obstacles or barriers before it can be transmitted to another host. The virus must survive or replicate in mosquito midgut epithelial cells and replicate in salivary glands for injection into a new host. A mosquito must also provide a suitable internal environment

and live long enough for virus replication, movement, and transmission.

Vector competence varies among different species and among different geographic populations of the same species (Goddard 2000, Goddard et al. 2002, Sardelis et al. 2002). Vector capacity implies, in addition, the modifying effect on vector competence of environmental factors. For example the frequency of vertebrate host-vector contacts and vector abundance (Goddard, 1999).

The primary objective of the studies described in the dissertation is to compare the vector competence of *Ochlerotatus trivittatus* (Coq), *Aedes albopictus* (Skuse), and *Culex pipiens* (L.). All 3 mosquito species have the capacity to transfer WNV from avian to mammalian species. *Ochlerotatus trivittatus* is of particular interest because it feeds primarily on mammal species such as cottontail rabbits and might play a role in maintaining WNV in these populations. These populations might serve as a source of WNV for human infection.

Dissertation Organization

This dissertation consists of five chapters. Three chapters (chapter 2, 3, and 4) will be submitted to a journal for publication. Chapter 1 is a general introduction consisting of an introduction, dissertation organization, and literature review. Chapter 2 is a comparison of the susceptibility of *Ochlerotatus trivittatus* (Coq), *Aedes albopictus* (Skuse), and *Culex pipiens* (L.) to WNV infection. Chapter 3 is a comparison of WNV transmission by *Oc. trivittatus*, *Ae. albopictus*, and *Cx. pipiens*. Chapter 4 is a comparison of *in vitro* and *in vivo* transmission of WNV by *Oc. trivittatus* and *Ae. albopictus* and Chapter 5 is the general conclusions of this dissertation. References cited are listed at the end of each chapter. All

results and conclusions from these studies were interpreted and written into manuscript form by the author.

Literature Review

Background and history of West Nile virus

West Nile virus (WNV) is an arthropod-borne virus widely distributed in humans, birds, and other vertebrates in Africa, the Middle East, parts of Europe, the former Soviet Union and Asia (Burke and Monath, 2001). It was originally isolated in 1937 from a female patient in the West Nile province of Uganda, East Africa (Smithburn et al., 1940). Early outbreaks of WNV occurred in many areas of the world. For example, there were outbreaks in Israel in 1950 (Bernkopf et al. 1953), France in 1962 (Panthier 1968), and South Africa in 1975 (Jupp et al. 1986).

In the last ten years, outbreaks of WNV have occurred in many areas in the world. There were more than 800 hospitalized cases in Romania in 1996 (Cernescu et al. 1997). Both human and animal cases particularly, American crows, occurred in New York in 1999 (Briese et al. 1999, Jia et al. 1999). Fourteen human cases were reported in Russia from July to October, 1999 (Platonov et al. 2001). Another 76 equine clinical cases plus positive samples from gulls, ducks, magpies, and neutralizing antibodies in humans were found in France in September 2000 (Murgue et al. 2001, Durand et al. 2002).

The outbreak of WNV during 1999 in the New York metropolitan area of the USA was the first known occurrence of this virus in North America. West Nile virus spread throughout the United States and parts of Canada in only three years (Ford-Jones et al. 2002, Pepperell et al. 2003, Weese et al. 2003).

General characteristics of West Nile virus

West Nile virus is a linear positive, single-stranded RNA virus. It belongs to the genus Flavivirus of the family *Flaviviridae*. The genome of this virus is 10,000-11,000 nucleotides long and the virion is spherical and enveloped, with a diameter of about 40-60 nm. The nucleocapsids are icosahedral and 25-30 nm in diameter. There are at least 70 arboviruses in the genus Flavivirus that infect humans and other animals. Some well known Flaviviruses are dengue, Japanese encephalitis, kokobera, koutango, kunjin, Murray Valley encephalitis, St. Louis encephalitis, tick-borne encephalitis, West Nile, and yellow fever virus. Flaviviruses can be transmitted by different arthropods however most Flaviviruses are transmitted by mosquitoes. Flaviviruses, their mosquito vectors, and known vertebrate hosts are shown in Table 1.1.

West Nile virus is in the Japanese encephalitis serocomplex which includes ten viruses; Alfuy, Japanese encephalitis, Koutango, Kokobera, Kunjin, Murray Valley encephalitis, Stratford, St. Louis encephalitis, and Usutu virus. These viruses have been isolated from Africa, southern Europe, Middle East, Asia, Australia, North, Central, and South America.

A phylogenetic study provided clues to the epidemiology and distribution of WNV. Nucleic acid sequence analysis of the New York 1999 strain of WNV indicated a common origin of this virus with the WNV isolated from a domestic goose in Israel in 1998 (Lanciotti et al. 1999). A genetic characterization was conducted on the envelope gene of eleven strains of WNV in New York State during 2000. The strains from mosquito pools and dead vertebrates are the same as the WNV that was isolated in 1999 (Ebel et al. 2001). The phylogenetic studies of isolates from Europe and Africa suggest the introduction of WNV by

immigrating birds from sub-Saharan Africa into Europe (Savage et al. 1999). Phylogenetic studies also indicated a close relationship between Alfuy and Murray Valley encephalitis virus, Kokobera and Stratford virus, Kunjin and WNV (Poidinger et al. 1996).

Table 1.1 Flaviviruses transmitted by mosquitoes and their vectors and vertebrate hosts

Virus	Vector	Vertebrate hosts
Dengue 1-4	*Aedes aegypti* *Aedes albopictus*	Humans Primates
Japanese encephalitis	*Culex tritaeniorhynchus*	Swine Birds
Murray Valley encephalitis	*Culex annulirostris*	Birds
Racio	Mosquitoes	Birds
St. Louis encephalitis	*Culex pipiens* *Culex tasalis* *Culex nigripalpus* *Culex* spp.	Birds
Wesselsbron	Mosquitoes	Sheep
West Nile	*Culex pipiens* *Culex* spp. *Aedes* spp. *Ochlerotatus* spp.	Birds
Yellow fever	*Aedes aegypti* *Aedes africanus* *Aedes simpsoni* *Haemagogus* spp.	Humans Primates

Vector competence for West Nile virus

Vector competence refers to the ability of an arthropod to acquire, maintain, and transmit a pathogen from one host to another (Goddard 2000). Not all arthropods are vectors of disease agents. Even blood-feeding arthropods are not always vectors, but vectors are almost always blood-feeding arthropods. Generally, insects or ticks may pick up pathogens with their blood meal. The pathogen must overcome many obstacles or barriers before being transmitted to another host. In many cases, the pathogen must survive or replicate in arthropod tissues such as the midgut epithelium, muscle, nervous tissue, reproductive organs, or salivary glands before being transmitted to a new host. In some insects, however, transmission occurs without the pathogen making its ways into the salivary glands. In such cases, they transmit the pathogen with their feces during feeding or biting.

Vector competence varies among different species and also among different geographic populations of the same species (Goddard et al. 2002, Sardelis et al. 2002). To evaluate vector competence of mosquitoes for WNV in nature, many criteria must be considered. They must provide a suitable internal environment and live long enough for WNV replication and transmission to take place. A competent vector must have a host-feeding pattern consistent for the target host. They must feed often and for extended periods, ingesting infected blood, and must readily disperse. Their geographic distribution must match the transmission pattern and they need to be abundant.

Not all species of mosquitoes or ticks are competent vectors for WNV. Mosquitoes become infected with WNV when they feed on WNV infected animals particularly birds that have a high viremia. The virus replicates in the mosquito and localizes in high concentrations in salivary glands and is transmitted during subsequent feedings.

Table 1.2 West Nile virus positive mosquito pools from field collections reported in the USA since 1999

Genus	Species	
Aedes	*Aedes albopictus*	*Aedes vexans*
	Aedes aegypti	*Aedes cinereus*
Anopheles	*Anopheles barberi*	*Anopheles punctipennis*
	Anopheles atropos	*Anopheles quadrimaculatus*
	Anopheles crucians/bradleyi	*Anopheles walkeri*
Coquillettidia	*Coquillettidia perturbans*	
Culiseta	*Culiseta inornata*	*Culiseta melanura*
Culex	*Culex erraticus*	*Culex restuans*
	Culex nigripalpus	*Culex salinarius*
	Culex pipiens	*Culex tarsalis*
	Culex quinquefasciatus	*Culex territans*
Deinocerites	*Deinocerites cancer*	
Ochlerotatus	*Ochlerotatus atropalpus*	*Ochlerotatus provocans*
	Ochlerotatus atlanticus/tormentor	*Ochlerotatus sollicitans*
	Ochlerotatus canadensis	*Ochlerotatus sticticus*
	Ochlerotatus cantator	*Ochlerotatus stimulans*
	Ochlerotatus dorsalis	*Ochlerotatus taeniorhynchus*
	Ochlerotatus fitchii	*Ochlerotatus triseriatus*
	Ochlerotatus infirmatus	*Ochlerotatus trivittatus*
	Ochlerotatus japonicus	
Orthopodomyia	*Orthopodomyia signifera*	
Psorophora	*Psorophora ciliata*	*Psorophora ferox*
	Psorophora columbiae	*Psorophora howardii*
Uranotaenia	*Uranotaenia sapphirina*	

There are 43 mosquito species known to have tested positive for WNV from field collections in the USA since 1999 (Table 1.2). These data were obtained by CDC field investigations or were reported by state surveillance programs to ArboNet as of August 23, 2003 (CDC, 2003a).

After infecting a mosquito, WNV replicates in mosquito midgut cells. Infective titers of the virus may be different for each mosquito species (Table 1.3). Even though the mosquito species take an infected blood meal with the same virus titer, the ability of WNV to replicate in each mosquito may vary.

Table 1.3 Reported West Nile virus titers in mosquito species after taking an infected blood meal

Species	Uptake WNV (Log_{10} PFU / ml)	N[a]	Tested tissue	Tested day (PBF)[b]	WNV titer per mosquito (Log_{10} PFU)
Cx. pipiens	6.5	6	leg	14	0 - 4.5
Cx. pipiens	6.5	6	body	14	2.6 - 6.4
Oc. j. japonicus	6.5	3	leg	14	4.1 - 5.3
Oc. j. japonicus	6.5	3	body	14	6.0 - 6.8

[a]No. tested mosquitoes

[b]PBF = post blood feeding

Modified from Sardelis and Turell (2001)

Not all WNV infected mosquitoes can transmit WNV. Different species or strains of mosquitoes may have the same or different levels or types of barriers for WNV. Presence of WNV in the hemolymph indicates that virus can move across the mosquito midgut barrier and virus in the saliva indicates that virus can move across the salivary gland barrier. The infection, dissemination (WNV in the hemolymph), and transmission of WNV also differ from one mosquito species to another (Table 1.4). Different strains of the same species of mosquito may also differ in their ability to become infected or transmit WNV (Table 1.5).

West Nile virus can be transmitted by more than one type of vector. Mosquitoes and ticks become infected and transmit WNV. This virus was isolated repeatedly from ticks in the genera *Argus* and *Hyalomma* in Egypt (Hoogstraal 1972).

Abbassy et al (1993) showed soft ticks have the potential to be competent vectors for WNV in laboratory conditions. *Argas persicus, A. hermanni* and *A. arboreus* were allowed to feed on blood with $10^{5.5} - 10^{6.2}$ CID_{50s} / ml of WNV (Eg 101 strain) which was isolated from a sick child in Sindbis Village, Egypt in 1950. The virus was passaged in sucking mouse brain and baby hamster kidney cells. The virus was detected only 3-8 days after feeding in *A. persicus* and *A. hermanni. Argas arboreus* had a titer of $10^{4.0}$ CID_{50s} / ml in whole tick homogenates at day 4. Titers remained at $10^{3.0}$ CID_{50s} / ml for 50 days after feeding. The virus was transmitted to clean chicks on day 20 and it was detected in the salivary glands, ovaries, synganglia and coxal fluid of the ticks using virus isolation, indirect fluorescent antibody and histochemical techniques. There was horizontal and vertical transmission but not transtadial (from nymph to adult) and venereal transmission in this tick (Abbassy et al. 1993)

Table 1.4 West Nile virus infection, dissemination, and estimated transmission rates for mosquitoes after taking an infected blood meal

Species	Uptake WNV[a]	N[b]	Infection rate	Dissemination rate	Estimated transmission rate
Ae. aegypti	7.2 ± 0.3	19	16	16	≤ 16
Ae. albopictus	7.2 ± 0.3	61	90	85	73
Ae. atropalpus	7.2 ± 0.3	12	92	92	92
Ae. japonicus	7.2 ± 0.3	36	69	64	64
Ae. sollicitans	5.2 ± 0.2	9	11	11	7
Ae. sollicitans	7.2 ± 0.3	50	70	16	11
Ae. taeniorhynchus	5.2 ± 0.2	45	2	0	0
Ae. taeniorhynchus	7.2 ± 0.3	75	12	3	3
Ae. vexans	5.2 ± 0.2	3	0	0	0
Ae. vexans	7.2 ± 0.3	13	46	8	8
Coquilletidia perturbans	6.6 ± 0.3	11	18	9	2
Cx. nigripalpus	4.6	7	29	0	0
Cx. nigripalpus	5.7 ± 0.5	132	78	8	7
Cx. nigripalpus	6.8 ± 0.4	127	84	12	10
Cx. pipiens	5.2 ± 0.2	46	17	2	2
Cx. pipiens	6.0 ± 0.5	17	82	23	20
Cx. pipiens	7.0 ± 0.4	78	79	24	21
Cx. pipiens	7.2 ± 0.3	95	81	23	20
Cx. quinquefasciatus	5.0	13	46	0	0
Cx. quinquefasciatus	5.5	16	50	6	6
Cx. quinquefasciatus	6.3	17	94	12	≤ 13
Cx. quinquefasciatus	7.0 ± 0.5	78	91	22	20
Cx. resturans	6.6 ± 0.3	11	100	55	55
Cx. salinarius	6.6 ± 0.3	20	95	60	34
Oc. j. japonicus	6.0 ± 0.5	92	57	56	54
Oc. j. japonicus	7.0 ± 0.4	83	80	77	75

