DISSERTATION

FLAVIVIRUS SURVEILLANCE IN MOSQUITOES FROM NORTHERN COLORADO, WITH THE DETECTION AND DESCRIPTION OF TWO INSECT-SPECIFIC FLAVIVIRUSES

Submitted by

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY BETHANY G. BOLLING ENTITLED FLAVIVIRUS SURVEILLANCE IN MOSQUITOES FROM NORTHERN COLORADO, WITH THE DETECTION AND DESCRIPTION OF TWO INSECT-SPECIFIC FLAVIVIRUSES BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

FLAVIVIRUS SURVEILLANCE IN MOSQUITOES FROM NORTHERN COLORADO, WITH THE DETECTION AND DESCRIPTION OF TWO INSECT-SPECIFIC FLAVIVIRUSES

Vector-borne diseases remain a major public health concern worldwide. The studies described here underline the importance of combining field surveillance activities with laboratory experiments to provide a comprehensive understanding of the dynamics of vector-borne disease systems.

Entomological measures of West Nile virus (WNV) risk were found to be strongly associated with human WNV disease cases in northeastern Colorado. Specifically, *Culex tarsalis* abundance and the Vector Index for WNV-infected *Cx. tarsalis* females (weekly mean per trap night x weekly proportion of WNV-infected females) were associated with weekly numbers of WNV human disease cases with lag times of 4-7 weeks and 1-2 weeks, respectively. This provides information that can be utilized for decision-making processes concerning when to initiate mosquito control activities and how to best utilize limited resources.

These studies also describe the first detection of insect-specific flaviviruses in mosquitoes collected in Colorado. *Culex* flavivirus (CXFV), first described in Japan in 2007, and a new insect-specific flavivirus, designated Calbertado virus, were detected in

Culex spp. mosquitoes. Experiments were conducted to explore the transmission dynamics of CXFV in a naturally infected *Culex pipiens* laboratory colony and also the potential effects of CXFV infection on vector competence for WNV. Results indicated that vertical transmission is the primary mechanism for viral persistence in the colony, with venereal transmission perhaps playing a supplemental role. Vector competence experiments suggested possible suppression of WNV replication by persistent CXFV infection. These findings are important as insect-specific flavivirus transmission dynamics have not been described yet and studies investigating putative interactions between insect-specific flaviviruses with arboviruses, like WNV, are lacking.

This work provides a better understanding of local vector-borne disease systems, providing a basis for additional studies to further characterize the dynamics of co-circulating flaviviruses in vector mosquitoes.

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CHAPTER 1

LITERATURE REVIEW

Introduction

Understanding factors that influence vector-borne disease systems is critical for modeling disease risk patterns, which in turn provide guidance for efficient use of prevention, surveillance, and control resources (Eisen and Eisen, 2008). Vector-borne diseases are complex systems, with different variables contributing to the interactions between pathogen, vector, and vertebrate host (Moore 2008). This research examines some of these variables by 1) determining the utility of different entomological risk measures for forecasting human West Nile virus disease cases; 2) describing the seasonal and spatial prevalence of co-circulating flaviviruses in *Culex* spp. mosquitoes; 3) investigating natural maintenance and transmission cycles of insect-specific flaviviruses; and, 4) by evaluating possible interactions of heterologous flaviviruses in cell culture and in mosquitoes.

Flaviviridae

Viruses in the genus *Flavivirus* (Family *Flaviridae*) are single-stranded, positive-sense, RNA viruses found on every continent except Antarctica (Gould et al., 2003). They can be divided into four groups based on ecological niches and phylogenetic analyses (Fig. 1.1): mosquito-borne, tick-borne, insect-specific, and no-known-vector groups (Gould et al., 2003, Cook and Holmes, 2006, Lobo et al., 2009). Most flaviviruses are arthropod-borne (arboviruses), maintained in nature by hematophagous arthropod transmission between susceptible vertebrate hosts, and many are human and veterinary pathogens. Arthropod-borne flaviviruses include the mosquito-borne and tick-borne groups, containing viruses capable of replicating in vertebrate and invertebrate cells

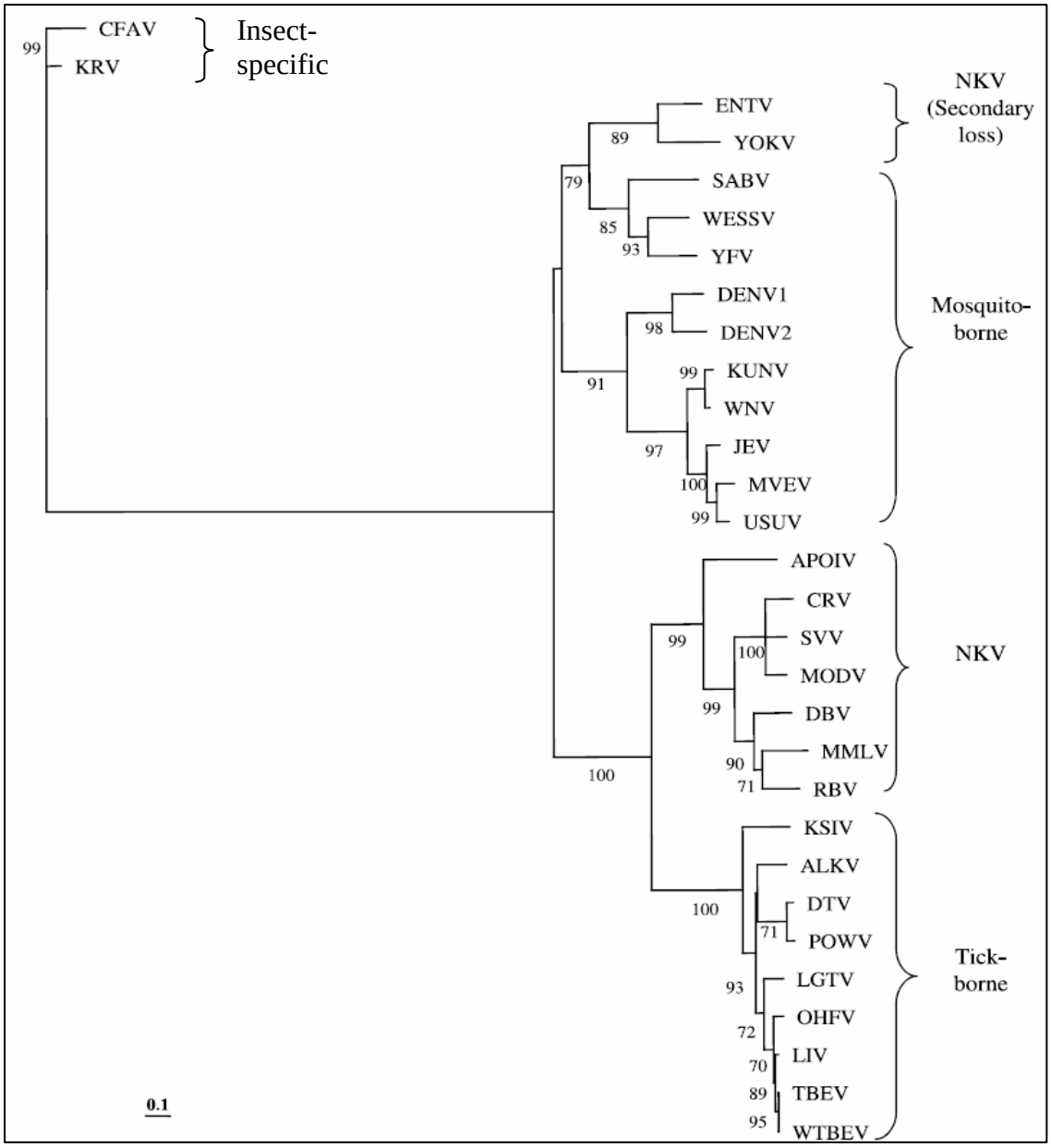

Figure 1.1. Phylogenetic relationships of the genus *Flavivirus* inferred using the NS3 gene (Cook and Holmes 2006).

(Cook and Holmes, 2006). Insect-specific flaviviruses are able to replicate solely in invertebrate cells and have been isolated from insect cell lines and numerous species of field-collected mosquitoes (Lobo et al., 2009). No-known vector flaviviruses are a paraphyletic group containing viruses that have only been found infecting vertebrate hosts, namely bats and rodents (Gaunt et al., 2001). Some examples of mosquito-borne flaviviruses that cause significant impacts on human health are dengue virus, yellow fever virus, and West Nile virus (WNV). Dengue virus alone is responsible for causing over 50 million cases of dengue fever worldwide per year, with approximately 2.5 billion people living in risk areas (WHO 2009).

The different transmission modes of flaviviruses are strongly correlated with phylogeny (Cook and Holmes, 2006), providing valuable insight into vector-pathogen relationships. The mosquito-borne flavivirus group can be divided into two distinct categories (Gaunt et al., 2001). The first category contains the neurotropic viruses, associated with encephalitic disease in humans, which are maintained in transmission cycles between *Culex* species and bird reservoirs. The second category contains the non-neurotropic viruses, which are maintained in transmission cycles involving *Aedes* species and primate hosts, and are more associated with hemorrhagic clinical manifestations in humans. These correlations provide evidence of the importance of the vector species and host species in flavivirus evolution (Gaunt et al., 2001). Flaviviruses are RNA viruses and thus demonstrate higher mutation rates and greater genetic plasticity, compared to DNA viruses, because of their error-prone polymerase and lack of proofreading capacity (Holland and Domingo, 1998). This provides RNA viruses with a mechanism to adapt to the selective constraints imposed by particular environments, vector species, and host

species. Components of arbovirus transmission cycles that also contribute to genetic variation include: 1) mosquitoes feed several times during their life, 2) virus titers can reach high levels in the mosquito and vertebrate host, and 3) viral infections in mosquitoes are persistent, resulting in a very dynamic system (Gould et al., 2003).

West Nile virus

West Nile virus (WNV) is a mosquito-borne flavivirus maintained in a natural transmission cycle involving birds and *Culex* mosquitoes (Hayes et al., 2005). It was first isolated in 1937 from a febrile woman in the West Nile district of Uganda (Smithburn et al., 1940). The emergence in 1999 (Lanciotti et al., 1999) and subsequent spread of WNV in the United States (Fig. 1.2), has demonstrated the need for improved vector control and disease surveillance programs (Beaty 2005). It has subsequently expanded its geographic range, now existing on all continents except Antarctica, making it the most widely distributed arbovirus in the world (Kramer et al., 2008). West Nile virus is capable of causing severe meningitis and encephalitis in humans and it continues to be an important public health concern in the United States, with 1,370 cases and 37 deaths occurring in 2008 (CDC 2008). Considerable resources have been directed toward prevention efforts, but these strategies have not been effective or sustainable in many settings (Petersen and Roehrig, 2007).

Knowledge of the spatial and temporal dynamics of mosquito vectors and WNV is important for both efficient mosquito control programs and for emphasizing personal protection measures for the public. Vector-borne disease systems are complex, with numerous factors contributing to the interactions between vector, vertebrate host, and

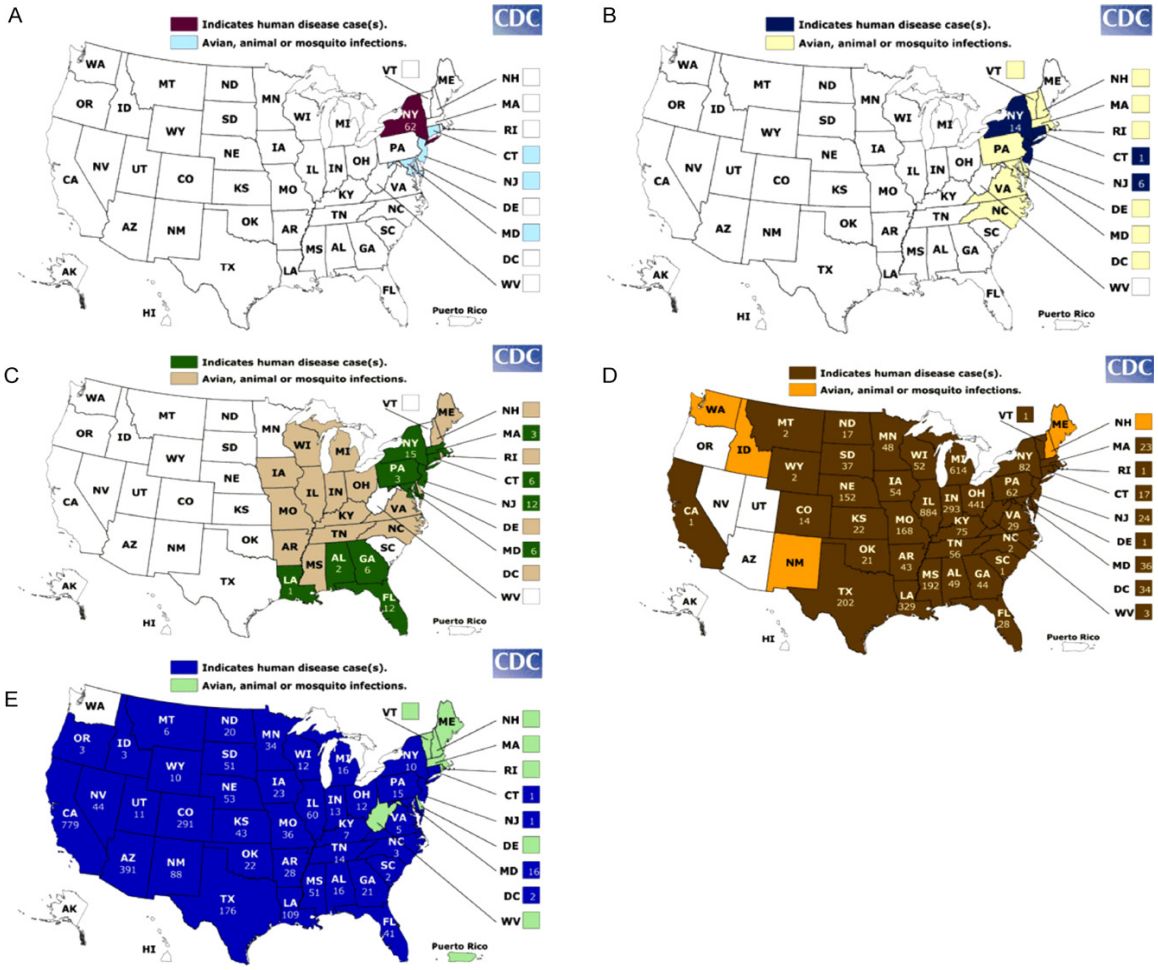

Figure 1.2. Dispersal of West Nile virus in the USA during: (A) 1999; (B) 2000; (C) 2001; (D) 2002; (E) 2003 (http://www.cdc.gov/ncidod/dvbid/westnile/background.htm).

pathogen (Fig. 1.3) (Moore 2008). Environmental variables are important to consider when predicting vector-borne disease risk. Climate is a major factor contributing to the distribution and abundance of arthropods, the duration of arthropod life cycles, the dispersal and evolution of arboviruses, and the vector transmission efficiency of arboviruses to vertebrate hosts (Gould and Higgs, 2009). Specifically, warm temperatures tend to enhance pathogen transmission by 1) increasing vector populations, 2) increasing the frequency of blood feeding and oviposition, thus increasing vector-host contact, and

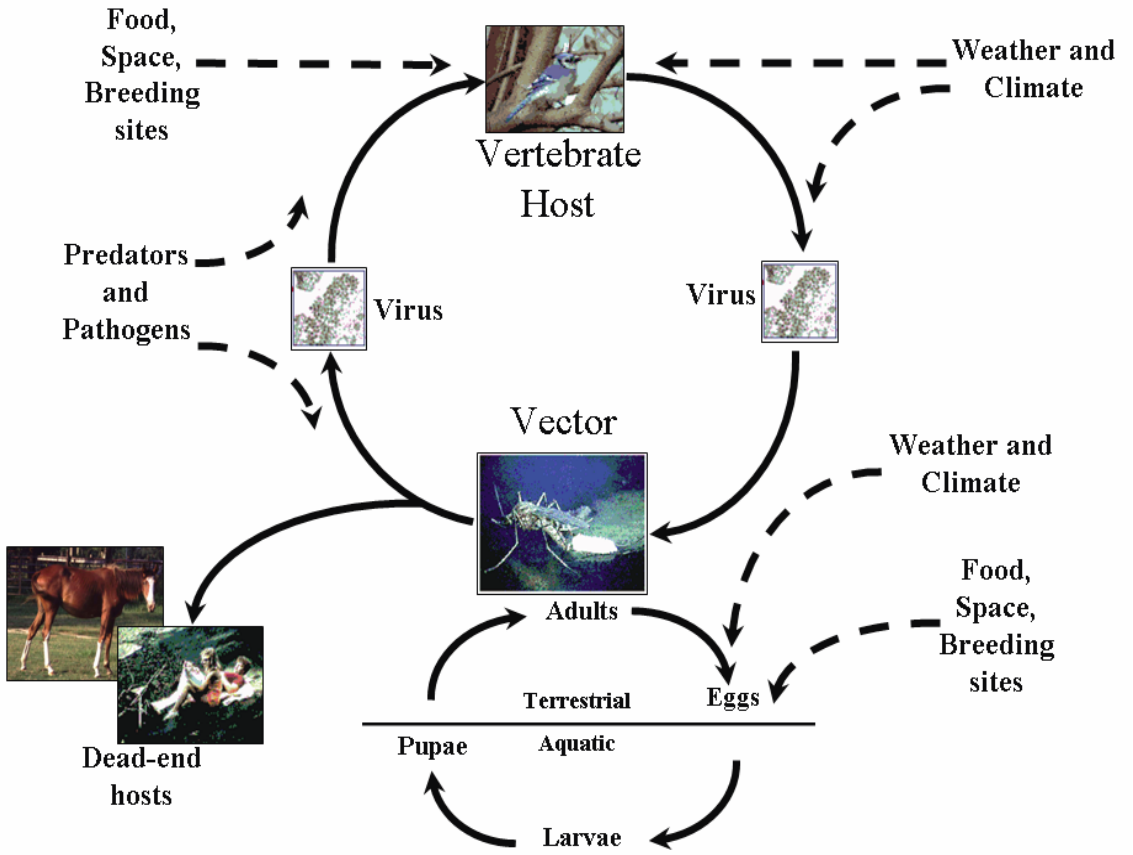

Figure 1.3. Typical transmission cycle of a vector-borne agent, in this case a mosquito-transmitted arbovirus such as West Nile virus (Moore 2008).

3) increasing the rate of pathogen development in the vector, resulting in a shorter extrinsic incubation period (Reisen 2010). Most vector-borne transmission occurs during the warmest periods of the year, but there is a threshold above which elevated temperatures can have negative effects on vector survival (Reisen 1995, 2010). Precipitation is another important environmental variable affecting mosquito-borne disease systems, as it determines the quantity and quality of larval habitats and therefore adult population size (Reisen et al., 2007). Recent studies have indicated that periods of drought can enhance arbovirus transmission (Monath 1980, DeGroote et al., 2008, Brault

2009, Jacob et al., 2009) by bringing avian hosts into close contact with vector mosquitoes (Shaman et al., 2004, Shaman et al., 2005). Landuse patterns can also play a role in the quantity and quality of larval breeding habitats (DeGroote et al., 2008, Winters et al., 2008a, Winters et al., 2008b, Jacob et al., 2009, Liu et al., 2009, Liu and Weng 2009, Ward et al., 2009). For example, irrigated agricultural areas can serve as productive larval habitats (Rapp 1985, Reisen et al., 1992a, Gates and Boston, 2009), as well as urban areas with bird baths and swimming pools left un-maintained (Reisen et al., 2009).

West Nile virus is maintained and amplified in nature in enzootic transmission cycles involving mainly *Culex* species mosquitoes and avian hosts, with humans and horses serving as incidental hosts. Ornithophilic mosquitoes tend to serve as amplifying hosts (*Culex pipiens* L., *Culex quinquefasciatus* Say, *Culex nigripalpus* Theobald, and *Culex tarsalis* Coquillett), while more catholic feeders (*Aedes albopictus* Skuse) and mosquitoes that change host preference during the transmission season (*Cx. nigripalpus* and *Cx. tarsalis*), may serve as important bridge vectors (Turell et al 2005). Primary vector species vary by geographic location (Konrad et al., 2009), with *Cx. tarsalis* considered to be the primary WNV vector in the western states (Goddard et al., 2002, Bolling et al., 2007), *Cx. pipiens* in the eastern states (Andreadis et al., 2004), and *Cx. quinquefasciatus* and *Cx. nigripalpus* in the southeastern states (Godsey et al., 2003, Rutledge et al., 2003). Other routes of human infection that have been documented are blood transfusions, organ transplantation, and breastfeeding, (Kramer et al., 2007) but it is unclear what effects these have on the overall dynamics of WNV transmission. West Nile virus continues to be an important public health concern in the U.S and understanding the complex transmission cycle of this disease will help to form the basis

for surveillance, prevention, and control programs.

Combining vector abundance data with WNV infection rates to estimate a vector index (Nasci et al., 2005) can provide a comprehensive tool to assess disease risk (Bolling et al., 2009), but few studies have taken this approach (Bell et al., 2005, Gujral et al., 2007). The vector index was developed by the CDC using the parameters of species composition, population density, and infection rate, which can be used as a threshold for vector management decisions. A recurring theme in recent WNV literature is the need for a predictive model to aid in efforts for reducing disease risk. Incorporation of the vector index in forecasting WNV risk spatially and temporally appears to be a promising system (Bolling et al., 2009).

Insect-specific flaviviruses

The genus *Flavivirus* contains over 70 single-stranded, positive-sense RNA viruses, most of which are arthropod-borne (arboviruses), transmitted between vertebrate hosts by mosquitoes or ticks. Arboviruses are capable of replicating in vertebrate and invertebrate cell lines. There is a group within the genus *Flavivirus*, referred to as insect-specific flaviviruses, which has been found to only replicate in invertebrate cells. Cell fusing agent virus (CFAV) was the first insect-specific flavivirus described (Stollar and Thomas 1975). It was isolated from an *Aedes aegypti* cell line that resulted in massive syncytia formation when co-cultivated with *Aedes albopictus* cells (Stollar and Thomas 1975). The complete nucleotide sequence for CFAV was determined approximately 15 years later and was found to be distantly related to other flaviviruses based on deduced amino acie sequences (Cammisa-Parks et al., 1992). Sequence identities between CFAV and other flaviviruses were highest for NS5 and NS3 genes, with 45% and 34%

similarities, respectively (Cammissa-Parks et al., 1992). Recently, isolates of CFAV have been detected in field-caught *Aedes* and *Culex* spp. mosquitoes from Puerto Rico (Cook et al., 2006). Phylogenetic analyses of the genus *Flavivirus* suggest that CFAV may represent a basal lineage of the genus (Cammissa-Parks et al., 1992, Marin et al., 1995).

Almost 25 years after the first insect-specific flavivirus was isolated, a second virus, Kamiti River virus (KRV), was isolated from *Aedes macintoshi* larvae and pupae collected from flooded dambos in Kenya and was found to be related to CFAV (Crabtree et al., 2003, Sang et al., 2003). As seen with CFAV, KRV did not antigenically cross-react with other arboviruses and it failed to replicate in vertebrate cells. Kamiti River virus did cause CPE, but not cell fusion in infected cells, a characteristic of CFAV (Crabtree et al., 2003). Comparison of amino acid sequences showed 81% sequence identity for NS5 between KRV and CFAV, but only ~45% identity for NS5 between KRV and other flaviviruses (Crabtree et al., 2003). Interestingly, sequences closely related to CFAV and KRV were found integrated into the genomes of laboratory-bred and field-caught *Aedes* spp. mosquitoes, generating questions about possible integration mechanisms involved and potential effects on flavivirus evolution (Crochu et al., 2004, Roiz et al., 2009).

A third insect-specific flavivirus, *Culex* flavivirus (CXFV), was detected in 2007 in *Culex* spp. mosquitoes collected in Japan (Hoshino et al., 2007). Isolates were made from *Cx. pipiens*, *Culex tritaeniorhynchus* Giles, and *Cx. quinquefasciatus* mosquito pools. Phylogenetic analysis of E protein amino acid sequences of CXFV and other flaviviruses revealed that CXFV clustered with CFAV and KRV in the insect-specific group (Hoshino et al., 2007). Similar to previously described insect-specific flaviviruses,

Culex flavivirus did not infect vertebrate cells, but it also did not cause severe CPE in C6/36 cells, as seen with CFAV and KRV (Hoshino et al., 2007). *Culex* flavivirus has since been detected in *Cx. pipiens* from Iowa (Blitvich et al., 2009), California (Tyler et al., 2010), and Colorado (Bolling et al., 2010), *Cx. quinquefasciatus* mosquitoes from Guatemala (Morales-Betoulle et al., 2008), Mexico (Farfan-Ale et al., 2009), Trinidad (Kim et al., 2009), Texas (Kim et al., 2009), and Uganda (Cook et al., 2009), *Cx. tarsalis* from Iowa (Blitvich et al., 2009), California (Tyler et al., 2010), and Colorado (Bolling et al., 2010), and *Cx. restuans* from Texas (Kim et al., 2009). The CXFV strains isolated from various geographic locations are genetically similar (< 10% nucleotide sequence difference) and yet there are phenotypic differences in the presence and type of cytopathic effect observed in C6/36 cells. The original isolate from Japan appeared to cause minor growth inhibition and cell aggregation in C6/36 cells, but only after 4 passages (Hoshino et al., 2007). Similar findings were also described for isolates from Guatemala (Morales-Betoulle et al., 2008), Mexico (Farfan-Ale et al., 2009), Iowa (Blitvich et al., 2009), Uganda (Cook et al., 2009), and Colorado (Bolling et al., 2010). Interestingly, further studies with the Guatemalan CXFV isolate revealed striking differences in CPE, with substantial cell death (Kent et al., 2010), contrary to the previous description (Morales-Betoulle et al., 2008). Differences in CPE were also seen among the 7 isolates from Texas, where one isolate produced marked CPE with syncytia formation, similar to CFAV, while the other 6 isolates did not (Kim et al., 2009). Further studies are needed to determine the genetic basis for these variable CPEs.

