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Studies on the vector competence of a Mid-western strain of Aedes vexans (Meigen) (Diptera: Culicidae) for West Nile Virus

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Studies on the vector competence of a Mid-western strain of *Aedes vexans* (Meigen) (Diptera: Culicidae) for West Nile Virus

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DEDICATION

This thesis is dedicated to Dr. Wayne A. Rowley. I was introduced to Medical Entomology and the social impacts mosquito-borne diseases have on society by Dr. Rowley during a biological field trip to Costa Rica in 1999. His guidance has helped me achieve goals that, at first, seemed very distant. He is my mentor, the one who taught me to look at science and life objectively. He has assisted my development as a scientist and as a public servant by allowing me opportunities one can only wish to have. Dr. Rowley has pushed me to be who I am today with his encouragement and he has given me all the tools necessary to be successful. For this, I sincerely thank you, Dr. Rowley, and will always be in your debt.

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CHAPTER 1. INTRODUCTION.

Thesis Organization.

This thesis is composed of 5 chapters. The first chapter gives a general introduction of West Nile Virus, *Aedes vexans*, and a brief summary of the research objectives. Chapter 2 is an extended review of literature pertaining to history, biology, and ecology of West Nile Virus, and the biology and ecology of *Aedes vexans* mosquitoes. Chapter 3 is a manuscript intended for publication. The manuscript includes an introduction, an account of the materials and methods, the results, and a discussion of the results. Chapter 4 is a preliminary manuscript intended to initiate further studies for publication. The manuscript includes an introduction, an account of the materials and methods, the results, and a discussion of the results. Chapter 5 contains general conclusions from the study and recommendations for future research. Literature cited in each chapter is at the conclusion of that chapter.

General Introduction.

Aedes vexans (Meigen) may bridge West Nile Virus (WNV) from the avian amplification cycle to a mammalian enzootic cycle, but it is unclear if *Ae. vexans* is a competent vector of WNV. Studies by Goddard et al. (2002) suggest that *Ae. vexans* is a low to moderately efficient laboratory vector of WNV. However, it is not known if *Ae. vexans* effectively transmits WNV in nature. Goddard et al. (2002) used hanging droplets of defibrinated rabbit blood instead of an infected animal to orally infect several species of mosquitoes. It's possible that WNV infection rates of *Ae. vexans* may be different if the mosquito feeds on viremic animals. Furthermore, studies on the dissemination of WNV in *Ae. vexans* and on the transmission of WNV by *Ae. vexans* during feeding are needed.

Female *Ae. vexans*, when infected with West Nile Virus (WNV), may also vertically transmit the virus from parent to progeny. Vertical transmission is a possible overwintering mechanism for several arthropod-borne viruses (arboviruses). La Crosse encephalitis and trivittatus virus are examples of viruses maintained within a vector population by vertical transmission (Christensen et al. 1978). A virus transmitted from one generation to the next through infected eggs is transovarial transmission (TOT) (Thrusfield 1995). Transovarial transmission may be a mechanism that helps maintain a virus in nature during inter-epidemic periods and *Ae. vexans* overwinter as diapausing eggs.. If *Ae. vexans* becomes infected while feeding on viremic animals and transovarially transmits the virus, this would be of considerable epidemiological importance in the natural history of this virus.

West Nile Virus.

The WNV outbreak in New York in 1999 was the first documented introduction of WNV in the Western Hemisphere. The spread of WNV from New York City to California in four years was an unprecedented spread of an imported mosquito-borne disease. The ability to infect 198 species of birds (CDC website 2004, Gould and Fikrig 2004) and 43 species of North American mosquitoes (CDC website 2004, Gould and Fikrig 2004) may explain how WNV spreads so rapidly (Personal Communication, Gubler 2003). West Nile Virus is a member of the Japanese encephalitis (JE) virus serogroup in the family *Flaviviridae*, and has the potential to cause febrile illness, encephalitis, and death in humans (Turell et al. 2001, Brinton 2002, Peterson 2004, CDC website 2004). West Nile Virus is recognized as the most widespread of the flaviviruses, with geographical distribution spanning North and Central America, Africa, Europe, and Asia (Hubálek and Halouzka 1999, Burke and Monath 2001, Brinton 2002, CDC website 2004).

***Aedes vexans*.**

Aedes vexans is widely distributed across eastern Asia, North America, western Africa, and much of Pacific Oceania (Horsfall et al. 1973, Briegel 2003). *Aedes vexans* is one of the most widespread and often locally abundant species in the north and north-central United States. It occurs in all 50 states of the U.S. and in all provinces of the Canada except Newfoundland, Labrador, and some areas of the Northwest Territory (Crans 2004). In addition to being abundant and wide spread, *Ae. vexans* is crepuscular. It is active from approximately 45 minutes before sunset to 120 minutes after sunset (Wright and Knight 1966). *Aedes vexans* is anthropophagic, but it is also an opportunistic feeder. It feeds on birds if mammalian hosts are not available (Shemanchuk 1969, Horsfall et al. 1973, Loftin et al. 1997). The opportunistic feeding habits and its ability to become infected with WNV makes *Ae. vexans* a possible bridge vector that carries WNV from the normal bird-mosquito-bird cycle to mammals. *Aedes vexans* is a known bridge vector of Eastern Equine Encephalitis virus (EEE) from birds to humans and horses (Moncayo and Edman 1999). Turell et al. (2001) and Goddard et al. (2002) found *Ae. vexans* to be a competent and moderately efficient laboratory vector of WNV. It is also a significant component in the natural maintenance of Tahyna virus in Europe and encephalitis viruses in North America (Horsfall et al. 1973). It is not known if TOT of these viruses occurs in *Ae. vexans* populations.

The mechanism that maintains WNV during winter in temperate regions remains in question. It is known that vertical transmission of closely related flaviviruses (JE, St. Louis encephalitis, Kunjin virus) by mosquitoes is possible (Baqar et al. 1993). The mechanisms for persistence of WNV through periods of vector inactivity also is unknown, Dohm,

Sardelis, and Turell (2002) found that WNV can be vertically transmitted by *Ae. albopictus*. After diapause in the egg stage, WNV was detected in *Ae. albopictus* (Dohm, Sardelis, and Turrell 2002). If *Ae. vexans* transmits WNV transovarially, even at a low minimal filial infection rate (MFIR) such as 1/1000 or less, it would still be important because of the large populations of *Ae. vexans* that occur in many areas of the U.S. As such, *Ae. vexans* could contribute to the persistence of WNV in the United States (Dohm, Sardelis, and Turell, 2002).

Objectives.

Hypothesis – *Ae. vexans*, after feeding on a WNV viremic host, will develop a disseminated infection and transmit WNV during feeding.

Null Hypothesis – *Ae. vexans*, after feeding on a WNV viremic host, will not develop a disseminated infection and will not transmit WNV during feeding.

Specific objectives:

1) Determine whether or not *Ae. vexans* becomes orally infected with WNV.
2) Determine whether or not WNV disseminates in *Ae. vexans*.
3) Determine whether or not *Ae. vexans* transmits WNV during feeding.
4) Determine if WNV is transovarially transmitted by orally infected *Ae. vexans*.

Summary.

Since *Ae. vexans* is widespread in the U. S. and is anthropophagic, it is important to determine if this species is a competent bridge vector for WNV. Transovarial transmission may function as a maintenance mechanism for WNV, and be an overwintering mechanism in temperate zones. As a candidate bridge vector for WNV in the U.S., and its wide distribution in the U.S., *Ae. vexans* is an important part of the natural history of WNV.

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CHAPTER 2. LITERATURE REVIEW.

History of WNV.