[a]Log_{10} PFU / ml

[b]No. tested mosquitoes

Modified from Sardelis and Turell (2001), Sardelis et al. (2001), Turell et al. (2001)

Table 1.5 West Nile virus infection and transmission rates after taking an infected blood meal in California mosquitoes

Species	Source by county	Uptake WNV	N[a]	Tested day	Infection rate	Transmission rate
Cx. erythrothorax	Orange	4.9 ± 0.1	47	7	15	0
		7.1 ± 01	15	7	67	0
		7.1 ± 01	48	14	77	19
	Riverside	4.9 ± 0.1	12	7	67	0
		4.9 ± 0.1	20	14	65	30
		7.1 ± 01	15	7	100	33
		7.1 ± 01	25	14	100	64
Cx. p. quinquefasciatus	Kern	4.9 ± 0.1	50	7	58	0
		4.9 ± 0.1	50	14	10	0
		7.1 ± 01	50	7	86	4
		7.1 ± 01	50	14	58	52
	Orange	7.1 ± 01	58	14	28	19
	Riverside	4.9 ± 0.1	50	7	0	0
		4.9 ± 0.1	55	14	0	0
		7.1 ± 01	60	7	8	0
		7.1 ± 01	60	14	13	2
Cx. tarsalis	Kern	4.9 ± 0.1	60	7	30	10
		4.9 ± 0.1	45	14	7	0
		7.1 ± 01	15	7	93	40
		7.1 ± 01	35	14	74	60
	Riverside	4.9 ± 0.1	40	7	13	0
		4.9 ± 0.1	10	14	0	0
		7.1 ± 01	49	7	94	10
		7.1 ± 01	55	14	85	62
	Yolo	4.9 ± 0.1	25	7	8	0
		4.9 ± 0.1	11	14	36	82
		7.1 ± 01	30	7	87	60
		7.1 ± 01	1	14	100	100

[a]No. tested mosquitoes

Modified from Goddard (2002)

Barriers for arbovirus infection, dissemination, and transmission by mosquitoes

A barrier refers to morphological and physiological factors of the internal structure of mosquitoes that prevent invasion of host cells by viruses. Barriers may be different in different species and in different strains of the same species.

Infection barrier

An infection barrier prevents a mosquito from becoming infected by a virus. To infect a mosquito, a virus must get past various infection barriers such as digestive enzymes, the peritrophic membrane, and the surface of midgut epithelial cells (Houk et al. 1986, Kramer et al. 1989, Bosio et al. 2000, Modlmaier et al. 2002).

Digestive enzymes. Some digestive enzymes produced by mosquitoes can inactivate or decrease the infectivity of arboviruses in ingested blood. Trypsin and chymotrysin are protease enzymes secreted by midgut epithelial cells of *Aedes* mosquitoes for example *Ae. taeniorhynchus* (Stoltz and Summers, 1971). Some mosquitoes such as *Cx. tarsalis* produce only trypsin (Houk and Hardy, 1982). The production, secretion, and concentration of trypsin and chymotrypsin are different in each species. The sensitivity of arboviruses to trypsin and chymotrypsin affects the integrity of the viral envelope. The viral envelope was removed in some viruses by trypsin and chymotrysin (Biddle, 1968). However, the attachment of some viruses may depend on a proteolytic cleavage of viral glycoproteins by midgut enzymes, for example the attachment of La Crosse virus to the midgut of *Ae. triseriatus* mosquitoes.

Peritrophic membrane. The digestive system or alimentary canal of mosquitoes may be easily divided into three distinguishable portions (regions). These are the foregut, midgut, and hindgut. The foregut and hindgut are ectodermal in origin but the midgut has an

endodermal origin. Since the foregut and hindgut are ectodermal, they are lined with a cuticle-like material similar to that covering the outside of an insect's body. Because the midgut does not have a cuticle, it is susceptible to infect by some viruses. Mosquitoes, however, produce a peritrophic membrane that protects midgut epithelial cells from damage and invasion by pathogens.

The peritrophic membrane is a 1-μm-thick tubular film that forms around the food. It is actually a loose lining inside the midgut (Daly et al. 1998) . It is made of a chitin fibril set in a protein-carbohydrate matrix and is usually made up of a number of separate laminae. The peritrophic membrane is permeable to the products of digestion and digestive enzymes released from the epithelial cells but it is not permeable to other large molecules such as undigested proteins and polysaccharides. This membrane is absent in unfed mosquitoes but forms within 20-24 hours after blood meal ingestion (Houk et al. 1979). Only a blood meal induces the formation of peritrophic membrane in adult mosquitoes.

The peritrophic membrane is formed by a ring of specialized cells at the anterior end of the midgut. It forms as a continuous envelope along the midgut. Formation and solubility of the peritrophic membrane in *Anopheles stephensi* depends on salt concentration in the gut lumen (Berner et al. 1983). Once produced, the matrix moves backward with the bolus of food (blood) and is eventually eliminated in the feces. The relative impermeability of the peritrophic membrane may also confer some degree of protection for the midgut from virus infection (Houk et al. 1979). Some viruses attach to receptor sites on midgut epithelial cells because they enter midgut cells within minutes or a few hours before secretion of the peritrophic membrane. The composition of the peritropic membrane is different among each

mosquito species. For example *Anopheles* mosquitoes have N-acetylgalactosamine and galactose in the matrix but *Aedes* mosquitoes do not (Berner et al. 1983).

Midgut epithelial cell surface. There are two main regions in the midgut, the anterior or thoracic midgut and the posterior or abdominal midgut. The site of initial infection in the midgut for each mosquito species is different, however the midgut is presumed to be the site of initial infection because most of a blood meal imbibed by mosquitoes is directed there for digestion. In the abdominal midgut, blood cells become concentrated and serum is expressed to the periphery soon after feeding. This process concentrates the ingested virus adjacent to the epithelium (Weaver et al. 1991). Virus concentration does not occur when mosquitoes are allowed to feed on an artificial infected blood meal that does not clot in the midgut which reduces the susceptibility of mosquitoes to virus infection. For example western equine encephalomyelitis (WEE) virus in *Culex tarsalis* concentrates in the abdominal midgut of *Cx. tarsalis* after taking a blood meal from an infected chick. However concentration occurs in the thoracic midgut when *Cx. tarsalis* takes an artificial infected blood meal (Weaver et al. 1993). Rift Valley fever virus however replicates in the thoracic midgut of *Cx. pipiens* and disseminates from there (Rosomer *et al.*, 1987).

The midgut barrier for WEE virus infection in *Cx. pipiens* is associated with an inability of the virus to adsorb and/or penetrate midgut epithelium when administered perorally. The barrier is not related to an inability of the *Cx. pipiens* midgut epithelial cells to support viral multiplication because these cells become infected with WEE virus when the virus is administered parenterally (Houk et al. 1986). The infection barrier to one virus can be different or the same in different strains of mosquitoes. The study by Kramar et al. (1989)

showed two different strains of *Cx. tarsalis* had different degrees of susceptibility to WEE virus but on the other hand both were equally susceptible to peroral infection with St. Louis encephalitis and some bunyaviruses.

Viruses only attach to specific cell receptors on the surface of midgut epithelial cells. Different species may have different or similar receptors. Western equine encephalomyelitis virus is unable to penetrate the midgut of *Cx. pipiens* but it is able to penetrate the midgut of *Cx. tarsalis* because *Cx. tarsalis* has a specific receptor for this virus (Hardy et al. 1978). Charge and charge distribution on the surface of midgut epithelial cells, blood meal content, and pH also have an effect on virus infectivity. *Culex tarsalis* was optimally infected with WEE virus when the pH of an infected blood meal was 8.0. However, the infection rate was significantly reduced when the pH of an infected blood meal was either lower than 6 or higher than 8.5 (Houk et al. 1986).

Dissemination barrier

The dissemination barrier or mesenteronal escape barrier prevents the movment of virus from midgut epithelial cells to the hemocoel of the mosquito. This barrier is a dose-dependent but not a time-dependent barrier. This means dissemination of the virus increases when the amount of virus in the infecting blood meal is increased. Some viruses infect and replicate in the midgut but cannot move across midgut epithelial cell basement membrane. Some viruses cannot complete the maturation process in the midgut cells because of the dissemination barrier. For example, the replication of the epizootic strain of Venezuelan encephalitis virus was confined to the midgut but did not occur in the hemocoel of *Cx. (Melanoconion) taeniopus* (Weaver et al. 1984). The accumulation of nonenveloped or

naked nucleocapsid virions along the margin of midgut epithelial cells is observed with some viruses.

Mosquitoes are similar to other insects in that they have only an innate immune system. The innate immune system of mosquitoes is composed of humeral immune responses, which are defensive proteins and cellular immune responses based on the activities of hemocytes. Fifteen to twenty different proteins including attacins, cecropins, and lysozymes with antiviral and antibacterial activity provide humoral immune responses in mosquitoes. *Aedes aegypti* and *Cx. pipiens* express defensin in hemolymph 9-10 days after being infected with Sindbis virus (Cheng *et al.*, 2001).

An antiviral melanization reaction occurs when mosquitoes are infected by both DNA and RNA viruses. The melanization reaction is activated by hemolymph phenoloxidase (mushroom tyrosinase). *Anopheles gambiae* produces five types of serine protease in its hemolymph for cleaving prophenoloxidase, which is a principle enzyme in the melanin synthesis process.

Mosquitoes also develop a cellular immune response to prevent replication of viruses such a dengue-2 virus. This occurs in both midgut and salivary gland cells (Olson et al. 1996). Some viruses for example, polyadnaviruses selectively disable the cellular immune response and alter host physiology, growth, and development (Shelby and Webb 1997).

Transmission barrier

The transmission or salivary gland escape barrier prevents the transmission of virus from the mosquito to other hosts. This barrier is both dose and time dependent. This means the transmission of a virus depends on the amount of disseminated virus in the hemocoel and the length of exposure time to the salivary glands. Virus will be transmitted to another host if

it can get into the salivary glands and can be released with the saliva when the mosquito takes another blood meal. Quality and quantity of specific components in the products from the salivary glands also increase transmission of viruses (Beaty and Marquardt 1996).

Biology and the importance of *Ochlerotatus trivittatus*

Ochlerotatus trivittatus (Coquillett) formally known as *Aedes trivittatus*, is a floodwater mosquito that is widely distributed in North America including Southern Canada and the USA. This mosquito is in the family *Culicidae* of the order Diptera.

It is a medium-sized mosquito with two distinctive features; black triangular bands on the lateral margins of the abdomen and two bands of dark scales on the mesonotum. These characteristics differentiate it from other mosquitoes. It is a rural mosquito and is an annoying, anthropophilic species. The typical larval habitat is a swamp forest. Larvae of this mosquito can be found in temporary pool sites that form by rain or floods, for example stream flood pools, grassy rain pools, and forest pools. Females bite in the late afternoon and at dusk however they will bite whenever their resting places are disturbed. This mosquito overwinters in the egg stage. Males have a short life span, about one week, but females may live five to six weeks. The period from hatching to emergence of the adult is 6-8 days. Females immediately start searching for a blood meal after emergence. Adults tend to stay relatively close to their breeding areas. The study by Duryea (1990) indicated that they are more likely to be caught by landing catch than light trap.

Rowley *et al.* (1973) found this mosquito could be the most abundant species in many areas of Iowa. *Ochlerotatus trivittatus* is an important mosquito because it feeds on many different hosts including mammals and birds (Pinger and Rowley 1975). Several studies

have shown the ability of *Oc. trivittatus* to be a competent vector for several viruses including EEE (Andreadis et al. 1998), WEE (Green et al. 1980), and trivittatus virus (Watts et al. 1976, Andrews et al. 1977, Christensen et al. 1978). It is also a major vector of *Dirofilaria immitis* (Dog heart worm) (Christensen and Andrew 1976, Andrews et al. 1977).

Biology and the importance of *Aedes albopictus*

Aedes albopictus (Skuse), the Asian tiger mosquito, is a nuisance and potential disease vector. This mosquito is in the family *Culicidae* of the order Diptera. Adults are covered with black scales with silver white bands on the palps and tarsi. A band of silver scales forms a distinct stripe on the dorsal surface of the thorax and head. In the USA, it was first discovered in Texas in 1985 and it has become a major pest mosquito in many communities in the southeastern United States. It was probably introduced to the US in shipments of scrap tires from northern Asia. This mosquito overwinters in the egg stage. Transovarial transmission of arboviruses also occurs in *Ae. albopictus*. It is a competent vector for many viruses in nature and under experimental conditions (Table 1.6). A study by Tesh and Gubler (1975) demonstrated transovarial transmission of La Crosse virus by experimentally infected *Ae. albopictus* females. However, only 2.7 % of the F1 generation of both sexes was infected. Transovarially infected mosquitoes contained less virus than mosquitoes infected by inoculation or ingestion (Tesh and Gubler 1975, Tesh and Shroyer 1980).