In the past few years, several new insect-specific flaviviruses have been isolated and characterized. Quang Binh virus was isolated in Vietnam from *Cx. tritaeniorhynchus*

mosquitoes collected in 2002 (Crabtree et al., 2009). *Aedes* flavivirus, isolated from *Aedes albopictus* and *Aedes flavopictus* mosquitoes in Japan, groups with other insect-specific flaviviruses based on full length genome sequences, but based on sequences of the NS1, NS2A, NS2B, and NS3 proteins and putative protein cleavage sites, it has a high degree of similarity to cell silent agent (CSA), which is a flavivirus-related nucleotide sequence found integrated into the genome of *Ae. albopictus* (Crochu et al., 2004). Two other recently described flaviviruses, Nounané virus, isolated from *Uranotaenia mashonaensis* in West Africa (Junglen et al., 2009), and Lammi virus, isolated from *Aedes cinereus* from Finland (Huhtamo et al., 2009), appear to represent a distinct group of insect-specific flaviviruses. Phylogenetic analyses group them with mosquito-borne flaviviruses that cause disease in vertebrates, such as West Nile and Japanese encephalitis viruses, yet they do not replicate in vertebrate cells (Junglen et al., 2009, Huhtamo et al., 2009). Calbertado virus, named from detections made in California, Alberta (Canada), and Colorado, shares approximately 70% identity to CXFV, based on NS5 nucleotide sequences, and has been detected in *Cx. tarsalis* and *Cx. pipiens* mosquitoes (Tyler et al., 2010, Bolling et al., 2010). Several insect-specific flaviviruses have also been isolated in Spain and described as KRV-and CFAV-related, but additional studies are needed to further characterize these isolates (Aranda et al., 2008, Sánchez-Seco et al., 2009). Of note, flavivirus RNA, closely related to described insect-specific flaviviruses, was detected in two pools of *Phlebotomus perniciosus* (sandflies) collected in Algeria, representing the first detection of an insect-specific flavivirus in a non-mosquito dipteran (Moureau et al., 2009). Research on insect-specific flaviviruses is rapidly expanding, and as new viruses are being isolated and characterized, it will be

interesting to see how the evolutionary implications affect current theories about flavivirus phylogeny.

Transmission

Arbovirus transmission cycles have three essential components: 1) the virus, 2) the hematophagous arthropod vector, and 3) the vertebrate host (Kuno and Chang, 2005). Arboviruses are maintained in nature by propagative biological transmission, where the virus replicates in the vector and vertebrate host, and remains in the same developmental form (Higgs and Beaty, 2005). Thus, arboviruses must be capable of infection and replication in two disparate systems: the poikilothermic invertebrate vector and the homeothermic vertebrate host (Higgs and Beaty, 2005). Arboviruses are maintained by ongoing transmission between arthropod vectors and vertebrate hosts by sustained infections in the vector, host, or both and by perpetuation through adverse seasons by using various survival mechanisms (Kramer and Ebel, 2003, Higgs and Beaty, 2005). Some arboviruses survive trans-seasonal periods by vertical and/or horizontal transmission strategies. Vertical transmission refers to the transfer of a pathogen from a parent to his or her progeny (Fine 1981). This can occur by a female arthropod infecting her offspring through transovarial transmission, where the virus infects the germ layer of the developing egg, or transovum transmission, where virus is on the egg surface, or by a male arthropod infecting progeny via seminal fluid (Higgs and Beaty, 2005). Horizontal transmission between male and female vectors can occur by venereal transmission during copulation or between female vectors via a viremic vertebrate host (Higgs and Beaty, 2005) or by co-feeding on a nonviremic host (McGee et al., 2007).

Arboviruses in the genus *Flavivirus* are primarily maintained in nature through biological transmission between blood feeding arthropods and susceptible vertebrate hosts, but vertical and horizontal transmission strategies appear to play a role as well. Vertical transmission of a flavivirus was first described in Senegal with isolation of Koutango virus from a male *Aedes aegypti* (Coz et al., 1976). Since then, there have been numerous descriptions of flaviviruses isolated from larvae or male mosquitoes, including Japanese encephalitis virus from *Culex tritaeniorhynchus* (Rosen et al., 1978), yellow fever from *Ae. aegypti* (Fontenille et al., 1997), and WNV from *Culex univittatus* (Miller et al., 2000), and also laboratory studies have demonstrated vertical transmission of flaviviruses (Tesh et al., 1977, Beaty et al., 1980, Nayar et al., 1986, Shroyer 1990b). Apparently, flaviviruses are vertically transmitted at the time of oviposition, during fertilization via the micropyle, as the fully developed egg passes through the oviduct (Higgs and Beaty, 2005). This is much less efficient compared to "true" transovarial transmission, where the virus infects the developing egg (Kramer and Ebel, 2003). Filial infection rates seen with vertically transmitted flaviviruses in mosquitoes are usually low (less than 1%), compared to much higher rates seen with bunyaviruses (Tesh 1984). Horizontal transmission of arthropod-borne flaviviruses between adult mosquitoes has also been documented. Venereal transmission was demonstrated with dengue-infected males transmitting to females (Rosen 1987, Tu et al., 1998), but females did not sexually transmit virus to males (Rosen 1987) and Saint Louis encephalitis virus was shown to be venereally transmitted from male to female mosquitoes (Nayar et al., 1986, Shroyer 1990).

There are few data regarding the transmission dynamics of insect-specific flaviviruses, which have been found to replicate only in invertebrate cells. The inability to infect and replicate in vertebrate cells indicates that this group of flaviviruses has a distinct transmission cycle compared to the arthropod-borne flaviviruses, which are maintained between arthropod vectors and vertebrate hosts. There is evidence that vertical transmission plays an important role in the transmission of insect-specific flaviviruses. Kamiti River virus was first isolated from *Aedes macintoshi* larvae and pupae collected from flooded dambos in Kenya (Sang et al., 2003) and laboratory experiments conducted with *Aedes aegypti* mosquitoes, orally exposed to KRV, indicated that vertical transmission was possible (Lutomiah et al 2007). *Culex* flavivirus and *Aedes* flavivirus were both detected in adult males and females during mosquito field surveys in Japan (Hoshino et al., 2007, 2009). Cell fusing agent virus, first isolated from an insect cell line, was recently detected in male and female mosquito pools collected in Puerto Rico (Cook et al., 2006). Detection of insect-specific flaviviruses in all life stages, including adult mosquitoes of both sexes, suggests vertical transmission as a probable mechanism of viral maintenance in nature (Cook et al., 2006).

Vectorial Capacity and Vector Competence

Vectorial capacity is the overall ability of a vector species, in a given location, at a specific time, to transmit a pathogen (Higgs and Beaty 2005). This is the main factor determining whether a vector-borne disease continues to spread. A more specific definition is the average number of potentially infective bites that will ultimately be delivered by all the vectors feeding on a single host in one day (Fine 1981). The

conceptual basis for this model was originally developed by Ronald Ross for malaria transmission (Ross 1915, Freier 1989). The model further developed with contributions made by Macdonald (1957) and Garrett-Jones (1964). Vectorial capacity has three basic components: feeding (a), survival rate (p), and extrinsic incubation period (n). These factors most strongly affect vectorial capacity because 'a' is squared, 'n' is the exponent of 'p', and 'p' is in the numerator and denominator. Of these, vectorial capacity is most sensitive to changes in 'p' (Black and Moore 2005).

$$\text{Vectorial capacity} = \frac{ma^2p^nb}{-\ln p}$$

Where 'm' = vector density in relation to the host

'a' = probability a vector feeds on a host in 1 day [host preference index (HPI) x feeding frequency (FF)]

'p' = probability the vector will survive 1 day

'n' = duration of the extrinsic incubation period (days)

'b' = vector competence (the proportion of vectors ingesting an infective meal that successfully become infective)

The vectorial capacity equation is useful in understanding transmission dynamics of a disease system, but it is not very practical for day-to day decision-making at a vector control agency. Some of the variables are difficult to quantify, especially survival rate, which is the most important one (Black and Moore, 2005). Many vector control agencies use certain variables from the vectorial capacity equation, or variations of it, as a tool for

making risk assessments (Crans and McCuiston 1993, Hornby and Opp, 1994). Sometimes decisions for larviciding and adulticiding are based simply on larval counts or adult collections meeting certain threshold levels. Other factors may be incorporated like sentinel flock data and minimum infection rates in vector mosquitoes.

Vector competence is part of the vectorial capacity equation and can be defined as the natural ability of a particular vector to biologically transmit a pathogen (Higgs and Beaty, 2005). Components of vector competence include susceptibility to infection, receptiveness to pathogen replication, duration of extrinsic incubation period (time required for the pathogen to develop to infectivity in the vector), and transmission efficiency (Higgs and Beaty 2005, Anderson and Rico-Hesse 2006). These components can be experimentally determined for specific vector-pathogen relationships by administering an infectious blood meal and testing for infection rates (susceptibility to infection), dissemination rates (receptiveness to pathogen replication), and transmission rates (transmission efficiency) at different time points (duration of extrinsic incubation period). Vector competence information can be useful in determining important species involved in pathogen transmission, but additional factors should be considered before incriminating a particular vector species, including repeated detection of a pathogen from field-collected individuals and a relationship between the vector and naturally infected vertebrate hosts (Reeves 1957, Turell et al., 2005).

Superinfection

In nature, arboviruses can have overlapping geographical ranges and share similar transmission cycles, allowing for potential interactions to occur in co-infected arthropod

vectors. Field studies in eastern India revealed dual infections of Chikungunya virus and dengue virus in *Aedes aegypti* mosquitoes (Mourya et al., 2001). It is probably rare that a mosquito would ingest two viruses simultaneously, but more likely would become infected with one virus and then be exposed to a second virus at a later time (Borucki et al., 1999). Superinfection exclusion, or homologous interference, is the ability of an established virus infection to prevent infection with a secondary virus (Tscherne et al., 2007). For example, *Aedes albopictus* cells persistently infected with Sindbis virus were refractory to infection with homologous strains of Sindbis and other heterologous alphaviruses (Eaton 1979, Karpf et al., 1997). Porcine kidney cells, persistently infected with louping ill virus, a tick-borne flavivirus, were resistant to superinfection with homologous flaviviruses and most heterologous flaviviruses, with yellow fever French neurotropic virus as the only flavivirus tested that was able to replicate (Venugopal and Gould 1992). Persistent infections with Saint Louis encephalitis virus were established in cell cultures derived from *Aedes albopictus* and *Aedes dorsalis* and both cultures were resistant to superinfection with homologous virus, but not with heterologous Japanese encephalitis virus and yellow fever virus (Randolph and Hardy, 1988). *In vivo* studies showed that dengue-infected *Ae. aegypti* were less likely to become infected and transmit yellow fever virus compared to mosquitoes not infected with dengue virus (Sabin 1952). *Aedes triseriatus*, orally infected with LaCrosse (LAC) virus, were resistant to superinfection with a second virus after 72 hr (Sundin and Beaty, 1988). In contrast, *Ae. triseriatus* mosquitoes transovarially infected with LAC virus, were susceptible to superinfection with a second LAC virus (Borucki et al., 1999). Similarly, dual infections occurred in *Cx. tarsalis* mosquitoes infected simultaneously or consecutively (1 week

lag) with eastern and western equine encephalitis viruses (Chamberlain and Sudia 1957). Within-host interaction among viruses varies based on the biological system of interest and the consequence of interaction is dependent upon the order and interval of infection by different strains (Pepin et al., 2008). Competitive suppression was documented between dengue virus serotypes (DENV2 and DENV4), where replication of both viruses was suppressed in superinfection of C6/36 cells, with asymmetric results, as DENV2 was more suppressed than DENV4 in mixed infections (Pepin et al., 2008). Superinfection experiments in *Cx. quinquefasciatus* with West Nile virus and Saint Louis encephalitis virus also resulted in competitive suppression, where infection rates and dissemination rates for both viruses were lower in co-infected mosquitoes compared to mosquitoes exposed to only one virus (Pesko and Mores 2009).

Multiple mechanisms have been described and hypothesized for superinfection exclusion, including competition for host cell receptors or intracellular host factors, production of interferon or similar substances by the host cell, or by defective interfering particles from the first virus (Karpf et al., 1997, Burivong et al., 2004). Recent studies indicate that mosquitoes use RNA interference pathways to modulate viral infections (Keene et al., 2004, Sanchez-Vargas et al., 2009). In terms of superinfection, the presence of one arbovirus could activate the RNAi pathway, causing it to target the superinfecting, related virus, leading to exclusion or suppression of the second virus (Pesko and Mores 2009). The molecular basis for superinfection exclusion or suppression in mosquitoes currently remains unclear.

Information about arbovirus vector species persistently infected with insect-specific flaviviruses and the implications of dual infections is lacking. There has been one

study to determine whether prior infection with an insect-specific flavivirus modulates vector competence for a heterologous arbovirus (Kent et al., 2010). Experiments were conducted in *Cx. quinquefasciatus* mosquitoes intrathoracically inoculated with *Culex* flavivirus (Izabal strain) and then challenged with WNV. No significant differences were seen for infection, dissemination, or transmission rates for WNV between CXFV+ and CXFV- mosquitoes (Kent et al., 2010). Additional studies are needed to explore superinfection in natural systems and also with other insect-specific flaviviruses and vector species.

Specific Aims

Initially, the main objective for this project was to describe the spatial and temporal patterns of flaviviruses, specifically WNV, circulating in *Culex* spp. mosquitoes in northern Colorado. Unexpectedly, two insect-specific flaviviruses were detected that generated new questions and thus new directions for this research. The detection of insect flaviviruses in local *Culex* populations, along with continual circulation of WNV in Northern Colorado, provided an excellent opportunity to study potential interactions between heterologous flaviviruses in vector mosquitoes. The specific aims addressed were to 1) compare the seasonal patterns for WNV infection rates in *Culex tarsalis* mosquitoes with human disease cases; 2) determine the prevalence of insect-specific flaviviruses in *Culex* spp. mosquitoes; 3) investigate the dynamics of *Culex* flavivirus, an insect-specific flavivirus, in a naturally infected *Cx. pipiens* laboratory colony; and 4) evaluate the interaction between *Culex* flavivirus and WNV, in cell culture and in mosquitoes. This study provides new insights into local flavivirus transmission in *Culex*

spp. mosquitoes which can be applied to viral surveillance activities and mosquito control efforts.

CHAPTER 2

SEASONAL PATTERNS FOR ENTOMOLOGICAL MEASURES OF RISK FOR EXPOSURE TO *CULEX* VECTORS AND WEST NILE VIRUS IN RELATION TO HUMAN DISEASE CASES IN NORTHEASTERN COLORADO

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Introduction

Colorado experienced a dramatic West Nile virus (WNV) disease outbreak in 2003 with 2,947 reported cases of human disease and a smaller outbreak in 2007 with 578 reported cases (http://www.cdphe.state.co.us/dc/zoonosis/wnv/). This included two major WNV disease foci: 1) the northern Front Range and northeastern plains; and 2) the Grand Junction area in the western part of the state. Knowledge of seasonal patterns of activity for mosquito vectors and WNV is critical for both effective implementation of mosquito control measures and to advise the public regarding critical time periods when use of personal protection measures, such as repellents, should be emphasized. Previous studies on seasonal patterns of risk for exposure to *Culex* vectors in eastern Colorado were focused narrowly on the Fort Collins-Loveland area and showed that abundance of *Culex* vectors peaked during July-August (Smith et al. 1993, Bolling et al. 2007). The study by Bolling et al. (2007) also reported on WNV infection rates in various mosquito species but did not specifically present data for seasonal patterns of WNV infection rates. Recently, Kent et al. (2009) reported an increase in WNV infection rates in *Cx. tarsalis* Coquillett from June to August in 2007 in Weld County in the northeastern Colorado plains. A similar pattern with increasing WNV infection rates over the summer has been recorded for this species also in other western states (Bell et al. 2005; DiMenna et al. 2006; Nielsen et al. 2008; Reisen et al. 2008a, 2009). However, studies combining mosquito abundance and WNV infection rate to generate a more comprehensive measure of entomological risk of exposure to WNV, such as the Vector Index for abundance of WNV-infected mosquitoes, have been scarce (Bell et al. 2005, Gujral et al. 2007).

This study focused on a 5-county area in northeastern Colorado and aimed to determine seasonal patterns for: 1) abundance of the primary WNV vectors, *Cx. pipiens* L. and *Cx. tarsalis*, and the nuisance-biter and potential secondary WNV vector, *Aedes vexans* (Meigen); 2) WNV infection rates in *Cx. pipiens* and *Cx. tarsalis* females, and 3) the Vector Index for abundance of WNV-infected *Cx. tarsalis* females. In addition, we determined if these entomological risk measures for *Cx. tarsalis* were associated with the seasonal occurrence of human WNV disease cases.

Materials and Methods

Study area. The study area in northeastern Colorado includes the western edge of the Great Plains and the eastern edge of the Rocky Mountains. The climate in this area is characterized by cold winters and hot summers with low humidity. The average annual rainfall in Fort Collins in Larimer County from 1971-2000 was 393 mm (Mountain States Weather Services, Fort Collins, CO). Mosquito sampling was conducted along two rivers that emerge from the Rocky Mountains in western Larimer County (Poudre River and Big Thompson River) and then flow into the prairie landscapes characteristic of eastern Colorado (Fig. 2.1). Both rivers merge into the South Platte River in Weld County (Fig. 2.1). In the plains, these rivers typically are bordered by a narrow band of forested riparian wetland, dominated by cottonwood (*Populus* spp.) and willow (*Salix* spp.), which, in turn, is commonly surrounded by irrigated agricultural land. In the foothills and low montane habitats, the rivers flow through a canyon landscape dominated by grass, shrub, conifers (primarily Ponderosa pine, *Pinus ponderosa* Dougl. ex Laws.), and aspen (*Populus tremuloides* Michx.). Mosquito sampling sites were selected within the

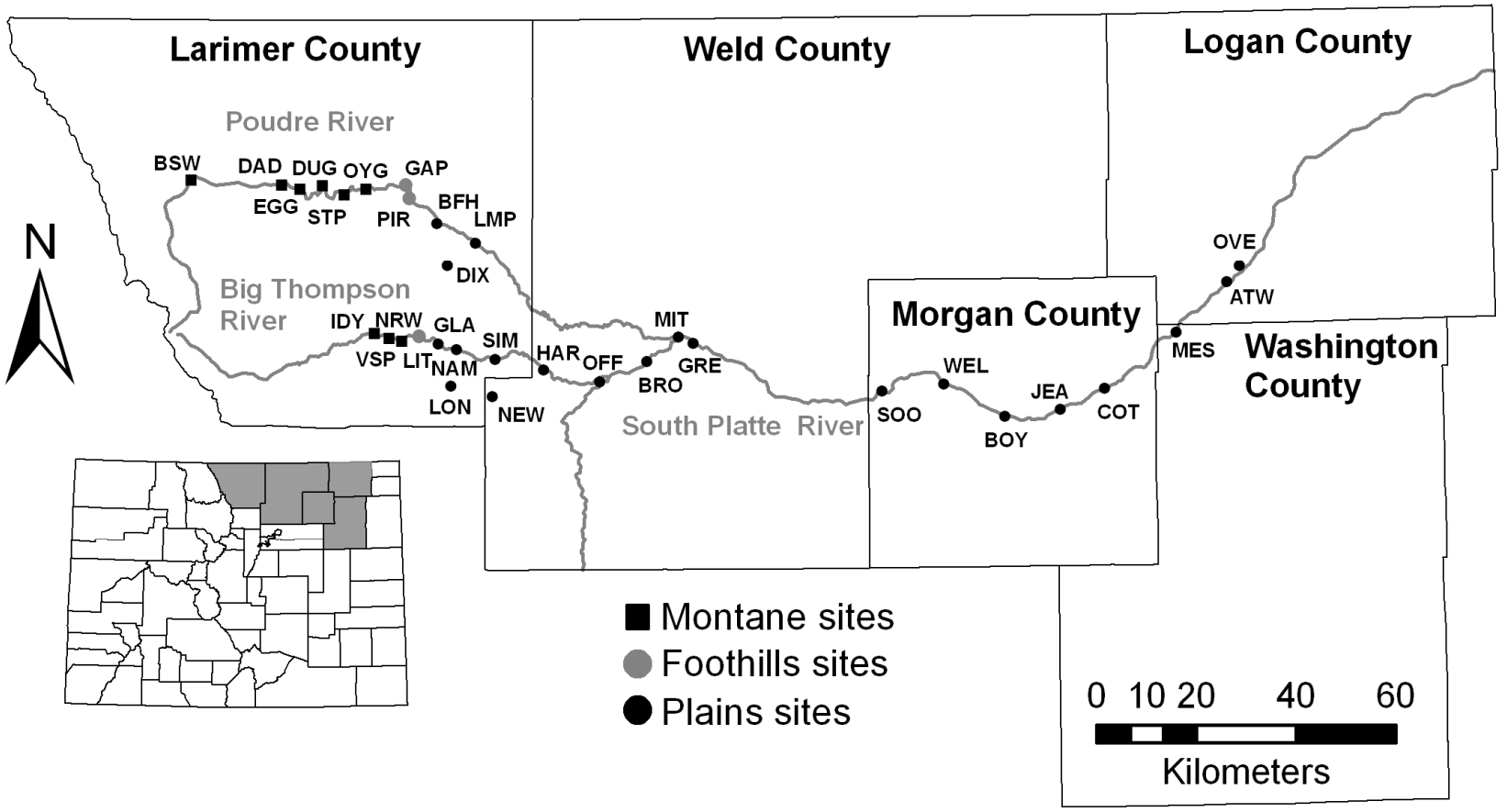


Figure 2.1. Location of mosquito sampling sites for 2006 (along the Poudre River) and 2007 (along the Big Thompson River and South Platte River). The location of the targeted 5-county area in Colorado is shown in the inset map.

relatively uniform riparian corridor at sites which could be accessed by automobile. Locations were mapped with a GPS receiver (Trimble Geo XT; Trimble Corp., Sunnyvale, CA) and visualized using ArcGIS 9.3 (ESRI, Redlands, CA). Selected environmental site characteristics are provided in Table 2.1. Latitude and longitude for sampling sites are included to facilitate future studies to determine if seasonal patterns were affected by climate change.

Mosquito collection and identification. Mosquitoes were collected using CO_2-baited CDC miniature light traps (John W. Hock Company, Gainesville, FL) that were suspended ~1.5 m above the ground and operated from afternoon (1500-1700 hours) until morning (0800-1000 hours). Sampling sites contained two traps baited with ~1 kg of dry ice and were located directly along the aforementioned rivers. Sampling in 2006 included 10 sites located along the Poudre River and 1 site by the Dixon Reservoir in Fort Collins. This spanned an elevation gradient from below 1,600 m in Fort Collins up to 2,360 m in the Poudre Canyon (Fig. 2.1). The sites were sampled every 2 wk from mid-April to late October 2006. Sampling in 2007 included 20 sites along the Big Thompson and South Platte rivers and two additional sites located south of the Big Thompson River in the Loveland area. This included an elevation gradient ranging from 1,215 m in the prairie landscape of eastern Colorado to 1,840 m in the montane habitat of the Big Thompson Canyon (Fig. 2.1). These sites were sampled every 2 wk from mid-June to mid-September 2007. Collected mosquitoes were examined with a dissecting microscope and identified to species using published keys (Harmston and Lawson 1967, Darsie and Ward 2005). Taxonomic nomenclature for Aedini genera follows Reinert et al. (2004).

Table 2.1. Characteristics of mosquito collection sites.

Site	Habitat	Site coordinates[a]		Elevation (m)	Elevation category (m)	Mean June-Aug. temp. (ºC)[b]
		Latitude (N)	Longitude (W)			
Poudre River sampling transect (2006)						
LMP	Plains	40.59456333	-105.07842459	1,510	1,501-1,600	20.5
BFH	Plains	40.63046744	-105.16906742	1,560	1,501-1,600	19.6
DIX[c]	Plains	40.55414868	-105.14104913	1,585	1,501-1,600	19.8
PIR	Foothills	40.67531706	-105.23651783	1,610	1,601-1,750	18.1
GAP	Foothills	40.70004260	-105.24424789	1,640	1,601-1,750	18.2
OYG	Montane	40.69214711	-105.33747484	1,750	1,601-1,750	16.6
STP	Montane	40.68193007	-105.38909379	1,860	>1,750	15.9
DUG	Montane	40.69854287	-105.44131068	2,000	>1,750	15.5
EGG	Montane	40.69110602	-105.49463206	2,110	>1,750	15.0
DAD	Montane	40.69959335	-105.53861231	2,130	>1,750	14.5
BSW	Montane	40.70755489	-105.75274554	2,360	>1,750	11.5
Big Thompson River-South Platte River sampling transect (2007)						
OVE	Plains	40.53981478	-103.26729278	1,215	1,201-1,300	22.1
ATW	Plains	40.51132441	-103.29860284	1,219	1,201-1,300	22.1
MES	Plains	40.42114686	-103.42058665	1,242	1,201-1,300	22.0
COT	Plains	40.32261249	-103.59151936	1,272	1,201-1,300	22.2
JEA	Plains	40.28480442	-103.69612552	1,286	1,201-1,300	22.3
BOY	Plains	40.27350806	-103.82703510	1,303	1,301-1,400	22.2
WEL	Plains	40.33373196	-103.97041874	1,321	1,301-1,400	21.9
SOO	Plains	40.32223738	-104.11763996	1,344	1,301-1,400	21.8
GRE	Plains	40.41074220	-104.56347789	1,397	1,301-1,400	21.8
MIT	Plains	40.42255709	-104.59813692	1,402	1,401-1,500	21.8
BRO	Plains	40.37795104	-104.67287258	1,418	1,401-1,500	21.8
OFF	Plains	40.34138892	-104.78297469	1,434	1,401-1,500	21.5
HAR	Plains	40.36346382	-104.91709610	1,459	1,401-1,500	21.3
SIM	Plains	40.38336774	-105.03109402	1,487	1,401-1,500	20.9
NEW[d]	Plains	40.31557104	-105.03709367	1,511	1,501-1,600	20.9
NAM	Plains	40.40073803	-105.12296546	1,524	1,501-1,600	20.6
GLA	Plains	40.41089097	-105.16606378	1,544	1,501-1,600	20.4
LON[e]	Plains	40.33409016	-105.13580524	1,566	1,501-1,600	20.6
LIT	Foothills	40.42509226	-105.21171030	1,598	1,501-1,600	19.9
NRW	Montane	40.41515686	-105.25146509	1,688	1,601-1,750	19.2
VSP	Montane	40.42004747	-105.28132071	1,737	1,601-1,750	18.6
IDY	Montane	40.42918494	-105.31700388	1,840	>1,750	17.3

[a]Site locations were determined with a GPS receiver; [b]Mean values for 1961-1990 were based on GIS-derived data from Climate Source LLC, Corvallis, OR (2x2 km spatial resolution). [c]Located by Dixon Reservoir, ~7 km south of the Poudre River; [d]Located by Newell Lake, ~8 km south of the Big Thompson River; [e]Located by Lonetree Reservoir ~7 km south of the Big Thompson River.

Detection of West Nile virus in *Culex* mosquitoes. *Culex* mosquitoes were examined for presence of WNV RNA following Bolling et al. (2007) with the modifications outlined below. Mosquitoes were identified on a chill table and placed in pools of 1 to 50 by species, sex, site, trap, and date. Mosquito pools were then stored at -70°C until processed for viral RNA detection.

Each pool was triturated for 45 sec with a vortex mixer in a 5-ml round-bottom polypropylene tube (Becton Dickinson Labware, Franklin Lakes, NJ) using 1.5 ml of diluent (1X minimum essential medium containing 2% fetal bovine serum, 100 µg/ml penicillin/streptomycin, supplemented with L-glutamine and nonessential amino acids) and 4 copper-coated steel shot (4.5-mm diameter; 0.177" caliber). Suspensions were then centrifuged at 3,000 rpm for 10 min at 4°C. Total RNA was extracted from 140 µl of the supernatant using the QIAamp viral RNA Mini kit (Qiagen Inc., Valencia, CA). RNA was then eluted in 60 µl of nuclease-free water (Ambion Inc., Austin, TX). Reverse transcription-PCR was used to detect viral RNA in the samples. Mosquito pools were first tested using universal flavivirus primers targeting a portion of the NS5 gene (forward MAMD: 5'-AACATGATGGGRAARAGRGARAA-3', reverse cFD2: 5'-GTGTCCCAGCCGGCGGTGTCATCAGC-3') (Scaramozzino et al. 2001). Pools testing positive for flavivirus RNA were then tested for WNV using primers developed and recommended by the CDC for use in WNV surveillance (forward WN212: 5'-TTGTGTTGGCTCTCTTGGCGTTCTT-3', reverse WN619c: 5'-CAGCCGACAGCACTGGACATTCATA-3') (Gubler et al. 2000, Lanciotti et al. 2000). PCR products were visualized following electrophoresis on a 1% agarose gel stained with

ethidium bromide. Negative (no template) and positive controls were included in each RT-PCR run.