West Nile Virus was isolated in the West Nile District of Uganda in 1937 (Brinton 2002, CDC website 2004, Gould and Fikrig 2004). The virus was isolated from an adult female displaying febrile illness (Smithburn et al. 1970, Brinton 2002, CDC website 2004, Gould and Fikrig 2004). Since its discovery, WNV has been recognized as the causative agent of infrequent disease outbreaks in humans. The ecology of WNV was characterized in the 1950s by scientists in Egypt (CDC website 2004). Severe human meningitis or encephalitis in elderly patients was the recognizing characteristics of an outbreak in Israel in 1957 (CDC website 2004). Before 1999, WNV was endemic in regions of Africa, the Middle East, Europe, and in areas of India (Hubálek and Halouzka 1999, Burke and Monath 2001, Brinton 2002, CDC website 2004, Gould and Fikrig 2004). Epizootics caused by WNV have occurred in, but are not limited to, France, Romania, Russia, Algeria, Madagascar, Senegal, and South Africa (Brinton 2002). The largest known human epidemic before the U.S. outbreaks in 2002 and 2003 occurred in 1974 in Cape Province, South Africa (Brinton 2002). The 1974 outbreak had approximately 3000 human cases (Brinton 2002). West Nile Virus was first isolated in the Western Hemisphere in 1999. The 1999 epidemic in New York City resulted in seven human deaths and several hundred dead birds and horses (Gould and Fikrig 2004). Transmission of WNV reoccurred during the summers from 2000 to 2004 and the virus is now endemic throughout the continental U.S. In 2002, there were 4,156 human cases in the U.S. and in 2003 there were 9,862 human cases in the U.S. (CDC website 2004).

Biology of WNV.

Classification: WNV is a member of the genus *Flavivirus* in the family *Flaviviridae*. Approximately 70 viruses are currently classified in the genus *Flavivirus* and are divided into twelve serogroups. WNV is in the Japanese encephalitis virus (JE) serogroup. There are two lineages of WNV based on signature amino acid substitutions or deletions in their envelope proteins (Burke and Monath 2001, Brinton 2002). Lineage 1 isolations of WNV have all been from human infections (Brinton 2002, Gould and Fikrig 2004). The second lineage is restricted to endemic enzootics in Africa (Brinton 2002, Gould and Fikrig 2004).

Structure: WNV is a single-stranded RNA virus. The genome of the virus is 11,029 nucleotides long and possesses a single open reading frame (ORF) of 10,301 nucleotides that produce ten viral proteins (Brinton 2002). Three viral proteins encoded at the 5' section of the ORF are structural proteins for the capsid, membrane, and envelope (Brinton 2002). The 3' portion of the ORF encodes seven nonstructural proteins (Brinton 2002).

Morphology: WNV virions are ~50 nm in diameter. The virions are spherical and have an envelope. The envelope and capsid have icosahedral symmetry as indicated by cryo-electron microscopy (Brinton 2002).

Replication: WNV replicates in many cell cultures. Cell culture varieties susceptible to WNV include primary chicken, duck, and mouse embryo cells, and monkey, human, pig, rodent, amphibian, and insect continuous cell lines (Burke and Monath 2001, Brinton 2002). Virions enter cells through receptor-mediated endocytosis (Brinton 2002). Translation and replication of viral RNA occurs in the cytoplasm. Progeny virions are released via exocytosis from infected mammalian cells within 10 to 12 h after infection (Brinton 2002). Extracellular virus titers are not normally observed until 24 h after infection (Brinton 2002).

Ecology of WNV.

Wild birds are the primary reservoir hosts in endemic areas and serve as the typical source of virus initiating epizootics outside endemic areas (Turell et al. 2001, Brinton 2002, Gould and Fikrig 2004). A large number of wild bird species develop viremias of 10^5 PFU / ml of serum and can sustain such viremic levels of WNV for days to weeks (Brinton 2002). Isolations of WNV have been made from *Aedes*, *Anopheles, Coquillettidia*, *Culex*, *Mansonia*, *Minomyia*, and *Ochlerotatus* mosquitoes on four continents (Burke and Monath 2001, Brinton 2002, Gould and Fikrig 2004). *Culex* species serve as the primary vector of WNV. The principal amplification cycle for WNV relies on the relationship between birds and ornithophilic/ornithophagic mosquitoes. Turrell et al. (2001) believe that for a mosquito to be an efficient enzootic vector, it needs to feed primarily on avian hosts (Turell et al 2001). *Culex* mosquitoes are ornithophagous and are highly susceptible to oral infection (Brinton 2002). To be a "bridge" vector and transmit WNV from the enzootic cycle (birds) to humans and other mammalian hosts, mosquitoes need to be general and opportunistic feeders (Turell et al. 2001). Species in the genera *Aedes* and *Ochlerotatus* can be bridge vectors of WNV as they feed primarily on mammals (Horsfall et al. 1973, Brinton 2002, Gould and Fikrig 2004).

Biology and Ecology of *Ae. vexans*.

Eggs and hatching stimuli: Eggs are elongate, fusiform, and ventrally arched. The exposed chorion is a bronze color that, when viewed at magnifications of 50X or more, allows for recognition of the species among populations in North America (Horsfall et al. 1973). Eggs range from 561 to 743 μ in length and 165 to 231 μ in diameter. Newly deposited eggs increase in diameter within 24 h and assume their definitive shape within 48 to 72 h (Horsfall et al 1973). Weight increases from 5 to 12 μg as embryonation progresses

(Horsfall et al. 1973). Eggs may become dormant but retain a latent capability for hatching, or they may hatch without delay. Embryos may remain viable a year or more, and survival for several years is possible (Horsfall et al. 1973). *Aedes vexans* eggs hatch in serial or partial broods from a population. Hatching may be partial at one inundation and subsequent inundations are necessary to induce hatching in a full brood (Wilson and Horsfall 1970, Novak and Shroyer 1978). This form of egg hatching is referred to as installment hatching. Aedine eggs have a hatching response correlated to a depression of dissolved oxygen levels (Judson 1960, Wilson and Horsfall 1970, Horsfall et al. 1973, Edgerly, Willey, and Livdahl 1993). Bacterial colonization of the egg surface stimulates hatching and Borg and Horsfall (1953) found that eggs that are sterilized do not hatch until bacteria are introduced into the hatching media. The presence of bacteria in hatching media reduce dissolved oxygen levels and stimulate hatching (Borg and Horsfall 1951). Eggs must be conditioned before hatching will occur. Conditioning is a term used to describe the process required to allow successful hatching. These steps include the completion of embryogenesis, a brief period of non-saturation of the substrate, and an exposure to temperatures greater than 18°C to bypass latency (Judson 1960, Horsfall et al. 1973). The process also refers to the flooding of eggs and the decrease of dissolved oxygen levels in the hatching medium (Borg and Horsfall 1951, Judson 1960, Horsfall et al. 1973). Changing the dissolved oxygen level is more effective in stimulating hatching than is a static concentration of dissolved oxygen (Judson 1960). Livdahl (1982) found that competition for food is a regulating factor of hatching, and competitiveness of large larvae on small ones can be severe. An inhibitory process may result from the grazing by larvae on bacteria from egg surfaces, thereby removing the source of hatching stimulation (Livdahl, Koenekoop, and Futterweit 1984). Inhibition of hatching in

the presence of large larvae enables embryos to avoid severe competition when in the first instar.