Table 1.6 Susceptibility of *Aedes albopictus* to oral infection with arboviruses and its ability to transmit by biting

Virus	Infection	Transmission
Chikungunya	+	+
Dengue 1, 2, 3, 4	+	+
Eastern equine encephalitis	+	+
Jamestown Canyon	+	+
Japanese encephalitis	+	+
Keystone	+	-
La Crosse	+	+
Mayaro	+	+
Nodamura	+	?
Oropouche	+	-
Orungo	+	+
Potosi	+	+
Rift Valley fever	+	+
Ross River	+	+
San Angelo	+	+
Sindbis	+	+
St. Louis encephalitis	+	+
Trivittatus	+	-
West Nile	+	+
Western equine encephalitis	+	+
Venezuelan equine encephalitis	+	+
Yellow fever	+	+

Modified from Mitchell (1991)

Biology and the importance of *Culex pipiens pipiens*

Culex pipiens pipiens (Linnaeus), the northern house mosquito, is one subspecies in the *Culex pipiens* complex. This mosquito belongs to the family *Culicidae* of the order Diptera. It is a bird feeding mosquito. It has a blunt abdomen which distinguishes it from *Aedes* and *Ochlerotatus* mosquitoes. Breeding habitats of *Cx. p. pipiens* are unique because they prefer polluted water or water with high organic content. Building construction sites with water accumulation in the urban area can be an important breeding place for this mosquito (Baumgartner 1987). This mosquito lays a group of eggs that are attached together to form an egg raft. There are two physiological forms. Autogenous mosquitoes do not need blood for egg development but anautogenous *Cx. p. pipiens* require blood for egg development.

This mosquito overwinters in the adult stage. Jaenson (1987) indicated that only non-blood fed, nulliparous females survived the winter. Fructose and other plant sugars may be the main energy sources for winter survival of the female. Overwintering *Cx. p. pipiens* in the United States may be important in the maintenance of WNV in the northeastern USA. West Nile viral RNA and live virus were found in pools of overwintering *Cx. p. pipiens* from Queens, New York (Nasci et al. 2001). The longevity of this mosquito is about forty to fifty days. However some viruses have an effect on the longevity or survival of infected mosquitoes. For example, *Cx. p. pipiens* infected with Rift Valley Fever virus (RVFV) had a 48 % decrease in survival (Faran et al. 1987).

Culex p. pipiens is a potential vector of many human and animal pathogens. These pathogens include lymphatic filaria, protozoans, and arboviruses. For example, *Wuchereria bancrofti, Brugia malayi, Brugia timori*, *Plasmodium spp*, WEE virus, RVFV, Sindbis virus,

Japanese encephalitis virus, St. Louis encephalitis virus, Tahyna virus, Oropouche virus and WNV. *Culex p. pipiens* was an important vector of WNV in many areas during outbreaks in the 1990s including the USA and Romania (Pitigoi et al. 1998, Bernard et al. 2001). *Cx. p. pipiens* are highly susceptible to WNV infection. Almost all infected individuals with a disseminated infections transmit WNV by bite (Turell et al. 2000).

Environmental temperature has an effect on susceptibility of *Cx. p. pipiens* adults to arbovirus infection and dissemination, such as RVFV and WNV. Holding temperatures for *Cx. p. pipiens* after taking RVFV infected blood meals affect their infection rate. RVFV infection rates in *Cx. p. pipiens* were 10 % at 13 °C, 20 % at 17 °C, 41 % at 19 °C, and 91 % at 26 °C (Brubaker and Turell 1998). Dohm et al. (2002) recovered WNV from nearly all *Cx. p. pipiens* tested and disseminated infections were detected as early as 4 days post blood feeding (PBF) in mosquitoes held at 30 °C. Disseminated infections were not detected until 25 days PBF and less than 30 % had a disseminated infection at 28 days PBF when held at 18 °C.

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CHAPTER 2. A COMPARISON OF THE SUSCEPTIBILITY OF *OCHLEROTATUS TRIVITTATUS* (COQ.), *AEDES ALBOPICTUS* (SKUSE), AND *CULEX PIPIENS* (L.) TO WEST NILE VIRUS INFECTION

A paper to be submitted to the Journal of Vector-Borne and Zoonotic Diseases

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ABSTRACT

Many species of large and small wild mammals are susceptible to West Nile virus (WNV) infection but develop viremias that seldom exceed $10^{5.0}$ CID_{50s} / ml. To evaluate the potential contribution of mammals to WNV infection of humans it is necessary to determine the likelihood of potential bridge vectors such as *Ochlerotatus trivittatus* and *Aedes albopictus* becoming infected with WNV after taking blood meals from viremic mammals. Accordingly, the WNV susceptibility of *Oc. trivittatus*, *Ae. albopictus* and *Culex pipiens,* a primary amplifying vector, were compared by feeding mosquitoes on viremic chicks with

blood meal titers ranging from $10^{2.5}$ to $10^{10.0}$ CID_{50s} / ml. The susceptibility of *Oc trivittatus* and *Cx. pipiens* to WNV were essentially the same but markedly greater than the susceptibility of *Ae. albopictus*. The lowest observed infection rates of *Oc. trivittatus, Ae. albopictus* and *Cx. pipiens* were 12.5, 10.8, and 2.2 % following blood meals containing $10^{4.5}$, $10^{5.5}$, and $10^{4.5}$ CID_{50s} / ml of virus respectively. No infection was observed among 41 *Ae. albopictus* that took blood meals from among 3 chicks with titers of $10^{5.0}$ CID_{50s} / ml nor among 9 mosquitoes that fed among 3 chicks with titers of $10^{4.5}$ CID_{50s} / ml. The 50 % infective doses for the three species as determined by logistic regression were $10^{6.0}$, $10^{6.6}$, and $10^{6.2}$ CID_{50s}/ml respectively.

KEY WORDS West Nile virus, infection, susceptibility, *Ochlerotatus trivittatus*, *Aedes albopictus*, *Culex pipiens*

INTRODUCTION

West Nile virus (WNV) is a single-stranded RNA virus in the Japanese encephalitis serogroup of the *Flaviviridae* that is of great public health and veterinary significant (CDC, 2003b). It was originally isolated from a woman in the West Nile district of Uganda in 1937 (Smithburn et al., 1940). The virus was subsequently reported throughout the Eastern hemisphere in the Middle East, Europe and Asia (Burke and Monath, 2001) and in the Western hemisphere. The virus was first recognized in North America in the summer of 1999 (CDC, 1999) and has since spread to Canada (Weese et al. 2003) and Mexico (Blitvich et al. 2003, Ulloa et al. 2003). The primary transmission cycle of WNV is bird-mosquito-

bird. The ornithophilic *Culex* species are considered to be the primary amplifying vectors and are among the 43 different mosquito species from which WNV has been isolated (CDC, 2003a). These species represent 10 genera including *Aedes* and *Ochlerotattus*. Some of these species may prove to be effective amplifying vectors. Others may prove to be important bridge vectors that not only transfer the virus from the avian reservoir to mammalian species but might also contribute to a low level maintenance of the virus in specific mammalian populations such as the cottontail rabbit which is often found in high abundance in suburban areas.

Logistic regression modeling was used in this study to compare the WNV susceptibility of two potential bridge vectors, *Ochlerotatus trivittatus* (Coq.) and *Aedes albopictus* (Skuse) to the WNV susceptibility of a principle amplifying vector *Culex pipiens* (L.). *Ochlerotatus trivittatus* is widely distributed in North America and parts of Mexico and Panama (Carpenter 1968, Carpenter 1970, Trimbel 1972, Howard et al. 1917), and is one of the most abundant species in the North and North Central regions of the United States of America. It is highly anthropophilic but will also feed on a wide variety of wild mammals and birds. In one field study 600 blood-fed mosquitoes were analyzed to determine host preference. Forty-eight percent had blood meals of rabbit origin and 7 % were of avian origin (Pinger and Rowley, 1975). Similarly *Ae. albopictus* is anthropophilic but will also feed on avian (Niebylski et al. 1994) and mammalian species (Gomes et al. 2003, Samui et al. 2003). The use of logistic regression modeling for these comparisons was particularly useful to predict probability of infection of bridge vectors at the lower viremias that characterize WNV infection of mammals.

MATERIALS AND METHODS

Experimental design and data analysis

The susceptibility of *Oc. trivittatus, Ae. albopictus,* and *Cx. pipiens* to WNV infection was compared by logistic regression modeling. Accordingly on different days groups of up to ten 1- to 3-day-old chicks were inoculated with doses of WNV ranging from $10^{2.0}$ to $10^{4.0}$ CID_{50s} / chick to generate a range of viremias extending from $10^{1.5}$ to $10^{10.5}$ CID_{50s} / ml. Subsequently, groups of *Oc. trivittatus, Ae. albopictus,* and *Cx. pipiens* were pooled or used separately and fed on individual chicks at times ranging from 12 to 72 hrs after inoculation. Blood was collected from the jugular vein of each chick for WNV assay immediately after blood-feeding. Mosquitoes that fed to repletion were removed from the pools by species, maintained separately for 14 days and then tested for the presence of WNV.

Infection rates were determined by species for each blood meal titer. Studies and virus titers effect on WNV infection were tested using Wald test (Sall et al. 2000). These data were used to construct logistic regression models for the 3 mosquito species using JMP version 5.0 (SAS Institute Inc., Cary, NC, USA) (Sall et al. 2000). These models were used to predict the probability (P) of WNV infection at specific blood-meal virus titers using the following formula: $P = \frac{1}{1+e^{-\beta_0-\beta_1(titer)}}$ where β_0 = the intercept, β_1 = slope and titer = Log_{10} CID_{50s} / ml.

Observed differences in infection rates for each blood-meal virus titer among mosquito species were analyzed using one-way ANOVA and Student's t-test. The area under the receiver operating characteristic (ROC) curve was calculated for each model to measure the degree of goodness of fit between predicted and observed data. The logistic

models for each species were tested for study and titer effect on infection rates. The logistic regression curves described by the models were compared by testing their β values for significant differences using the z-test statistic (Hosmer and Lemeshow 2000).

Challenging the model

The ability of logistic regression models to predict infection was evaluated by feeding 5 groups of *Ae. albopictus* on 2- to 5-day-old chicks with WNV blood-meal titers ranging from $10^{5.0}$ to $10^{9.5}$ CID_{50s} / ml. The percent agreement was determined for observed and predicted infection rates for each blood meal titer.

Mosquitoes

Ochlerotatus trivittatus were first generation mosquitoes derived from adults collected in Iowa. *Aedes albopictus* were the 10^{th} to 20^{th} generations of parents that were originally collected in Missouri and colonized by the Illinois Natural History Survey. *Culex pipiens* were the 8^{th} to 10^{th} generations of parents that were originally collected in Iowa and colonized at Iowa State University in 2002. All mosquitoes were maintained in controlled environmental conditions (27 ± 1°C and 80 ± 5 % RH with a 16:8 hr photoperiod) and fed on a 10 % sucrose solution. Mosquitoes were deprived of sucrose for 48 hrs before blood-feeding on viremic chicks or feeding from capillary tubes.

Chicks

One- to 2-day-old WNV specific antibody-free broiler chicks (Ross × Ross) were obtained from a commercial hatchery (Hoover's hatchery, Inc., Rudd, IA) and housed in biosafety level 3 facilities.

Cells and medium

Vero-76 cells were used for virus propagation and assay. Two different cell culture mediums were used. Carbon dioxide dependent growth medium (CDM) consisted of Dulbecco's modified Eagle's medium (GIBCO®, Invitrogen Corp.) with 2.0 mM of L-glutamine, 20 mg gentamicin sulfate (GentaMax™100, Phoenix Pharmaceutical Inc.) per 100 ml of medium and supplemented with 10 % fetal calf serum (FCS). Maintenance medium (MM) used in virus assays consisted of one part CDM with 1 % FCS and one part CO_2-independent medium (CIM) (GIBCO®, Invitrogen Corp.) that was supplemented with 4.0 mM of L-glutamine, (Cellgro®, Mediatech 800, Cellgro, Inc.) 1 % FCS and 20 mg of gentamicin sulfate per 100 ml medium. Maintenance medium was supplemented with 20 % FCS when used to process mosquito specimens for virus.

Virus

West Nile virus (NY 1999-crow) was supplied by the National Veterinary Services Laboratory, Ames, Iowa. The virus was passaged 6 times in Vero-76 cells and once in *Aedes albopictus* by feeding mosquitoes on blood containing $10^{6.0}$ CID_{50s} WNV / ml using a feeding apparatus covered with a swine intestinal membrane (Rutledge, 1964). Virus-infected mosquitoes were killed at day14 after feeding by freezing at - 60 °C and were triturated in cold MM. The virus preparations were filtered through 450 nm filters and stored in liquid nitrogen until used.

Virus assay

Chick serums were assayed for WNV on Vero-76 cells by both the quantal and quantitative methods. Twenty-five cm^2 cell culture flasks containing confluent cell monolayers were inoculated with 1 ml aliquots of serial 10-fold dilutions of virus prepared in

MM containing 1 % FCS. For quantal assays an additional 6 ml of MM with 1 % FCS was added to each flask after a 1 hr incubation period. Cell cultures were observed for cytopathic effect (CPE) for up to 8 days. Cell cultures with CPE were tested by RT-PCR to confirm presence of WNV. Quantal assays were expressed as CID_{50s} / ml. For quantitative assays, inoculums were replaced after a 1 hr incubation period with 4 ml of MM containing 1 % Agar Noble (DIFCO®, Becton Dickinson). Four ml of a second overlay identical to the first but containing 0.004 % neutral red dye was added to cell cultures 4 days later. Plaques were counted and titer expressed as plaque forming units (PFU) / ml.