Infection rates per 1,000 individuals were calculated as bias-corrected Maximum Likelihood Estimates using the Excel Add-In PooledInfRate, version 3.0 (Biggerstaff 2006).

Epidemiological data. Data for 219 WNV disease cases reported in 2007 from the targeted 5-county area (Larimer, Weld, Morgan, Washington, and Logan) were provided by the Colorado Department of Public Health and Environment. This included date of onset for each case, located by county, zip code and census tract of residence, but did not include any personal identifiers.

Presentation and analysis of data. Presented data for mosquito abundance in 2006 are restricted to the WNV vector *Cx. tarsalis* and the nuisance biter *Ae. vexans* because the other locally important WNV vector, *Cx. pipiens*, was not collected in sufficient numbers for meaningful inclusion in the presentation. To simplify the presentation, seasonal patterns for mosquito abundance and temperature were aggregated into three elevation classes: 1,501-1,600 m, 1,601-1,750 m, and > 1,750 m (sites falling into each of these categories are shown in Table 2.1). Mosquito abundance data from 2006 are shown together with mean weekly temperatures determined using HOBO H8 Pro series loggers (Onset Computer Corporation, Pocasset, MA). Seasonal data for mosquito abundance in 2007, which did not span the entire active season, are presented in the context of comparison with WNV infection rates in mosquitoes and human WNV disease cases, and therefore are restricted to the primary WNV vectors *Cx. tarsalis* and *Cx. pipiens*.

Presented data for WNV infection in mosquitoes for 2006-2007 are restricted to *Cx. tarsalis* and *Cx. pipiens* females. We also present a series of seasonal data for June-September 2007 (aggregated data for the 18 sites in the plains and excluding the foothills-montane sites [LIT, NRW, VSP and IDY], which yielded very few *Cx. tarsalis*) including abundance of *Cx. tarsalis* females (weekly means per trap night), infection rate with WNV per 1,000 *Cx. tarsalis* females (weekly Maximum Likelihood Estimate for infection rate), and the Vector Index (Gujral et al. 2007) for abundance of WNV-infected *Cx. tarsalis* females (weekly mean per trap night x weekly proportion of WNV-infected females).

Statistical tests used are indicated in the text. All statistical analyses were carried out using the JMP® 7.0.1 statistical package (SAS Institute Inc., Cary, NC) and results were considered significant when $P < 0.05$.

Results

Seasonal patterns of mosquito abundance along elevation gradients in the Colorado Front Range area – 2006. Examination of seasonal abundance patterns for the WNV vector *Cx. tarsalis* along an elevation gradient extending from plains to montane habitats in the Colorado Front Range area from April to October 2006 showed that: 1) peak abundance of females was greater below 1,600 m compared to 1,601-1,750 m or > 1,750 m; 2) females were collected over a longer time period below 1,600 m; and 3) peak abundance occurred earlier below 1,600 m (Fig. 2.2). *Cx. tarsalis* females were first recorded during mid-April (on the first sampling occasion of the year) for sites located below 1,600 m and between 1,601-1,750 m; these mosquitoes probably

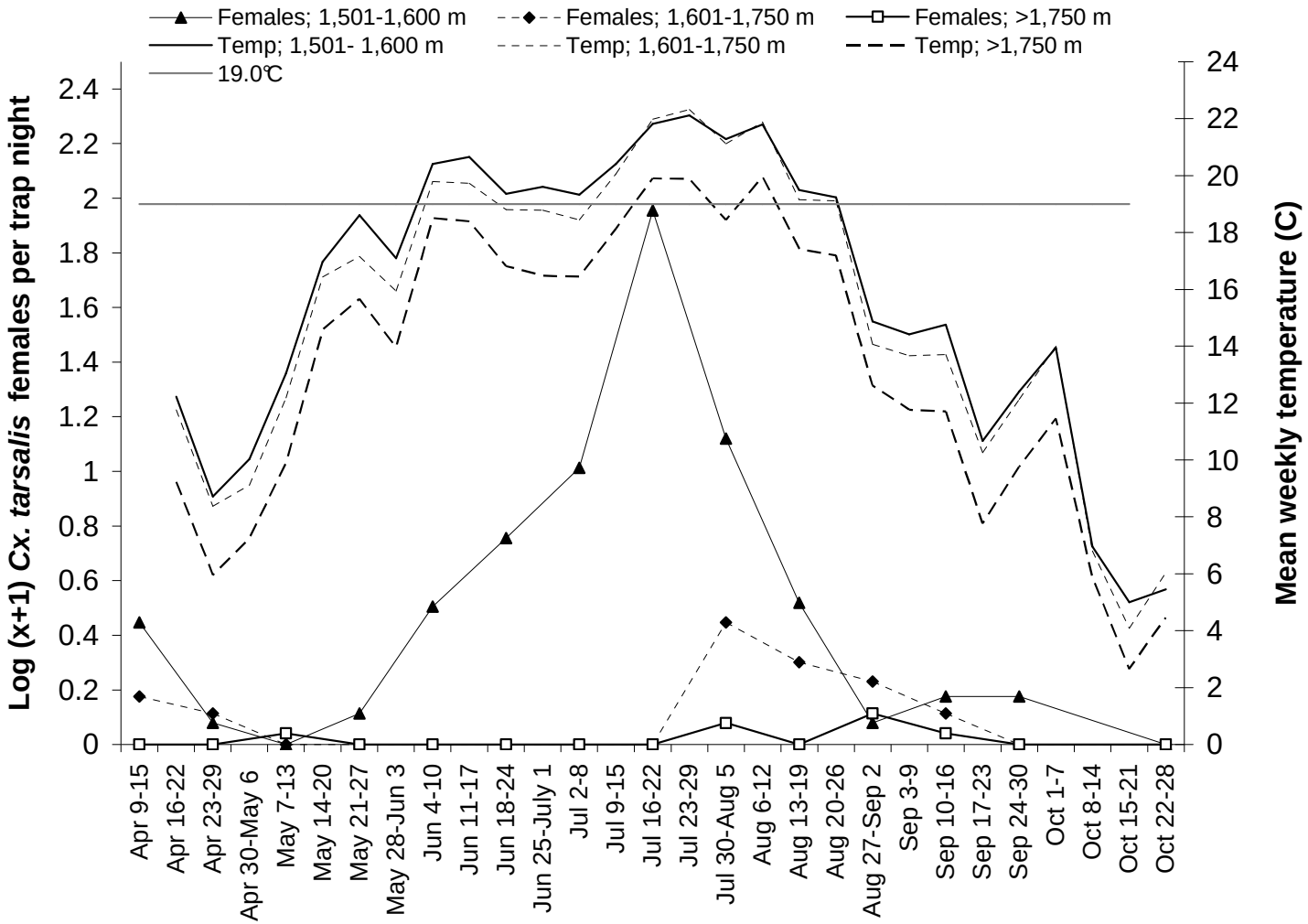


Figure 2.2. Seasonal patterns of abundance of *Cx. tarsalis* females in relation to mean weekly temperature by elevation category (1,501-1,600; 1,601-1,750; and > 1,750 m) along the Poudre River, April-October 2006.

represented overwintered females that emerged and sought blood meals during warm spring weather (Fig. 2.2). The earliest collection of *Cx. tarsalis* females from sites above 1,750 m occurred in early May.

Below 1,600 m, *Cx. tarsalis* females were collected on each sampling occasion from late May to late September with a distinct peak in mid-July. In contrast, not a single *Cx. tarsalis* female was collected from late May to mid-July between 1,601-1,750 m or above 1,750 m. At these higher elevations, peak abundances occurred in early August (1,601-1,750 m) or late August (above 1,750 m). The period with consecutive collections of *Cx. tarsalis* females extended from late July to mid-September for the sites between 1,601-1,750 m. As illustrated in Fig. 2.2, *Cx. tarsalis* abundance increased rapidly when weekly mean air temperatures consistently exceeded 18.5-19.5°C, occurring in late May at elevations below 1,600 m and in mid-July at higher elevations.

The seasonal abundance patterns for *Ae. vexans* were similar to *Cx. tarsalis* below 1,600 m, with consistent collections of females from late May to late September and peak abundance occurring in mid-July (Fig. 2.3). However, at higher elevations the patterns differed between the two species. *Ae. vexans* females peaked sharply in late May between 1,601-1,750 m and declined gradually in abundance thereafter, compared to *Cx. tarsalis*, which peaked later in the season at 1,601-1,750 m than at 1,600 m.

Seasonal patterns of *Cx. tarsalis* and *Cx. pipiens* in the northeastern Colorado plains – 2007. Data from 2007 provided an opportunity to compare seasonal patterns for *Cx. tarsalis* and *Cx. pipiens* from late June to mid-September in the northeastern Colorado plains (Fig. 2.4). This showed that abundance of *Cx. tarsalis* females peaked in

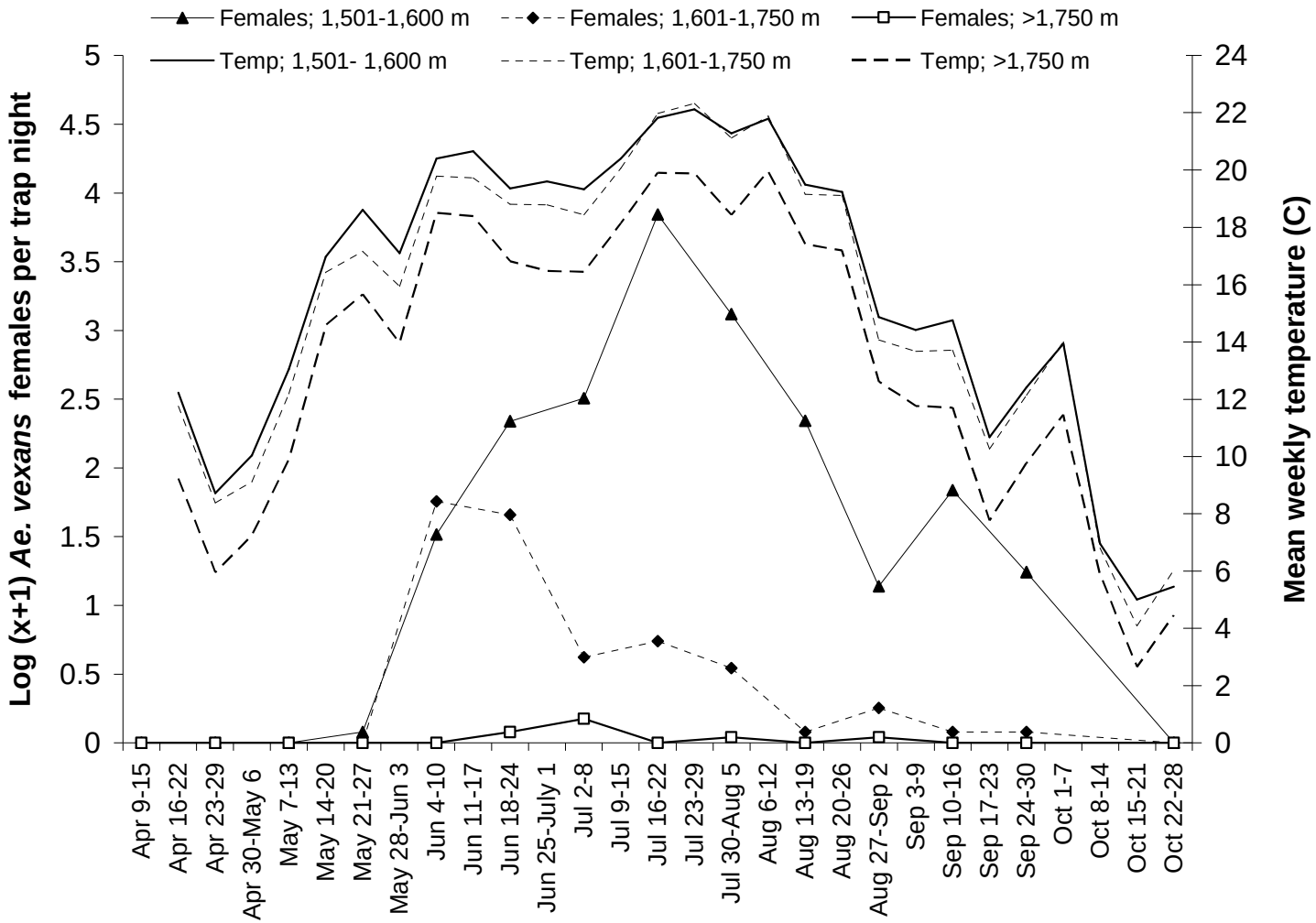


Figure 2.3. Seasonal patterns of abundance of *Ae. vexans* females in relation to mean weekly temperature by elevation category (1,501-1,600; 1,601-1,750; and > 1,750 m) along the Poudre River, April-October 2006.

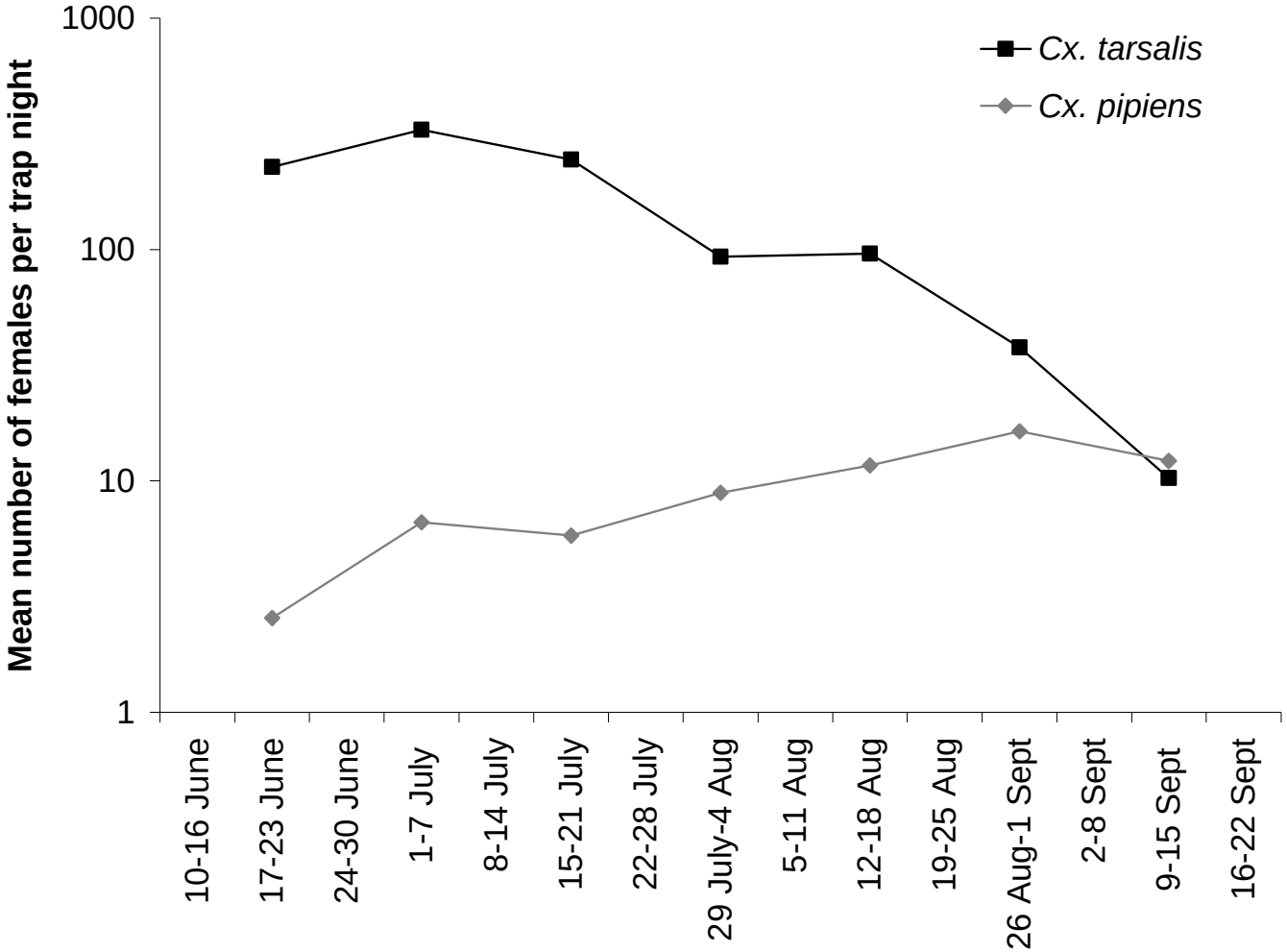


Figure 2.4. Seasonal patterns of abundance of *Cx. tarsalis* and *Cx. pipiens* females for 18 sites in the northeastern Colorado plains, June-September 2007.

early July and declined gradually thereafter. In contrast, *Cx. pipiens* females gradually increased in abundance over the sampling period to reach peak numbers in late August.

Seasonal patterns of WNV infection in *Culex* mosquitoes – 2006 and 2007. In both 2006 and 2007, WNV-infected *Cx. tarsalis* females were collected in the plains but not in foothills-montane areas above 1,600 m (Tables 2.2-2.3). WNV activity was limited in the plains sites examined in 2006, with WNV infection in *Cx. tarsalis* females recorded only in mid-July (Table 2.2). Sampling in the plains in 2007 included a different set of collection sites and yielded far greater numbers of *Cx. tarsalis* females and more intense WNV activity compared to 2006. Only two out of the 18 sites examined in the plains in 2007 failed to produce infected *Cx. tarsalis* females. The proportion of plains sites producing infected *Cx. tarsalis* females ranged from 22% in late June to ≥67% in mid-July and mid-August.

Overall WNV infection rates for *Cx. tarsalis* females in the 18 plains sites in 2007 increased gradually from late June (MLE of 0.53 per 1,000 females) to reach a peak in mid-August (8.29 per 1,000 females) and then remained high through mid-September (4.49-5.41 per 1,000 females) (Table 2.3; Fig 2.5). Infection rates for individual collections by site and date, where ≥500 *Cx. tarsalis* females were examined, ranged from 0 to 8.90 per 1,000 females, with most collections that produced infected females falling in the infection rate range of 1-3 per 1,000 females (Table 2.3). Very high rates of WNV infection, >10 per 1,000 females, were associated with smaller sample sizes.

Fewer *Cx. pipiens* females were collected at most sites, and only five pools were infected with WNV. Infected pools were recorded from 20-22 June, 17-19 July, 15-17

Table 2.2. Seasonal pattern of infection with West Nile virus in *Cx. tarsalis* females in Larimer County, April-September 2006.

	Maximum Likelihood Estimate (MLE) for WNV infection rate per 1,000 females[a] (total number of females examined)												
Elevation category; Habitat; Sites	14 April	28 April	9 May	25 May	7 June	21 June	8 July	19 July	2 Aug	16 Aug	30 Aug	13 Sept	27 Sept
<1,600 m; Plains; DIX	0 (11)	0 (1)	-----	0 (2)	0 (9)	0 (15)	0 (40)	15.00 (355)	0 (12)	0 (6)	0 (1)	0 (3)	0 (3)
<1,600 m; Plains; LMP	-----	-----	-----	-----	0 (1)	0 (12)	0 (1)	0 (31)	0 (16)	0 (1)	-----	-----	-----
<1,600 m; Plains; BFH	-----	-----	-----	-----	0 (1)	0 (1)	0 (15)	6.99 (149)	0 (45)	0 (7)	0 (1)	-----	-----
<1,600 m; Plains sites combined; DIX, LMP, BFH	0 (11)	0 (1)	-----	0 (2)	0 (11)	0 (28)	0 (56)	11.91 (535)	0 (73)	0 (14)	0 (2)	0 (3)	0 (3)
1,601-1,750 m; Foothills-Montane; PIR, GAP, OYG	0 (3)	0 (2)	-----	-----	-----	-----	-----	-----	0 (11)	0 (6)	0 (4)	0 (2)	-----
>1,750 m; Montane; STP, DUG, EGG, DAD, BSW	-----	-----	0 (1)	-----	-----	-----	-----	-----	0 (2)	-----	0 (3)	0 (1)	-----

[a]Females were tested in pools of 1-50. Bias-corrected MLEs were calculated with the Excel Add-In PooledInfRate, version 3.0 (Biggerstaff 2006).

Table 2.3. Seasonal patterns of infection with West Nile virus and Vector Index for *Cx. tarsalis* females along the South Platte River-Big Thompson River corridor, June-September 2007.

	WNV infection rate (IR) per 1,000 females[a] (total number of females examined) and Vector Index (VI)[b]													
	20-22 June		4-6 July		17-19 July		1-3 August		15-17 August		29-31 August		12-14 September	
Site	IR	VI	IR	VI	IR	VI	IR	VI	IR	VI	IR	VI	IR	VI
Plains														
OVE	0 (129)	0	0 (155)	0	6.62 (507)	1.68	19.5 (208)	2.03	12.4 (257)	1.60	67.1 (44)	1.48	0 (51)	0
ATW	1.29 (769)	0.50	0 (1078)	0	13.9 (461)	3.20	0 (285)	0	50.0 (20)	1.90	18.8 (62)	0.58	0 (3)	0
MES	0 (812)	0	0.65 (1545)	0.50	4.48 (474)	1.06	0 (28)	0	28.6 (107)	1.53	0 (37)	0	38.9 (31)	0.60
COT	0 (105)	0	1.13 (887)	0.50	4.45 (200)	0.45	4.84 (181)	0.44	0 (113)	0	0 (34)	0	0 (13)	0
JEA	0 (453)	0	2.41 (1735)	2.09	7.23 (470)	1.70	22.0 (176)	1.94	4.87 (422)	1.03	2.60 (386)	0.50	0 (43)	0
BOY	0 (49)	0	0 (74)	0	0 (113)	0	0 (55)	0	0 (22)	0	0 (7)	0	0 (4)	0
WEL	0.83 (1175)	0.49	2.31 (891)	1.03	2.56 (1224)	1.57	8.47 (554)	2.35	3.50 (287)	0.50	0 (325)	0	0 (15)	0
SOO	1.72 (582)	0.50	0.78 (1275)	0.50	5.20 (1066)	2.77	10.3 (465)	2.40	3.65 (277)	0.51	0 (44)	0	0 (27)	0
GRE	0 (412)	0	0 (375)	0	1.05 (951)	0.50	0 (72)	0	0 (62)	0	83.3 (12)	0.50	0 (6)	0
MIT	0 (1304)	0	1.39 (1467)	1.02	1.52 (660)	0.50	0 (90)	0	5.33 (376)	1.00	0 (267)	0	0 (79)	0
BRO	0 (611)	0	0 (579)	0	1.15 (867)	0.25	2.28 (439)	0.50	8.36 (119)	0.50	0 (32)	0	0 (27)	0
OFF	0 (570)	0	0 (980)	0	2.78 (1132)	1.57	4.01 (511)	1.02	8.90 (809)	3.60	0 (37)	0	0 (53)	0
HAR	0 (127)	0	5.94 (171)	0.51	5.45 (395)	1.08	8.16 (132)	0.54	0 (87)	0	0 (6)	0	0 (3)	0
SIM	0 (43)	0	8.76 (86)	0.60	0 (139)	0	0 (42)	0	29.2 (33)	0.32	0 (10)	0	0 (5)	0
NEW	4.07 (249)	0.51	0 (217)	0	2.39 (419)	0.50	0 (45)	0	14.1 (369)	2.60	0 (38)	0	200.0 (5)	0.50
NAM	0 (38)	0	0 (51)	0	0 (68)	0	166.7 (6)	1.00	0 (15)	0	----	0	0 (1)	0
GLA	0 (31)	0	0 (88)	0	0 (66)	0	0 (62)	0	0 (4)	0	0 (3)	0	0 (2)	0
LON	0 (65)	0	0 (135)	0	0 (154)	0	0 (74)	0	8.85 (94)	0.42	0 (17)	0	0 (3)	0
Total	**0.53**	**0.12**	**1.13**	**0.37**	**3.59**	**0.88**	**6.59**	**0.61**	**8.29**	**0.80**	**4.49**	**0.17**	**5.41**	**0.06**
Foothills-Montane														
LIT	0 (1)	0	0 (4)	0	0 (18)	0	0 (18)	0	0 (4)	0	0 (3)	0	0 (2)	0
NRW	----	0	0 (3)	0	0 (1)	0	0 (1)	0	----	0	0 (1)	0	----	0
VSP	----	0	0 (20)	0	0 (14)	0	0 (4)	0	0 (2)	0	0 (1)	0	----	0
IDY	0 (1)	0	0 (2)	0	0 (1)	0	----	0	----	0	----	0	----	0
Total	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**

[a]Females were tested in pools of 1-50. Bias-corrected Maximum Likelihood Estimates for WNV infection rates were calculated with the Excel Add-In PooledInfRate, version 3.0 (Biggerstaff 2006); [b]Vector Index was calculated as the mean number of females per trap night times the proportion of WNV-infected females.