Larvae and Pupae: *Aedes vexans* larvae are aquatic and go through four instars. Larvae have a sclerotized head capsule and a soft, membranous abdomen. Maximum size is achieved by larvae that develop in vernal pools at 20°C (Horsfall et al. 1973). Larvae in sunlit pools are nearly half the size and are much lighter in color than larvae that develop in vernal pools (Horsfall et al. 1973). Larvae can be present in newly flooded depressions in the ground. They appear in flood plains, woodland pools, wet prairies, ditches, cattail sedge marshes, and canals that have lentic aquatic habitat (Horsfall et al. 1973, Sharkey, Sjogren, and Kulman 1988). Larval concentration varies with depth of the water and age of larvae (Horsfall et al. 1973, Livdahl, Koenekoop, and Futterweit 1984). First instar larvae congregate in the margins of bodies of water, where water is shallow and has floating detritus (Horsfall et al. 1973). Fourth instars typically occupy entire flooded areas and are widely dispersed (Horsfall et al. 1973). *Aedes vexans* larvae occur in water that is 15°C or warmer (Horsfall et al. 1973). They are omnivorous, feeding on suspended matter when young and on matter attached to or lying on submerged objects when older (Horsfall et al. 1973). The mouthbrushes of young larvae are strictly of the filter feeder type (Horsfall et al. 1973). The mouthbrushes of older larvae vary between browsing and filter feeding (Horsfall et al. 1973). *Aedes vexans* respires by diffusion of dissolved oxygen through the cuticle and by aerial oxygen through spiracles in the siphon tube (Horsfall et al. 1973). Young larvae can survive by diffusion of oxygen through the cuticle, whereas older larvae rely on aerial oxygen for respiration (Horsfall et al. 1973). Fourth instars form dense masses of individuals, but aggregation is rare in larvae that are in the third or younger instars (Horsfall et al. 1973).

Pupae are aquatic, non-feeding, and contain the pharate adult. Adult emergence is temperature dependent. Development and emergence ceases at 10°C or less (Horsfall et al. 1973). At temperatures between 15°C and 22°C, adult emergence occurs within 3 to 9 days (Horsfall et al. 1973). At temperatures of 27°C to 37°C, development and emergence is complete in 1 to 3 days (Horsfall et al. 1973). At 40°C and higher, mortality occurs in at least 50 percent of pupae (Horsfall et al. 1973).

Adults and Oviposition: *Aedes vexans* are yellowish-brown or tawny brown in color. Close examination shows narrow bands of white scales on the base of the tarsal segments (Figure 1). *Ae. vexans* is multivoltine and large numbers are found in areas after periodic rains (Read and Moon 1996). Shortly after emergence, males and females are found in vegetation proximal to the larval habitat. At that time, males form swarms above the vegetation, especially at dusk (Horsfall et al. 1973). When females fly into male swarms, a male grasps a female, but it is not determined whether copulation occurs in the air or the pair fall from the swarm to the substrate to complete copulation (Horsfall et al. 1973). Copulation occurs before females make their exodus flight (Horsfall et al. 1973, Briegel, Waltert, and Kuhn 2001). After emergence, adult female *Ae. vexans* form aggregations during diurnal massing in vegetation and near ovipositional sites (Horsfall et al 1973, Boxmeyer and Palchick 1999). During the first seven days of imaginal life, females must feed on sugar or otherwise die, as they are low in teneral protein and lipids after emergence (Briegel 2003). Both males and females feed on nectar from plants to acquire carbohydrate meals. Only females take blood meals, and the blood protein is used for egg production. Host seeking and blood feeding occurs during the crepuscular period with a preferred time near sunset (Wright and Knight 1966, Briegel, Waltert, and Kuhn 2001, Gingrich and Casillas 2004). Horsfall et

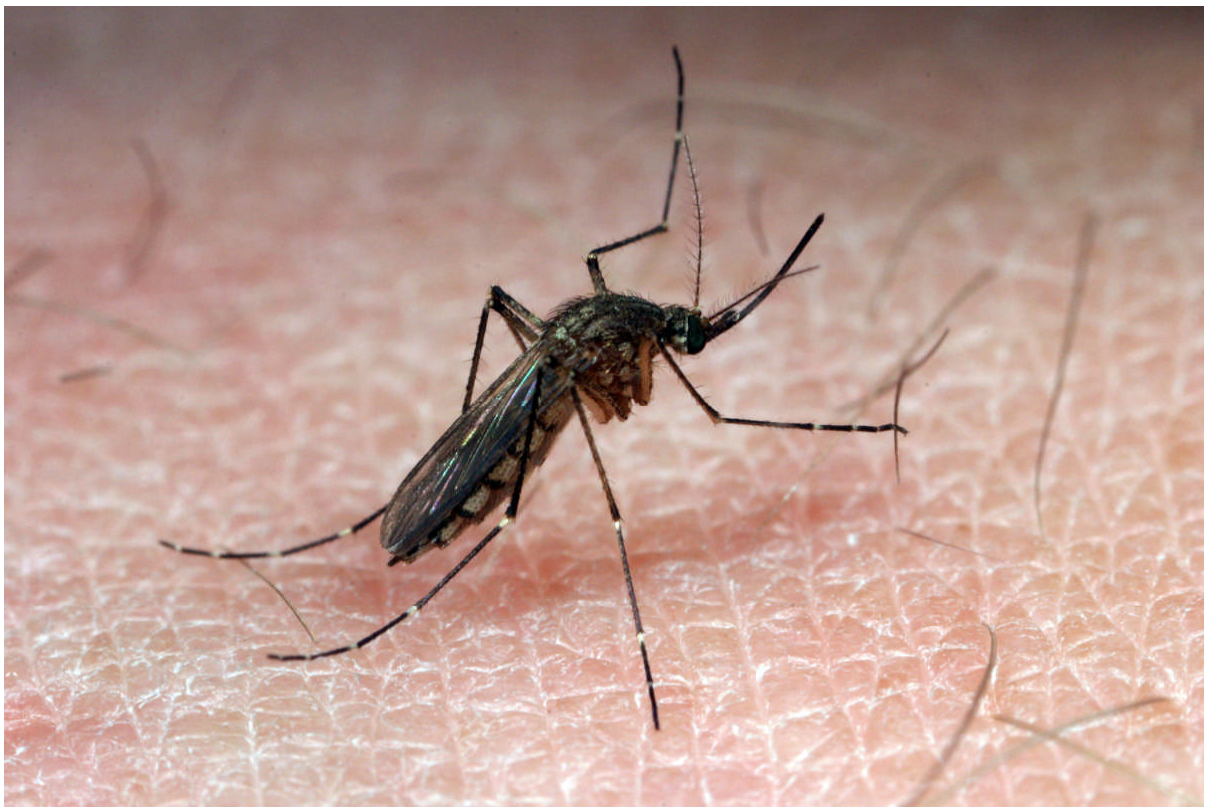

Figure 1. Digital image of *Aedes vexans* female.

al. (1973) have reared hundreds of *Ae. vexans* and none were autogenous and no records of autogeny can be found. Females feed on the blood of mammals and birds (Shemanchuk 1969, Horsfall et al. 1973, Loftin et al. 1997). *Aedes vexans* is ecologically categorized as a floodwater mosquito because eggs are deposited on soil that will be inundated by rain or runoff water that forms pools on the ground (Novak 1981, Read and Moon 1996). Eggs can be located on blades of grass, small stems of plants, leaf litter, and soil that is subject to inundation with water (Gjullin et al. 1941, Horsfall et al. 1973, Strickman 1982, Friederich 1984). Eggs can also be found below ground level in cracks in soil, arthropod burrows, and hoof prints (Horsfall et al. 1973, Novak 1981). Heavy densities of *Ae. vexans* eggs can be found in shallow depressions in fields, forests, and flood plains where runoff rainwater collects from earlier weather events (Gjullin et al. 1941, Horsfall et al. 1973). Abundance of eggs is a function of the length of time the soil surface has moisture levels attractive for oviposition (Horsfall et al. 1973, Novak 1981, Strickman 1982, Friederich 1984). Low, dense herbal shade and loose layers of detritus on the soil surface are especially attractive to ovipositing *Ae. vexans* (Horsfall et al. 1973, Novak 1981, Strickman 1982, Sharkey, Sjogren, and Kulman 1988). Bare, unshaded, smooth soil rarely has substantial numbers of eggs (Horsfall et al. 1973, Strickman 1982). Egg deposition occurs along fallen trees, branches, and stumps of trees that act as barriers to the outflow of pooled water (Russo 1979, Novak 1981).