Virus was detected in torsos of individual mosquitoes by separately triturating the specimens in 300 µl of cold MM. These volumes were increased to 2 ml in MM containing 1 % FCS. The trituration products were passed through 450 nm filters directly into 25 cm^2 cell culture flasks containing cell monolayers from which medium was removed. An additional 5 ml of MM containing 1 % FCS was added to individual flasks after a 1 hour incubation period. The cell cultures were observed for the presence of CPE for up to 8 days. Cell cultures with CPE were tested by RT-PCR to confirm the presence of WNV.

RT-PCR

RNA was extracted from cell culture medium using the QIAamp viral RNA kit (QIAGEN Inc.). The RT-PCR for WNV specific RNA was conducted as described by Lanciotti et al. (2000) with the following modifications. The amplifying cycle was increased from 40 to 45 cycles and the RT-PCR product (408-bp-size nucleic acid) was electrophoresed (Wide Mini Sub® Cell, Bio-Rad) through a 0.8 % agarose gel (NuSieve®, FMC Bioproducts) prepared with 1X Tris-Acetate-EDTA buffer (Fisher Scientific) containing 0.3 mg ethidium bromide per 100 ml gel (Sigma-Aldrich Co.).

RESULTS

Relative host preference

A summary of the feeding success rates of *Oc. trivittatus, Ae. albopictus,* and *Cx. pipiens* on 2- to 5-day-old virus-infected chicks is presented in Table 2.1. The success rates of the 3 species were 22.8, 67.2, and 77.4 % respectively. The difference in feeding success rates between *Cx. pipiens* and *Ae. albopictus* was not statistically significant. However the feeding success rate of *Oc. trivittatus* was significantly less than that of *Ae. albopictus* and *Cx. pipiens* ($p < 0.001$).

Susceptibility of *Oc. trivittatus, Ae. albopictus* and *Cx. pipiens* to WNV (NY1999-crow)

The susceptibility of each mosquito species to WNV infection is summarized by blood-meal titer in Table 2.2. Blood meal titers ranging up to $10^{7.0}$ to $10^{7.5}$ CID_{50s} / ml had a significant effect ($p < 0.05$) on infection rates of *Oc. trivittatus, Ae. albopictus* and *Cx. pipiens.* No blood meal titer effect was observed among *Oc. trivittatus* ($p = 0.053$) and *Cx pipiens* ($p = 0.56$) that took blood meal with titers > $10^{7.0}$ CID_{50s} / ml nor among *Ae. albopictus* that took blood meals with titers > $10^{7.5}$ CID_{50s} / ml ($p = 0.36$).

The lowest observed infection rates of *Oc. trivittatus, Ae. albopictus*, and *Cx. pipiens* were 12.5, 10.8 and 2.2 % following blood meals containing $10^{4.5}$, $10^{5.5}$, and $10^{4.5}$ CID_{50s} / ml of virus respectively. No infection was observed among 41 *Ae. albopictus* that took blood meals from among 3 chicks with titers of $10^{5.0}$ CID_{50s} / ml nor among 9 mosquitoes that fed among 3 chicks with titers of $10^{4.5}$ CID_{50s} / ml.

The infection rates of *Ae. albopictus* were consistently less than the infection rates of *Oc. trivittatus* and *Cx. pipiens* that took blood-meal with titers that ranged from $10^{4.5}$ to $10^{8.5}$ CID_{50s} / ml. However this apparent difference between the observed susceptibility of *Ae.*

albopictus and that of *Oc. trivittatus* and *Cx. pipiens* was only significant among mosquitoes that took blood meals with a titer of $10^{7.0}$ CID_{50s} / ml ($p < 0.001$). The infection rate of *Ae. albopictus* following blood meals with a titer of $10^{5.5}$ CID_{50s} / ml was also significantly less than the infection rate of *Oc. trivittatus* ($p = 0.02$).

Logistic regression models of the susceptibility of *Ochlerotatus trivittatus, Aedes albopictus*, and *Culex pipiens* to West Nile virus

The estimated coefficients and summaries of areas under the ROC curves described by the 3 models are summarized in Table 2.3. The relationship demonstrating the effect of blood meal titer on infection rate of *Oc. trivittatus, Ae. albopictus*, and *Cx. pipiens* is shown in Figure 2.1. Data from all experiments conducted on all days were combined and used in constructing the models because no experiment effect on virus infection rates was observed. The ROC values of the models ranged from 0.917 to 0.967 on a scale from 0.0 to 1.0 demonstrating a high degree of fit between predicted and observed values.

Blood-meal titers predicted to infect the 3 mosquito species at rates ranging from 1 to 90 % are summarized in Table 2.4. The predicted infection rates were compared by determining if there was overlapping of the 95 % confidence intervals (CI). Titers predicted to infect 50 and 80 % of *Ae. albopictus* were significantly greater than the predicted infective titers for *Cx. pipiens* and *Oc. trivittatus* at these levels. The predicted blood meal infective $dose_{50}$ for *Oc. trivittatus*, *Ae. albopictus*, and *Cx. pipiens* were $10^{6.0}$, $10^{6.6}$ and $10^{6.2}$ CID_{50s} / ml respectively. No significant differences were observed among infective blood meal titers at the 5 and 10 % predicted rates of infection.

Challenging the model

Percent agreement between observed and predicted value are summarized by blood meal titer in Table 2.5. The overall percent agreement was 94.8 %.

DISCUSSION

Ochlerotatus trivittatus and *Ae. albopictus* might be important bridge vectors for WNV and also contribute to its maintenance among mammalian species. Both mosquito species are anthropophilic and also readily feed on mammals such as cottontail rabbits and occasionally on wild birds (Pinger and Rowley 1975, Niebylski et al. 1994, Gomes et al. 2003, Samui et al. 2003). Field specimens of WNV-infected *Oc. trivittatus* and *Ae. albopictus* have been reported (CDC, 2003a). Consequently the preceding study was done to determine the relative degree of susceptibility of *Oc. trivittatus, Ae. albopictus*, and *Cx. pipiens* to WNV infection especially at low levels of viremia that characterize mammalian WNV infections (Bunning et al. 2002).

Results of this study clearly demonstrated that *Oc. trivitattus* will actively feed on avian species (Table 2.1) and is essentially as susceptible to WNV infection as *Cx. pipiens*. These observations also suggest that *Oc. trivitattus* and *Cx. pipiens* were more susceptible to WNV infection than *Ae. albopictus*. These conclusions are based on the following observations. Firstly, the lowest blood-meal titer observed to infect *Oc. trivittatus* and *Cx. pipiens* was $10^{4.5}$ CID_{50s} / ml which infected 12.5 and 2.2 % of the blood-fed mosquitoes respectively (Table 2.2). In contrast the lowest blood meal titer observed to infect *Ae. albopictus* was $10^{5.5}$ CID_{50s} / ml. Secondly, the observed infection rates of *Ae. albopictus* were consistently lower than the observed infection rates for *Oc. trivittatus* and *Cx. pipiens* at

blood meal titers ranging from $10^{4.5}$ to $10^{8.5}$ CID_{50s} / ml (Table 2.2). Infection rates of all 3 mosquito species that took blood meals with titers $>10^{8.5}$ CID_{50s} / ml were essentially the same. Thirdly, blood meal titers predicted by the logistic regression models to infect 50 and 80 % of *Oc. trivittatus* and *Cx. pipiens* were not significantly different (Table 2.4). However these predicted titers were different at the 95 % level of confidence from the blood meal titers predicted to infect *Ae albopictus* at the same rates.

Preliminary experiments in our laboratory indicate that viremias of WNV in cottontail rabbits can reach titers as high as $10^{5.5}$ CID_{50s} / ml. Consequently *Oc. trivittatus* and possibly *Ae. albopictus* might play a role in maintaining WNV in wild mammalian populations. Before definitive conclusions can be made regarding the potential role of these two mosquito species as possible maintenance vectors in small mammal populations, it will be necessary to determine the efficacy by which each mosquito species transmits the virus, and the degree to which strains of each species vary in their susceptibility to WNV infection.

The Iowa strain of *Cx. pipiens* used in this study may be more susceptible to WNV than strains of *Cx. pipiens* from New York, California, and Maryland. Infection rates of these strains were 79 (n=78), 66 (n=50) and 81 % (n=95) respectively after feeding on blood with titers ranging from $10^{7.0}$ to $10^{7.2}$ PFU / ml (Sardelis and Turell, 2001, Turell et al. 2001, Goddard et al. 2002,). Titers predicted at the 95 % confidence level by the Iowa *Cx. pipiens* model and adjusted to PFU / ml to yield the same infection rates as the New York, California, and Maryland stains were 0.9 to 1.2, 1.1 to 1.4, and 0.7 to 1.1 $\log_{10}$ less respectively. The susceptibility of the Missouri strain of *Ae. albopictus* to WNV used in the current study appears to be similar to the susceptibility of Oahu strain of *Ae. albopictus* (Turell et al. 2001). Ninety percent (n=61) of the Oahu strain became infected after feeding

on blood containing $10^{7.2\pm0.3}$ PFU /ml. This titer falls within the 95 % CI of the blood meal titer predicted by the Missouri *Ae. albopictus* model to infect at the same rate. No reports are currently available regarding susceptibility of different strains of *Oc trivittatus* to WNV.

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Table 2.1 A comparison of the feeding success rate of *Ochlerotatus trivittatus*, *Aedes albopictus*, and *Culex pipiens* that fed on 1- to 5-day-old chicks

Experiment	Feeding success rate[a]		
	Oc. trivittatus	*Ae. albopictus*	*Cx pipiens*
	% (N)	% (N)	% (N)
1	30.8 (107)	81.4 (70)	59.5 (247)
2	29.5 (193)	66.3 (80)	77.8 (54)
3	21.0 (295)	66.3 (98)	84.7 (209)
4	7.0 (128)	45.3 (150)	94.8 (210)
5	25.7 (354)	76.5 (200)	70.0 (210)
Mean[b] ± SE	22.8 ± 4.30	67.2 ± 6.20	77.4 ± 6.05

[a]feeding success rate is the number of mosquitoes feeding to repletion divided by total number of mosquitoes and multiply by 100

[b]the feeding success rate of *Oc. trivittatus* was significant less than feeding success rates of *Ae. albopictus* and *Cx. pipiens* which were not significant different ($p < 0.001$)

Table 2.2 Susceptibility of *Ochlerotatus trivittatus, Aedes albopictus*, and *Culex pipiens* to WNV (New York 1999) determined 14 days after blood-feeding on viremic chicks

Blood meal titer[a]	*Oc.trivittatus*			*Ae. albopictus*			*Cx. pipiens*		
	No. infect. chicks	No. tested mosq.	% infected mosq.	No. infect. chicks	No. tested mosq.	% infected mosq.	No. infect. chicks	No. tested mosq.	% infected mosq.
0	1	3	0^{c}	1	5	$0^{4,5}$	-	-	-
1.5	1	14	0^{c}	1	4	$0^{4,5}$	-	-	-
2.5	-	-	-	2	19	$0^{4,5}$	1	45	0^{5}
3.5	4	30	0^{c}	4	25	$0^{4,5}$	-	-	-
4.5	2	32	12.5^{3}	3	9	$0^{4,5}$	1	45	2.2^{5}
5.0		-	-	3	41	0^{5}	1	45	2.2^{5}
5.5[b]	3	14	42.9^{2}	5	37	$10.8^{4,5}$	1	40	15.0^{4}
6.0	2	9	44.4^{2}	3	16	18.8^{4}	1	24	50.0^{3}
6.5	7	59	57.6^{2}	12	32	50.0^{3}	3	37	64.9^{2}
7.0[b]	7	42	85.7^{1}	12	78	70.5^{2}	2	46	97.8^{1}
7.5	10	59	94.9^{1}	17	70	87.1^{1}	4	58	96.6^{1}
8.0	5	44	93.2^{1}	9	71	91.6^{1}	2	48	95.8^{1}
8.5	3	10	100.0^{1}	12	90	94.4^{1}	1	26	96.2^{1}
9.0	2	8	100.0^{1}	4	20	100.0^{1}	1	19	100.0^{1}
9.5	1	10	100.0^{1}	2	10	$90.0^{1,2}$	1	19	100.0^{1}
10.5	-	-	-	1	4	$100.0^{1,2}$	-	-	-

[a]blood-meal virus titer expressed as Log_{10} CID_{50s} of WNV / ml serum. Titers can be converted to PFU / ml by the following formula: PFU/ml = 0.935(CID_{50s} / ml) - 0.174, (r^2 = 0.956, n=196)

[b]indicate the statistically significant different of the infection among species ($p < 0.05$)

Percentage of infected mosquitoes within each species that are not connected by the same superscript number are significantly different ($p = 0.05$)

Table 2.3 Estimated coefficients and area under receiver operating characteristic curves used to construct logistic regression models and evaluate the goodness of fit

Species	β_0[a] ± SE[c]	β_1[b] ± SE	Area under ROC curve[d]
Oc. trivittatus	-9.42 ± 1.19	1.56 ± 0.18	0.917
Ae. albopictus	-12.22 ± 1.45	1.85 ± 0.16	0.924
Cx. pipiens	-14.94 ± 1.46	2.43 ± 0.23	0.967

Probability of WNV infection in the mosquito = $\frac{1}{1+e^{-\beta_0-\beta_1(titer)}}$

[a]β_0 = intercept

[b]β_1 = slope

[c]SE = standard error

[d]ROC = receiver operating characteristic which is an indicator of the goodness of fit between observed value and curve from logistic regression model (scale from 0 to 1)

Table 2.4 West Nile virus infection rate and predicted virus titers in *Ochlerotatus trivittatus, Aedes albopictus* and *Culex pipiens* from logistic regression models