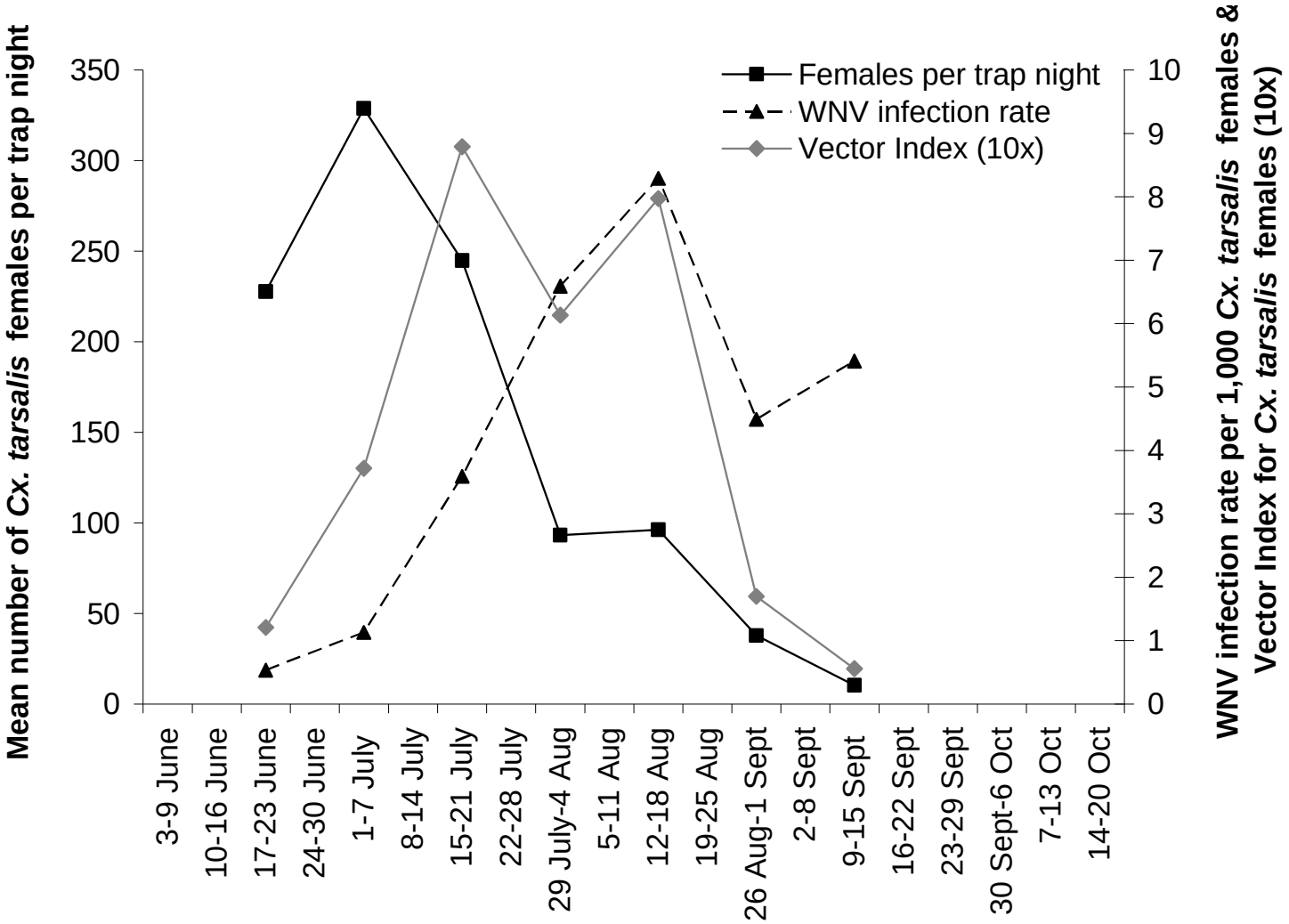


Figure 2.5. Seasonal patterns for mean number of *Cx. tarsalis* females per trap night, WNV infection rate in the females, and Vector Index for abundance of WNV-infected *Cx. tarsalis* females (10x) in the northeastern Colorado plains, June-September 2007.

August, 29-31 August and 12-14 September. The overall infection rate (MLE per 1,000 females) for the plains sites during June-September was 2.10.

Seasonal patterns of entomological risk measures in relation to occurrence of WNV disease cases – 2007. For the 2007 data, we explored potential relationships between the seasonal pattern of WNV disease cases in the targeted 5-county area (Larimer-Weld-Morgan-Washington-Logan) and the seasonal patterns for three entomological risk measures combined for the 18 plains sites located within these counties: 1) mean number of *Cx. tarsalis* females per trap night (Fig. 2.5); 2) WNV infection rate per 1,000 *Cx. tarsalis* females (Table 2.3, Fig. 2.5); and 3) Vector Index for abundance of infected *Cx. tarsalis* females (mean per trap night x proportion of WNV-infected females) (Table 2.3, Fig. 2.5). The seasonal patterns for the three different entomological risk measures are shown together in Fig. 2.5. Abundance of *Cx. tarsalis* females peaked in early July and declined gradually thereafter, whereas the WNV infection rate in the females increased gradually to reach a peak in mid-August. This resulted in the Vector Index peaking in mid-July and mid-August. The Vector Index for all plains sites combined exceeded 0.50 from mid-July to mid-August, and at least one site recorded a Vector Index ≥0.50 from late June to mid-September and ≥1.00 from early July to late August.

Linear regression models where entomological risk measures were used to predict WNV disease in subsequent weeks, with time-lags ranging from 0 to 8 wk, showed that abundance of *Cx. tarsalis* females was strongly associated with weekly numbers of WNV disease cases 4-7 wk later and that the Vector Index was strongly associated with weekly numbers of WNV disease cases 1-2 wk later (Table 2.4). Weekly patterns for WNV

Table 2.4. Results for linear regression models to predict numbers of WNV disease cases in subsequent weeks from entomological risk measures, northeastern Colorado, 2007.

Lag time for comparison with weekly numbers of WNV disease cases	Abundance of *Cx. tarsalis* females[a]				WNV infection rate in *Cx. tarsalis* females[b]				Vector Index for *Cx. tarsalis* females[c]			
	Model fit		ANOVA		Model fit		ANOVA		Model fit		ANOVA	
	Type[d]	r^2	$F_{1,5}$	P	Type[d]	r^2	$F_{1,5}$	P	Type[d]	r^2	$F_{1,5}$	P
+ 0 wk	NA	0.187	1.15	0.33	Pos	0.712	12.39	0.02	NA	0.488	4.76	0.08
+ 1 wk	NA	0.007	0.04	0.86	NA	0.373	2.97	0.14	Pos	0.786	18.37	0.008
+ 2 wk	NA	0.102	0.57	0.48	NA	0.061	0.33	0.59	Pos	0.725	13.18	0.02
+ 3 wk	NA	0.371	2.95	0.14	NA	0.001	0.01	0.98	NA	0.534	5.72	0.06
+ 4 wk	Pos	0.907	49.01	<0.001	NA	0.401	3.35	0.12	NA	0.130	0.75	0.43
+ 5 wk	Pos	0.729	13.47	0.01	NA	0.477	4.55	0.08	NA	0.054	0.28	0.61
+ 6 wk	Pos	0.801	20.18	0.006	Neg	0.738	14.05	0.01	NA	0.009	0.04	0.84
+ 7 wk	Pos	0.911	51.30	<0.001	Neg	0.781	17.80	0.008	NA	0.001	0.01	0.95
+ 8 wk	NA	0.498	4.95	0.07	Neg	0.678	10.51	0.02	NA	0.075	0.40	0.55

All comparisons were based on seven data points for entomological risk measures (taken every 2 wk from late June to mid-September); these data points combined data for 18 plains sites located within the 5-county area in northeastern Colorado that was used to determine weekly WNV disease cases; [a]Mean number of *Cx. tarsalis* females per trap night; [b]Maximum Likelihood Estimate for WNV infection rate per 1,000 *Cx. tarsalis* females; [c]Vector Index for abundance of WNV-infected *Cx. tarsalis* females (weekly mean per trap night x weekly proportion of WNV-infected females); [d] Pos – Positive linear relationship, Neg – Negative linear relationship, NA – no significant relationship.

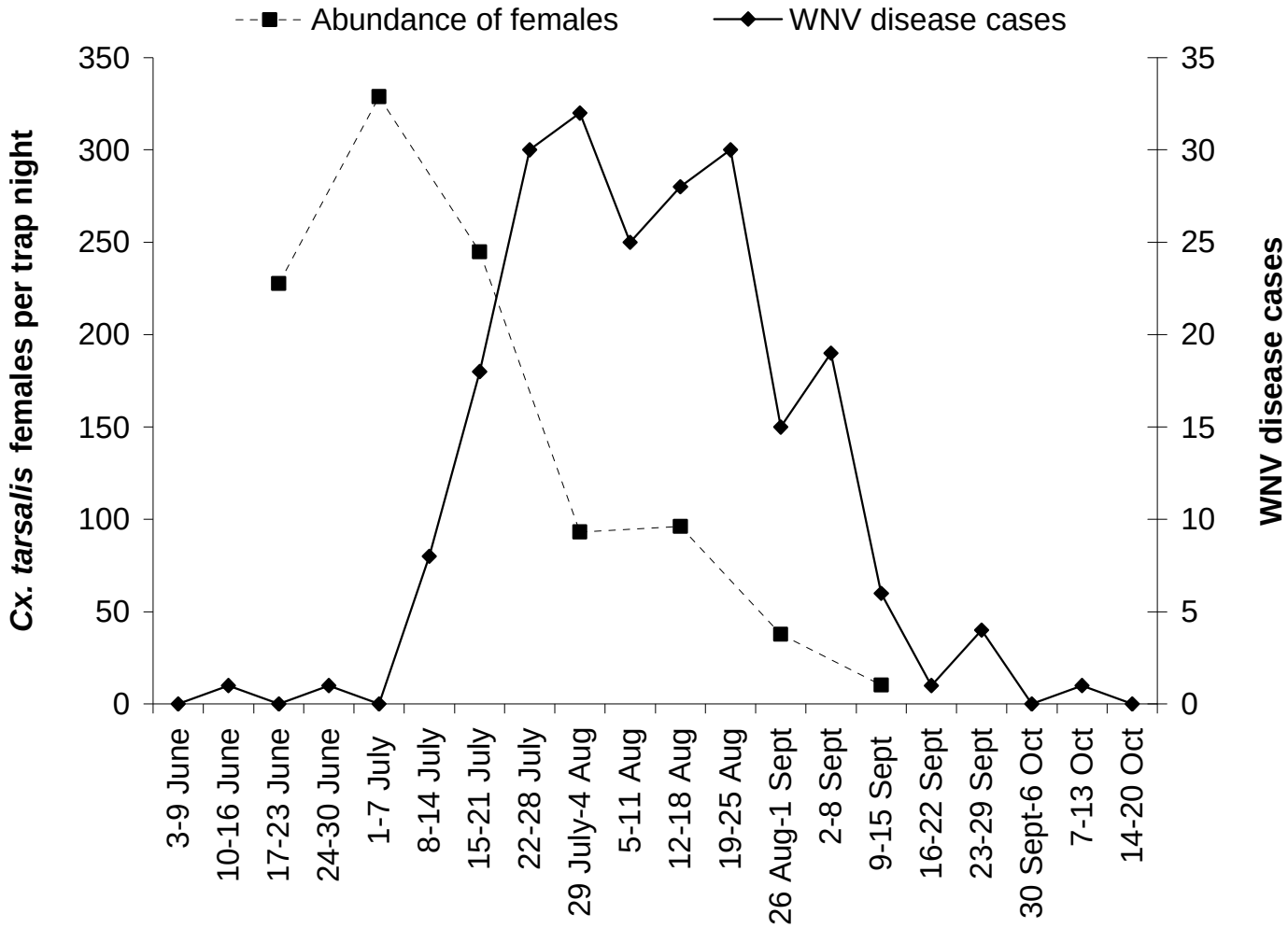


Figure 2.6. Seasonal pattern of WNV disease cases in a 5-county area (Larimer, Weld, Morgan, Washington, and Logan) in northeastern Colorado, June-September 2007, in relation to abundance of *Cx. tarsalis* females.

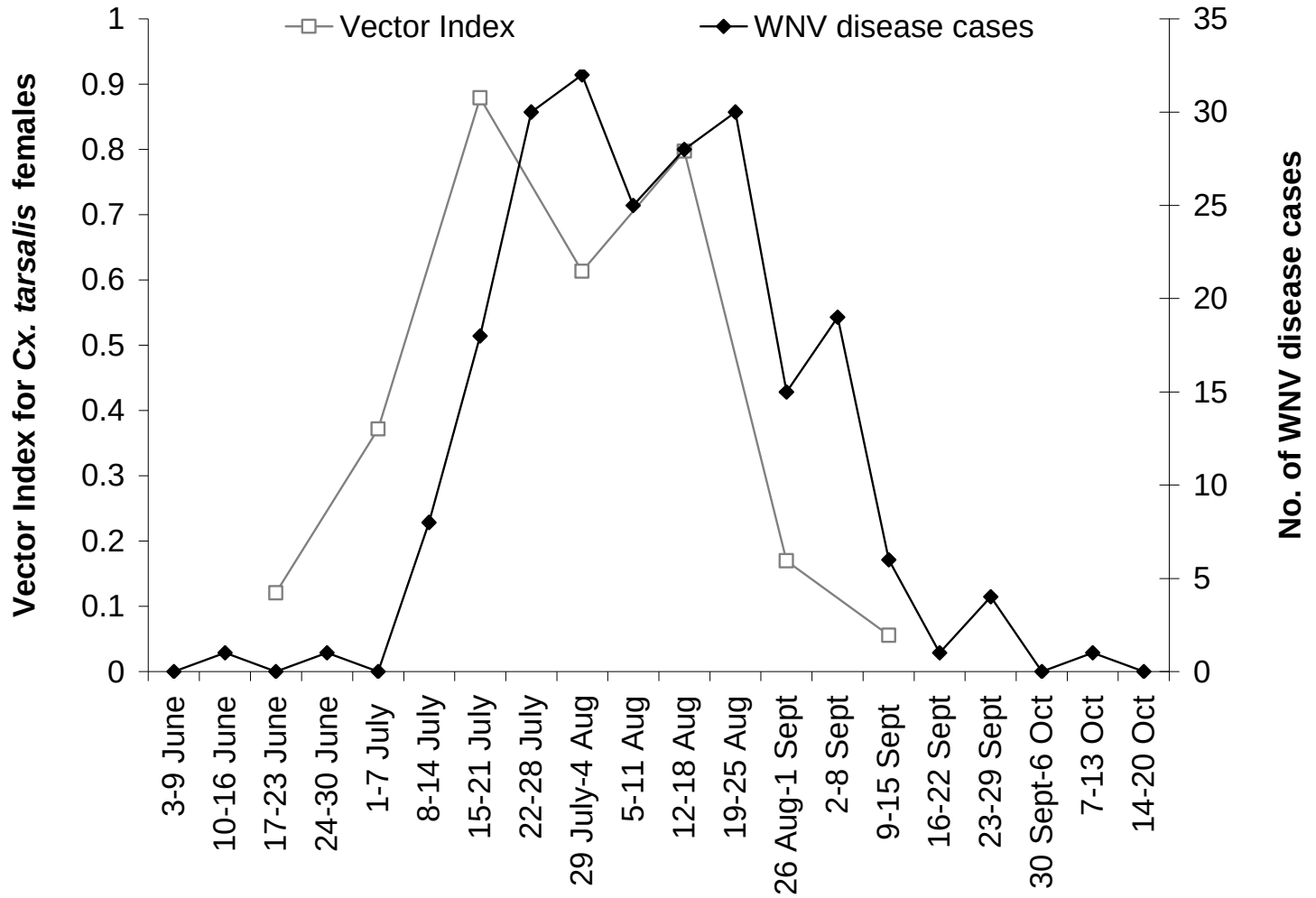


Figure 2.7. Seasonal pattern of WNV disease cases in a 5-county area (Larimer, Weld, Morgan, Washington, and Logan) in northeastern Colorado, June-September 2007, in relation to the Vector Index for abundance of WNV-infected *Cx. tarsalis* females.

disease cases in relation to abundance of *Cx. tarsalis* females or Vector Index are shown in Figs. 2.6-2.7.

Discussion

This study provided detailed descriptions of seasonal risk patterns for exposure to mosquitoes and WNV in a wide range of habitat types in Colorado, including prairie landscapes in the Great Plains and foothills and montane areas in the Rocky Mountains. Key findings included that: 1) the seasonal activity period is shortened and peak numbers occur later in the summer for *Cx. tarsalis* females in foothills-montane areas above 1,600 m compared to plains areas below 1,600 m along Colorado's Front Range; 2) seasonal patterns of abundance for *Cx. tarsalis* and *Cx. pipiens* females in the northeastern Colorado plains in 2007 differed in that *Cx. tarsalis* reached peak abundance in early July whereas the peak for *Cx. pipiens* did not occur until late August; 3) WNV-infected *Cx. tarsalis* females were recorded from nearly all sites sampled in the plains in 2007 with infection rates commonly exceeding 1 infected female per 1,000 examined; 4) the Vector Index for abundance of WNV-infected *Cx. tarsalis* females exceeded 0.50 for the plains sites combined from mid-July to mid-August, with values for at least one individual site exceeding 1.00 from early July to late August; and 5) abundance of *Cx. tarsalis* females and the Vector Index for abundance of infected females were strongly associated with weekly numbers of WNV disease cases with onset 4-7 weeks later (female abundance) or 1-2 wk later (Vector Index).

An important limitation of this study is that we were not able to sample all sites during both years. Some of the key findings outlined above, especially the associations

between entomological risk measures and human disease cases, need to be corroborated not only in other parts of the western U.S. but also in future studies in Colorado that span multiple years and can account for between-year variability in weather conditions, mosquito population dynamics, and WNV transmission intensity. Another issue that needs to be addressed in future studies is to clarify the relative roles of *Cx. tarsalis* versus *Cx. pipiens* as bridge vectors of WNV to humans in eastern Colorado. Our findings suggest that *Cx. tarsalis* should be considered a primary vector of WNV to humans in eastern Colorado but further work is needed to define the local circumstances under which *Cx. pipiens* also may play an important role in this respect.

Seasonal patterns of mosquito abundance along elevation gradients in the Colorado Front Range. To our knowledge, this is the first study from North America exploiting a natural elevation/climate gradient to determine how seasonal patterns of abundance of adult nuisance-biting or vector mosquitoes change with elevation at the cool edge of the range of the mosquitoes. For the WNV vector *Cx. tarsalis*, we found dramatic changes in seasonal abundance patterns above 1,600 m. Above this elevation threshold, the seasonal activity period for *Cx. tarsalis* females was shortened (Fig. 2.2), peak numbers were lower and occurred later in the summer (Fig. 2.2), and WNV was not detected from the females (Tables 2.2-2.3). We speculate that these differences were due to: 1) temperature conditions at elevations above 1,600 m in the Front Range that limited population growth of *Cx. tarsalis* by slowing larval development rates and gonotrophic cycles, and 2) a reduction in the number, size, and persistence of larval habitats due to land use changes such as a lack of irrigation and other human-managed water inputs. This also may keep the overall abundance of *Culex* vectors below a critical threshold for

enzootic WNV transmission to occur. These speculations provoke interesting questions regarding how climate warming in coming decades, should it occur, may impact risk of exposure to *Culex* vectors and WNV in mountainous areas of the western U.S. where current climate conditions are marginally suitable for *Culex* vectors and viral replication, but where nearby lower elevation areas have active WNV transmission foci.

Variability in the seasonal abundance pattern for *Ae. vexans* along the same elevation gradient was less dramatic. The seasonal pattern observed below 1,600 m, with increased abundance from late June to early August, was expected from previous studies conducted in Great Plains landscapes in eastern Colorado and Nebraska (Janousek and Kramer 1999, Bolling et al. 2007). In foothills-montane areas above 1,600 m, *Ae. vexans* exhibited a similar rate of increase as seen in the lower elevation sites from late May to early June. Abundance then stabilized briefly before starting to decline in late June, which differed from the lower elevation sites where abundance continued to increase sharply until mid-July. This resulted in a distinct seasonal pattern above 1,600 m characterized by a short period of increasing abundance in late May, a brief peak during the first two weeks of June, and a slow decline thereafter. We speculate that the seasonal pattern above 1,600 m results from a combination of: 1) limited access to larval development sites in dry foothills-montane canyon landscapes beyond the initial spring river flooding event; and 2) cooler temperatures negatively impacting larval development rates and female gonotrophic cycles.

Seasonal patterns of *Culex* abundance in the northeastern Colorado plains. Seasonal patterns of abundance of *Cx. tarsalis* and *Cx. pipiens* have been described previously from many parts of the western U.S. The single peak seasonal pattern for

abundance of *Cx. tarsalis* females observed in this study in 2006-2007, with elevated abundances occurring from late June to mid-August, agrees with previous studies from Colorado (e.g., Tsai et al. 1988, Smith et al. 1993, Bolling et al. 2007), Nebraska (Janousek and Kramer 1999), North Dakota (Bell et al. 2005), Utah (Beadle 1959), Washington (Pecoraro et al. 2007), and northern California (Reisen et al. 1995a). A different seasonal pattern with an earlier spring peak and a second distinct peak in the fall can occur in warmer areas such as southeastern California (e.g., Reisen et al. 1995b, 2008a, 2009). Further, an intermediate pattern, with a peak for *Cx. tarsalis* females in July and a smaller but distinct second peak in September, was reported from the Davis area in central California (Nielsen et al. 2008).

The seasonal pattern observed by us for *Cx. pipiens* females in 2007, with gradually increasing abundance reaching a peak in late August, agrees with a previous study using light traps in western Colorado (Tsai et al. 1988). Other studies have reported earlier peaks for *Cx. pipiens* females in late June and July in Washington (Pecoraro et al. 2007) and the Central Valley of California (Nielsen et al. 2008). We also recognize that the data for *Cx. pipiens* in our study should be interpreted with care because use of CDC light traps can underestimate the abundance of this species compared to efforts that also include gravid traps (Tsai et al. 1988).

With the exception of California (e.g., Kliewer et al. 1969; Olson et al. 1979; Reisen et al. 1992b, 2008b; Wegbreit and Resien 2000), there is a lack of long-term studies from the western U.S. to determine the extent of between-year variability in seasonal abundance patterns for *Culex* vectors, especially in relation to weather patterns. This is unfortunate because such studies are critical for developing models to forecast

Culex vector abundance based on weather patterns. For example, Reisen and colleagues used long-term (1950-2000) data for *Cx. tarsalis* from mosquito control programs in California to determine impacts of climate variation on mosquito abundance and found strong correlations between spring abundance of *Cx. tarsalis* and winter-spring precipitation, winter snow pack and winter-spring temperature (Reisen et al. 2008b). Similar studies are needed from the Great Plains WNV disease focus.

Seasonal patterns for WNV infection rates and Vector Index for *Cx. tarsalis*. The overall seasonal pattern for WNV infection rates in *Cx. tarsalis* females in the northeastern Colorado plains in 2007 was characterized by a gradual increase in infection rates from late June to late July, peak values occurring during the first half of August, and infection rates remaining high until the study was concluded in mid-September (Table 2.3). A similar monthly pattern was recorded from June to August for WNV infection rates in *Cx. tarsalis* females in other parts of Weld County in 2007 (Kent et al. 2009). Other studies have produced similar seasonal patterns for infection of *Cx. tarsalis* with WNV in California, New Mexico, and North Dakota (Bell et al. 2005; DiMenna et al. 2006; Nielsen et al. 2008; Reisen et al. 2008a, 2009), and with western equine or St. Louis encephalitis viruses in Colorado (e.g., Hess and Hayes 1967, Tsai et al. 1988, Smith et al. 1993). We also found considerable variation among trap sites in seasonal patterns for WNV infection rates (Table 2.3) which underscores the importance of operating multiple trap stations for mosquito-based WNV surveillance. Additional studies are needed to determine optimal combinations of trap densities and trap locations for mosquito-based WNV surveillance in the Great Plains landscape to minimize operational cost without compromising data quality.

The general pattern for WNV infection rates in *Cx. tarsalis* observed in our study likely reflects the seasonal pattern of intensity of enzootic transmission of WNV, which can be expected to increase over the summer as *Culex* vectors become more abundant and new generations of WNV-susceptible birds emerge. Further, a temporal shift in feeding behavior of *Cx. tarsalis* towards increased feeding on mammals from spring to summer has been observed in California and northeastern Colorado (Tempelis et al. 1965, 1967). This phenomenon has been hypothesized to impact seasonal risk of human exposure to *Cx. tarsalis* and other *Culex* species (Edman and Taylor 1968, Kilpatrick et al. 2006). However, a recent study from Weld County showed that the percentage of *Cx. tarsalis* that fed on humans increased from June to July/August, but remained below 7% for all months examined (Kent et al. 2009). During each month, >75% of *Cx. tarsalis* blood meals came from birds. The epidemiological importance of temporal shifts in feeding behavior remains unclear. We caution against attempting to adjust risk indices for human exposure to WNV-infected *Cx. tarsalis* based on perceived seasonal changes in feeding behaviors until we have gained a better understanding of the underlying mechanisms. For example, increased feeding on humans in summer may simply reflect changes in human behavior rather than changes in mosquito feeding habits.

The Vector Index for abundance of WNV-infected vectors, which was developed by the Centers for Disease Control and Prevention, is in operational use in mosquito control programs in some parts of the western U.S., including Colorado, but has not received much attention in the published literature. In fact, we are only aware of two previously published studies presenting data on the Vector Index, or variations of the Vector Index, for *Cx. tarsalis* (Bell et al. 2005, Gujral et al. 2007). Our study, which uses

a Vector Index for *Cx. tarsalis* females to assess risk of exposure to WNV-infected females, clearly demonstrates the value of combining information for vector abundance and WNV infection rates to generate a more meaningful risk index. In Fig. 2.5, we illustrate how the seasonal pattern for the Vector Index differs from that of mosquito abundance alone or WNV infection rate alone. In fact, we find it surprising that this type of risk index has taken so long to permeate the WNV literature. Similar risk indices that combine vector abundance and vector infection rate are used extensively to assess risk for exposure to tick-borne pathogens such as *Borrelia burgdorferi* (e.g., Mather et al. 1996, Stafford et al. 1998, Eisen et al. 2004) and also were used previously as measures of risk for exposure to *Cx. tarsalis* infected with western equine or St. Louis encephalitis viruses (e.g., Reeves et al. 1962, Hess and Hayes 1967, Tsai et al. 1988)

Predicting seasonal patterns for WNV disease cases from entomological risk measures. Our study demonstrates that the number of weekly human cases of WNV disease within the targeted 5-county area in 2007 could be predicted by the abundance of *Cx. tarsalis* females (4-7 weeks previously) and by the Vector Index for WNV-infected *Cx. tarsalis* females (1-2 wk previously) (Table 2.4; Figs 2.6-2.7). Interestingly, a previous study on western equine encephalitis virus (WEEV) in eastern Colorado in 1965 showed a similar pattern where the weekly numbers of human WEE cases were predicted by abundance of *Cx. tarsalis* females with a 4-wk time-lag and by abundance of WEEV-infected *Cx. tarsalis* per trap night with a 2-wk time lag (Hess and Hayes 1967). A recent large-scale study of WEEV transmission to sentinel chickens in central and southeastern California also identified 4-6 weeks as the critical time lag between *Cx. tarsalis* abundance and sentinel chicken seroconversions (Barker 2008).

Our data for time-lags between entomological risk measures and human WNV disease cases provide critical information for operational surveillance programs to determine time-lags for which these entomological risk measures are meaningful and how they should be used to guide emergency vector control activities. One distinct drawback of using the Vector Index is the short lead time of 1-2 weeks for reliable prediction of human case loads. This underscores the critical need for rapid turn-around of WNV testing of mosquito pools in order for the Vector Index to be operationally useful. The longer lead time for abundance of *Cx. tarsalis* females (4 wk) to predict human case loads for WNV disease argues for use of this entomological risk measure. However, the robustness of the predictive capability of this risk measure needs to be evaluated prospectively and corroborated in other areas and over multiple years because the longer time-lag, relative to the Vector Index, may result in greater sensitivity to weather events such as cold spells that can affect vector population growth and intensity of enzootic WNV transmission. Advances in statistical models for early detection or warning systems (e.g., Chaves and Pascual 2007) also provide new opportunities to explore how entomological data can be used to predict human cases.