Diapause and overwintering: Diapause defined by Beck (1962) is a state of development in which growth and metabolic processes slow. It is initiated by physiological changes rather than by adverse environmental conditions. Environmental factors that induce diapause act as cues that reliably occur and predict the oncoming of unfavorable climatic

conditions. The principle stimulus for the induction of diapause is a change in photoperiod, however, lower temperatures enhance the effects of short photoperiods (Beck 1962, McHaffey 1972, Mitchell 1988). Photoperiodism is a response to daily and seasonal daylight rhythms. The combination of short photoperiods and low temperatures causes greater numbers of mosquitoes to enter diapause than does a single stimulus (McHaffey 1972, Mitchell 1988). Diapause decreases in intensity as the season progresses and overwintering diapause may end by midwinter rather than by spring (Mitchell 1988). Overwintering, the term used to describe the behavior of hibernation in mosquitoes, is a result of the initiation of diapause and occurs in response to diapause (Beck 1962, Mitchell 1988). In mosquito species that overwinter in the egg stage, diapause occurs after completion of embryogenesis, when the embryo is fully developed as a pharate larva (Horsfall et al. 1973, Shroyer and Craig 1980, Mitchell 1988). In multivoltine species such as *Ae. vexans*, diapause is facultative and eggs laid in late summer and autumn enter diapause (McHaffey 1972, Horsfall et al. 1973, Mitchell 1988). For *Ae. vexans*, induction of diapause in eggs can be a result of photoperiod exposure on adult females. Vinogradova (1965) showed that reared adult *Ae. togoi* maintained under short photoperiods laid diapausing eggs. Other reports of photoperiodic induction of diapause in eggs have been made for *Ochlerotatus atropalpus*, *Oc. epactius*, *Oc. caspius*, and *Ae. vexans* (McHaffey 1972, Shroyer and Craig 1980, Mitchell 1988). Diapause can also be initiated in eggs without influence from adult female photoperiod exposure. McHaffey (1972) and Shroyer and Craig (1980) showed that eggs exposed to short photoperiods (11 hr day length) laid by females maintained at a 16L:8D photoperiod went into diapause. Studies on durability show that most eggs that survive

winter will hatch the following spring or summer if appropriate hatching stimuli are present (Horsfall et al. 1973).

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CHAPTER 3. STUDIES ON THE VECTOR COMPETENCE OF A MID-WESTERN STRAIN OF *AEDES VEXANS* (MEIGEN) (DIPTERA: CULICIDAE) FOR WEST NILE VIRUS

A paper to be submitted to the Journal of Medical Entomology

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ABSTRACT

Aedes vexans (Meigen) mosquitoes were given access to 2- to 4-day-old chickens inoculated with $10^{3.0}$ CID_{50s} / ml West Nile Virus (WNV) at 1 day old. Mosquitoes were given access to chickens at different intervals after inoculation to make certain that blood meals from chickens were of varying virus titers. No differences occurred in infection rates of *Ae. vexans* and *Culex pipiens* (L.) fed on the same WNV viremic chicken. Infection rates of *Ae. vexans* were higher (79%) when fed on chickens with viremias of 6.0 $\log_{10}$ WNV PFUs / ml or higher than they were (27%) in mosquitoes fed on chickens with viremias below 6.0 $\log_{10}$ WNV PFUs / ml. Dissemination rates were similar in *Ae. vexans* 14 and 21 days post blood feeding (PBF) (74 % and 74 %, respectively) . Likewise, transmission rates were similar in *Ae. vexans* 14 days and 21 days PBF (25 % and 27 %, respectively). *Aedes vexans* fed on viremic chickens became infected, developed disseminated infections,

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and transmitted WNV during feeding. This indicated that, under laboratory conditions, *Ae. vexans* is a competent vector of WNV.

INTRODUCTION

The spread of WNV from New York City to California in only 4 years represents an unprecedented spread of an imported mosquito-borne disease. The ability of WNV to infect 198 species of birds (CDC website 2004, Gould and Fikrig 2004) and 43 species of North American mosquitoes (CDC website 2004, Gould and Fikrig 2004) may explain how WNV spread so rapidly (Personal Communication, Gubler 2003). *Aedes vexans* (Meigen) is one of the most widespread and locally abundant nuisance mosquitoes in North America. It occurs in all 50 states of the U.S. and in all provinces of the Canada except Newfoundland, Labrador, and some areas of the Northwest Territory (Crans 2004). In addition to being abundant and wide spread, *Ae. vexans* is active from approximately 45 minutes before sunset to 120 minutes after sunset (Wright and Knight 1966). *Aedes vexans* is anthropophagic, but it also can be an opportunistic feeder. It feeds on birds if mammalian hosts are not available (Shemanchuk 1969, Horsfall et al. 1973, Loftin et al. 1997). *Aedes vexans* is a bridge vector of Eastern Equine Encephalitis virus (EEE) from birds to humans and horses (Moncayo and Edman 1999). It may also bridge WNV from the avian amplification cycle to mammals such as humans and horses. It is unclear if *Ae. vexans* is a competent vector of WNV. Studies by Goddard et al. (2002) indicated that *Ae. vexans* is a "low to moderately" efficient laboratory vector of WNV, but it is unclear if *Ae. vexans* is a vector in nature. Goddard et al. (2002) used hanging drops of defibrinated rabbit blood instead of an infected animal to infect mosquitoes. Blood droplets with 2 different viremias ($10^{7.1}$ and $10^{4.9}$ WNV PFUs/1.0 ml of

blood) were used in Goddard et al's. studies. These authors used only 22 *Ae. vexans* in the study.

The objectives of this study were to determine if *Ae. vexans* becomes infected and develops a disseminated infection after feeding on a viremic avian host. A second objective was to determine the WNV viremia in an avian host necessary to infect *Ae. vexans*. A third objective was to determine if *Ae. vexans* transmits WNV by bite.

MATERIALS AND METHODS

Mosquitoes. Female *Ae. vexans* were trapped with CO_2-baited CDC light traps at several locations in central Iowa. Field-collected mosquitoes were placed into 0.5 L paper cups with a cloth mesh screen on the top. Random pools of trapped mosquitoes were tested for WNV using VecTest® to determine whether any field collected *Ae. vexans* were infected with the virus. Mosquitoes were allowed to take a blood meal from an anesthetized rabbit. Engorged mosquitoes were separated from those that did not feed and were held in cups for 72 h at 26°C ± 1° and 80 % ± 5 % RH. A cotton pad saturated with 0.3 M sucrose was placed on each cup. Gravid mosquitoes were transferred to oviposition cages (OP cages) described by Christensen et al. (1978). One end is clear plexiglass and the other is plexiglass with a hole that can be fitted with a rubber stopper. Ten mosquitoes were placed into an OP cage. Oviposition cages were placed in trays (34.3 x 25.4 cm x 3.8 cm) lined on the bottom with moist absorbent cotton wrapped (covered) with cheesecloth. Gravid mosquitoes had access to cotton pads saturated with 0.3 M sucrose. Mosquitoes were held at 26°C ± 1°C and 80 % ± 5 % RH in a 16:8 photoperiod. Eggs were collected and held for 14 days at to allow for embryonation after which, they were stored at 4°C until hatched. Eggs were placed at 26°C ± 1°C and 80 % ± 5 % RH for 14 days before being submerged in deoxygenated water.