West Nile virus infection rate	Predicted virus titer[a] (95 % confidence interval)		
	Oc. trivittatus	*Ae. albopictus*	*Cx. pipiens*
1	3.1 (2.1-3.8)	4.1 (3.5-4.6)	4.3 (3.8-4.6)
2	3.5 (2.6-4.1)	4.5 (4.0-4.9)	4.6 (4..1-4.9)
5	4.2 (3.4-4.6)	5.0 (4.6-5.3)	4.9 (4.6-5.2)
10	4.6 (4.0-5.0)	5.4 (5.1-5.7)	5.3 (5.0-5.5)
50	6.0 (5.8-6.3)	6.6 (6.5-6.8)	6.2 (6.0-6.3)
80	6.9 (6.7-7.2)	7.4 (7.2-7.6)	6.7 (6.6-6.9)
90	7.5 (7.2-7.8)	7.8 (7.6-8.1)	7.1 (6.9-7.3)

[a]titer expressed as Log_{10} CID_{50s} / ml

Table 2.5 A comparison of the observed and predicted West Nile virus infection rate (%) of *Aedes albopictus*

WNV titer[a]	Observed[b] percent infection	Predicted percent infection	Percent agreement[c,d]
5	0	4.8	NC[e]
5.5	10	11.2	88.0
7	70	66.8	95.4
8	90	92.7	97.0
9	100	98.8	98.8

[a]titer expressed as Log_{10} CID_{50s} / ml serum

[b]N = 20 for each group

[c]percent agreement = 100- [((predicted value -observed value)/observed value) ×100]

[d]the overall percent agreement for blood-meal titers $10^{5.5} - 10^{9.0}$ CID_{50s} / ml was 94.8 %

[e]not calculate

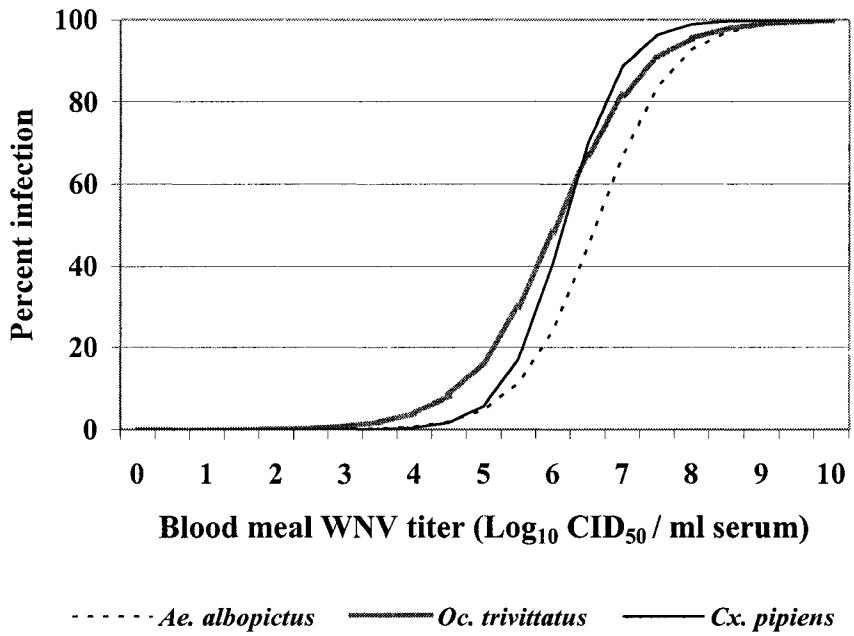

Figure 2.1 The relationship between blood meal West Nile virus titers and infection rates of *Ochlerotatus trivittatus, Aedes albopictus*, and *Culex pipiens* as demonstrated by logistic regression models

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CHAPTER 3. A COMPARISON OF WEST NILE VIRUS TRANSMISSION BY *OCHLEROTATUS TRIVITTATUS* (COQ.), *AEDES ALBOPICTUS* (SKUSE), AND *CULEX PIPIENS* (L.)

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ABSTRACT

Ochlerotatus trivittatus is widely distributed within the USA and feeds primarily on small mammals but is opportunistic and will also feed on humans and birds. Consequently it may prove to be an important bridge vector and might also serve to maintain WNV in small mammal populations such as cottontail rabbits. In a previous study an Iowa strain of *Oc. trivittatus* was shown to be essentially as susceptible to WNV infection as the Iowa strain of *Culex pipiens*, a primary amplifying vector, but more susceptible than a Missouri strain of *Aedes albopictus*, a known bridge vector. The present study compared WNV transmission

by *Oc. trivittatus, Ae. albopictus*, and *Cx. pipiens* using the capillary tube feeding method. Blood-meal titers had no effect on transmission rates within individual species at titers $\geq 10^{7.0}$ CID_{50s} / ml. The cumulative mean ± SE transmission rates 14 days after taking blood meals with titers $\geq 10^{7.0}$ CID_{50s} / ml were 41.7 ± 2.6, 72.4 ± 5.6, and 46.8 ± 4.5 % for *Oc. trivittatus*, *Ae. albopictus,* and *Cx. pipiens* respectively. These data clearly indicated that *Oc. trivittatus* can transmit WNV at essentially the same rate as *Cx. pipiens* but less than *Ae. albopictus* which transmits WNV 1.7 to 1.5 times more efficiently than *Oc. trivittatus* or *Cx. pipiens* ($p < 0.01$). However *Ae. albopictus* only transmitted WNV after taking blood meals with titers $\geq 10^{7.0}$ CID_{50s} / ml whereas *Oc. trivittatus* and *Cx. pipiens* transmitted WNV after feeding on blood with titers $\geq 10^{5.5}$ CID_{50s} / ml. These observations suggest that *Oc. trivittatus* is not as important as *Ae. albopictus* as a bridge vector but might play a more significant role in maintaining WNV in populations of small mammals which typically develop a low level viremia.

KEY WORDS West Nile virus, infection, dissemination, transmission, *Ochlerotatus trivittatus, Aedes albopictus*, *Culex pipiens*,

INTRODUCTION

West Nile Virus (WNV) is a single-stranded RNA virus in the Japanese encephalitis serogroup of the family *Flaviviridae*. It was originally isolated from a woman in the West Nile district of Uganda in 1937 (Smithburn et al. 1940). The virus is present in Africa, the Middle East, parts of Europe, and Asia (Burke and Monath, 2001). West Nile virus was first isolated in North America during an outbreak in New York city in August 1999

(CDC,1999). Subsequently WNV has spread throughout the USA, some parts of Canada (Weese et al. 2003), and Mexico (Blitvich et al. 2003, Ulloa et al. 2003).

Since the outbreak of WNV in the USA, WNV has been isolated from 43 species of mosquitoes representing 10 genera (CDC, 2003). A few studies indicate some of these species could be competent vectors of WNV (Goddard et al. 2002, Sardelis et al. 2002, Turell et al. 2002). To be a competent vector for WNV, the mosquito must provide a suitable internal environment to facilitate virus replication, dissemination, and transmission. Infection of a mosquito with WNV does not mean a mosquito can transmit the virus and be a competent vector. To be a competent vector the mosquito must transmit the virus in the saliva.

In a previous study *Ochlerotatus trivittatus* was shown to be as susceptible to WNV infection as *Cx. pipiens* which is considered to be a primary WNV amplifying vector (Andreadis et al. 2001) and more susceptible than *Ae. albopictus* that most likely serves as a bridge vector (Sardelis et al. 2002). *Ochlerotatus trivittatus* (Coq.) is an abundant species in many areas of Iowa (Rowley et al. 1973). This species also feeds on many different hosts including birds and mammals such as the cottontail rabbit (Pinger and Rowley 1975). Vector competency of *Oc. trivittatus* has been demonstrated for other arboviruses such as eastern equine encephalitis virus (Andreadis et al. 1998), western equine encephalitis virus (Green et al. 1980), and trivittatus virus (Watts et al. 1976, Andrews et al. 1977, Christensen et al. 1978). The objective of the following study was to determine if *Oc. trivittatus* can be a competent WNV vector. For this purpose the efficacy of WNV transmission by *Oc. trivittatus* was compared to WNV transmission by *Cx. pipiens* and *Ae. albopictus*.

MATERIALS AND METHODS

Experimental design and data analysis

Transmission rates for *Ochlerotatus trivittatus, Aedes albopictus* and *Culex pipiens* were determined by the capillary tube feeding method at day14 after taking blood meals with WNV titers ranging from $10^{1.5}$ to $10^{10.0}$ CID_{50s} / ml. This broad range of titers was generated by injecting groups of up to 8 chicks on different days with doses of WNV ranging from $10^{2.0}$ to $10^{4.0}$ CID_{50s}. The chicks were subsequently used as blood meal sources 12 to 72 hrs later for mixed and homogenous group of *Oc. trivittatus, Ae albopictus*, and *Cx. pipiens*. Chicks were bled immediately after blood-feeding for virus assay.

Virus infection of individual mosquitoes was confirmed by detecting virus in torsos. Disseminated infections in the same mosquitoes were detected by virus assay of hemolymph contained in legs. Virus transmission was determined by detecting virus in saliva deposited during a 20 min feeding period into a 5 % sucrose solution in PBS with 0.5 % FCS contained in a capillary tube. These data were used to construct logistic regression models for infection, dissemination and transmission rates of the 3 mosquito species using JMP version 5.0 (SAS Institute Inc., Cary, NC, USA) as previously described (Tiawsirisup et al. 2004). Studies and virus titers effect on the infection, dissemination, and transmission were tested using Wald test (Sall et al. 2000).

Observed differences between transmission rates of different species by blood-meal titer were compared by one-way ANOVA and Student's t-test at $p \leq 0.05$. The area under the receiver operating characteristic (ROC) curve was calculated for each model to measure the degree of goodness of fit between predicted and observed data. The logistic models representing the 3 parameters for each mosquito species were tested for study effect and titer

effect on infection, dissemination and transmission rates. The logistic regression curves of the models were compared by testing their beta values by the z-test (Hosmer and Lemeshow 2000).

Mosquitoes

Ochlerotatus trivittatus were first generation mosquitoes derived from adults collected in Iowa. *Aedes albopictus* were the 10^{th} to 20^{th} generations of parents that were originally collected in Missouri and colonized by the Illinois Natural History Survey. *Culex pipiens* were the 8^{th} to 10^{th} generations of parents that were originally collected in Iowa and colonized at Iowa State University in 2002. All mosquitoes were maintained in controlled environmental conditions (27 ± 1°C and 80 ± 5 % RH with a 16:8 hr photoperiod) and fed on a 10 % sucrose solution. Mosquitoes were deprived of sucrose for 48 hrs before blood-feeding on viremic chicks or feeding from capillary tubes.

Chicks

One- to 2-day-old WNV specific antibody-free broiler chicks (Ross × Ross) were obtained from a commercial hatchery (Hoover's hatchery, Inc., Rudd, IA) and housed in biosafety level 3 facilities.

Cells and medium

Vero-76 cells were used for virus propagation and assay. Two different cell culture mediums were used. Carbon dioxide dependent growth medium (CDM) consisted of Dulbecco's modified Eagle's medium (GIBCO®, Invitrogen Corp.) with 2.0 mM of L-glutamine, 20 mg gentamicin sulfate (GentaMax™100, Phoenix Pharmaceutical Inc.) per 100 ml of medium and supplemented with 10 % fetal calf serum (FCS). Maintenance medium (MM) used in virus assays consisted of one part CDM with 1 % FCS and one part

CO_2-independent medium (CIM) (GIBCO®, Invitrogen Corp.) that was supplemented with 4.0 mM of L-glutamine, (Cellgro®, Mediatech 800, Cellgro, Inc.) 1 % FCS and 20 mg of gentamicin sulfate per 100 ml medium. Maintenance medium was supplemented with 20 % FCS when used to process mosquito specimens for virus.

Virus

West Nile virus (NY 1999-crow) was supplied by the National Veterinary Services Laboratory, Ames, Iowa. The virus was passaged 6 times in Vero-76 cells and once in *Aedes albopictus* by feeding mosquitoes on blood containing $10^{6.0}$ CID_{50s} WNV / ml using a feeding apparatus covered with a swine intestinal (Rutledge, 1964). Virus-infected mosquitoes were killed at day14 after feeding by freezing at - 60 °C and were triturated in cold MM. The virus preparations were filtered through 450 nm filters and stored in liquid nitrogen until used.

Virus assays

Chick serums were assayed for WNV onVero-76 cells by both the quantal and quantitative methods. Twenty-five cm^2 cell culture flasks containing confluent cell monolayers were inoculated with 1 ml aliquots of serial 10-fold dilutions of virus prepared in MM containing 1 % FCS. For quantal assays an additional 6 ml of MM with 1 % FCS was added to each flask after a 1 hr incubation period. Cell cultures were observed for cytopathic effect (CPE) for up to 8 days. Cell cultures with CPE were tested by RT-PCR to confirm presence of WNV. Quantal assays were expressed as CID_{50s} / ml. For quantitative assays, inoculums were replaced after a 1 hr incubation period with 4 ml of MM containing 1 % Agar Noble (DIFCO®, Becton Dickinson). Four ml of a second overlay identical to the first

but containing 0.004 % neutral red dye was added to cell cultures 4 days later. Plaques were counted and titer expressed as plaque forming units (PFU) / ml.

Virus was detected in torsos and legs of individual mosquitoes by separately triturating the specimens in 300 µl of cold MM. These volumes were increased to 2 ml in MM containing 1 % FCS. Virus was detected in saliva by transferring the contents of capillary feeding tubes to 2 ml of cold MM. The expanded contents of the capillary tubes and the triturating products were passed through 450 nm filters directly into 25 cm^2 cell culture flasks containing cell monolayers from which medium was removed. An additional 5ml of MM containing 1 % FCS was added to individual flasks after a 1 hour incubation period. The cell cultures were observed for the presence of CPE for up to 8 days. Cell cultures with CPE were tested by RT-PCR to confirm the presence of WNV.