CHAPTER 3

INSECT-SPECIFIC FLAVIVIRUSES FROM *CULEX* MOSQUITOES IN COLORADO, WITH EVIDENCE OF VERTICAL TRANSMISSION

Introduction

The genus *Flavivirus* contains numerous arthropod-borne viruses (arboviruses) that are associated with disease in vertebrates. These arboviruses are capable of replicating in both vertebrate and invertebrate cells (Cook and Holmes, 2006). There is, however, a group of viruses within the genus *Flavivirus* that appear to replicate only in invertebrate cells. These are considered insect-specific flaviviruses and include cell fusing agent virus (CFAV) (Stollar and Thomas, 1975), Kamiti River virus (KRV) (Crabtree et al., 2003, Sang et al., 2003), *Culex* flavivirus (CXFV) (Hoshino et al., 2007), Quang Binh virus (Crabtree et al., 2009), *Aedes* flavivirus (AEFV) (Hoshino et al., 2009), Nounané virus (NOUV) (Junglen et al., 2009), Lammi virus (LAMV) (Huhtamo et al., 2009), and Nakiwogo virus (Cook et al., 2009). Phylogenetic analyses suggest that insect-specific viruses are the most divergent group within the genus *Flavivirus* and may represent the earliest forms of flaviviruses (Cook and Holmes, 2006, Hoshino et al., 2007).

Cell fusing agent virus, the first insect-specific flavivirus described, was isolated from a cultured line of *Ae. aegypti* mosquito cells (Stollar and Thomas, 1975). It has recently been isolated from field-collected mosquitoes in Puerto Rico (Cook et al., 2006). Kamiti River virus was isolated in 2003 from *Ae. macintoshi* larvae and pupae from Kenya and described as a CFAV-related flavivirus (Crabtree et al., 2003, Sang et al., 2003). *Culex* flavivirus was first isolated in Japan from *Cx. pipiens* and other *Culex* species (Hoshino et al., 2007), and has since been described in *Culex* mosquitoes from Guatemala (Morales-Betoulle et al., 2008), Mexico (Farfan-Ale et al., 2009), Trinidad (Kim et al., 2009), Texas (Kim et al., 2009), Iowa (Blitvich et al., 2009), and Uganda

(Cook et al., 2009). Quang Binh virus was isolated from *Cx. tritaeniorhynchus* collected in Vietnam in 2002 (Crabtree et al., 2009).

Aedes flavivirus, isolated from *Aedes* mosquitoes in Japan, groups with other insect-specific flaviviruses in phylogenetic analyses, but, interestingly, has a high degree of similarity to cell silent agent (CSA), which is a flavivirus-related nucleotide sequence found integrated into the genome of *Ae. albopictus* (Hoshino et al., 2009, Crochu et al., 2004). Two other recently described flaviviruses, NOUV (Junglen et al., 2009) and LAMV (Huhtamo et al., 2009), appear to represent a distinct group of insect-specific flaviviruses. Phylogenetic analyses group them with mosquito-borne flaviviruses that cause disease in vertebrates, such as West Nile and Japanese encephalitis viruses, yet they do not appear to grow in vertebrate cells (Junglen et al., 2009, Huhtamo et al., 2009). Research on insect-specific flaviviruses is rapidly expanding, as new viruses are being isolated and characterized.

The findings described here originated from adult mosquito collections conducted along riparian corridors in northeastern Colorado during 2006-2007 to investigate spatial and temporal risk patterns for exposure to West Nile virus (WNV)-infected *Culex* spp. mosquitoes. *Culex* flavivirus (CXFV) and a novel insect-specific flavivirus (Calbertado virus, CLBOV) were detected from *Cx. tarsalis* and *Cx. pipiens* using universal flavivirus primers targeting the NS5 gene.

Materials and Methods

Study area. The 5-county study area (Larimer, Weld, Morgan, Logan, and Washington) is located in northeastern Colorado and has been previously described

(Bolling et al.,2009). Briefly, mosquito sampling was conducted along three rivers: the Poudre River and the Big Thompson River, which both emerge from the Rocky Mountains in western Larimer County, and the South Platte River into which the other two rivers merge in the eastern Colorado plains (Fig. 3.1). Mosquito collections were conducted in plains, foothills, and montane areas along these riparian corridors in sampling sites which could be accessed by automobile. Site locations were mapped with a GPS receiver (Trimble Geo XT; Trimble Corp., Sunnyvale, CA) and visualized using ArcGIS 9.3 (ESRI, Redlands, CA).

Mosquito collection and identification. Mosquitoes were collected using CO_2-baited CDC miniature light traps (John W. Hock Company, Gainesville, FL) that were suspended ~1.5 m above the ground and operated from afternoon (1500-1700 hours) until morning (0800-1000 hours). Sampling sites contained two traps baited with ~1 kg of dry ice and were located directly along the aforementioned rivers. Sampling during the summer of 2006 included 10 sites located along the Poudre River and 1 site by the Dixon Reservoir in Fort Collins (Fig. 3.1). This spanned an elevation gradient from below 1,600 m in Fort Collins up to 2,360 m in the Poudre Canyon. The sites were sampled every 2 wk from mid-April to late October 2006.

Sampling in 2007 included 20 sites along the Big Thompson and South Platte rivers and two additional sites located south of the Big Thompson River in the Loveland area (Fig. 3.1). This included an elevation gradient ranging from 1,215 m in the prairie landscape of eastern Colorado to 1,840 m in the montane habitat of the Big Thompson Canyon. The sites were sampled every 2 wk from mid-June to mid-September 2007. Collected mosquitoes were examined with a dissecting microscope and identified to

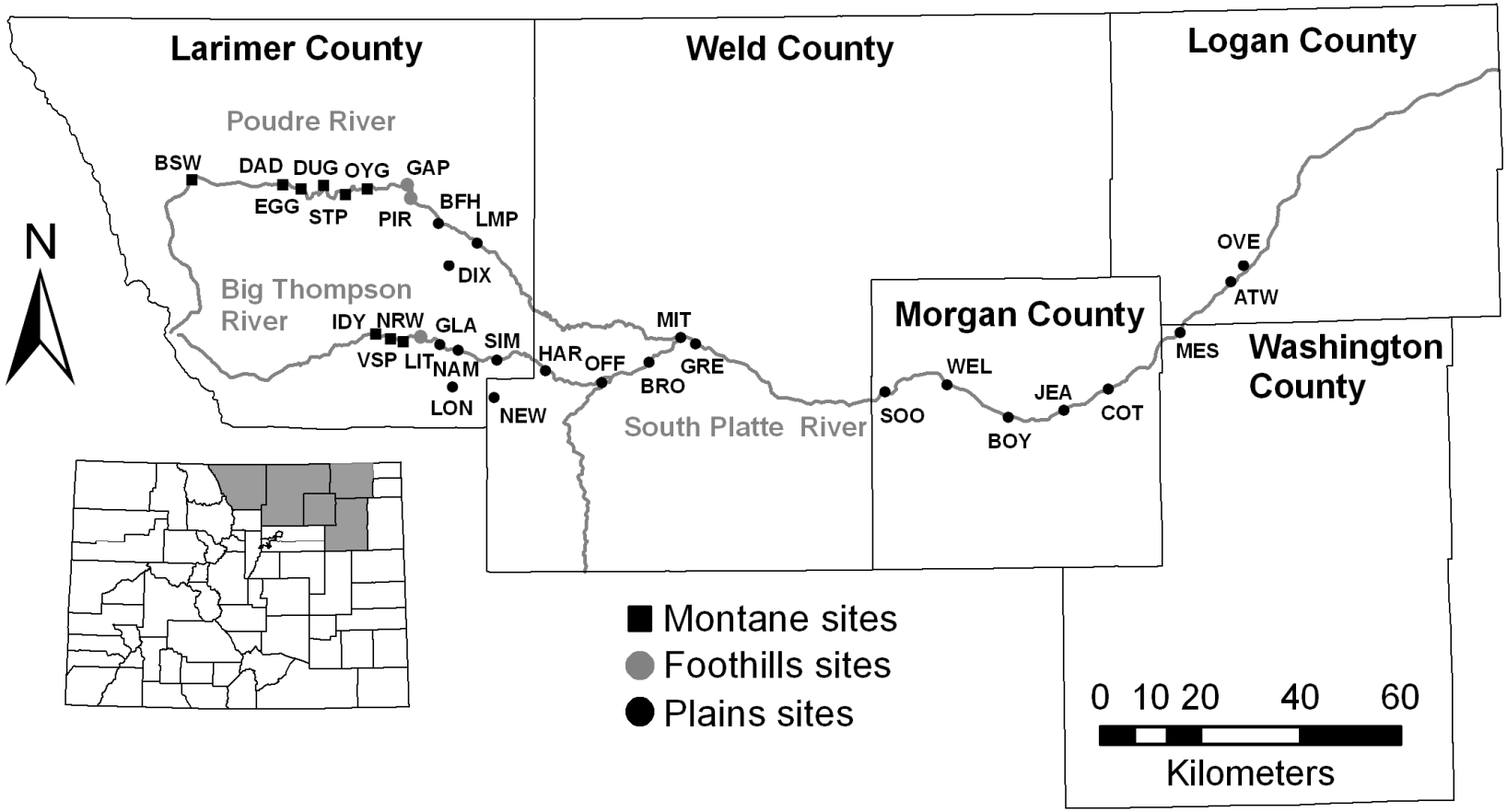


Figure 3.1. Location of mosquito sampling sites for 2006 (along the Poudre River) and 2007 (along the Big Thompson River and South Platte River). The location of the targeted 5-county area in Colorado is shown in the inset map.

species using published identification keys (Harmsten and Lawson, 1967, Darsie and Ward, 2005).

Detection of flavivirus RNA and nucleotide sequencing. *Culex* mosquitoes were examined for presence of viral RNA following Bolling et al. (2007), with the modifications outlined below. Mosquitoes were identified on a chill table and placed in pools of 1 to 50 by species, sex, site, trap location, and date. Mosquito pools were then stored at -70°C until processed for viral RNA detection.

Each pool was triturated for 45 sec with a vortex mixer in a 5-ml round-bottom polypropylene tube (Becton Dickinson, Franklin Lakes, NJ) using 1.5 ml of diluent (minimum essential medium [MEM] containing 2% fetal bovine serum [FBS], 100 µg/ml penicillin/streptomycin, supplemented with L-glutamine and nonessential amino acids) and 4 copper-coated steel shot (4.5-mm diameter; 0.177" caliber). Suspensions were centrifuged at 3,000 rpm for 10 min at 4°C. Total RNA was extracted from 140 µl of the supernatant using the QIAamp viral RNA Mini kit (Qiagen Inc., Valencia, CA). RNA was eluted in 60 µl of nuclease-free water (Ambion Inc., Austin, TX).

Reverse transcription-polymerase chain reaction (RT-PCR) was carried out using SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA) and GoTaq DNA polymerase (Promega, Madison, WI). Mosquito pools were first tested using universal flavivirus primers (cFD2 and MAMD) targeting a portion of the NS5 gene (Scaramozzino et al., 2001). Pools testing positive for flavivirus RNA were tested with virus-specific primers for WNV (Gubler et al., 2000, Lanciotti et al., 2000), CXFV (Hoshino et al., 2007), and CLBOV. PCR products were visualized following

electrophoresis on a 1% agarose gel stained with ethidium bromide. Negative (no template) and positive controls were included in each RT-PCR.

Infection rates per 1,000 individuals were calculated as bias-corrected Maximum Likelihood Estimates using the Excel Add-In PooledInfRate, version 3.0 (Biggerstaff 2006). Presented infection rates are based on males and females combined. The most common *Culex* spp. found in our light trap collections were *Cx. tarsalis* and *Cx. pipiens*. Because *Cx. tarsalis* are easily identifiable, even when badly damaged, mosquitoes identified only as *Culex* spp. were grouped with *Cx. pipiens* for analyses.

For nucleotide sequencing, RT-PCR was carried out as described above using primers FU1 or FU2 and cFD3 (Kuno et al., 1998). The PCR products were purified using a QIAquick Gel Extraction kit (Qiagen) and submitted for sequencing to the CSU Proteomics and Metabolomics Facility, which uses the ABI 3130 Genetic Analyzer. Sequences obtained for the NS5 genome region were compared to other flavivirus sequences using the BLASTn program (blast.ncbi.nlm.gov/Blast.cgi) (Altschul et al., 1997).

Phylogenetic analysis. Nucleotide sequences of insect-specific flavivirus genomes detected from *Culex* spp. in Colorado were compared to known flavivirus sequences as follows. The ~1kb sequences from NS5 genes were aligned using ClustalX 1.81 (Thompson et al., 1997). The ClustalX program aligns each sequence to each of the other sequences and uses the pairwise alignments to create a guide tree, which is then used to help create a multiple alignment (Hall 2004). Phylogenetic trees were constructed from the alignment using various methods including neighbor-

joining, maximum parsimony, and maximum likelihood analyses in PAUP 4.0 (Swofford 1991), and also with Bayesian analysis using MrBayes 3.1 (Huelsenbeck and Ronquist, 2001). Neighbor joining is a distance-based method that calculates a distance matrix from the differences between sequence comparisons and uses that matrix to construct a tree showing the net divergence between taxa. Maximum parsimony, maximum likelihood, and Bayesian analyses are character-based methods where characters are compared within each column of a multiple alignment (Hall 2004). The maximum parsimony method selects a tree that requires the least number of evolutionary changes to explain the data. Maximum likelihood analysis produces a tree by evaluating the probability of a specified evolutionary model generating the observed sequences. The evolutionary model used for this study was the general time-reversible (GTR) model, which estimates base frequencies that are site-specific by codon position. Bayesian phylogenetic analysis is a variant of maximum likelihood where a set of trees is chosen based on a sampling method called Monte Carlo Markov Chain, which samples trees from the distribution of posterior probabilities (Hall 2004). Resulting trees were midpoint rooted to show relationships between sequences using RETREE from the Phylip package (Felsenstein 1989) and displayed using Treeview 1.6.6 (Page 1996).

Virus isolation. To isolate viruses, 100µl of homogenate supernatants from infected pools were inoculated onto Vero cells (African green monkey kidney), DF-1 cells (chicken embryo fibroblast), and C6/36 cells (*Ae. albopictus*) in 25 cm^2 flasks. After addition of 1 ml of medium, the flasks were rocked for 1 hr at room temperature. Four ml of medium were then added and cells were monitored for

cytopathic effects (CPE). The Vero and DF-1 cells were maintained at 37°C and 7% CO_2 with MEM supplemented with 10 % FBS, penicillin/streptomycin, L-glutamine, and nonessential amino acids. The C6/36 cells were maintained at 28°C with L-15 medium supplemented with 7% FBS, penicillin/streptomycin, L-glutamine, and nonessential amino acids. Cell culture medium was harvested after each passage for 4 passages and tested by RT-PCR to assess virus propagation.

Immunofluorescense assay. Indirect fluorescent-antibody assays (IFA) were conducted on spot slides of infected C6/36 cells using polyclonal anti Japanese encephalitis virus (JEV), St. Louis encephalitis virus (SLEV), WNV, CXFV, and CLBOV as primary antibody. Murine hyperimmune ascitic fluids for JEV (M30178ABY), SLEV (VS0102), and WNV (M30200ABY) were obtained from the Division of Vector-Borne Infectious Diseases, Centers for Disease Control and Prevention, Fort Collins, CO. Virus-specific antisera for the Colorado strains of CXFV and CLBOV were produced by subcutaneous immunization of ICR mice with infected C6/36 cell culture medium, clarified by centrifugation. Mice were immunized 3 times, at 2 wk intervals, with complete Freund's adjuvant for the first immunization and incomplete Freund's adjuvant for the remaining immunizations. An indirect ELISA on mouse sera 2 wk after the final immunization confirmed antibody production. Serum was cross-absorbed twice to sonicated C6/36 cells to reduce non-specific binding. Secondary antibody for IFA was biotinylated sheep anti-mouse IgG, followed by streptavidin-fluorescein (GE Healthcare Bio-Sciences Corp., Piscataway, NJ).

Persistently CXFV-infected mosquito colony. A *Cx. pipiens* colony was established from egg rafts collected in Fort Collins during summer 2005. After discovery of CXFV and CLBOV in local field populations, the colony was determined to be infected with CXFV by RT-PCR as described above and by virus isolation in C6/36 cells. Mosquito homogenates taken from the colony in 2005 and stored at -80°C tested positive for CXFV, indicating that the mosquitoes were infected at the time the colony was established. The colony is maintained in a 60cm x 60cm x 60cm cage at 25°C, 75% relative humidity, with a 16:8 light:dark cycle. To investigate viral maintenance within the colony, a quantitative RT-PCR assay was designed using a QuantiTect SYBR Green RT-PCR kit (Qiagen Inc., Valencia, CA) and CXFV-specific primers. Total RNA was extracted from individual egg rafts, individual 4th instar larvae, and individual adult mosquitoes using Trizol (Invitrogen, Carlsbad, CA).

Results

Discovery of insect-specific flaviviruses in *Culex* spp. In 2006, ~1,300 *Culex* mosquitoes from northeastern Colorado were tested for viral RNA by RT-PCR using universal flavivirus primers. Unexpectedly, numerous *Culex* pools testing positive for flavivirus RNA were negative for the flaviviruses that most commonly are found in *Culex* in Colorado: WNV and SLEV. To rule out contamination, 80 pools (89 mosquitoes) of *Culiseta* spp. mosquitoes and 64 pools (1,997 mosquitoes) of *Ae. vexans* were tested by RT-PCR with the universal flavivirus primers. All these pools tested negative.

PCR products from flavivirus-positive *Culex* pools were then sequenced, revealing similarity in some cases to a previously described insect-specific flavivirus, CXFV. In addition, we discovered sequences from a novel insect-specific flavivirus, referred to herein as Calbertado virus (CLBOV), as this virus has been found in California, Alberta, Canada (Pabbaraju et al., 2009, Tyler et al., 2010), and Colorado. Specific primers were designed to detect these insect-specific flaviviruses in mosquito pools.

In 2007, we collected ~43,000 *Culex* spp. mosquitoes. All *Culex* pools were first tested with the flavivirus primers. Thereafter, all flavivirus-positive pools were tested with WNV-specific, CXFV-specific, and CLBOV-specific primers.

Trends for insect-specific flaviviruses in *Culex* spp. in time and space. Infection rates for WNV, CXFV, and CLBOV in *Cx. tarsalis* and *Cx. pipiens* were calculated by month (Table 3.1) and by site (Table 3.2) for both 2006 and 2007. Infection rates include male and female pools of mosquitoes combined, as viral RNA was detected in both. The seasonal patterns of infection rates are shown for 2007 (Fig. 3.2), when *Culex* abundance was higher and virus detection was based on larger numbers of mosquitoes than in 2006.

In the case of the 2006 collections, most CXFV-positive pools (36/37) came from *Cx. pipiens*, with only a single *Cx. tarsalis* pool positive for CXFV. Conversely, all (34) CLBOV-positive pools were from *Cx. tarsalis* (Table 3.1). This pattern was less clear in 2007 when both CXFV and CLBOV were found in *Cx. tarsalis* as well as *Cx. pipiens*. However, there still was a trend towards most CXFV-positive pools being recorded from *Cx. pipiens* (contributing 125 of 178 CXFV-positive pools) and

most CLBOV-positive pools coming from *Cx. tarsalis* (contributing 113 of 121 CLBOV-positive pools).

The overall infection rates per 1,000 *Cx. tarsalis* in 2006 were 5.47 for WNV, 0.83 for CXFV, and 40.13 for CLBOV. The corresponding infection rates for *Cx. tarsalis* in 2007 were 2.65 for WNV, 1.34 for CXFV, and 2.95 for CLBOV. Differences between years are likely related, in part, to the fact that different sites were sampled in 2006 and 2007. The overall infection rates per 1,000 *Cx. pipiens* in 2006 were 0 for WNV, 462.42 for CXFV, and 0 for CLBOV. The corresponding infection rates for *Cx. pipiens* in 2007 were 2.41 for WNV, 72.09 for CXFV, and 3.18 for CLBOV. Notably, CXFV infection rates in *Cx. pipiens* were very high compared to the other two viruses.

Table 3.1. Monthly infection rates with West Nile virus (WNV), *Culex* flavivirus (CXFV), and Calbertado virus (CLBOV) for *Cx. tarsalis* and *Cx. pipiens*, Colorado, 2006-2007.

Yr	Month	*Culex tarsalis*					*Culex pipiens*				
		Total Mosq.	No. Pools	Infection Rates (MLE)			Total Mosq.	No. Pools	Infection Rates (MLE)		
				WNV	CXFV	CLBOV			WNV	CXFV	CLBOV
2006	April	17	7	0	0	154.98	1	1	0	1000*	0
	May	4	3	0	0	0	0	0	0	0	0
	June	45	12	0	0	133.48	9	3	0	76.46	0
	July	889	40	7.71	1.12	33.37	56	12	0	247.49	0
	Aug	189	52	0	0	32.70	43	20	0	638.14	0
	Sept	77	21	0	0	36.56	18	8	0	777.73	0
	Oct	0	0	0	0	0	2	1	0	500*	0
2007	June	7909	546	0.51	0.25	3.28	110	25	18.94	211.95	17.00
	July	23420	1308	1.96	0.86	2.91	568	71	1.72	101.53	3.45
	Aug	8662	1233	6.58	3.31	2.61	1407	108	1.44	124.35	2.18
	Sept	444	225	6.80	8.91	2.22	454	38	2.23	67.19	2.15

Adult mosquitoes were tested in pools of 1-50. Bias-corrected Maximum Likelihood Estimates for infection rates per 1000 mosquitoes were calculated with Excel Add-In PooledInfRate 3.0 (Biggerstaff 2006).
[1]Based in part on data published previously by Bolling et al (2009).
[2]One single pool that was examined tested positive, so a minimum infection rate was calculated ([no. of positive pools/no. of mosquitoes tested] x 1,000).

In 2007, the WNV and CXFV infection rates in *Cx. tarsalis* increased gradually from June to September, while the CLBOV infection rates were highest in June (3.28) and then decreased slightly from July to September (Table 3.1, Fig. 3.2). West Nile virus and CLBOV infection rates in *Cx. pipiens* followed similar seasonal patterns, with the highest infection rates occurring in June, when mosquito counts were low (Table 3.1, Fig. 3.2).

Table 3.2. Infection rates by trapping site with West Nile virus (WNV), *Culex* flavivirus (CXFV), and Calbertado virus (CLBOV) for *Cx. tarsalis* and *Cx. pipiens*, Colorado, 2006-2007.

Year	Site	Habitat	Elevation (m)	*Culex tarsalis*					*Culex pipiens*				
				Total Mosq.	No. Pools	Infection Rates			Total Mosq.	No. Pools	Infection Rates		
						WNV[1]	CXFV	CLBOV			WNV[1]	CXFV	CLBOV
Poudre River transect (2006)	LMP	Plains	1,510	108	18	0	0	45.05	23	11	0	400.08	0
	BFH	Plains	1,560	436	37	2.34	2.35	36.95	63	23	0	902.52	0
	DIX	Plains	1,585	610	41	9.83	0	37.40	33	7	0	189.75	0
	PIR	Foothills	1,610	38	14	0	0	59.40	10	4	0	150.08	0
	GAP	Foothills	1,640	10	7	0	0	102.37	0	0	0	0	0
	OYG	Montane	1,750	7	7	0	0	0	0	0	0	0	0
	STP	Montane	1,860	5	4	0	0	0	0	0	0	0	0
	DUG	Montane	2,000	4	4	0	0	0	0	0	0	0	0
	EGG	Montane	2,110	1	1	0	0	0	0	0	0	0	0
	DAD	Montane	2,130	1	1	0	0	0	0	0	0	0	0
	BSW	Montane	2,360	1	1	0	0	0	0	0	0	0	0
Big Thompson River-South Platte River transect (2007)	OVE	Plains	1,215	1,628	189	8.22	1.25	1.23	60	19	0	33.02	0
	ATW	Plains	1,219	2,799	192	2.99	0	0.36	15	9	0	132.11	0
	MES	Plains	1,242	3,170	204	1.96	0.32	0.96	24	7	0	0	42.55
	COT	Plains	1,272	1,631	178	1.84	1.25	0	115	17	8.32	169.15	0
	JEA	Plains	1,286	4,436	242	3.12	0.68	1.39	63	13	17.08	14.92	0
	BOY	Plains	1,303	335	124	0	3.04	0	241	14	0	63.56	8.24
	WEL	Plains	1,321	4,862	241	2.37	0.21	1.26	40	10	0	27.03	0
	SOO	Plains	1,344	3,900	229	3.31	0	1.31	40	10	0	45.88	0
	GRE	Plains	1,397	2,035	170	0.98	0.49	3.60	14	8	68.82	235.53	0
	MIT	Plains	1,402	4,356	231	1.17	0.93	1.17	137	15	0	173.16	7.06
	BRO	Plains	1,418	2,846	209	1.07	5.39	6.40	588	22	1.68	84.48	0
	OFF	Plains	1,434	4,528	234	2.56	3.56	4.11	317	17	3.23	141.78	3.24
	HAR	Plains	1,459	975	139	4.49	3.19	13.72	193	16	0	207.95	4.88
	SIM	Plains	1,487	371	122	5.22	7.85	16.03	266	15	0	286.95	3.72
	NEW	Plains	1,511	1,387	165	5.59	1.47	12.83	328	16	3.07	73.60	0
	NAM	Plains	1,524	238	98	8.17	0	12.22	21	8	0	143.95	47.77
	GLA	Plains	1,544	262	97	0	0	3.66	21	10	0	385.73	0
	LON	Plains	1,566	555	135	1.75	1.75	7.13	49	12	0	298.85	0
	LIT	Foothills	1,598	64	57	0	0	0	7	4	0	359.92	0
	NRW	Montane	1,688	7	7	0	0	0	0	0	0	0	0
	VSP	Montane	1,737	46	45	0	0	0	0	0	0	0	0
	IDY	Montane	1,840	4	4	0	0	0	0	0	0	0	0

Adult mosquitoes were tested in pools of 1-50. Bias-corrected Maximum Likelihood Estimates for infection rates per 1000 mosquitoes were calculated with Excel Add-In PooledInfRate 3.0 (Biggerstaff 2006)

[1]Based in part on data published previously by Barker et al (2009).