Brewers' yeast (0.02 g) was added to the water to stimulate hatching. Larvae were fed pulverized Tetramin®. Pupae were removed from rearing pans and placed in 0.5 L cups containing a small amount of water (50 pupae per cup). After emergence, adults were separated by date of emergence and sex.

Culex pipiens pipiens (L.) used in this study were 14th generation from a laboratory colony maintained at Iowa State University. The original generation was started from egg rafts collected from sod buckets located at several locations in central Iowa in August 2003.

Chickens. One-day-old WNV antibody-free white leghorn chickens (*Gallus gallus*) were obtained from a commercial chicken hatchery (Hoover's Hatchery, Inc., Rudd, IA) and housed in Biosafety Level 3 (BSL3) containment facilities.

Cells and Media. Vero-76 cell cultures were used for virus propagation and isolation. The cell culture medium was a CO_2 independent growth medium (CIM) consisting of Dulbecco's modified Eagle's medium (GIBCO®, Invitrogen Corp.) with 10 % heat inactivated fetal bovine serum (FBS), 2.0 mM of L-glutamine, and 20 mg gentamicin sulfate (GentaMax™100, Phoenix Pharmaceutical Inc.) per 100 ml of medium (Tiawsirisup et al. 2004). Mosquitoes were processed and tested for virus in CIM supplemented with 20 % FBS.

Virus. West Nile Virus (IA 2002-crow) was obtained from the brain of a crow, *Corvus brachyrhynchos* Brehm, found dead in Ames, Iowa. The virus was passed 3 times in Vero-76 cell cultures and then froze at -70°C until used to inoculate chickens.

Virus Assay. Virus was assayed in Vero-76 cell cultures to determine titer levels in individual chicken serum. Twenty-five cm^2 cell culture flasks were inoculated with 1 ml of serial 10-fold dilutions of serum prepared in CIM containing 1 % FBS. After a 1 h

incubation period, 4 ml of CIM with 1 % FBS, 1 % Agar Noble (DIFCO®, Becton Dickinson), 3.0 mM of L-glutamine, and 20 mg of gentamicin per 100 ml were added to the flasks (Tiawsirisup et al. 2004). Four days later, 5 ml of identical agar with 0.004 % neutral red dye was overlaid on the cell cultures (Tiawsirisup et al. 2004). Plaques were counted and titers were expressed as plaque forming units (PFU) / ml.

Mosquitoes were placed in 300 µl of CIM supplemented with 20 % FBS and pulverized with a mechanical pestle. Samples were placed in 1.7 ml of CIM with 10 % FBS and was used to inoculate Vero-76 cells via a 0.45 µm filtered syringe. After 4, 6, and 8 days post inoculation CPE was noted. After day 8 CPE, all cell cultures were frozen at -70°C. West Nile Virus presence or absence in cell cultures was confirmed by RT-PCR.

RT-PCR. RNA was extracted from cell culture medium using QIAamp viral RNA kits (QIAGEN Inc.). Reverse transcriptase-polymerase chain reaction for WNV specific RNA was conducted as described by Lanciotti et al. (2000) with 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.).

Infection and dissemination. Virgin 2- to 6-day-old *Ae. vexans* and 2- to 6-day-old *Cx. pipiens* were held in 0.5 l cups. Cotton pads with 0.3 M sucrose were removed 48 h and water was removed 12 h prior to blood feeding. Mosquitoes were provided access to 2- to 4-day-old viremic chickens by placing the chicken in a nylon sock. Mosquitoes were allowed to feed for 30 min. Engorged *Ae. vexans* and *Cx. pipiens* were aspirated from the paper cups using a venturi aspiration system, and held individually at 26°C ± 1°C and 80 % ± 5 % RH

for either 14 or 21 PBF. On day 14 or 21 PBF, individual mosquitoes were processed to determine infection and dissemination rates. Individual mosquitoes, minus their legs were placed in 300 μL of cold CIM supplemented with 20 % FBS and pulverized with a mechanical pestle. The legs of individual mosquitoes were processed the same way as the bodies of mosquitoes were processed.

Transmission. Mosquitoes were allowed to feed on 0.3 M sucrose mixed with equal parts of CIM containing 10 % FBS media in a capillary tube (Aitken 1977, Cornel and Jupp 1989). Mosquitoes were lightly anesthetized with wet ice and the wings and legs were removed. The legs of individual mosquitoes were processed in the same manner as previously described. The proboscis of individual mosquitoes was inserted into a capillary tube containing the sucrose/CIM media. Mosquitoes were allowed to feed for 15 min or until engorgement was observed. Individual mosquitoes were processed as previously indicated. The sucrose/CIM solution was extracted from the capillary tube into a micro-centrifuge tube with 300 μL of cold CIM supplemented with 20 % FBS media and was processed the same as mosquitoes were processed.

Experimental Design and Data Analysis. Mosquitoes were given access to chickens inoculated with $10^{3.0}$ CID_{50s} / ml WNV at different intervals after inoculation to make certain that both species took a blood meal from chickens with varying virus titers. Blood was drawn from each chicken immediately after the mosquitoes finished feeding and assayed to determine the WNV titer.

The JMP 5.0 statistical software (SAS Institute Inc., Cary, NC, USA) was used to analyze all data. Differences in infection rates between *Ae. vexans* and *Cx. pipiens* were determined by the Fisher's Exact Test at the 0.05 confidence level. Differences in infection

rates, dissemination rates, and transmission rates of *Ae. vexans* fed on chickens with high and low viremias were determined by the Fisher's Exact Test at the 0.05 confidence level.

RESULTS

West Nile virus infection rates of *Ae. vexans* and *Cx. pipiens*. Infection rates of *Ae. vexans* and *Cx. pipiens* determined 14 days after feeding on baby chickens with viremias that ranged from $10^{3.4}$ PFU / ml to $10^{7.0}$ PFU / ml are summarized in Table 1. The lowest observed infective serum WNV titer for *Ae. vexans* was $10^{4.5}$ PFU / ml. Two of 4 mosquitoes were infected. Infection rates of 100% were observed at serum WNV titers $\geq 10^{6.7}$ PFU / ml. The lowest infective serum WNV titer for *Cx. pipiens* was $10^{3.4}$ PFU / ml. One of 6 (17%) mosquitoes was infected. Six of 6 (100%) *Cx. pipiens* were infected after feeding on a baby chicken with a serum WNV titer of $10^{6.7}$ PFU / ml.

Infection $dose_{50s}$ of *Ae. vexans* and *Cx. pipiens* were $10^{5.9}$ and $10^{5.6}$ PFU / ml respectively. The linear regression models that were used to calculate these values were: $y = 24.4x - 94.4$ ($r^2 = 0.527$), and $y = 16.1x - 40.5$ ($r^2 = 0.464$) respectively, where y = % of mosquitoes infected, and x = the serum WNV titer of the chicken on which mosquitoes fed (Table 4).