RT-PCR

RNA was extracted from cell culture medium using the QIAamp viral RNA kit (QIAGEN Inc.). The RT-PCR for WNV specific RNA was conducted as described by Lanciotti et al. (2000) with the following modifications. The amplifying cycle was increased from 40 to 45 cycles and the RT-PCR product (408-bp-size nucleic acid) was electrophoresed (Wide Mini Sub® Cell, Bio-Rad) through a 0.8 % agarose gel (NuSieve®, FMC Bioproducts) prepared with 1X Tris-Acetate-EDTA buffer (Fisher Scientific) containing 0.3 mg ethidium bromide per 100 ml gel (Sigma-Aldrich Co.).

RESULTS

Transmission, infection, and dissemination rates are summarized by blood-meal titer for *Oc. trivittatus*, *Ae. albopictus,* and *Cx. pipiens* in Tables 3.1, 3.2 and 3.3 respectively. No

study effect was detected by logistic regression models for any of the 3 parameters. Consequently all data for each parameter was combined for analysis. The estimated coefficients used in constructing the logistic regression models for each parameter of each mosquito species are summarized in Table 3.4. The areas under the receiver operating characteristic (ROC) curves for each model are summarized in Table 3.5. The relationship between the 3 parameters and blood meal titers for each mosquito species is shown in Figure 3.1.

Transmission rates for all 3 mosquito species were significantly affected by WNV titers of blood-meals over the broad range tested. However no effect was observed at levels $\geq 10^{7.0}$ CID_{50s} / ml. Transmission rates for each mosquito species that took infective blood-meals with WNV titers < $10^{7.0}$ CID_{50s} / ml were markedly less than transmission rates among mosquitoes that feed on blood with titers $\geq 10^{7.0}$ CID_{50s} / ml. Transmission rates of *Oc. trivittatus* after taking blood meals with titers of $10^{5.5}$ and $10^{6.5}$ CID_{50s} / ml were 25 and 15.4 % respectively (Table 3.1). No transmission by *Ae. albopictus* was observed after taking infective blood meals with virus titers of $10^{6.5}$ CID_{50s} / ml (Table 3.2). Transmission rates for *Cx. pipiens* after taking blood meals with titers of $10^{5.5}$ and $10^{6.5}$ CID_{50s} / ml were 4.0 and 7.7 % respectively (Table 3.3). In contrast the cumulative mean of all transmission rates and ranges for *Oc. trivittatus*, *Ae. albopictus,* and *Cx. pipiens* after taking blood meals containing $\geq 10^{7.0}$ CID_{50s} / ml of virus were 41.7 (37.5 to 51.7), 72.4 (60.0 to 90.0), and 46.8 (32 to 57.7) percent respectively (Table 3.6). The cumulative mean transmission rate of *Ae. albopictus* was 1.7 and 1.5 times greater than the transmission rates of *Oc. trivittatus* and *Cx. pipiens* respectively. This difference was significant ($p < 0.01$).

Marked differences were also observed between the slopes of the logistic regression curves describing the effect of blood meal virus titer and infection and dissemination rates of *Cx. pipiens* and *Oc. trivittatus* (Figure 3.1). These differences were significant for *Cx. pipiens* ($p < 0.002$) but not for *Oc. trivittatus* ($p = 0.21$). In contrast there was no marked difference between the slopes of the regression curves describing infection and dissemination rates of *Ae. albopictus* ($p = 0.51$).

DISCUSSION

Ochlerotatus trivitatus is a ubiquitous mosquito that feeds primarily on small mammals. It is also opportunistic and will feed on humans and birds. It is widely distributed within the USA. The susceptibility of *Oc. trivittatus* to WNV infection was characterized in a previous study and shown to be essentially the same as the susceptibility of *Cx. pipiens* which is considered to be a primary amplifying vector of WNV (Andreadis et al. 2001). *Ochlerotatus trivittatus* was also shown to be more susceptible to WNV than *Ae. albopictus* which is considered a bridge vector since it readily feeds on humans, mammals, and birds (Niebylski et al. 1994, Gomes et al. 2003, Samui et al. 2003). The feeding pattern of *Oc. trivittatus* and its susceptibility to WNV infection make it a possible bridge vector that might also be involved in maintaining the virus in specific mammalian populations provided that it can transmit the virus.

The present study clearly demonstrated that *Oc. trivittatus* can transmit WNV (Table 3.1). The cumulative mean transmission rates of *Oc. trivittatus* and *Cx. pipiens* after taking blood meals with titers ranging from $10^{7.0}$ to $10^{9.5}$ were essentially the same and ranged from 37.5 to 51.7 % and 32.0 to 57.7 % respectively. In contrast the mean cumulative

transmission rate and range of *Ae. albopictus* was 72.4 (60 – 90) % which was 1.7 and 1.5 times greater than the cumulative transmission rates of *Oc trivitattus* and *Cx. pipiens.* This difference was highly significant ($p<0.01$).

The transmission rates of the Missouri strain of *Ae albopictus* used in the present study were similar to the transmission rates reported for the Oahu, TAMU, FRED, and CHEV strains which varied from 36 to 92% (Turell et al. 2001, Sardelis et al. 2002). Similarly the transmission rates of a California strain of *Cx. pipiens* following blood meals with titers of $10^{4.9}$ and $10^{7.1}$ PFU / ml were 60 and 36% respectively which was in close agreement with the transmission rates of the Iowa strain of *Cx. pipiens* used in the present study (Gooddard et al. 2000). In contrast the transmission rates of a New York and Maryland strain of *Cx. pipien*s after infection by blood meals with titers ranging from $10^{5.2}$ to $10^{7.2}$ PFU / ml ranged from 2 to 21% (Turell et al. 2001, Sardelis and Turell, 2001) which might mean that the Iowa strain of *Cx pipiens* can transmit WNV more efficiently than the New York and Maryland strains.

The ability of *Ae. albopictus* to transmit WNV more efficiently than *Oc. trivittatus* and *Cx. pipiens* could be due to less efficient salivary gland and / or dissemination barriers in *Ae albopictus*. The presence of relatively larger areas between the infection and dissemination curves of *Oc. trivtitatus* and *Cx. pipiens* than is present between the infection and dissemination curves of *Ae albopictus* (Figure 3.1) suggests that the most likely explanation is the absence of an effective dissemination barrier in *Ae. albopictus*.

The above study clearly indicates that *Ae albopictus* can transmit WNV more efficiently than *Oc. trivitattus*. However *Oc. trivittatus* was shown to transmit virus after being infected blood meals with titers $\geq 10^{5.5}$ CID_{50}s/ml (Table 3.1). Transmission by *Ae*

albopictus only occurred after infection by blood meals with titers of $\geq 10^{7.0}$ CID_{50}s/ml (Tables 3.2). Consequently *Oc. trivittatus* might play a more significant role in maintaining WNV in populations of small mammals which typically develop low levels of viremia. Additional studies to characterize the magnitude and duration of viremias in wild mammals are needed before definitive conclusions can be made regarding the role of *Oc.trivitattus* and *Ae. albopictus* in maintaining WNV in mammalian populations.

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Table 3.1 West Nile virus infection, dissemination, and transmission by *Ochlerotatus trivittatus* determined 14 days after blood-feeding on viremic chicks

Blood meal virus titer[a]	No. tested mosquitoes	Percent infection[b]	Percent dissemination[c]	Percent transmission[d]
1.5	7	0	0	0
3.5	8	0	0	0
4.5	16	12.5	0	0
5.5	4	75.0	75.0	25.0
6.5	26	61.5	42.3	15.4
7	16	81.3	56.3	37.5
7.5	43	95.4	76.7	41.9
8	29	96.6	86.2	51.7
8.5	8	100.0	62.5	37.5
9	8	100.0	87.5	62.5
9.5	10	100.0	100.0	40.0

[a]titer expressed as Log_{10} CID_{50s} / ml

[b]percent infection is defined as the percentage of blood-fed mosquitoes with virus in their torsos

[c]percent dissemination is defined as the percentage of blood-fed mosquitoes with virus in the hemocoel as indicated by detecting virus in legs

[d]percent transmission is defined as the percentage of blood-fed mosquitoes with virus in their saliva

Table 3.2 West Nile virus infection, dissemination, and transmission by *Aedes albopictus* determined 14 days after blood-feeding on viremic chicks

Blood meal virus titer[a]	No. tested mosquitoes	Percent infection[b]	Percent dissemination[c]	Percent transmission[d]
2.5	19	0	0	0
5	21	0	0	0
6.5	31	6.5	6.5	0
7	28	89.3	82.1	60.7
7.5	40	97.5	95.0	75.0
8	30	93.3	86.7	60.0
8.5	55	90.9	89.1	76.4
9.5	10	90.0	90.0	90.0
10.5	4	100.0	100.0	100.00

[a]percent infection is defined as the percentage of blood-fed mosquitoes with virus in their torso.

[b]percent dissemination is defined as the percentage of blood-fed mosquitoes with virus in the hemocoel as indicated by detecting virus in legs.

[c]percent transmission is defined as the percentage of blood-fed mosquitoes with virus in their saliva.

Table 3.3 West Nile virus infection, dissemination, and transmission by *Culex pipiens* determined 14 days after blood-feeding on viremic chicks

Blood meal virus titer[a]	No. tested mosquitoes	Percent infection[b]	Percent dissemination[c]	Percent transmission[d]
2.5	25	0	0	0
4.5	25	4.0	0	0
5	25	4.0	0	0
5.5	25	16.0	8.0	4.0
6.5	13	76.9	30.8	7.7
7	21	95.2	66.7	42.9
7.5	25	96.0	76.0	32.0
8	24	100.0	70.8	54.2
8.5	26	96.2	80.8	57.7
9.5	19	100.0	79.0	47.4

[a]titer expressed as Log $_{10}$ CID_{50s} / ml

[b]percent infection is defined as the percentage of blood-fed mosquitoes with virus in their torsos

[c]percent dissemination is defined as the percentage of blood-fed mosquitoes with virus in the hemocoel as indicated by detecting virus in legs

[d]percent transmission is defined as the percentage of blood-fed mosquitoes with virus in their saliva

Table 3.4 The estimated coefficients from logistic regression analyses used to construct West Nile virus infection, dissemination, and transmission models for *Ochlerotatus trivittatus, Aedes albopictus,* and *Culex pipiens*

Model	*Oc. trivittatus*		*Ae. albopictus*		*Cx. pipiens*	
	β_0[a] ± SE[c]	β_1[b] ± SE	β_0 ± SE	β_1 ± SE	β_0 ± SE	β_1 ± SE
Infection	-9.12 ± 1.69	1.55 ± 0.25	-14.98 ± 2.34	2.21 ± 0.33	-14.53 ± 1.96	2.38 ± 0.32
Dissemination	-7.60 ± 1.43	1.14 ± 0.20	-13.20 ± 2.02	1.92 ± 0.28	-8.74 ± 1.15	1.22 ± 0.16
Transmission	-5.55 ± 1.19	0.66 ± 0.16	-10.22 ± 1.55	1.38 ± 0.20	-7.38 ± 1.10	0.88 ± 1.42

Probability of WNV infection in the mosquito = $\frac{1}{1+e^{-\beta_0-\beta_1(titer)}}$

[a]β_0 = intercept

[b]β_1 = slope

[c]SE = standard error

Table 3.5 Area under receiver operating characteristic curve value for the logistic regression models of the West Nile virus infection, dissemination, and transmission by *Ochlerotatus trivittatus, Aedes albopictus* and *Culex pipiens*

Model	Area under ROC[a] curve value		
	Oc. trivittatus	*Ae. albopictus*	*Cx. pipiens*
Infection	0.928	0.920	0.974
Dissemination	0.829	0.900	0.894
Transmission	0.721	0.837	0.834

[a]ROC = receiver operating characteristic which area under ROC curve indicates the goodness of fit between observed values and the logistic regression models (scale from 0 and 1)

Table 3.6 A comparison of West Nile virus transmission rate by *Ochlerotatus trivittatus, Aedes albopictus* and *Culex pipiens* after blood-feeding on viremic chick with virus titer $10^{7.0} - 10^{9.5}$ CID_{50s} / ml

Blood meal virus titer[a]	Transmission rate (%)		
	Oc. trivittatus	*Ae. albopictus*	*Cx. pipiens*
7.0	37.5	60.7	42.9
7.5	41.9	75.0	32.0
8.0	51.7	60.0	54.2
8.5	37.5	76.4	57.7
9.5	40.0	90.0	47.4
Mean[b] ± SE	41.7 ± 2.6	72.4 ± 5.6	46.8 ± 4.5

[a]titer expressed as Log_{10} CID_{50s} / ml

[b]the transmission rate of *Ae. albopictus* was significant greater than the transmission rate of *Oc. trivittatus* and *Cx. pipiens* which were not significant different ($p < 0.001$)