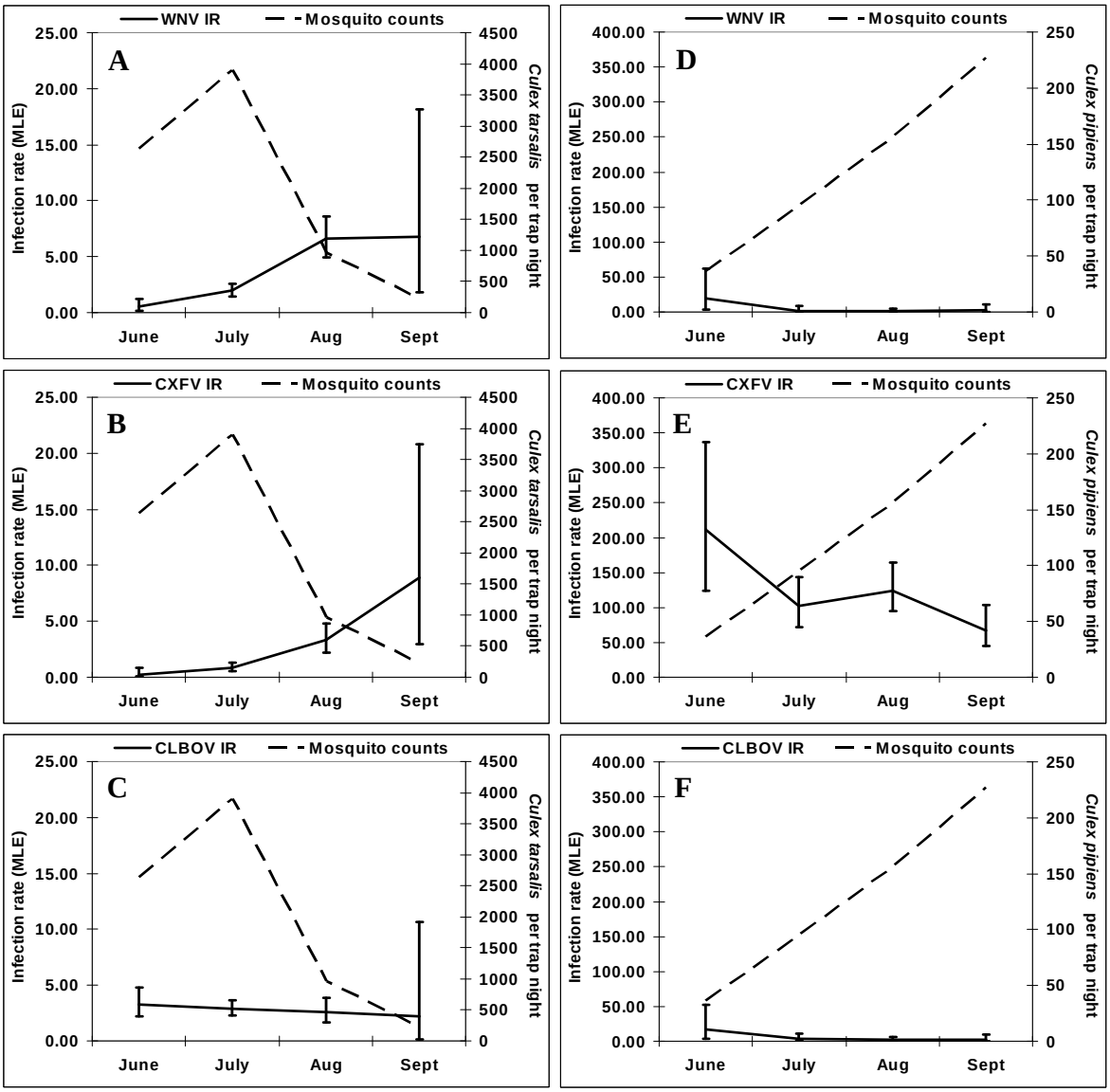


Figure 3.2. Monthly infection rates from June-September 2007 for *Cx. tarsalis* (A-C) and *Cx. pipiens* (D-F) with West Nile virus (WNV) (A and D), *Culex* flavivirus (CXFV) (B and E), Calbertado virus (CLBOV) (C and F). Error bars indicate 95% skewness-corrected confidence intervals. Estimates for infection rates per 1000 mosquitoes were calculated with Excel Add-In PooledInfRate 3.0 (Biggerstaff 2006).

Both insect-specific flaviviruses occurred widely in the 5-county study area. CXFV was detected from all 21 sites in the plains sampled in 2006-2007 and CLBOV was recorded from 20 of these sites. The viruses also occurred in foothills sites but were not recorded from montane sites where mosquito counts are very low (Table 3.2). Site-specific infection rates for WNV were compared to site-specific CXFV and CLBOV infection rates by graphing, but there were no apparent associations (data not shown).

Phylogenetic analyses. Bayesian analysis was conducted using a 1 kb region of the NS5 gene sequence to assess phylogenetic relationships between insect-specific flavivirus isolates from *Culex* spp. mosquitoes in Colorado and other selected flaviviruses (Fig. 3.3). Neighbor-joining, maximum parsimony and maximum likelihood analyses resulted in similar tree topologies (data not shown). The CXFV isolate from this study was most similar to CXFV isolates from Texas, and grouped with other CXFV isolates from Iowa, Japan, Mexico, and Guatemala (Fig. 3.3). The Colorado CLBOV isolate shared closest phylogenetic relationships with CLBOV sequences detected in California and Alberta, Canada (Pabbaraju et al., 2009, Tyler et al., 2010), with blast results indicating 97% NS5 amino acid sequence similarity. Based on Bayesian analysis, the insect-specific flavivirus clade contains two subclades, which correspond with insect host genus. The first subclade contains CLBOV, Quang Binh virus, and the *Culex* flavivirus isolates, which have all been detected in *Culex* mosquitoes. The second subclade contains KRV, CFAV, and AEFV, which have all been described in *Aedes* mosquitoes. Interestingly, Nounané virus, which was recently isolated from *Uranotaenia* mosquitoes in Côte d'Ivoire

(Junglen et al., 2009), appears to only replicate in insect cells, and yet based on our phylogenetic analysis, groups with the arthropod-borne flaviviruses.

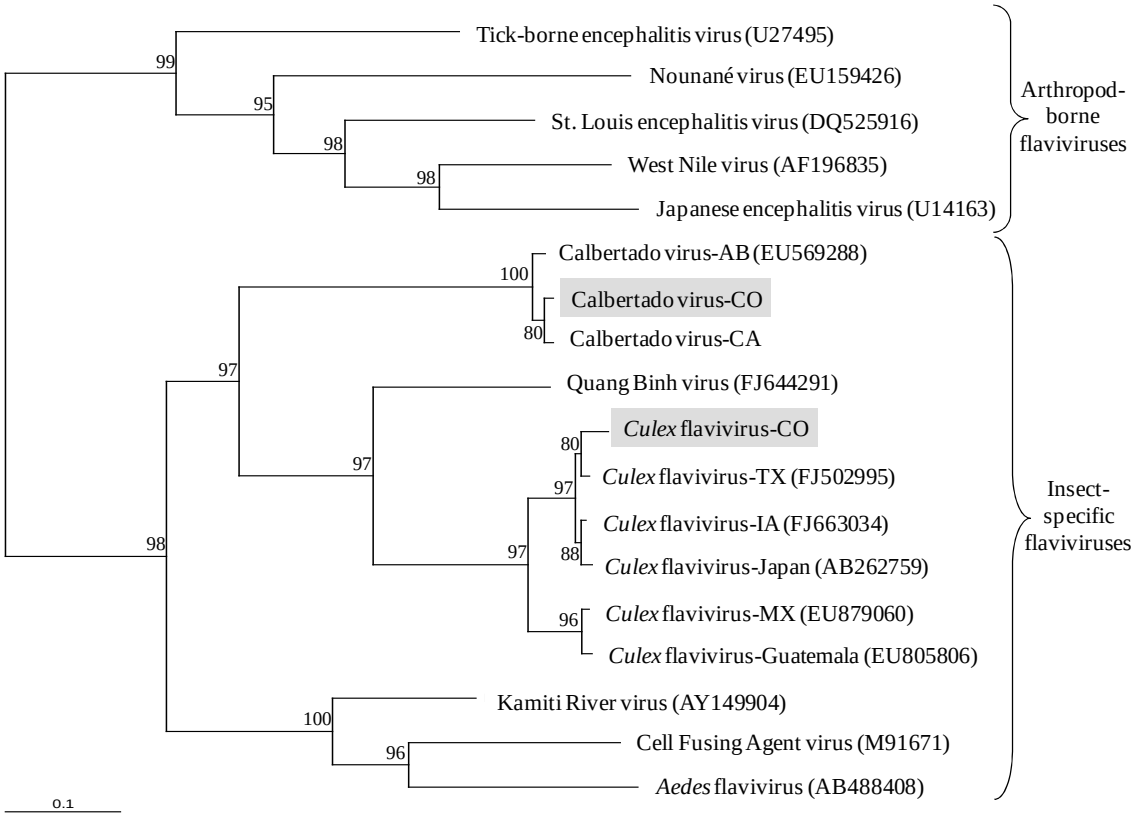


Figure 3.3. Phylogenetic tree inferred from Bayesian analysis and midpoint-rooted, showing relationships between insect-specific flaviviruses from *Culex* species mosquitoes collected in Colorado (shaded), with other flaviviruses, based on a 1 kb segment of the NS5 gene. The numbers at the nodes represent clade credibility values and the scale bar indicates the number of estimated substitutions per site. GenBank accession numbers for the sequences used in the analysis are listed in parentheses.

Virus isolation. Isolation of insect-specific flaviviruses from *Culex* in Colorado was attempted by blind passages in Vero, DF-1, and C6/36 cell cultures. Vero (mammalian) and DF-1 (avian) cells inoculated with mosquito homogenate supernatants and cell culture media did not exhibit any CPE after 1-4 passages. RNA

extractions were performed on Vero and DF-1 cell culture medium after each passage and these tested negative by RT-PCR using virus-specific primers. In contrast, CXFV-infected *Cx. pipiens* homogenates caused minor growth inhibition for C6/36 (mosquito) cells after several passages. RNA extracted from cell culture medium was CXFV-positive by RT-PCR after each passage. CLBOV-infected *Cx. tarsalis* homogenates caused apparent CPE in C6/36 cells on 5 dpi only after 10 passages. RNA extractions of C6/36 cell culture medium for passages 1-3 were negative by RT-PCR using virus-specific primers but after the 4th passage were positive. Spot slides of CXFV- and CLBOV-infected C6/36 cells were tested by IFA with JE, SLE, and WNV antibodies based on a previous report (Kim et al., 2009), but antigens were undetectable. IFA using virus specific antibodies for CXFV and CLBOV produced positive results (Fig. 3.4), confirming C6/36 cell infections.

CXFV infection in a *Cx. pipiens* laboratory colony. After isolation of CXFV from adult mosquitoes collected in 2006 and 2007, our *Cx. pipiens* laboratory colony, established during the summer of 2005 from egg rafts collected in Fort Collins, was tested in March of 2007 and found to be infected with CXFV by RT-PCR and virus isolation. A subset of mosquitoes had been taken from the colony in September of 2005 and stored at -80°C. These stored specimens also tested positive for CXFV, indicating that the mosquitoes were infected at the time the colony was established. To examine viral maintenance within this naturally infected colony, total RNA was extracted from individual egg rafts, individual 4th instar larvae, and individual adult (male and female) mosquitoes and tested by a qRT-PCR assay with virus-specific primers. All life stages were found to be positive for CXFV RNA.

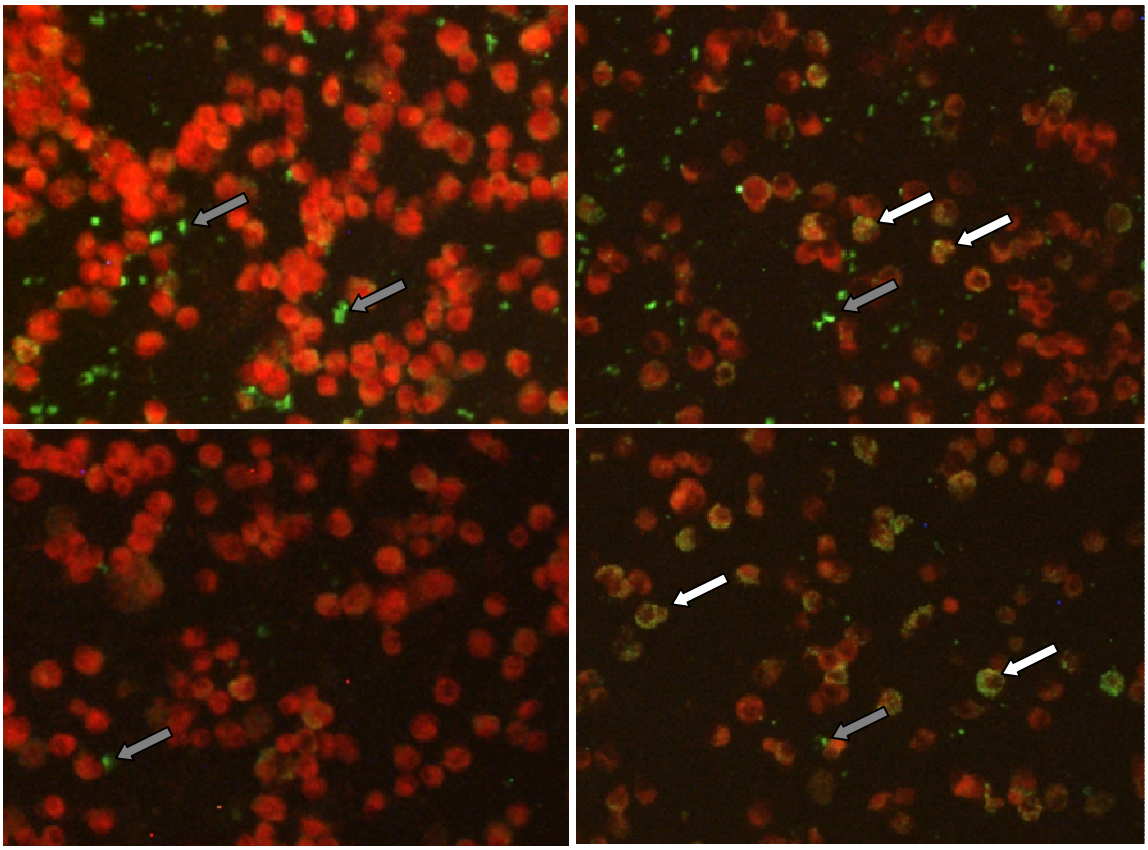


Figure 3.4. Indirect fluorescent-antibody assays performed on C6/36 cells using mouse antisera to CXFV (A, uninfected, B, CXFV-infected) and CLBOV (C, uninfected, D, CLBOV-infected). Cells were counterstained with Evans blue, so that uninfected cells appear red and viral antigen in infected cells appears yellow-green. Arrows indicate infected cells (white arrows) vs. non-specific extracellular staining artifacts (gray arrows).

Discussion

Culex mosquitoes collected in 2006-2007 in northeastern Colorado, as part of ongoing studies to investigate entomological patterns of risk for exposure to WNV, were found to be commonly infected with two insect-specific flaviviruses. Both viruses were isolated in C6/36 mosquito cells, but failed to replicate in Vero mammalian cells or DF-1 avian cells. Phylogenetic analyses, based on a 1 kb sequence from the NS5 gene, revealed that these viruses group with previously described insect-specific flaviviruses. We isolated a strain of *Culex* flavivirus

(CXFV) from *Cx. pipiens* and a novel insect-specific flavivirus, Calbertado virus (CLBOV), from *Cx. tarsalis*. The 1 kb sequence from the NS5 gene of the Colorado CLBOV isolate shares 97% identity with amino acid sequences from California and Alberta, Canada (Pabbaraju et al., 2009, Tyler et al., 2010). The next most closely related viruses to CLBOV are *Culex* flavivirus isolates, with NS5 nucleotide sequences ranging from 65-68% similarity. After detecting these insect-specific flaviviruses in *Culex* from Colorado, we initiated studies to investigate seasonal and spatial patterns of infection, determine cell culture host range, and examine viral prevalence within a naturally infected laboratory colony.

Trends for insect-specific flaviviruses in *Culex* spp in time and space. We found that infection rates for CXFV and CLBOV varied by *Culex* species, month, and site. In 2006, there was a strong species-specific pattern with CXFV being detected almost exclusively in *Cx. pipiens* and all records of CLBOV coming from *Cx. tarsalis.* In 2007, we collected and examined far greater numbers of *Culex* mosquitoes, and both insect-specific flaviviruses were detected in *Cx. tarsalis* as well as *Cx. pipiens*. These findings suggest that CXFV and CLBOV circulate in both species of mosquitoes in northern Colorado, although we cannot entirely rule out the possibility that a body part from one species sometimes was accidentally combined with a pool from the other species. *Culex* flavivirus has previously been detected in a variety of *Culex* spp. including *Cx. pipiens* (Hoshino et al., 2007, Blitvich et al., 2009), *Cx. tarsalis* (Blitvich et al., 2009), *Cx. quinquefasciatus* (Hoshino et al., 2007, Morales-Betoulle et al., 2008, Kim et al., 2009), *Cx. tritaeniorhynchus* (Hoshino et al., 2007), and *Cx. restuans* (Kim et al., 2009). These findings indicate that CXFV

occurs in numerous *Culex* species. We also tested a subset of *Ae. vexans* and *Culiseta* spp. pools, but these were negative with the pan-flavivirus primers used. Other studies also found *Aedes* species pools to be negative for CXFV RNA (Hoshino et al., 2007, Blitvich et al., 2009).

We detected CLBOV RNA in both *Cx. tarsalis* and *Cx. pipiens* from Colorado, and isolated the virus from several *Cx tarsalis* pools. A portion of the NS5 gene of this virus shares a high similarity to viral sequences found in *C. tarsalis* mosquitoes in Alberta and other western provinces of Canada where this mosquito is found (Pabbaraju et al., 2009, Tyler et al., 2010). Although a virus isolate has not yet been obtained from Alberta, detection of this sequence in a number of Canadian collections of *Culex* species suggests that CLBOV may have a wide geographic range. Field studies in California also indicate that CLBOV is present in *Cx. tarsalis* and *Cx. pipiens* populations there (Tyler et al., 2010).

Our data suggest that CXFV and CLBOV are prevalent in *Cx. tarsalis* and *Cx. pipiens* throughout the northeastern Colorado plains. In 2006, CXFV RNA was detected in April and in every month from June through October (Table 3.1). In 2007, CXFV was detected in both species from June through September. In Texas, Kim et al. (2009) recorded CXFV positive mosquito pools during February and March, but continued surveillance from April to August resulted in only negative pools. In Iowa, CXFV RNA was detected from July through October, but not in May or June, possibly because of low numbers of *Culex* collected early in the season (Blitvich et al., 2009).

Our study yielded very high infection rates for CXFV in *Cx. pipiens*. Other studies have reported variable infection rates for CXFV, with minimum infection rates (MIR) per 1,000 *Cx. quinquefasciatus* ranging from 4.7 in Guatemala (Morales-Betoulle et al., 2008) to 20.8 in Mexico (Farfan-Ale et al., 2009), and overall MIRs in Iowa ranging from 1.2 in *Cx. tarsalis* to 10.3 for *Cx. pipiens* (Blitvich et al., 2009). Both our study (Table 3.2) and the Iowa study recorded substantial site-specific variability for CXFV infection rates in a given species. Further research is needed to determine the mechanisms resulting in spatial and temporal variability in CXFV infection rates.

We present the first data regarding seasonality of CLBOV, which was recorded in April 2006 and then in every month from June - September in 2006 and 2007 (Table 3.1). Infection rates with CLBOV for months when at least 100 *Cx. tarsalis* or *Cx. pipiens* were examined ranged from 2.15 to 36.79. As a crude comparison, in Alberta, Canada, CLBOV was detected in 67 of 140 (48 %) *Cx. tarsalis* mosquito pools tested from 2003-2005 (Pabbaraju et al., 2009, Tyler et al., 2010).

Comparison of seasonal infection rates for CXFV, CLBOV and WNV. Sample sizes for *Culex* mosquitoes in 2007 were adequate to compare the seasonal (monthly) patterns for infection rates with CXFV, CLBOV and WNV from June-September (Fig. 3.2). This produced some unexpected and intriguing results. For instance, the infection rates in *Cx. tarsalis* for WNV and CXFV followed similar patterns, gradually increasing throughout the study period (Fig. 3.2A-3.2B), while the CLBOV infection rate showed an opposite, slightly decreasing trend from June

through September (Fig. 3.2C). The infection rates in *Cx. pipiens* with WNV, CXFV, and CLBOV followed the same general decreasing trend from June to September (Fig. 3.2D-3.2F), but the infection rates observed for CXFV were much higher compared to WNV and CLBOV.

Natural maintenance of CXFV and CLBOV. *Culex* flavivirus and CLBOV were detected in both male and female mosquito pools in our studies. Other studies investigating insect-specific flaviviruses have reported similar findings (Hoshino et al., 2007, Hoshino et al., 2009, Cook et al., 2006). Presence of insect-specific flaviviruses in mosquitoes of both sexes suggests vertical transmission as a mechanism of viral maintenance in nature (Cook et al., 2006). Evidence supporting vertical transmission for Kamiti River virus (KRV) include that: (1) the first isolates came from adult *Ae. macintoshi* mosquitoes that were collected as larvae and pupae from flooded dambos, (Sang et al., 2003) and (2) female *Ae. aegypti*, infected with KRV by oral exposure, transmitted the virus to their offspring (Lutomiah et al., 2007). In order to investigate how insect-specific flaviviruses are maintained in mosquito populations, quantitative RT-PCR was performed on specimens from our laboratory colony of *Cx. pipiens* that was established from egg rafts collected in Fort Collins in 2005 and later found to be naturally and persistently infected with CXFV. Total RNA was extracted from individual egg rafts, single larvae, and single adults with high proportions of all stages testing positive for CXFV. These results provide more evidence supporting vertical transmission as a means of viral maintenance in natural populations.

If CXFV and CLBOV are indeed maintained in nature exclusively by vertical transmission, one might expect very high infection rates throughout the study area. This, however, was not observed by us or in other studies on CXFV. An important consideration is the sensitivity of the surveillance testing, which involved column-based RNA extractions, followed by standard RT-PCR assays. Initial studies with the CXFV-infected laboratory colony followed these methods, resulting in very few CXFV-positive specimens. In order to increase sensitivity, RNA extractions were performed using Trizol (Invitrogen), followed by quantitative RT-PCR on individual mosquitoes from the colony. These methods revealed a much higher infection rate, with variable titers among individuals, in the naturally infected colony. These observations suggest that viral titers of insect-specific flaviviruses in naturally infected mosquitoes may sometimes be below the threshold of sensitivity for certain testing methods.

Insect-specific flaviviruses are now being discovered and detected all over the world in various mosquito species. These viruses appear to replicate only in mosquito cells, so they do not pose a direct health risk to humans. However, the common occurrence of these viruses in natural *Culex* populations raises questions regarding possible interactions with other flaviviruses that do cause disease in humans, such as WNV (Crabtree et al., 2003). Future studies are needed to determine how insect-specific flaviviruses may interact with arboviruses in a co-infected mosquito and potentially impact vector competence.

CHAPTER 4

CHARACTERIZATION OF *CULEX* FLAVIVIRUS IN A NATURALLY INFECTED *CULEX PIPIENS* LABORATORY COLONY AND EFFECTS ON VECTOR COMPETENCE FOR WEST NILE

Introduction

The genus *Flavivirus* (family *Flaviviridae*) comprises over 70 single-stranded, positive-sense RNA viruses. Flaviviruses have a genome of about 11 kb that contains a single open reading frame encoding a polyprotein that is co- and post-translationally processed to produce three structural proteins [the capsid (C) protein, the membrane (M) protein, and the envelope (E) protein] and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5). The nonstructural proteins have proteolytic and replicative functions (Lindenbach and Rice, 2003) and also play a role in maturation (Mukhopadhyay et al., 2005). The virion is 40-60 nm in diameter including its lipid bilayer envelope. Most flaviviruses are arthropod-borne and are considered important human and veterinary pathogens, causing considerable morbidity and mortality associated with febrile illness, hemorrhagic fevers, and encephalitides. Some examples of flaviviruses causing human disease are West Nile virus (WNV), dengue virus, and Japanese encephalitis virus. The flaviviruses have been classified into three main ecological groups: mosquito-borne, tick-borne, and no-known-vector (Heinz et al., 2000). The mosquito-borne viruses have been further subdivided based on the main vector genus, with the *Culex*-borne viruses often associated with encephalitic disease in humans, and the *Aedes*-borne viruses more correlated with hemorrhagic disease (Gaunt et al., 2001). Another group, termed insect-specific flaviviruses, has been tentatively placed in this genus, consisting of viruses that replicate only in invertebrate cells and are antigenically unrelated to other flaviviruses (Gritsun and Gould, 2006). Recent isolations of insect-specific flaviviruses in numerous mosquito species, suggest that these viruses are widespread in nature.

Cell fusing agent virus (CFAV) was the first insect-specific flavivirus characterized (Stollar and Thomas, 1975). It was isolated from an *Aedes aegypti* mosquito cell culture line and has recently been isolated from field-caught mosquitoes in Puerto Rico (Cook et al., 2006). Other viruses tentatively placed in the insect-specific flavivirus group include Kamiti River virus (KRV) (Crabtree et al., 2003; Sang et al., 2003), *Culex* flavivirus (CXFV) (Hoshino et al., 2007), Quang Binh virus (Crabtree et al., 2009), *Aedes* flavivirus (Hoshino et al., 2009), Nounané virus (Junglen et al., 2009), Lammi virus (Huhtamo et al., 2009), and Nakiwogo virus (Cook et al., 2009). These viruses only replicate in invertebrate cells, in contrast to many other flaviviruses that are capable of replicating in both vertebrate and invertebrate cells (Cook and Holmes, 2006).

Culex flavivirus and a novel insect-specific flavivirus, Calbertado virus were isolated from *Culex* species mosquitoes collected during field studies conducted in northern Colorado, from 2006 to 2007. Viral RNA sequences were detected in male and female mosquito pools. After the detection of two insect-specific flaviviruses circulating in local mosquitoes, all laboratory mosquito colonies at AIDL (Arthropod-borne and Infectious Diseases Laboratory, Colorado State University) were tested with universal flavivirus primers cFD2/MAMD (Scaramozzino et al., 2001) by standard RT-PCR. A *Culex pipiens* laboratory colony established from egg raft collections in Fort Collins during the summer months of 2005, was found to be persistently infected with *Culex* flavivirus. In order to investigate transmission dynamics of CXFV in the naturally infected laboratory colony, egg rafts, individual larvae and individual adults were tested for CXFV by qRT-PCR. Experiments were also initiated to identify other modes of transmission, including larval horizontal transmission, and adult transmission, both

venereal and casual contact. The colonies used for these experiments were the *Cx. pipiens* colony established from Fort Collins, CO collections (CPCO), persistently infected with CXFV, and a *Cx. pipiens* colony established from mosquitoes collected in Iowa in 2002 (CPIA), which are not infected with CXFV.

The circulation of insect viruses in nature raises questions regarding possible interactions between these viruses and other flaviviruses in vector populations (Crabtree et al., 2003). To begin exploring the dynamics of co-infection with an insect-specific flavivirus and a heterologous flavivirus, in vitro studies were conducted in C6/36 cells infected with CXFV and WNV. To further investigate potential interactions, vector competence experiments were performed to determine the effects of persistent infection with CXFV on infection, dissemination, and transmission rates for WNV in *Cx. pipiens.*

Materials and Methods

Mosquitoes. The Colorado *Cx. pipiens* laboratory colony (CPCO) was established in 2005 by collecting egg rafts along lake margins at Dixon Reservoir and Riverbend Ponds Natural Area in Fort Collins, Colorado. Egg rafts were hatched in individual pans and larvae were reared to fourth instar for species identification. Approximately thirty-five egg rafts were used to begin the *Cx. pipiens* laboratory colony. After the adults emerged into the cage, 20 individuals were removed and pooled by sex. Total RNA was extracted and stored at -80°C for WNV testing by standard RT-PCR. After it was determined that two insect-specific flaviviruses were circulating in local mosquito species from 2006-2007 collections, the *Cx. pipiens* colony was tested using universal flavivirus primers, and tested positive. The RNA originally isolated at the time

of colony establishment was tested using CXFV-specific primers (Hoshino et al., 2007), resulting in a positive PCR product. A subset was removed from the CPCO colony in 2008 and triturated as previously described. The mosquito suspensions were filtered through a 0.45 µm filter and the filtrate placed on C6/36 cells to attempt virus isolation. Four blind passages were completed and cell culture medium was harvested after each passage. Total RNA was extracted from these cell culture medium samples using a QIAamp Viral RNA Minikit (Qiagen) and tested by standard RT-PCR using CXFV-specific primers. Supernatants were positive for CXFV after all four passages, confirming a virus isolate from the CO *Cx. pipiens* colony. The Iowa *Cx. pipiens* colony (CPIA) was established from egg rafts sent from a laboratory colony at Iowa State University in 2002. Adult mosquitoes from the Iowa colony have consistently tested negative for the presence of CXFV by standard RT-PCR. All *Culex* colonies are maintained at 27°C, 75% relative humidity, with a photoperiod of 16:8 (L:D).