West Nile virus dissemination rates in *Ae. vexans*. The dissemination rates of WNV in *Ae. vexans* at 14 and 21 days after feeding on viremic baby chickens are summarized in Table 2. Dissemination rates among *Ae. vexans* 14 days after feeding on baby chickens with serum WNV titers ranging from $10^{6.5}$ to $10^{8.2}$ PFU / ml ranged from 59 to 100%. The mean dissemination rate of *Ae. vexans* that fed on chickens with WNV titers $\leq 10^{6.8}$ PFU /ml was 59.5 ± 0.5 and mosquitoes that fed on chickens with serum WNV titers

Table 1. West Nile virus (WNV) infection rates of *Aedes vexans* and *Culex pipiens* 14 days after feeding on viremic baby chickens

Blood meal titer[a]	*Number chickens / titer*	*Infection rate*[b] *Aedes vexans (n)*	*Number chickens / titer*	*Infection rate Culex pipiens (n)*
3.4	1	0 (6)	1	17 (6)
4.5	1	50 (4)	1	33 (3)
5.0	1	0 (1)	1	100 (1)
5.4	1	60 (5)	1	67 (3)
5.6	1	0 (3)	-[c]	-
5.7	2	22 (18)	1	25 (4)
5.8	1	50 (4)	-	-
6.0	1	0 (1)	1	33 (6)
6.2	2	61 (9)	1	100 (1)
6.5	1	80 (5)	-	-
6.6	1	40 (5)	-	-
6.7	1	100 (6)	1	100 (6)
6.8	2	84 (7)	-	-
7.0	2	100 (5)	1	63 (8)
7.3	1	100 (5)	-	-
8.9	1	100 (5)	-	-

[a]Titer expressed as log10 PFUs / ml serum
[b]Infection rate = percentage of blood feeding mosquitoes that became infected after feeding on viremic chickens
[c]Not done

Table 2. Dissemination rates of West Nile virus (WNV) in *Aedes vexans* at 14 and 21 days after feeding on viremic chickens

Titer of blood meals [a]	*14 days after feeding*		*21 days after feeding*	
	Infection rate (n) [b]	*Dissemination rate(n)* [c]	*Infection rate (n)*	*Dissemination rate(n)*
5.2	-[d]	-	80 (5)	60 (5)
6.5	80 (5)	60 (5)	67 (3)	33 (3)
6.8	84 (7)	59 (7)	67 (6)	17 (6)
6.9	-	-	100 (4)	50 (4)
7.3	100 (5)	100 (5)	100 (2)	100 (2)
8.2	100 (5)	100 (5)	100 (7)	71 (7)
8.3	-	-	64 (28)	70 (25)
8.6	-	-	100 (3)	67 (3)
8.9	-	-	100 (2)	50 (2)

[a]Titer expressed as $\log_{10}$ PFUs / ml serum.
[b]Infection rate = percentage of blood-feeding mosquitoes that became infected with WNV after feeding on viremic chickens.
[c]Dissemination rate = percentage of blood-feeding mosquitoes with a disseminated infection as determined by detecting virus in legs.
[d]Not done

Table 3. The estimated transmission rates of West Nile virus (WNV) by *Aedes vexans* 21 days after feeding on viremic chickens

Titer of blood meals [a]	*Infection rate (n)* [b]	*Dissemination rate(n)* [c]	*Disseminated transmission rate(n)* [d]	*Estimated transmission rate* [e]
5.2	80 (5)	60 (5)	25 (4)	15
8.6	100 (3)	67 (3)	44 (12)	31

[a]Titer expressed as $\log_{10}$ PFUs / ml serum.
[b]Infection rate = percentage of blood-feeding mosquitoes that became infected with WNV after feeding on viremic chickens.
[c]Dissemination rate = percentage of blood-feeding mosquitoes with a disseminated infection as determined by detecting virus in legs.
[d]Disseminated transmission rate = percentage of mosquitoes with a disseminated infection with virus in saliva.
[e]Estimated transmission rate = dissemination rate (%) multiplied by the disseminated transmission rate (%) divide by 100.

Table 4. Estimated coefficients used to construct *Aedes vexans and Culex pipiens* logistic regression models describing WNV infection, and areas under the receiver operating characteristic curves.

Model	$ß_o$[a] ± SE[c]	$ß_1$[b] ± SE	Area under ROC curve[d]
Aedes vexans	3.28	-0.56	0.70
Culex pipiens	3.87	-0.69	0.70

[a]$ß_o$ = intercept
[b]$ß_1$ = slope
[c]SE = standard error
[d]Area under the receiver operating characteristic (ROC) curve indicates the goodness of fit between observed and predicted values. Values approaching 1 indicate a high degree of fit.

$\geq 10^{7.3}$ PFU / ml was 100%. The mean dissemination rate among all mosquitoes 14 days after feeding on viremic baby chickens was 79.8 ± 11.7%.

Dissemination rates among *Ae. vexans* determined 21 days after feeding on baby chickens with serum WNV titers ranging from $10^{5.2}$ to $10^{8.9}$ PFU / ml varied from a low of 17% (1/6) for mosquitoes that fed on a baby chicken with a titer of $10^{6.8}$ PFU / ml to 100% (2/2) for mosquitoes that fed on a baby chicken with a titer of $10^{7.3}$ PFU / ml. The mean dissemination rate among all mosquitoes 21 days after feeding on viremic baby chickens was 57.5 ± 8.0 %.

West Nile virus transmission by *Ae. vexans.* The estimated transmission rates of WNV by *Ae. vexans* after feeding on viremic baby chickens with serum WNV titers of $10^{5.2}$ PFU and $10^{8.6}$ PFU were 15 and 31% respectively (Table 3).

Estimated coefficients used to construct *Aedes vexans* and *Culex pipiens* logistic regression models describing WNV infection. Logistical regression models were generated using the data summarized in Tables 1 and 2 to predict the ID_{50s} and 95% confidence intervals of *Aedes vexans* and *Culex pipiens.* The estimated coefficients used to construct the *Aedes vexans and Culex pipiens* logistic regression models and areas under the receiver operating characteristic curves are summarized in Table 4. The ID_{50s} and 95% CI of the 2 species were 5.9 (4.7 – 6.5) and 5.6 (1.4 – 7.3) pfu / ml serum respectively.

DISCUSSION

The vector competence of *Ae. vexans* for WNV was evaluated in this study. It was unknown as to whether or not *Ae. vexans* becomes infected with WNV by feeding on viremic hosts. It was also unknown if this species develops a disseminated infection, and transmits WNV by bite. *Culex pipiens* was used as a control because it readily becomes infected when

fed on a viremic host (Turell et al. 2001, Dohm, Sardelis, and Turell 2002, Goddard et al. 2002).

Infection rates of *Ae. vexans* and *Cx. pipiens* fed on the same WNV viremic chickens were similar (Table 1). Both species became infected and at similar rates. Infection rates increased when both species were fed on hosts with high viremias (6.0 – 7.0 $\log_{10}$ PFUs / ml). For example, 1 of 3 (33 %) *Cx. pipiens* and 1 of 4 (25 %) *Ae. vexans* became infected at a titer of 4.5 $\log_{10}$ PFUs / ml. However 6 of 6 (100 %) *Cx. pipiens* and 6 of 6 (100 %) *Ae. vexans* became infected when fed on chickens with viremias of 6.7 $\log_{10}$ PFUs / ml. Goddard et al. (2002) noted that *Cx. pipiens* had higher infection rates (58 % to 100 %) than *Ae. vexans* (32 %) at 7.1 $\log_{10}$ PFUs / ml. This study proves that *Ae. vexans* becomes infected with WNV when fed on a viremic avian host.

Significantly different infection rates occurred when *Ae. vexans* were fed on chickens with high viremias and on chickens with low viremias. Birds maintain and amplify WNV in nature. Many avian species such as House Sparrows, House Finches, American Robins, American Crows, and Blue Jays typically develop high WNV titers (Brinton 2002, Komar 2003). Because *Ae. vexans* feeds on birds (Shemanchuk 1969, Horsfall et al. 1973, Loftin et al. 1997) it could be an important part of the natural history of this virus.

Dissemination rates of WNV in *Ae. vexans* fed on chickens with viremias of 6.0 $\log_{10}$ PFUs / ml or greater did not differ from those fed on chickens with viremias of less than 6.0 $\log_{10}$ PFUs / ml. Approximately 75 % of infected *Ae. vexans* developed disseminated infections (Table 2). Therefore, of *Ae. vexans* that feed and become infected, 75 % of the mosquitoes will develop disseminated infections, regardless of the amount of virus in the blood of the host.