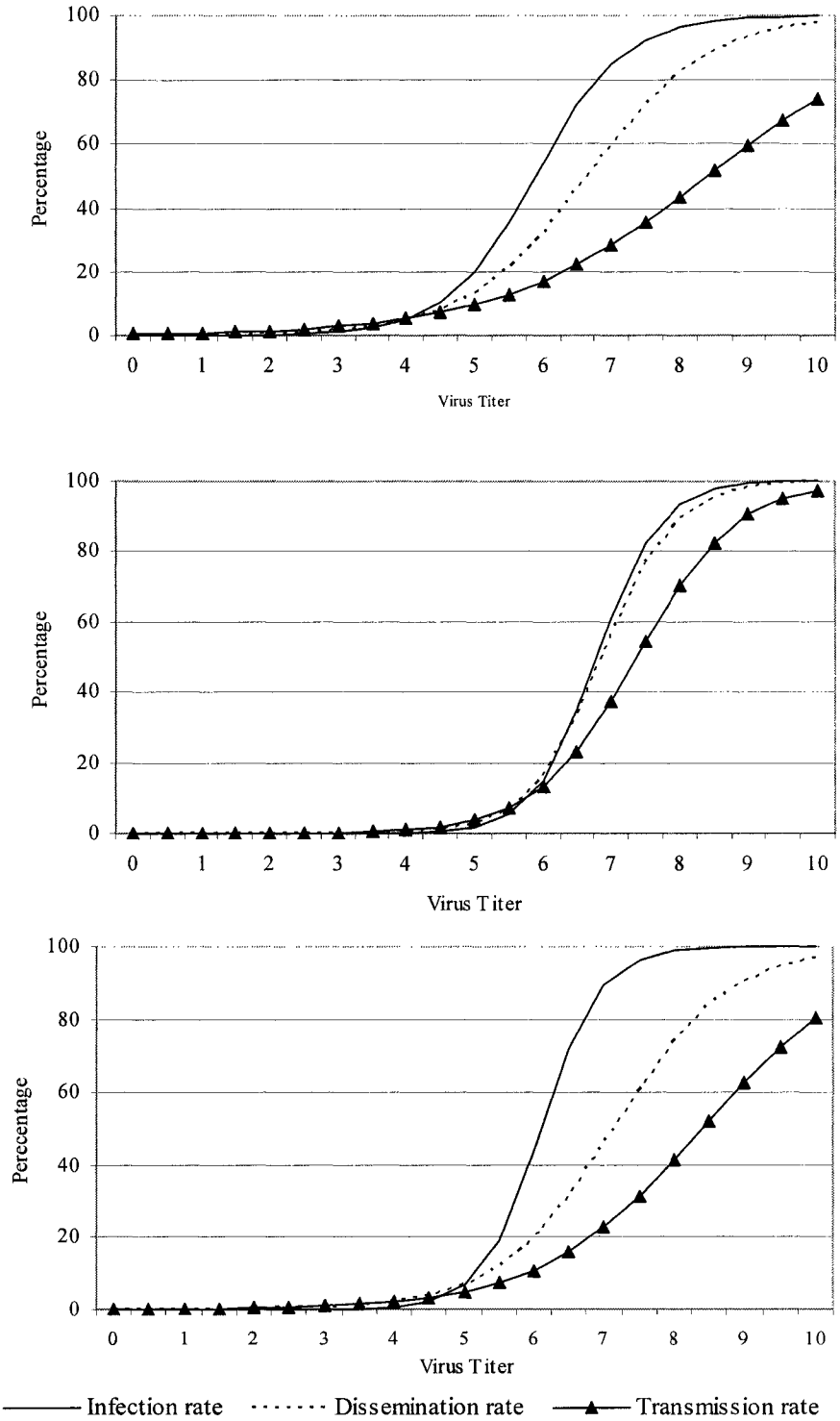

Figure 3.1 West Nile virus infection, dissemination, and transmission rates for *Ochlerotatus trivittatus* (above), *Aedes albopictus* (middle), and *Culex pipiens* (below) 14 days after blood-feeding on viremic chicks (titer titer expressed as Log_{10} CID_{50s} / ml)

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CHAPTER 4. A COMPARISON OF *IN VITRO* AND *IN VIVO* TRANSMISSION OF WEST NILE VIRUS BY *OCHLEROTATUS TRIVITTATUS* (COQ.) AND *AEDES ALBOPICTUS* (SKUSE)

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ABSTRACT

West Nile virus (WNV) is transmitted from infected mosquitoes to other hosts by biting. Virus levels in the saliva of individual mosquitoes vary. Low levels of WNV in mosquito saliva may be difficult to detect, therefore a sensitive assay is needed. *In vitro* and *in vivo* transmission of WNV-NY 1999 strain by *Ochlerotatus trivittatus* (Coq.) and *Aedes albopictus* (Skuse) were compared. Capillary tube feeding (*in vitro*) and chick feeding (*in vivo*) were used to establish if one or the other produced a higher transmission rate of WNV. Three groups of *Ae. albopictus* were fed on 3-day-old chicks with a viremia of $10^{7.5}$ CID_{50s} /

ml of WNV. Their ability to transmit the virus was examined at day 7, 12, and 13 post blood feeding (PBF). Two groups of *Oc. trivittatus* were also allowed to feed on 3-day-old chicks with viremias of $10^{8.0}$ and $10^{8.5}$ CID_{50s} / ml. Transmission by these mosquitoes was tested at day 13 and 19 PBF. Differences in the transmission rates of mosquitoes fed on capillary tubes or susceptible hosts (2-day-old chicks) were defined as the percentage of the blood-fed mosquitoes that transmitted WNV. The percentage of mosquitoes that transmitted WNV when fed on capillary tubes was higher than percentage of transmission when mosquitoes fed on susceptible chicks. *Ae. albopictus* at 13 days PBF transmitted WNV at a 12 % higher transmission rate using the capillary tube method. *Oc. trivittatus* feeding on capillary tube 13 days PBF had only a 5 % higher transmission rate than they did when feeding on chicks. The difference in transmission ranged between 5 and 20 % which was not statistically significant ($p > 0.05$). The data were analyzed and compared using the Student's t test at the 0.05 level of confidence using JMP 5.0.

KEY WORDS West Nile virus, *in vivo*, *in vitro*, capillary tubes, chicks, transmission, *Aedes albopictus*, *Ochlerotatus trivittatus*

INTRODUCTION

West Nile virus (WNV) is a re-emerging arbovirus widely distributed in many areas of the world including Northern America (CDC 1999, Lanciotti et al. 1999). The majority of the competent vectors of WNV are mosquitoes, however, *Argas* ticks (soft ticks) are reported be competent vectors under laboratory conditions (Abbassy et al. 1993). Many genera and species of mosquito become infected with WNV and seemingly transmit this virus. Ten

genera with 43 species of mosquitoes have been reported to be infected with WNV since 1999 (CDC, 2003). However, infected mosquitoes are not always competent vectors that can transmit the virus to other hosts. To be a competent vector for WNV, a mosquito has to provide an environment that facilitates the infection, dissemination, and transmission of the virus.

In vitro and *in vivo* methods have been used to evaluate transmission of virus by mosquitoes. A droplet method was used to study dengue-2 virus transmission by *Aedes albopictus* (Skuse) (Gubler and Rosen 1976). Similarly, the transmission of WNV (H442 strain) and Sindbis virus by *Culex univittatus* (Theobald) was examined (Cornel and Jupp 1989). A capillary tube feeding method was used to determine the transmission rate of yellow fever virus by *Ae. aegypti* (Linn.) (Beaty and Aitken, 1979). The transmission rate of WNV (H442 strain) and Sindbis virus by *Cx. univittatus* was also evaluated using the capillary tube method (Cornel and Jupp 1989). Cornel and Jupp (1989) found that the transmission of WNV (H442 strain) and Sindbis virus by *Cx. univittatus* when using the capillary tube feeding method was significantly lower than it was when mosquitoes were fed on hamsters.

Sardelis and Turell (2001) and Turell et al. (2001) used chicks to determine WNV transmission while Goddard et al. (2002) use the capillary tube feeding method. However, there are no data that show the difference between these two methods. This study was designed to evaluate the difference between *in vitro* (capillary tube feeding) and *in vivo* (chick feeding) as methods for studying the transmission of WNV (NY 1999 strain) by mosquitoes.

MATERIALS AND METHODS

Chicks

One- to 2-day-old WNV specific antibody-free broiler chicks (Ross × Ross) were obtained from a commercial hatchery (Hoover's hatchery, Inc., Rudd, IA) and housed in biosafety level 3 facilities.

Mosquitoes

Ochlerotatus trivittatus were first generation mosquitoes derived from field collected adults in Iowa. *Aedes albopictus* were the 10^{th} to 20^{th} generation originally collected in Missouri and colonized by the Illinois Natural History Survey. All mosquitoes were maintained in controlled environmental conditions (27 ± 1°C and 80 ± 5% RH with a 16:8 hr photoperiod) and fed a 10 % sucrose solution. Mosquitoes were deprived of the sucrose solution for 48 hrs before being blood-fed on viremic chicks or feeding from capillary tubes.

Cells and medium

Vero-76 cells were used for virus propagation and assay. Two different cell culture mediums were used. Carbon dioxide dependent growth medium (CDM) consisted of Dulbecco's modified Eagle's medium (GIBCO®, Invitrogen Corp.) with 2.0 mM of L-glutamine, 20 mg gentamicin sulfate (GentaMax™100, Phoenix Pharmaceutical Inc.) per 100 ml of medium and supplemented with 10 % fetal calf serum (FCS). Maintenance medium (MM) used in virus assays consisted of one part CDM with 1 % FCS and one part CO_2-independent medium (CIM) (GIBCO®, Invitrogen Corp.) that was supplemented with 4.0 mM of L-glutamine, (Cellgro®, Mediatech 800, Cellgro, Inc.) 1 % FCS and 20 mg of gentamicin sulfate per 100 ml medium. Maintenance medium was supplemented with 20 % FCS when used to process mosquito specimens for virus.

Virus

West Nile virus (NY 1999-crow) was supplied by the National Veterinary Services Laboratory, Ames, Iowa. The virus was passaged 6 times in Vero-76 cells and once in *Aedes albopictus* by feeding mosquitoes on blood containing $10^{6.0}$ CID_{50s} WNV / ml using a feeding apparatus covered with a swine intestinal membrane (Rutledge, 1964). Virus-infected mosquitoes were killed at day14 after feeding by freezing at - 60 °C and were triturated in cold MM. The virus preparations were filtered through 450 nm filters and stored in liquid nitrogen until used.

Virus assays

Virus was assayed in Vero-76 cells by both the quantal and quantitative methods using 25 cm^2 cell culture flasks inoculated with 1 ml of serial 10-fold dilutions of virus prepared in MM containing 1% FCS. For quantal assays an additional 6 ml of MM with 1 % FCS was added to each flask after 1 hr incubation. Cell cultures were observed for cytopathic effect (CPE) for up to 8 days. Cell cultures with CPE were tested by RT-PCR to confirm the presence of WNV. Quantal assays were expressed as CID_{50s} / ml. For quantitative assays, inoculums were replaced after a1 hr incubation period with 4 ml of MM containing 1 % Agar Noble (DIFCO®, Becton Dickinson), 1 % FCS, 3.0 mM of L-glutamine and 20 mg of gentamicin per100 ml. Five ml of a second overlay identical to the first but containing 0.004 % neutral red dye was added to cell cultures 4 days later. Plaques were counted and titer expressed as plaque forming units (PFU) / ml.

RT-PCR

RNA was extracted from cell culture medium using the QIAamp viral RNA kit (QIAGEN Inc.). The RT-PCR for WNV specific RNA was conducted as described by

Lanciotti et al. (2000) with the following modifications. The amplifying cycle was increased from 40 to 45 cycles and the RT-PCR product (408-bp-size nucleic acid) was electrophoresed (Wide Mini Sub® Cell, Bio-Rad) through a 0.8 % agarose gel (NuSieve®, FMC Bioproducts) prepared with 1X Tris-Acetate-EDTA buffer (Fisher Scientific) containing 0.3 mg ethidium bromide per 100 ml gel (Sigma-Aldrich Co.).

West Nile virus transmission

***In vitro* transmission (capillary tube).** Saliva from individual mosquitoes was collected for virus assay on specific day post blood feeding (PBF) by permitting the sucrose-deprived mosquitoes to feed on 0.5 % FCS in 5 % sucrose in a 1.5 × 50 mm-capillary tube for 20 min. The contents of the capillary tubes were added to 2 ml of cold MM. Torsos and legs removed from individual mosquitoes that fed on capillary tubes were triturated separately in 300 µl of cold MM. This volume was increased to 2 ml. The MM containing the contents of the capillary tubes and the triturating products were passed through 450 nm filters directly into 25 cm^2 cell culture flasks containing Vero-75 cell cultures from which the medium were removed. An additional 5 ml of MM were added to individual flasks after a 1 hr incubation period. The cell cultures were observed for CPE for up to 8 days. Cell cultures with CPE were tested by RT-PCR to confirm the presence of WNV.

***In vivo* transmission (chick).** Individual mosquitoes were allowed to feed on 2-day-old chicks for 30 min. The torsos and legs from individual blood-fed mosquito were also tested for the presence of WNV as described above. At day 3 PBF, blood was collected from each chick and was tested for WNV in Vero cells and by RT-PCR.

Experimental design and data analysis

Viremia chicks were obtained by inoculating a group of 1-day-old chicks with $10^{3.0}$

CID_{50s} WNV. Mosquitoes were allowed to feed on infected chicks at different times after inoculation in order to be infected by blood meals with different virus titers in different chicks. Blood was collected from each chick immediately after blood feeding and the serum was assayed to determine the WNV titer. Mosquitoes in each group were separated and tested for *in vitro* or *in vivo* transmission of WNV at day 7, 12, 13, or 19 PBF.

The JMP 5.0 (SAS Institute Inc., Cary, NC, USA) was used to analyze all data. Differences of the means between *in vitro* and *in vivo* transmission of WNV for each group of the mosquitoes were analyzed using the Student's t test at the 0.05 confidence level.

RESULTS

Transmission rates determined by *in vitro* and *in vivo* methods are summarized in Table 4.1. Percent infection and percent dissemination are also summarized in the table.