RNA extractions. Total RNA was extracted from different life stages (individual egg rafts, larvae, and adults) using Trizol Reagent (Invitrogen). Briefly, individual specimens were homogenized in 500 µl of Trizol Reagent using a motorized pestle and microcentrifuge tube. RNA was extracted following the manufacturer's protocol and eluted in 20 µl of nuclease-free water (Ambion). Samples were stored at -80°C until quantified by a real-time one-step RT-PCR assay.

Real-time reverse transcription (RT)-PCR. Primers were designed to target a 168nt region of the NS5 gene for CXFV detection (CXFV-Forward 5'-

CTACGCTCTCAACACCGTGA-3', CXFV-Reverse 5'-GTTGCCACAACCACATCATC-3'). Primers used to quantify WNV targeted a 70nt portion of the envelope gene (WNENV-Forward 5'-TCAGCGATCTCTCCACCAAAG-3',WNENV-Reverse 5'-GGGTCAGCACGTTTGTCATTG-3') (Lanciotti et al 2000). Standards were prepared by cloning the PCR products into the pCR 2.1 plasmid with the TA cloning kit (Invitrogen). DNA plasmids were purified by the QIAprep Spin Miniprep kit (Qiagen) and concentrations were measured spectrophotometrically. Plasmids were diluted to 10^{10} copies/µl and 10-fold serial dilutions were used to construct standard curves, ranging from 10 to 10^9 copies. RNA samples were quantified using the Quantitect SYBR Green RT-PCR kit (Qiagen) on a Bio-Rad iCycler iQ5 real-time PCR detection system (Bio-Rad). Duplicate reactions were set up for each sample, containing 10 µl of Quantitect SYBR Green RT-PCR Master Mix, 0.2 µl Quantitect RT Mix, 1 µleach of forward and reverse primers (10 µM), 3.8 µl of nuclease-free water (Ambion), and 100 ng of template RNA. No template and uninfected mosquito RNA controls were included for each run. The thermal profile consisted of reverse transcription at 50°C for 30 min, RT inactivation at 95°C for 15min, and 40 cycles of polymerase chain reaction at 94°C for 15 sec, 60°C for 30 sec, and 72°C for 30sec. Dissociation analysis was conducted to detect non-specific amplicons and primer dimers. To avoid the incorporation of non-specific fluorescence into quantitative measurements, the temperatures at which fluorescence detection was acquired were adjusted to quantify specific products only. Fluorescence profiles from the standard curves were used to estimate initial copy numbers of viral genomes in the RNA samples.

In order to correlate qRT-PCR quantification with infectious virus assays, a subset of WNV samples were tested by plaque assay in Vero cells. A subset of *Culex* flavivirus samples were also compared by testing with qRT-PCR and in cell culture. Briefly, for the CXFV infectivity assay, twelve-well plates of confluent C6/36 cells were inoculated with a 10-fold dilution series of CXFV virus suspension. Plates were incubated for 1hr at 28°C and then 2ml of medium were added to each well and plates were returned to the 28°C incubator. At 7dpi, medium was removed from plates and cells were washed twice with PBS. Cells were scraped from wells into fresh PBS and total RNA was extracted using Trizol Reagent. Standard RT-PCR was performed using CXFV-specific primers and the last dilution to produce a positive PCR product was used to estimate the infectious virus titer.

Transmission studies. *Vertical transmission.* Egg rafts, 4th instar larvae, and adults were removed from the CPCO colony and tested individually for the presence of CXFV. Male and female adult mosquitoes were tested at 5, 10, 20, and 30 days post emergence. Bloodfed females, varying in age, were removed from the colony 24 hr post bloodmeal for testing. *Culex* flavivirus titers per individual were estimated as genome equivalents using qRT-PCR.

Larval horizontal transmission. Fifteen first instar larvae from the CPCO colony were combined with 15 first instar larvae from the CPIA colony into one plastic larval pan (15 x 25 x 15 cm), containing approximately 500 ml of tap water. This was conducted in triplicate. Larvae were fed *ad libitum* with a mixture of fish food and rabbit food. Mosquitoes were reared to 4th instar larvae and then removed from the larval pan

and placed in ethanol at -80°C. Larvae were processed individually to detect CXFV infection by qRT-PCR. Additionally, water samples (1 ml) were collected from CPCO larval pans containing approximately 100 larvae per pan. Total RNA was extracted using Trizol Reagent (Invitrogen) and tested for the presence of CXFV RNA by qRT-PCR.

Venereal transmission. Mosquitoes from the CPCO and CPIA colonies were sexed and separated as pupae. After the adults emerged and sex determinations were confirmed, CPCO females (n = 39) were placed in a 30 x 30 x 30 cm cage with CPIA males (n = 38). In a separate cage, CPCO males (n = 40) were combined with CPIA females (n = 42). Mosquitoes were combined into cages at 1-2 days old with access to water via a soaked cotton wick protruding from a glass bottle and sugar cubes as a nutritional source. After 20 days mosquitoes were removed from the cages and females were dissected to determine insemination rates. Females were considered inseminated if spermatozoa were observed in the spermathecae. Mosquitoes were tested individually for CXFV RNA by qRT-PCR using CXFV-specific primers.

Contact transmission. Mosquitoes from the CPCO and CPIA colonies were sexed and separated as pupae. After the adults emerged and sex determinations were confirmed, CPCO females (n = 41) were placed in a 30 x 30 x 30 cm cage with CPIA females (n = 29). In a separate cage, CPCO males (n = 44) were combined with CPIA males (n = 40). Mosquitoes were combined into cages at 1-2 days old with access to water via a soaked cotton wick protruding from a glass bottle and sugar cubes as a nutritional source. After 20 days mosquitoes were removed from the cages and stored at -80°C. Mosquitoes were tested individually for CXFV RNA by qRT-PCR using CXFV-specific primers.

Vector Competence. *Viral interference in cell culture*. *Aedes albopictus* (C6/36) cells were maintained at 28°C with L-15 medium supplemented with 7% FBS, penicillin/streptomycin, L-glutamine, and nonessential amino acids. The WNV isolate used for the experiment was isolated from a pool of *Culex tarsalis* mosquitoes collected in Fort Collins, CO in 2004. It was passaged 5 times in Vero cells with a final titer of 1.0 x 10^7 pfu/ml, as determined by plaque assay in Vero cells. The CXFV isolate used for the experiment was obtained from *Cx. pipiens* mosquitoes in the CPCO laboratory colony, and was passaged 4 times in C6/36 cells, with a titer of 8.79 x 10^7 genome equivalents per ml, as determined by qRT-PCR. Twelve-well plates of confluent C6/36 cells were inoculated with CXFV at an MOI of 0.1 genome equivalents and placed on a rocker for 1 hr at room temperature. Two ml of medium were added to each well and plates were placed in a 28°C incubator. At 48 hr post infection, all medium was removed and cells were challenged with WNV at MOIs of 0.1 and 0.01. Briefly, medium was removed, with 1 ml aliquots taken for the 48 hr timepoint and then cells were inoculated with WNV at two different MOIs. After rocking at room temperature for 1 hr, inocula were removed and cells were washed with PBS. Fresh medium was added to each well and an aliquot for the 48 hr timepoint was taken. Aliquots were removed every 12 hr for an additional 5 days. Titers for CXFV were determined as genome equivalents by qRT-PCR and titers for WNV were determined by plaque assay in Vero cells.

Viral interference in mosquitoes. Mosquitoes from the CPCO and CPIA laboratory colonies were transferred to a BSL-3 environmental chamber as pupae and maintained at 28°C with 75% relative humidity. At approximately 3-5 days old, mosquitoes were offered a bloodmeal containing WNV-infected cell culture medium,

with a titer of 1 x 10^7 pfu/ml, mixed 1:1 with defibrinated sheep blood (Colorado Serum Co., Boulder, CO) and supplemented to 1 mM ATP. Mosquitoes were allowed 2 hr to feed and then all males and unengorged females were discarded. Bloodfed females were returned to the environmental chamber and had access to sugar and water. This experiment was done in duplicate. For the first experiment, 24 females from each colony were processed for infection rates at 14 days post infection. For the second experiment, approximately 30 females were removed from each colony at 7 days post infection for processing, and 24 females from each colony were processed at 14 days post infection. Each specimen was assayed to determine WNV infection (body-thorax and abdomen), dissemination (head, legs, and wings), and transmission (saliva). Females were immobilized by cold, wings and legs were removed, and the proboscis was inserted into a capillary tube containing 5 µl of immersion oil (Type B) for saliva collection. After 30 min of salivation, females were removed from the capillary tubes and heads were separated and placed in a microcentrifuge tube containing legs and wings. Bodies were placed in separate tubes. Immersion oil was expelled from the capillary tubes into 200 µl MEM, containing 2% FBS, and mixed by vortexing for 30 sec. Saliva samples were then centrifuged at 12000 rpm for 10 min at 4°C to allow separation of the immersion oil from the mixture. The medium, containing the saliva, was removed by pipetting and stored at -80°C. Total RNA was extracted from each sample using Trizol reagent, as follows. Saliva samples were mixed 1:1 with Trizol reagent. Bodies (thorax and abdomen) and legs (with heads and wings) were triturated in 500 µl of Trizol reagent. All samples were tested for WNV RNA by qRT-PCR and all CPCO mosquito samples were additionally tested for CXFV RNA by qRT-PCR.

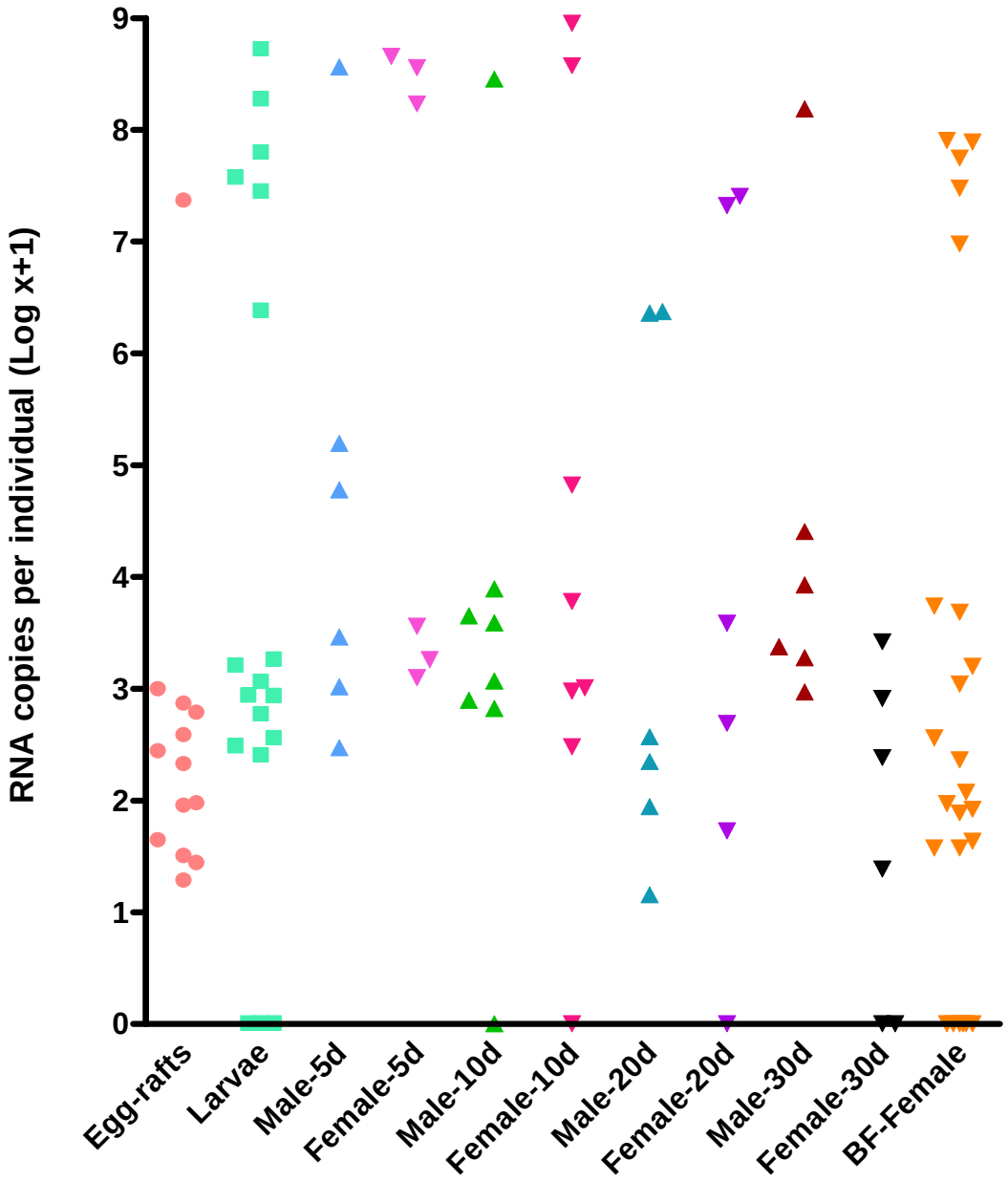
Results

Real-time reverse transcription (RT)-PCR. Comparison of WNV titers using qRT-PCR and Vero plaque assays resulted in a 4 log difference between assays with the qRT-PCR assay being more sensitive and detecting higher titers. There was also a difference between qRT-PCR results for CXFV and the endpoint dilution assay in C6/36 cells, with approximately a 5 log higher titer detected by qRT-PCR.

Transmission studies. *Vertical transmission. Culex* flavivirus RNA was detected in egg rafts, larvae, adult males, and adult females from the CPCO mosquito colony. Viral titers, determined as RNA copies, were variable within and among life stages (Fig. 4.1). There was a distinct pattern with many of the specimens containing low to moderate titers and a few from each group with very high titers.

Larval horizontal transmission. Three pans of mosquito larvae, containing 15 1st instar larvae from the CPCO colony and 15 1st instar larvae from the CPIA colony, were reared to 4th instar and then tested for the presence of CXFV RNA by qRT-PCR. In pan 1, 11 individual larvae were CXFV-positive, in pan 2, 12 larvae were CXFV-positive, and in pan 3, 15 larvae were CXFV-positive. Based on this experiment, performed in triplicate, combining CXFV-infected and uninfected larvae, did not result in transmission. To further investigate the possibility of CXFV transmission in larval pans, water was collected from CPCO colony pans containing approximately 100 larvae per pan. RNA was extracted from 1 ml aliquots of water from six larval pans and all tested

Figure 4.1. *Culex* flavivirus titers, shown as log_{10} RNA copies per individual, for *Culex pipiens* (Colorado colony, naturally infected with CXFV) at different life stages.



Titers were determined by qRT-PCR, using CXFV RNA-specific primers. Specimens were tested individually, except for eggs, which were tested as rafts. (BF = blood fed)

negative for CXFV RNA by qRT-PCR. These data further suggest that larval horizontal transmission is unlikely contributing to the maintenance of CXFV in natural populations.

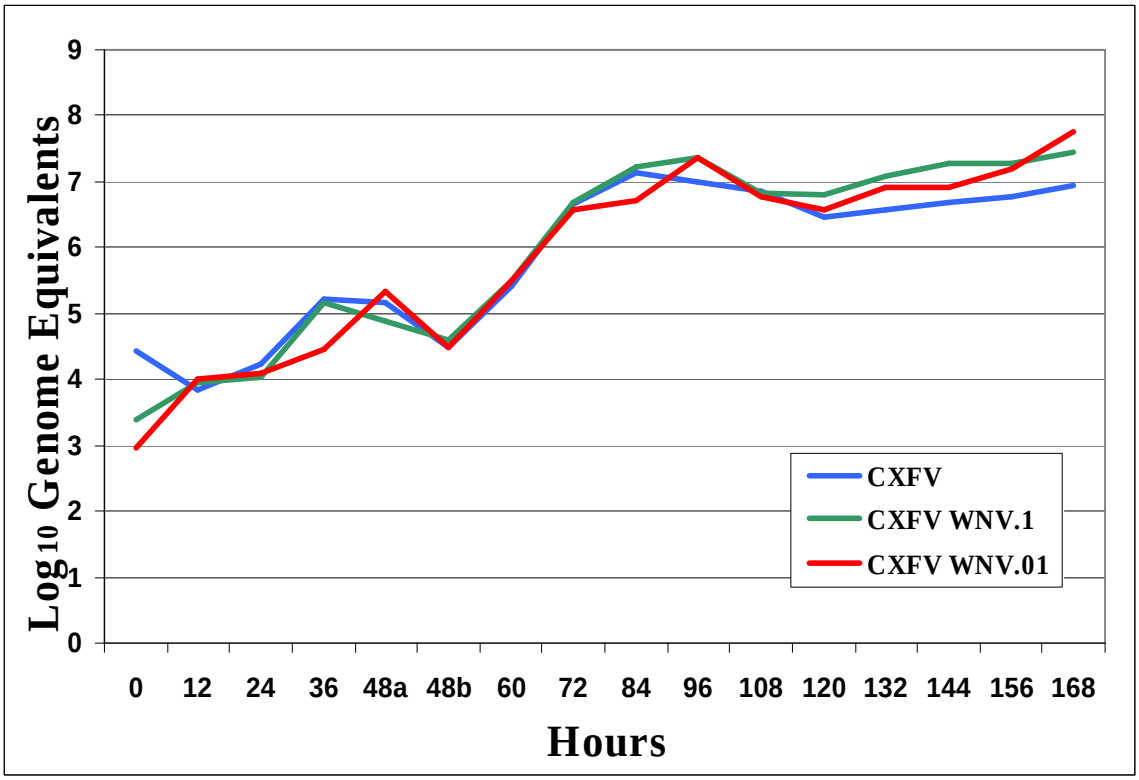
Venereal transmission. CPCO male mosquitoes (n = 40) were combined with CPIA female mosquitoes (n = 42) in one cage. After 20 days, mosquitoes were removed and all females were found to be inseminated. Mosquitoes were tested individually for CXFV RNA by qRT-PCR and one CPIA female (2.4%) was found to be infected. The reverse experiment was also set up with CPCO female mosquitoes (n = 39) combined with CPIA males (n = 38) in one cage. Again, all females were found to be inseminated after 20 days. Mosquitoes were processed individually for CXFV RNA by qRT-PCR and 2 CPIA males (5.3%) tested positive. Based on these observations, it appears that venereal transmission may play a role in CXFV maintenance.

Contact transmission. Male mosquitoes from the CPCO (n = 44) and CPIA (n = 40) colonies were combined into one cage for 20 days. Female mosquitoes from the CPCO (n = 41) and CPIA (n = 29) colonies were also combined into one cage for 20 days. RNA was extracted from individual mosquitoes and tested by qRT-PCR with CXFV primers. Twenty-eight males tested positive for CXFV RNA from the male contact transmission cage and 28 females tested positive for CXFV RNA from the female contact transmission cage. The number of CXFV-positive mosquitoes per cage did not exceed the number of CPCO colony mosquitoes added, so these infection rates indicate that contact transmission did not take place between adults of the same sex, based on our detection methods.

Vector competence. *Viral interference in cell culture. Aedes albopictus* (C6/36) cells were infected with CXFV at an MOI of 0.1 genome equivalents and then challenged with WNV at two different MOIs: 0.1 and 0.01. *Culex* flavivirus and WNV growth curves were determined by qRT-PCR and plaque assay in Vero cells, respectively. *Culex* flavivirus growth curves (Fig. 4.2) in cells co-infected with WNV, were similar to the CXFV growth curve in cells not co-infected with WNV. When the cells were challenged at 48 hours with WNV, all growth medium was removed, causing the dip seen at the 48b timepoint in the growth curves. *Culex* flaviviviral titers, estimated as genome equivalents, appeared to plateau around 7 logs at 84 hr post infection. West Nile virus growth curves in C6/36 cells co-infected with CXFV (Fig. 4.3), were similar to the WNV growth curves in cells not co-infected with CXFV until 84 hr post-infection, but by 108 hr pi, WNV titers in co-infected cultures were ~1 log lower than cultures infected with WNV only. However, by 168 hr post-infection, when the experiment was terminated, WNV titers in co-infected and solely-infected cultures were equivalent. All growth curves were analyzed using linear regression (Stata, Stata Statistical Software: Release 10, College Station, TX), controlling for time, to compare co-infection growth rates to virus growth rates in cultures that were not co-infected. Overall, WNV growth rates in cell cultures co-infected with CXFV, were significantly lower compared to cultures infected with WNV alone (WNV MOI 0.01, $p < 0.001$ and WNV MOI 0.1, $p = 0.042$).

Viral interference in mosquitoes. West Nile virus infection rates were compared between two *Cx. pipiens* laboratory colonies, CPCO (Colorado), which is persistently infected with CXFV, and CPIA (Iowa), which is not infected with CXFV. For the first experiment, a WNV infectious bloodmeal was administered to mosquitoes and after

Figure 4.2. *Culex* flavivirus growth curves in C6/36 cells challenged with West Nile virus at 48h post infection.



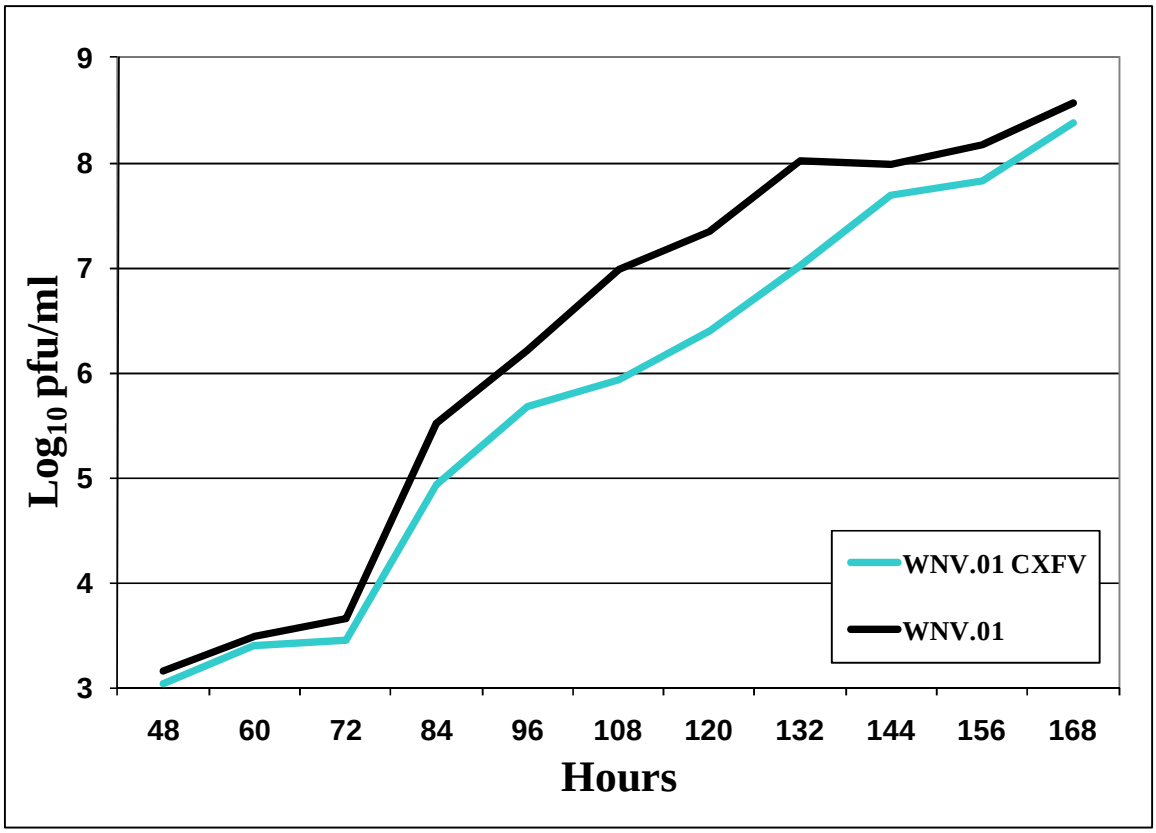
Culex flavivirus infections were at an MOI of 0.1. West Nile virus infections were done at MOIs of 0.1 and 0.01. Growth curves for CXFV were estimated using qRT-PCR to detect RNA with CXFV-specific primers. *Culex* flavivirus growth curves in cells challenged with WNV, were compared to those in cells infected with CXFV alone, using linear regression, and were not significantly different.
(48a = 48hr timepoint, pre-WNV challenge and 48b = 48hr timepoint, post-WNV challenge)

14dpi, 24 females from each colony were processed for infection rates. All 24 CPCO females were WNV RNA positive and 22/24 of the CPIA females were WNV RNA positive by qRT-PCR (Table 4.1). The WNV titers of the positive mosquitoes, determined as genome equivalents and $\log_{10}$ transformed, were compared between the two colonies and were not significantly different (Table 4.2) ($p = 0.75$, Student's two-tailed t-test). For the second experiment, mosquitoes from each colony were processed at

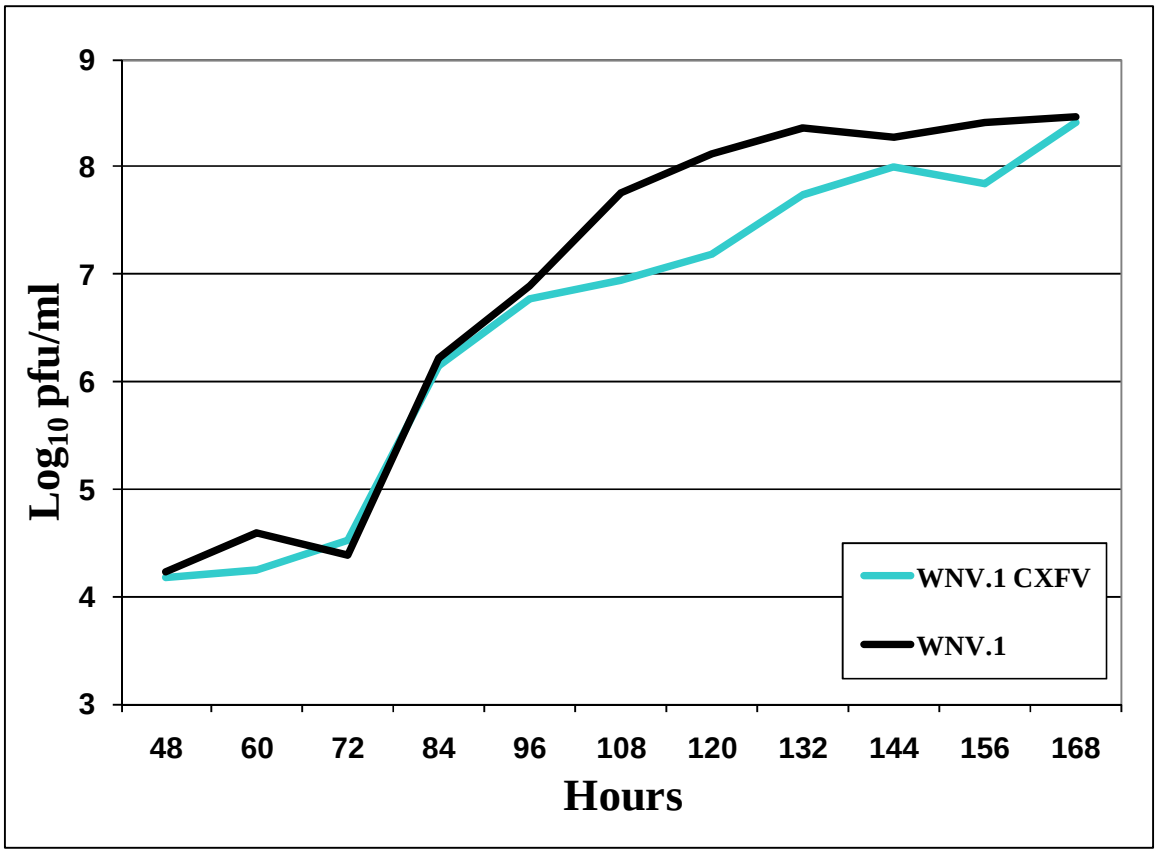
7 and 14dpi. Saliva from each female was tested to estimate transmission, and bodies (thorax and abdomen) and legs, heads and wings (together) were tested to determine

Figure 4.3. West Nile virus growth curves in C6/36 cells, co-infected with *Culex* flavivirus.