Transmission rates were determined using the capillary tube method (Aitken 1977, Cornel and Jupp 1989). Transmission by *Ae. vexans* fed on chickens with low (< 6.0) WNV viremias and for those fed on chickens with high (≥ 6.0) WNV viremias were similar. Approximately 25 % (4 / 15) infected *Ae. vexans* transmitted WNV during feeding from capillary tubes. *Aedes vexans* is anthropophagic and, if infected, could transmit the virus to humans. *Aedes vexans* is also widespread and at the same time locally abundant in much of the U.S. A transmission rate of 25 % would seem to have serious epidemiologic consequences.

Capillary tube feeding was chosen to determine transmission rates for *Ae. vexans* rather than chickens or other animals because non-mated *Ae. vexans* are reluctant to feed a second time. *Aedes vexans* does not mate under laboratory conditions. Consequently, the numbers of *Ae. vexans* available for this type of study were limited. Furthermore, using animals for transmission studies involves other considerations, such as susceptibility of the animal to infection, immunity status, and the BSL-3 facilities required to properly hold the animals.

This study demonstrates that *Ae. vexans* is a competent and moderately efficient vector for WNV. It is susceptible to oral infection through feeding on viremic hosts and develops a disseminated infection. *Aedes vexans* also transmits WNV by bite and could be important in an epizootic transmission cycle of WNV. There is also the possibility *Ae. vexans* is a bridge vector of WNV, transmitting the virus from the mosquito-bird-mosquito cycle to mammalian hosts including humans.

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CHAPTER 4. PRELIMINARY STUDIES ON THE VERTICAL TRANSMISSION OF WEST NILE VIRUS BY *AEDES VEXANS* (MEIGEN) (DIPTERA: CULICIDAE)

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ABSTRACT

Aedes vexans (Meigen) mosquitoes were given access to 2- to 4-day-old chickens inoculated with $10^{6.0}CID_{50s}$ / ml West Nile Virus (WNV) to $10^{9.0}CID_{50s}$ / ml WNV and at 1 day old. Mosquitoes of varying age were given access to blood meals and allowed to lay eggs. Eggs were collected and hatched 14 days after deposition to ensure embryonation. Hatching rates were low; only 1.7% of the total eggs collected hatched. Additionally, lack of survival of progeny to the adult stage impacted the numbers of mosquitoes available for testing to evaluate vertical transmission of WNV. No infection of progeny with WNV was detected.

INTRODUCTION

The spread of WNV from New York City to California in only 4 years represents an unprecedented spread of an imported mosquito-borne disease. The ability of WNV to infect 198 species of birds (CDC website 2004, Gould and Fikrig 2004) and 43 species of North American mosquitoes (CDC website 2004, Gould and Fikrig 2004) may explain how WNV spread so rapidly (Personal Communication, Gubler 2003). *Aedes vexans* (Meigen) is one of the most widespread and locally abundant nuisance mosquitoes in North America. It occurs

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in all 50 states of the U.S. and in all provinces of the Canada except Newfoundland, Labrador, and some areas of the Northwest Territory (Crans 2004). In addition to being abundant and wide spread, *Ae. vexans* is active from approximately 45 minutes before sunset to 120 minutes after sunset (Wright and Knight 1966). *Ae. vexans* is anthropophagic, but it also can be an opportunistic feeder. It feeds on birds if mammalian hosts are not available (Shemanchuk 1969, Horsfall et al. 1973, Loftin et al. 1997). *Aedes vexans* is a bridge vector of Eastern Equine Encephalitis virus (EEE) from birds to humans and horses (Moncayo and Edman 1999). It may also bridge WNV from the avian amplification cycle to mammals such as humans and horses. Female *Ae. vexans*, when infected with West Nile Virus, may vertically transmit the virus from parent to progeny. A virus transmitted from one generation to the next through infected eggs is transovarial transmission (TOT) (Thrusfield 1995). TOT in *Ae. vexans* is a possible overwintering mechanism for WNV in nature and may maintain the virus in nature during inter-epidemic periods as *Ae. vexans* overwinter as diapausing eggs. If *Ae. vexans* transovarially transmits the virus, this would be of considerable epidemiological importance in the natural history of the virus. The objective of this study was to determine if *Ae. vexans* transovarially transmits WNV after being fed on a viremic host.

MATERIALS AND METHODS

Mosquitoes. Female *Ae. vexans* were trapped with CO_2-baited CDC light traps at several locations in central Iowa. Field-collected mosquitoes were placed into 0.5 L paper cups with a cloth mesh screen on the top. Random pools of trapped mosquitoes were tested for WNV using VecTest® to determine whether any field collected *Ae. vexans* were infected with the virus. Mosquitoes were allowed to take a blood meal from an anesthetized rabbit.

Engorged mosquitoes were separated from those that did not feed and were held in cups for 72 h at 26°C ± 1° and 80 % ± 5 % RH. A cotton pad saturated with 0.3 M sucrose was placed on each cup. Gravid mosquitoes were transferred to oviposition cages (OP cages) described by Christensen et al. (1978). One end is clear plexiglass and the other is plexiglass with a hole that can be fitted with a rubber stopper. Ten mosquitoes were placed into an OP cage. Oviposition cages were placed in trays (34.3 x 25.4 cm x 3.8 cm) lined on the bottom with moist absorbent cotton wrapped (covered) with cheesecloth. Gravid mosquitoes had access to cotton pads saturated with 0.3 M sucrose. Mosquitoes were held at 26°C ± 1°C and 80 % ± 5 % RH in a 16:8 photoperiod. Eggs were collected and held for 14 days at to allow for embryonation after which, they were stored at 4°C until hatched. Eggs were placed at 26°C ± 1°C and 80 % ± 5 % RH for 14 days before being submerged in deoxygenated water. Brewers' yeast (0.02 g) was added to the water to stimulate hatching. Larvae were fed pulverized Tetramin®. Pupae were removed from rearing pans and placed in 0.5 L cups containing a small amount of water (50 pupae per cup). After emergence, adults were separated by date of emergence and sex.

Ae. vexans does not mate in captivity, therefore, to obtain fertile eggs from laboratory-reared mosquitoes, each female must be manually copulated. A technique modified from techniques developed by McDaniel and Horsfall (1957) and Yang et al. (1963) was used. Males and females were maintained separately in lots of 50. Four-day-old (± 2 days) male mosquitoes were aspirated individually between 2 layers of cheesecloth. A minuten pin attached to a wooden applicator stick was inserted through lateral side of the thorax. The legs, wings, and head were carefully removed. Three to 5 males were prepared at a time. One- to 3-day-old (± 1 day) females were aspirated into clear plastic 15 ml tubes,

lightly anesthetized with nitrogen gas and placed dorsum down under a dissecting microscope. A pinned male was presented ventral side down and at an angle of 45 degrees to the female. After copulation was initiated (i.e. after the male's claspers have grasped the female genitalia), the pair was lifted off the substrate and held until the male released the female. Mated females were transferred to cups.

Chickens. One-day-old WNV antibody-free white leghorn chickens (*Gallus gallus*) were obtained from a commercial chicken hatchery (Hoover's Hatchery, Inc., Rudd, IA) and housed in Biosafety Level 3 (BSL3) containment facilities.