The WNV infected blood-fed mosquitoes in each group were separated and tested for *in vitro* and *in vivo* transmission of WNV using capillary tube feeding and chick feeding. The results are shown in Table 4.1. Although some mosquitoes came from the same group, they were all tested individually for WNV infection and dissemination.

Three groups of *Ae. albopictus* fed on infected chicks with a virus titer of $10^{7.5}$ CID_{50s} / ml. The percent transmitting virus was determined at day 7, 12, and 13 PBF. Two groups of *Oc. trivittatus* were fed on infected chicks with virus titer $10^{8.0}$ and $10^{8.5}$ CID_{50s} / ml. Transmission of WNV by *Oc. trivittatus* was measured at day 13 and 19 PBF. The percentage of *in vitro* (capillary tube) transmission was higher than the percentage transmitting WNV when feeding on 2-day-old chicks (*in vivo*). The range was from 5 to 20 % . However, these differences were not statistically different ($p = 0.44 - 0.69$). In *Ae.*

albopictus, the percentage of mosquitoes transmitting WNV increased as the incubation time increased. The percent *Oc. trivittatus* transmitting WNV increased when both virus titer in the blood meal and the incubation time increased.

West Nile virus transmission by *Ae. albopictus* increased 45 % (capillary tube feeding) and 30 % (chick feeding) when the time after infection was increased from 7 to 12 days PBF. Transmission by these mosquitoes also increased 12 % (capillary tube feeding) and 20 % (chick feeding) when incubation time increased from 12 to 13 days PBF. Transmission rates of *Oc. trivitattus* also increased 48 % (capillary tube feeding) and 42 % (chick feeding) when the time after infection was increased from 13 to 19 days.

DISCUSSION

In vitro and *in vivo* transmission of WNV by the mosquitoes was compared using capillary tube feeding and chick feeding. *Ochlerotatus trivittatus* and *Ae. albopictus* were used in this study because earlier studies showed that these mosquitoes become infected with and transmit the virus. . The percentage of WNV transmission as determined by feeding mosquitoes on chicks and by feeding from a capillary tube was compared. The percentage of WNV transmission is defined as the percentage of blood-fed mosquitoes that transmitted WNV.

There were slight but not significant differences between transmission rates of WNV that were determined by the *in vitro* and *in vivo* methods. Our results are different from those of Cornel and Jupp (1989) who reported that determining transmission rates by feeding infected mosquitoes on hamsters (100 % transmission rate, n = 20) was more sensitive than feeding mosquitoes from capillary tubes (78 % transmission rate, n = 28).

A marked increase in WNV transmission by *Oc. trivittatus* was observed when the time that infected mosquitoes were maintained before testing for transmission was increased from 13 to 19 days. Transmission rates determined by the capillary tube method increased from 23 to 71 % and transmission rates determined by feeding on susceptible chicks increased from 18 to 60 %. The increased rates may have been due to WNV having more time to reach the salivary glands of infected mosquitoes. The increased transmission that can occur when incubation times are increased demonstrates the need to use standard times to determine transmission rates for purposes of comparison.

Chicks were used in the *in vivo* transmission study because birds are the amplifying hosts for WNV. We did not find significant differences in transmission rates between the *in vitro* and *in vivo* methods. Capillary tube feeding is simpler and easier to use than animals for determining transmission rates. However cross contamination is a concern and must be monitored at all times. Virus in capillary tubes also needs to be tested immediately after a mosquito feeds to avoid loss in infectivity. Cornel and Jupp (1989) were unable to isolate virus after a few days storage in capillary tubes at - 70°C. Using animals to study WNV transmission by mosquitoes must take into consideration the susceptibility of the animal species, their age, immunity status, and the necessary facilities required for animal holding

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Table 4.1 A comparison of *in vitro* (capillary tube feeding) and *in vivo* (chick feeding) transmission of West Nile virus by *Aedes albopictus* and *Ochlerotatus trivittatus*

Species	WNV titer[a] of infective blood meal	Day tested (PBF)[b]	*In vitro* method			*In vivo* method		
			Percent infection	Percent dissemination	Percent transmission[c]	Percent infection	Percent dissemination	Percent transmission[c]
Ae. albopictus	7.5	7	84 (26/31)	74 (23/31)	19 (6/31)	83 (24/29)	79 (23/29)	14 (4/29)
Ae. albopictus	7.5	12	80 (20/25)	80 (20/25)	64 (16/25)	72 (18/25)	64 (16/25)	44 (11/25)
Ae. albopictus	7.5	13	96 (24/25)	92 (23/25)	76 (19/25)	88 (22/25)	88 (22/25)	64 (16/25)
Oc. trivittatus	8	13	100 (22/22)	59 (13/22)	23 (5/22)	88.24 (15/17)	59 (10/17)	18 (3/17)
Oc. trivittatus	8.5	19	100 (14/14)	100 (14/14)	71 (10/14)	100 (10/10)	100 (10/10)	60 (6/10)

[a]$\text{Log}_{10}\ \text{CID}_{50s}$ / ml

[b]PBF = post blood feeding

[c]there was no statistically significant difference between transmission rate determined by the *in vitro* and *in vivo* methods ($p > 0.05$)

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CHAPTER 5. GENERAL CONCLUSIONS

General Discussion

The vector competence of *Ochlerotatus trivittatus* (Coq.) for West Nile virus (WNV) NY-1999 strain was compared to *Aedes albopictus* (Skuse), and *Culex pipiens* (L.). These species are considered to be bridge and amplifying vectors for WNV (Andreadis et al. 2001, Sardelis et al. 2002).

Infection, dissemination, and transmission of WNV by these 3 species were compared to evaluate their vector competence for WNV. Models for infection, dissemination, and transmission were constructed using logistic regression (Sall et al. 2000). These models can be used to predict the percentage of infection, dissemination, and transmission given the virus titer of an infected blood meal, particularly at lower levels of virus.

The area under the receiver operating characteristic (ROC) curve (scale from 0-1) was used as a measure of the goodness of fit between observed values and the logistic regression curves. Values close to 1 indicate a high degree of fit of the observed values with those of the logistic regression curve. The values of areas under the ROC curve for all models in these studies ranged from 0.721 - 0.967. The area under the ROC curve values for *Oc. trivittatus*, *Ae. albopictus* and *Cx. pipiens* infection models in this study were 0.92, 0.92, and 0.97 respectively. The infection model was a better model to predict rates than either the dissemination or transmission models.

In these studies, virus titers were expressed as CID_{50s} / ml. However samples also were plaque assayed. One hundred and ninety-six samples were used to compare virus titers obtained with both methods. The formula for relating PFU to CID_{50s} was developed using

linear regression in JMP (version 5.0). The formula is PFU / ml = 0.935 (CID_{50s} / ml) - 0.174.

Twenty-three percent of *Oc. trivittatus*, 66 % of *Ae. albopictus*, and 77 % of *Cx. pipiens* took a blood meal from chicks. In nature, mosquitoes can "select" a preferred host. *Oc. trivittatus* and *Ae. albopictus* normally are mammalophilic species but if mammalian hosts are limited and the mosquitoes have access to birds, both will feed on bird (Pinger and Rowley 1975, Niebylski et al. 1994). Thus, both *Oc. trivittatus* and *Ae. albopictus* are capable of being bridge vector for WNV.

In this study, the observed minimum WNV titer needed to infect *Oc. trivittatus*, *Ae. albopictus* and *Cx. pipiens* was $10^{4.5}$, $10^{5.5}$, and $10^{4.5}$ CID_{50s} / ml respectively. Dissemination and transmission of WNV by *Oc. trivittatus* and *Cx. pipiens* occurred after they took an infected blood meal with a virus titer $10^{5.5}$ CID_{50s} / ml. Dissemination and transmission of WNV in *Ae. albopictus* was detected after they took an infected blood meal with virus titers of $10^{6.5}$ and 10^{7} CID_{50s} / ml respectively. These observations indicate that these mosquitoes are readily infected with WNV however logistic regression models indicate that these species can become infected at lower levels of virus. This is particularly the case for *Oc. trivittatus*. Based on the logistic regression models, *Oc. trivittatus* is a possible maintenance vector for WNV in small mammals that tend to develop low level of WNV titers.

These studies indicate that *Oc. trivittatus*, *Ae. albopictus* and *Cx. pipiens* are competent vectors for WNV. Mean transmission rates of WNV by *Oc. trivittatus*, *Ae. albopictus*, and *Cx. pipiens* after they took a blood meal with virus titers between 10^{7} - $10^{9.5}$ CID_{50s} / ml were 41.7 ± 2.6, 72.4 ± 5.6, and 46.8 ± 4.5 % respectively. However *Ae. albopictus* was a better vector of WNV than either *Oc. trivittatus* or *Cx. pipiens* based on the

transmission rates of WNV observed in these studies. Logistic regression models, however, suggest that WNV transmission by *Oc. trivittatus* is higher than the transmission by *Ae. albopictus* and *Cx. pipiens* when they take blood meals with low levels of WNV ($\leq 10^{6.0}$ CID_{50s} / ml).

Capillary tube feeding proved to be a good method to study WNV transmission. It was compared with allowing mosquitoes to feed on 2-day-old chicks. The transmission rate by capillary tube feeding was about 5 - 20 % higher than percent transmission determined by feeding infected mosquitoes on chicks. However, these differences were not statistically significant different.

Recommendations for Future Research

The involvement of *Oc. trivittatus* and *Ae. albopictus* in the transmission and maintenance of WNV in nature needs to be determined. The ability of these species to transmit WNV transovarially also needs to be evaluated. The ability of these mosquitoes to transmit WNV to other animals particularly local animals such as cottontail rabbits and deer mice needs to be determined.

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APPENDIX. WEST NILE VIRUS CHARACTERISTICS IN CHICKS

Objective

To study West Nile virus titers in chicks after inoculation with different doses of WNV-NY 1999 strain.

Materials and Methods

Chicks

One- to 2-day-old WNV specific antibody-free broiler chicks (Ross × Ross) were obtained from a commercial hatchery (Hoover's hatchery, Inc., Rudd, IA) and housed in biosafety level 3 facilities.

Cells and medium

Vero-76 cells were used for virus propagation and assay. Two different cell culture mediums were used. Carbon dioxide dependent growth medium (CDM) consisted of Dulbecco's modified Eagle's medium (GIBCO®, Invitrogen Corp.) with 2.0 mM of L-glutamine, 20 mg gentamicin sulfate (GentaMax™100, Phoenix Pharmaceutical Inc.) per 100 ml of medium and supplemented with 10 % fetal calf serum (FCS). Maintenance medium (MM) used in virus assays consisted of one part CDM with 1% FCS and one part CO_2-independent medium (CIM) (GIBCO®, Invitrogen Corp.) that was supplemented with 4.0 mM of L-glutamine, (Cellgro®, Mediatech 800, Cellgro, Inc.) 1 % FCS and 20 mg of gentamicin sulfate per 100 ml medium.

Virus

West Nile virus (NY 1999-crow) was supplied by the National Veterinary Services Laboratory, Ames, Iowa. The virus was passaged 6 times in Vero-76 cells and once in *Aedes*

albopictus by feeding mosquitoes on blood containing $10^{6.0}$ CID_{50s} WNV / ml using a feeding apparatus covered with a swine intestinal membrane (Rutledge, 1964). Virus-infected mosquitoes were killed at day14 after feeding by freezing at - 60 °C and were triturated in cold MM. The virus preparations were filtered through 450 nm filters and stored in liquid nitrogen until used.

Virus assays

Chick serums were assayed for WNV on Vero-76 cells by the quantal method. Twenty-five cm^2 cell culture flasks containing confluent cell monolayers were inoculated with 1 ml aliquots of serial 10-fold dilutions of virus prepared in MM containing 1 % FCS. An additional 6 ml of MM with 1 % FCS was added to each flask after a 1 hr incubation period. Cell cultures were observed for CPE for up to 8 days. Cell cultures with CPE were tested by RT-PCR to confirm presence of WNV. Titers were expressed as CID_{50s} / ml.

RT-PCR

RNA was extracted from cell culture medium using the QIAamp viral RNA kit (QIAGEN Inc.). The RT-PCR for WNV specific RNA was conducted as described by Lanciotti et al. (2000) with the following modifications. The amplifying cycle was increased from 40 to 45 cycles and the RT-PCR product (408-bp-size nucleic acid) was electrophoresed (Wide Mini Sub® Cell, Bio-Rad) through a 0.8 % agarose gel (NuSieve®, FMC Bioproducts) prepared with 1X Tris-Acetate-EDTA buffer (Fisher Scientific) containing 0.3 mg ethidium bromide per 100 ml gel (Sigma-Aldrich Co.).

Results

Virus titers in chicks after inoculation with $10^{2.0}$-$10^{4.0}$ CID_{50s} WNV / chick at different times post inoculation are shown in Table A1.

Table A1. West Nile virus titers in 1- to 5-day-old chicks after inoculation with $10^{2.0}$-$10^{4.0}$ CID_{50s} of WNV

Time post inoculation (Hrs)	West Nile virus titers in chicks[a] (N)[b]		
	Inoc. with $10^{2.0}$ CID_{50s}	Inoc. with $10^{3.0}$ CID_{50s}	Inoc. with $10^{4.0}$ CID_{50s}
6	-	0 (2)	-
12	2.5 (1)	3.25 (4)	5 (1)
18	-	3.63 (4)	-
24	4.5 (1)	5.75 (4)	6 (1)
36	6.5 (1)	-	8 (1)
48	7 (1)	7.5 (4)	8 (1)
60	8 (1)	-	8 (1)
72	-	8.38 (4)	-
96	-	7.63 (4)	-

[a]titer expressed as Log_{10} CID_{50s} / ml

[b]number of chicks inoculated

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