Cells and Media. Vero-76 cell cultures were used for virus propagation and isolation. The cell culture medium was a CO_2 independent growth medium (CIM) consisting of Dulbecco's modified Eagle's medium (GIBCO®, Invitrogen Corp.) with 10 % heat inactivated fetal bovine serum (FBS), 2.0 mM of L-glutamine, and 20 mg gentamicin sulfate (GentaMax™100, Phoenix Pharmaceutical Inc.) per 100 ml of medium (Tiawsirisup et al. 2004). Mosquitoes were processed and tested for virus in CIM supplemented with 20 % FBS.

Virus. West Nile Virus (IA 2002-crow) was obtained from the brain of a crow, *Corvus brachyrhynchos* Brehm, found dead in Ames, Iowa. The virus was passed 3 times in Vero-76 cell cultures and then froze at -70°C until used to inoculate chickens.

Virus Assay. Virus was assayed in Vero-76 cell cultures to determine titer levels in individual chicken serum. Twenty-five cm^2 cell culture flasks were inoculated with 1 ml of serial 10-fold dilutions of serum prepared in CIM containing 1 % FBS. After a 1 h incubation period, 4 ml of CIM with 1 % FBS, 1 % Agar Noble (DIFCO®, Becton Dickinson), 3.0 mM of L-glutamine, and 20 mg of gentamicin per 100 ml were added to the

flasks (Tiawsirisup et al. 2004). Four days later, 5 ml of identical agar with 0.004 % neutral red dye was overlaid on the cell cultures (Tiawsirisup et al. 2004). Plaques were counted and titers were expressed as plaque forming units (PFU) / ml.

Mosquitoes were placed in 300 µl of CIM supplemented with 20 % FBS and pulverized with a mechanical pestle. Samples were placed in 1.7 ml of CIM with 10 % FBS and was used to inoculate Vero-76 cells via a 0.45 µm filtered syringe. After 4, 6, and 8 days post inoculation CPE was noted. After day 8 CPE, all cell cultures were frozen at -70°C. West Nile Virus presence or absence in cell cultures was confirmed by RT-PCR.

RT-PCR. RNA was extracted from cell culture medium using QIAamp viral RNA kits (QIAGEN Inc.). Reverse transcriptase-polymerase chain reaction for WNV specific RNA was conducted as described by Lanciotti et al. (2000) with 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.).

Infection. Manually copulated 5- to 15-day-old *Ae. vexans* and were held in 0.5 L cups. Cotton pads with 0.3 M sucrose were removed 48 h and water was removed 12 h prior to blood feeding. Mosquitoes were provided access to 2- to 4-day-old viremic chickens by placing the chicken in a nylon sock. Mosquitoes were allowed to feed for 30 min. Engorged *Ae. vexans* were aspirated from the paper cups using a venturi aspiration system, and held individually at 26°C ± 1°C and 80 % ± 5 % RH with a 16:8 photoperiod in ovipositional cages (OP cages) for 3 days. OP cages have sides of 1/8" plexiglass, the top was a fine mesh screen and the bottom was a metal screen large enough to allow eggs to fall through

(Christensen et al. 1978). One end is solid, clear plexiglass and the other end is plexiglass with a hole that can be fitted with a rubber stopper. The cages were placed in trays (34.3 x 25.4 cm) lined on the bottom with moist absorbent cotton wrapped (covered) with cheesecloth. Cotton pads (approx. 4 cm in diameter) saturated with 0.3 M sucrose solution were placed on top of the ovipositional cages. Eggs were collected and held 14 days at 26°C to allow for embryonation after which, they were submerged in deoxygenated water with 0.02 g of Brewers' yeast for 24 hrs to stimulate hatching. Larvae were provided pulverized Tetramin® as a food source. Pupae were picked out of the ceramic larval rearing pans and placed in 0.5 L cups containing a small amount of water. After emergence, adults were held for 4 – 5 days and then separated by emergence date and sex and tested for the presence of WNV.

RESULTS

141 of 353 *Ae. vexans* fed to repletion on viremic chicks and were allowed to deposit eggs. The time required for first clutch egg deposition varied from 13- to 25-days-post-feeding. A total of 1762 eggs were deposited. Hatching rates of *Ae. vexans* were low. Only 30 of 1762 eggs hatched and only 8 of the 30 hatched eggs developed and emerged as adults available for WNV testing. West Nile Virus was not detected in any progeny adults. Ultimately, not enough mosquitoes were available for testing to determine if vertical transmission occurs. In other examples of vertical transmission, rates were very low, such as a minimum infection rate of 1 infected mosquito per 1000 mosquitoes tested (Dohm, Sardelis, and Turell, 2002). More infected egg laying mosquitoes and more viable eggs were needed to conclusively complete this study.

DISCUSSION

Vertical transmission of WNV in *Ae. vexans* was evaluated in this study. It is unknown if *Ae. vexans* transmits WNV vertically. Only 1.7% of the 1762 eggs laid by 141 *Ae. vexans* fed on viremic chicks hatched and survived to the adult stage for testing. Vertical transmission of WNV in mosquitoes may serve as an important maintenance mechanism in temperate regions of the world and during periods of vector inactivity. Additionally, the role of vertical transmission in the amplification cycle may explain the early onset of virus activity in many areas where the WNV has become endemic. When considering the host preferences of *Ae. vexans*, it is important to continue evaluating the role of vertical transmission of WNV in *Ae. vexans*. Evidence of vertical transmission has been shown for many mosquito species and with many viruses.

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CHAPTER 5. GENERAL CONCLUSIONS

Summary of Experimental Results.

Aedes vexans is a competent and moderately efficient vector for WNV. *Aedes vexans* readily fed on avian hosts and became infected with WNV by feeding on WNV viremic chickens. Infection rates of *Ae. vexans* were not significantly different from infection rates of *Cx. pipiens* fed on the same WNV viremic chicken. This shows that *Ae. vexans* feeds on avian hosts, the primary reservoirs for WNV, and is susceptible to infection at rates similar to *Cx. pipiens*, a primary vector for WNV. There was a difference in infection rates of *Ae. vexans* fed on chickens with low WNV viremias and infection rates of those fed on chickens with high WNV viremias.

When fed on a viremic avian host, *Ae. vexans* developed a disseminated infection. Dissemination rates in *Ae. vexans* infected at low WNV viremias were not different from dissemination rates in *Ae. vexans* infected at high WNV viremias. A disseminated infection, although important for determining vector competence, does not mean *Ae. vexans* is capable of transmitting WNV. It does show that WNV is disseminating through the body of the mosquito.

Aedes vexans transmitted WNV during feeding using a capillary tube method developed by Aitken (1977) and Cornel and Jupp (1989). There was not a difference in transmission rates of *Ae. vexans* infected at low WNV viremias and transmission rates of *Ae. vexans* infected at high WNV viremias. Transmission of WNV by *Ae. vexans* is a function of infection, not the level of virus in host blood.

Aedes vexans could be a competent bridge vector and could contribute to an epizootic cycle of WNV in the U.S. Since *Ae. vexans* primarily feeds on mammals, but will opportunistically feed on birds, the mosquito could transmit WNV from the normal bird-mosquito-bird cycle to humans, causing outbreaks of WNV in humans.

Recommendations for Future Research.

Because *Ae. vexans* is an important nuisance mosquito in much of the U.S. and was determined to be a competent vector for WNV, future research needs to focus on the mosquito's role as a bridge vector for WNV. The first step is to demonstrate transmission by feeding infected *Ae. vexans* on animals. Using animals is time-consuming, expensive, difficult to conduct due to animal care restrictions, and requires the need for proper BSL-3 facilities. However, animal feeding studies are required to generate data that are comparable to what occurs in nature. Studies are needed to determine the extrinsic incubation period to completely define the vector competence of *Ae. vexans*. Finally, an examination of the contribution *Ae. vexans* makes to the maintenance of WNV in nature, whether it be through vertical transmission to progeny, or through transmission in a mammalian enzootic cycle is necessary.

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