A.



B.



C6/36 cells were infected with CXFV at an MOI of 0.1 and 48hr post infection, were challenged with WNV at MOIs of 0.01 (A) and 0.1 (B). West Nile virus growth curves were determined by plaque assay in Vero cells. West Nile virus growth curves in cells co-infected with CXFV were compared to growth curves in cells infected with WNV alone using linear regression and were significantly different (WNV MOI 0.01, $p < 0.001$ and WNV MOI 0.1, $p = 0.042$).

infection and dissemination rates, respectively. At 7dpi, the WNV dissemination rate for CPIA was significantly higher ($p = 0.04$, Fisher's Exact test) compared to CPCO. The infection rate for CPIA (97%) was also higher than the infection rate for CPCO (86%), but it was not significantly different ($p = 0.18$, Fisher's Exact test). Transmission rates were the same (3%) for both colonies at 7dpi. At 14 dpi, there were no significant differences detected between the two colonies for infection, dissemination, or

Table 4.1. Infection, dissemination, and transmission rates for West Nile virus by *Culex pipiens* mosquitoes.

	Experiment 1	Experiment 2					
	14dpi	7 dpi			14dpi		
	Infection	Infection	Dissemination	Transmission	Infection	Dissemination	Transmission
CPCO	100% (24/24)	86% (25/29)	72% (21/29)	3% (1/29)	71% (17/24)	67% (16/24)	21% (5/24)
CPIA	92% (22/24)	97% (32/33)	94% (31/33)	3% (1/33)	79% (19/24)	67% (16/24)	13% (3/24)
p-value	0.49	0.18	0.04*	1.00	0.74	1.00	0.70

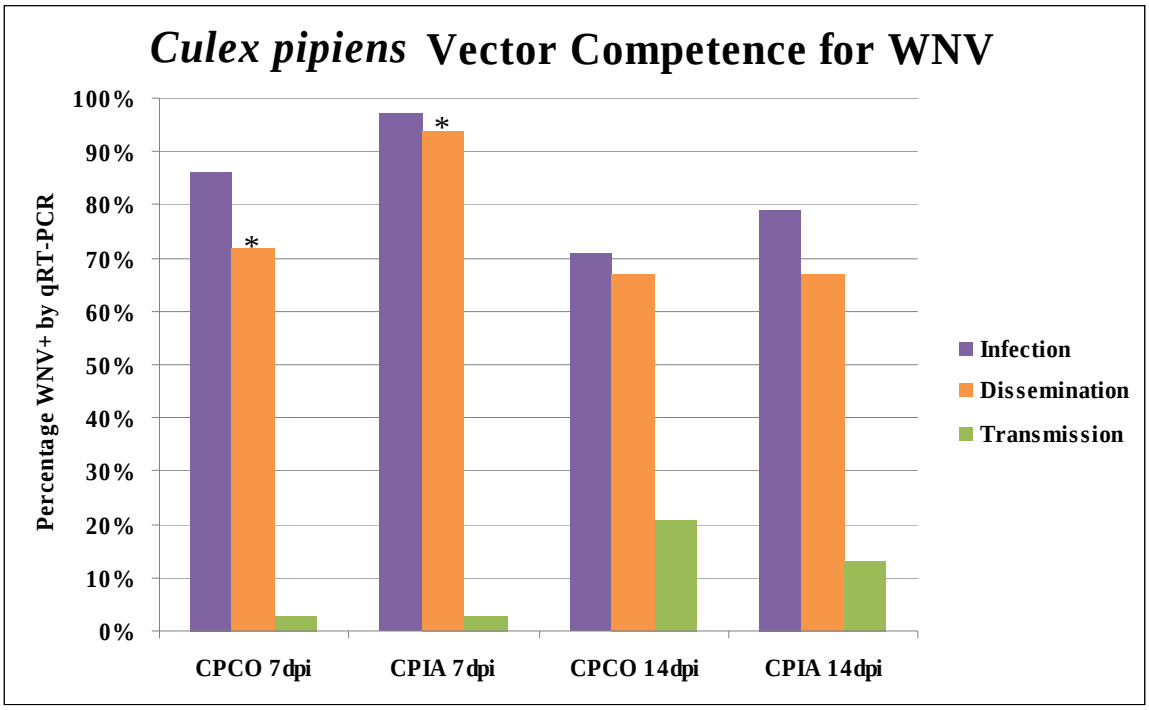
CPCO (*Culex pipiens*-Colorado, naturally infected with *Culex* flavivirus) and CPIA (*Culex pipiens*-Iowa, not infected with *Culex* flavivirus) colonies were administered a West Nile virus infectious blood meal. Infection (abdomen and thorax), dissemination (legs, head, and wings), and transmission (saliva) rates for WNV were determined by qRT-PCR and compared between the colonies by Fisher's Exact test.
*Statistically significant at $\alpha \leq 0.05$

Table 2. West Nile virus titers (mean $\log_{10}$ genome equivalents per mosquito ± standard error) in *Culex pipiens* mosquitoes.

	Experiment 1	Experiment 2					
	14dpi	7 dpi			14dpi		
	Bodies	Bodies	Legs	Saliva	Bodies	Legs	Saliva
CPCO	6.58 ± 0.43	6.72 ± 0.28	2.29 ± 0.25	0.22 ± 0.00*	7.18 ± 0.54	5.24 ± 0.62	2.98 ± 0.27
CPIA	6.77 ± 0.30	5.94 ± 0.32	3.35 ± 0.25	0.29 ± 0.00*	6.20 ± 0.51	4.52 ± 0.56	2.53 ± 0.17
p-value	0.75	0.08	0.01**	n/a	0.19	0.40	0.29

CPCO (*Culex pipiens*-Colorado, naturally infected with *Culex* flavivirus) and CPIA (*Culex pipiens*-Iowa, not infected with *Culex* flavivirus) colonies were administered a West Nile virus infectious blood meal. Bodies (abdomen and thorax), legs (with head and wings), and saliva samples were tested for WNV RNA and titers were compared between the colonies by the Student's two-tailed t-test.
*One saliva sample from each colony was WNV-positive, so these titers were not compared. **Statistically significant at $\alpha \leq 0.05$.

Figure 4.4. Infection, dissemination, and transmission rates for West Nile virus by *Culex pipiens* mosquitoes.



CPCO (*Culex pipiens*-Colorado, naturally infected with *Culex* flavivirus) and CPIA (*Culex pipiens*-Iowa, not infected with *Culex* flavivirus) colonies were administered a West Nile virus infectious blood meal. Infection (abdomen and thorax), dissemination (legs, head, and wings), and transmission (saliva) rates for WNV were compared between the colonies by Fisher's Exact test.
*Statistically significant at $\alpha \leq 0.05$

transmission rates. The infection rate for CPIA was slightly higher for CPIA (79%) compared to CPCO (71%). Dissemination rates for the two colonies were exactly the same at 67% and the transmission rate for CPCO (21%) was higher than was seen for CPIA (13%). West Nile virus genome titers for positive samples were compared between colonies. At 7dpi, the average WNV titer for CPCO bodies (thorax and abdomen) was 6.72 $\log_{10}$ genome equivalents per mosquito, which was higher than the average titer for CPIA with 5.94 $\log_{10}$ genome equivalents per mosquito. The difference between CPIA and CPCO WNV body titers was, however, not

significantly different (p = 0.08, Student's two-tailed t-test). The WNV titers for CPIA dissemination samples (legs, head, and wings) were significantly higher than CPCO dissemination samples (p = 0.01, Student's two-tailed t-test). The average titers were 3.35 and 2.29 $\log_{10}$ genome equivalents per mosquito for CPIA and CPCO, respectively. At 14dpi, overall, WNV titers were higher in CPCO mosquitoes compared to CPIA mosquitoes, but these differences were not statistically significant (Table 4.2).

Discussion

It is important to understand the dynamics of vector-borne disease systems in order to make efficient use of arbovirus surveillance and pest management efforts. The isolation and description of numerous insect-specific flaviviruses in recent years has prompted interest in how these viruses may interact with pathogenic arboviruses (transmitted to vertebrates) in vector mosquitoes. Data are lacking on how insect-specific flaviviruses are maintained in nature and what effects they may have on arbovirus transmission. The goals of this study were to investigate how *Culex* flavivirus is maintained in a naturally infected *Cx. pipiens* laboratory colony and whether prior infection with CXFV alters vector competence for WNV.

Transmission studies. *Vertical transmission*. We detected CXFV in egg rafts, larvae, adult males and adult females from a *Cx. pipiens* colony established from field collections in Fort Collins, Colorado in 2005. Detection of insect-specific flaviviruses in all life stages, including adult mosquitoes of both sexes, suggests vertical transmission as a probable mechanism of viral maintenance in nature (Cook et al.,

2006). Similarly, Kamiti River virus, an insect-specific flavivirus, was first isolated from *Aedes macintoshi* larvae and pupae collected from flooded dambos in Kenya (Sang et al). Additional studies were conducted with laboratory bred *Aedes aegypti* mosquitoes, orally exposed to KRV, indicating that vertical transmission can occur (Lutomiah et al 2007). *Culex* flavivirus, first isolated during a field survey of mosquito-borne viruses in Japan, was detected in adult males and females (Hoshino et al 2007). *Aedes* flavivirus, another insect-specific flavivirus isolated by the same researchers in Japan, was also found in both male and female adult mosquitoes (Hoshino et al 2009). These data and our findings support the idea that vertical transmission plays an important role in the maintenance of insect-specific flaviviruses in nature.

Culex flavivirus titers in mosquitoes from a naturally infected colony, estimated as RNA copies, were variable across life stages (Fig. 4.1), ranging from 1 to 9 logs per individual. All egg rafts (n = 13) tested were positive for CXFV RNA, but 3 out of 18 larvae and 11 out of 76 adults tested were negative for CXFV RNA. It is unclear if virus was absent in these individuals, or if the titers were below the threshold of detection. *Culex* flavivirus titers in individual CPCO colony mosquitoes revealed an interesting pattern (Fig. 4.1), with the majority of genome equivalent titers falling between 2-4 logs and several individuals within each life stage reaching as high as 9 logs. Similarly, a small proportion of field-collected *Aedes triseriatus* females were described as being super-infected with La Crosse virus, meaning the individuals contained infectious virus and large amounts of viral antigen and RNA, compared to other LAC-infected females (Reese et al., 2010). The presence of

individuals with higher titers may represent a mechanism of viral maintenance in nature.

Larval horizontal transmission. Transmission experiments were conducted to investigate viral maintenance mechanisms other than vertical transmission. Larval horizontal transmission experiments were conducted by combining first instar larvae from CPCO (CXFV-positive) and CPIA (CXFV-negative) colonies and rearing to 4th instar larvae in a shared pan. Larvae were tested individually for CXFV RNA by q RT-PCR. There was no evidence of CXFV transmission between infected and uninfected larvae. Additionally, CXFV RNA was not detected in water samples from CPCO colony pans containing approximately 100 larvae each. These findings suggest that although we have detected CXFV positive larvae from the CPCO colony, larval horizontal transmission does not play a role in viral maintenance. This was not unexpected, as flaviviruses are unlikely to be stable in water.

Venereal and contact transmission. Adult mosquitoes from CPCO and CPIA colonies were combined in order to investigate venereal transmission. One out of 42 (2.4%) CPIA females tested positive for CXFV RNA by qRT-PCR after sharing a cage and mating with CPCO males. Venereal transmission of flaviviruses from male to female mosquitoes has been documented (Nayar et al., 1986, Shroyer 1990a), but occurs at a low frequency. Surprisingly, 2 out of 38 (5.3%) CPIA males were positive for CXFV after sharing a cage and mating with CPCO females. Research is lacking on venereal transmission of flaviviruses from female to male mosquitoes. Studies with dengue virus in *Aedes albopictus* (Rosen 1987) showed that experimentally infected females did not transmit their infection sexually to males. It is possible that

the CPIA males from our experiments became infected with CXFV by other means than sexual transmission, such as sharing food sources with CXFV-positive females. To test this theory, we also initiated contact transmission studies, where CPCO males were combined with CPIA males and CPCO females were combined with CPIA females. Based on these experiments, CXFV transmission did not occur between same-sex mosquitoes while sharing cage space and food sources. These data support our findings of venereal transmission occurring from CXFV-positive females to uninfected males. Further studies are warranted to confirm these observations.

Vector competence. *Viral interference in cell culture.* Possible interactions between CXFV and WNV during co-infection were investigated with cell culture and adult mosquito experiments. Overall, WNV growth curves in C6/36 cells co-infected with CXFV, were significantly lower than WNV growth curves in singly-infected cells (Fig. 4.3). Differences were seen between 84 and 156 hr post infection, which may represent possible interference between CXFV and WNV during this time period. At 168 hr post infection, all WNV titers reached approximately 8.5 $\log_{10}$ pfu/ml, regardless of co-infection status. The differences seen at earlier timepoints may not be important, as titers were the same at168 hr post infection. If extrapolated to a mosquito-borne disease system though, this difference could represent an increase in the extrinsic incubation period for WNV in vectors co-infected with CXFV. Similar experiments in C6/36 cells with CXFV (Izabal strain) resulted in lower titers in a WNV growth curve in CXFV-positive cells compared to WNV alone, but the difference was not statistically significant (Kent et al., 2010). Additional

experiments are warranted to further explore possible interactions between CXFV and WNV in cell cultures.

Within-host interaction among viruses varies depending on the biological system of interest (Pepin et al., 2008). Some systems demonstrate superinfection exclusion, where a cell infected with one virus cannot be productively infected with the same or closely-related virus. For example, *Aedes albopictus* cells persistently infected with Sindbis virus were refractory to infection with homologous strains of Sindbis and other heterologous alphaviruses (Eaton 1979, Karpf et al., 1997). Competitive suppression has been documented between dengue virus serotypes (DENV2 and DENV4), where replication of both viruses was suppressed in superinfection of C6/36 cells (Pepin et al., 2008). Information about dual infections in vector host populations with heterologous flaviviruses is lacking. It would be beneficial to perform future studies with CXFV in a *Culex* cell line, as opposed to C6/36 (*Aedes albopictus*) cells, as the virus appears to be exclusively associated with *Culex* species mosquitoes in nature (Hoshino et al., 2007, Morales-Betoulle et al., 2008, Farfan-Ale et al., 2009, Kim et al., 2009, Blitvich et al., 2009, Cook et al., 2009).

Viral interference in mosquitoes. Vector competence experiments were conducted using two *Cx. pipiens* laboratory colonies: CPCO (*Cx. pipiens*-Colorado, CXFV-positive) and CPIA (*Cx. pipiens*-Iowa, CXFV-negative). Mosquitoes were administered a WNV infectious bloodmeal and infection, dissemination, and transmission rates were compared at 7 and 14dpi (Table 4.1, Fig. 4.4). The only significant difference detected between the two colonies was the WNV dissemination

rate at 7dpi. The WNV dissemination rate for CPIA (94%) was significantly higher than was seen for CPCO (72%) ($p = 0.04$). West Nile virus titers were also compared (Table 4.2), and once again, the only significant difference between the two colonies was seen at 7dpi, with mean titers for CPIA (3.35 $\log_{10}$ genome equivalents per mosquito) being higher than for CPCO (2.29 $\log_{10}$ genome equivalents per mosquito) ($p = 0.01$). To further examine possible interactions between CXFV and WNV in *Cx. pipiens*, CXFV and WNV titers ($\log_{10}$ genome equivalents per mosquito) were analyzed by correlation analysis (data not shown), with no evidence of a linear relationship. Kent et al (2010) investigated vector competence for WNV of *Culex quinquefasciatus* mosquitoes inoculated with CXFV (Izabal strain) 7 days prior to receiving a WNV infectious blood meal, with no significant differences detected between CXFV-positive and CXFV-negative mosquitoes at 14dpi. The significant differences we detected in dissemination rates were seen at 7dpi and these differences disappeared at 14dpi, as seen with the CXFV (Izabal strain) study. The results form our vector competence study suggest a competitive interaction between CXFV and WNV at 7dpi, as WNV in CPCO (CXFV-positive) mosquitoes had significantly lower dissemination rates and leg titers, compared to WNV in CPIA (CXFV-negative) mosquitoes. Infection rates were also higher for WNV in CPIA at 7dpi compared to CPCO, but the difference was not significant ($p = 0.18$). It should be noted that the *Cx. pipiens* colonies used for these experiments are from different geographic locations (Colorado and Iowa), so differences detected in vector competence for WNV could be due to factors other than co-infection with CXFV. Vector competence studies for dengue virus conducted in *Aedes aegypti* moquitoes

collected from different geographic regions in Mexico revealed infection rates ranging from 24% to 83%, perhaps resulting from genetic differences between populations (Bennett et al., 2002).

The significant differences seen in our study could indicate that CXFV is somehow suppressing WNV replication in mosquitoes at 7dpi. Experiments with C6/36 cells persistently infected with *Aedes albopictus* densovirus, suggested that viral suppression was occurring, as DENV2 infections were significantly lower in superinfected cell cultures, compared to naïve cell cultures (Burivong et al 2004). In contrast, *Ae. triseriatus* mosquitoes transovarially infected with LaCrosse (LAC) virus were susceptible to superinfection with a second LAC virus (Borucki et al., 1999). It is questionable whether the differences seen in our study at 7dpi are biologically relevant. For within-host competition among viruses to occur, they must infect the same cells in the host (Pepin et al., 2008). It is unclear at this time which mosquito tissues are targeted by insect-specific flaviviruses. Further studies are warranted to examine tissue tropisms of CXFV in naturally infected mosquitoes to determine if WNV interactions are even possible. With numerous insect-specific flaviviruses apparently causing persistent infections in natural populations worldwide, it will be important to investigate and determine mechanisms of interactions between these viruses and other viruses, particularly arboviruses transmissible to vertebrates.

CHAPTER 5

SUMMARY

Vector-borne diseases remain a major public health concern as new diseases are emerging and previously controlled diseases are now resurging (Beaty 2005). Many factors contribute to the spatial and temporal dynamics of disease outbreaks. Vector-borne disease systems are especially complex, as there are numerous components that affect the interactions between vector, vertebrate host, and pathogen (Moore 2008), including environmental variables, human behavior, and the genetics of particular vector and pathogen species. Vaccines are not available for many vector-borne diseases, so new strategies are needed to predict risk in order to reduce vector populations and prevent vector-host interactions (Eisen et al., 2009). Field surveillance programs are essential for understanding vector-borne disease transmission patterns. Knowledge gained through surveillance programs can be used to determine important factors for predicting disease risk.

Studies described in this dissertation developed from mosquito collections initiated in northern Colorado to investigate the distribution and abundance of primary WNV vector species. Seasonal patterns for entomological measures of risk for exposure to *Culex* vectors and West Nile virus (WNV) were examined in relation to human WNV disease cases. Mosquito collections in 2006 showed that the seasonal activity period is shorter and the peak abundance for *Cx. tarsalis* females occurs later in the summer above 1,600 m compared to plains areas below 1,600 m. Possible reasons for these differences are that the temperatures are lower at higher elevations and this can decrease the developmental rates of the mosquitoes as well as viral replication in the mosquitoes. Also, the land use patterns above 1,600 m are very different compared to the plains region, one main difference being the lack of irrigated agricultural fields. Mosquito

collections conducted in 2007 in the plains of northeastern Colorado showed that seasonal patterns of abundance for *Cx. tarsalis* and *Cx. pipiens* females were different in that *Cx. tarsalis* abundance peaked in early July whereas the peak for *Cx. pipiens* occurred in late August. The numbers of *Cx. pipiens* collected were markedly lower compared to the *Cx. tarsalis* counts for several reasons. First, the collection sites were located along riparian corridors and *Cx. pipiens* are more associated with urban-peridomestic sites. Also, the use of CDC light traps alone, without gravid traps, has been shown to underestimate the abundance of *Cx. pipiens* (Tsai et al., 1988). Future field studies should include gravid traps and also collection sites in urban areas to further characterize the role of *Cx. pipiens* in WNV transmission cycles in northern Colorado. During June-September in 2007, WNV-infected *Cx. tarsalis* females were collected from 16 of 18 sites in the plains, with no WNV detections at higher elevation sites, indicating that WNV activity was widespread throughout the study area below 1,600 m. West Nile virus infection rates in *Cx. tarsalis* females increased gradually from late June to peak in mid-August.

Linear regression analysis revealed that abundance of *Cx. tarsalis* females and the Vector Index (VI) for infected females were strongly associated with weekly numbers of WNV disease cases with onset 4-7 weeks later (female abundance) or 1-2 wk later (VI). These data support the notion that ongoing field surveillance projects can be useful for predicting human WNV disease risk. The VI combines information about vector species presence, density, and infection rate, producing a comprehensive estimate of the number of infectious vectors in the surveillance area (Nasci et al., 2005). Our studies demonstrate

that VI can be used by public health departments and vector control agencies to set thresholds for WNV epidemic risk.

Culex mosquitoes collected in Colorado during 2006-2007 were tested with universal flavivirus primers by standard RT-PCR. This led to the detection and subsequent isolation of two insect-specific flaviviruses: *Culex* flavivirus (CXFV), which was first described from Japan, and a novel insect flavivirus, uniquely designated Calbertado virus (CLBOV) to represent geographic regions where initial detections were made (California, Alberta, Canada, and Colorado). We recorded both viruses in *Cx. tarsalis* and *Cx. pipiens* from Colorado. In 2006, there was a strong species-specific pattern with CXFV being detected almost exclusively in *Cx. pipiens* and all records of CLBOV coming from *Cx. tarsalis.* In 2007, far greater numbers of *Culex* mosquitoes were identified and processed, and both insect-specific flaviviruses were detected in *Cx. tarsalis* as well as *Cx. pipiens*. These findings suggest that CXFV and CLBOV circulate in both species of mosquitoes in northern Colorado, although we cannot entirely rule out the possibility that a body part from one species sometimes was accidentally combined with a pool from the other species.

Site specific infection rates for insect-specific flaviviruses and WNV were examined to see if there were any obvious patterns of association, but none were detected. Our study did reveal very high infection rates for CXFV in *Cx. pipiens*, compared to much lower infection rates seen for CLBOV in *Cx. tarsalis.* One reason for this could be that smaller numbers of *Cx. pipiens* were collected and analyzed, which can result in overestimation of minimum infection rates. We did however, try to establish a CXFV-negative colony during the spring of 2009 and were unable to collect females

negative for CXFV. This suggests that CXFV is perhaps more prevalent in local mosquitoes than our field studies indicate. An important consideration is the sensitivity of the surveillance testing used for the field studies, which involved column-based RNA extractions, followed by standard RT-PCR assays. Initial studies with a CXFV-infected laboratory colony followed these methods, resulting in very few CXFV-positive specimens. In order to increase sensitivity, RNA extractions were performed using Trizol (Invitrogen), followed by quantitative RT-PCR, which is more sensitive than standard RT-PCR, on individual mosquitoes from the colony. These methods revealed a much higher infection rate, with variable titers among individuals, in the naturally infected colony. These observations suggest that viral titers of insect-specific flaviviruses in naturally infected mosquitoes may sometimes be below the threshold of sensitivity for certain testing methods.

Phylogenetic analyses were conducted to determine the relationships between insect-specific flaviviruses detected in *Culex* spp. collected in Colorado with other selected flaviviruses. The CXFV isolate from this study was most similar to CXFV isolates from Texas, and grouped with other CXFV isolates from Iowa, Japan, Mexico, and Guatemala. The Colorado CLBOV isolate shared closest phylogenetic relationships with CLBOV sequences detected in mosquitoes in California and Alberta, Canada, with blast results indicating 97% similarity. The Colorado CXFV and CLBOV isolates grouped with the other insect-specific flaviviruses associated with *Culex* spp. mosquitoes.

Insect-specific flaviviral RNA was detected in both male and female mosquitoes. Furthermore, quantitative RT-PCR revealed the presence of CXFV RNA in *Cx. pipiens* eggs, individual larvae, and individual adults from a CXFV naturally infected laboratory

colony established in 2005. These findings suggest vertical transmission as a means of viral maintenance in natural *Culex* populations. Interestingly, the CXFV viral titers, estimated as genome equivalents, were variable across and within life stages, with most individuals having genome equivalent titers falling between 2-4 logs per individual and several individuals within each life stage reaching as high as 9 logs. The presence of individuals with higher titers may represent a mechanism of viral persistence in nature.

Transmission experiments were conducted to investigate larval horizontal transmission and adult transmission, both venereal and casual contact. The colonies used for these experiments were the *Cx. pipiens* colony established from Fort Collins, CO collections (CPCO), persistently infected with CXFV, and a *Cx. pipiens* colony established from mosquitoes collected in Iowa in 2002 (CPIA), which are not infected with CXFV. Based on our studies, there was no evidence of larval transmission. There was evidence of venereal transmission, from male to female, and female to male. These findings were unexpected as female to male transmission of flaviviruses has not been documented. This may be a unique characteristic of insect-specific flaviviruses. Furthur studies are needed to confirm these observations, including examination of mosquito tissue tropisms for insect-specific flaviviruses by immunofluorescense assays.

Numerous insect-specific flaviviruses have been recently isolated and characterized raising questions about possible interactions with heterologous flaviviruses. To explore these potential interactions, *in vitro* studies were conducted in C6/36 cells infected with CXFV and WNV. West Nile virus growth curve titers in co-infected cultures were ~1 log lower than in cultures infected with WNV only and analyzed by linear regression, the growth curves were significantly different. When the experiments

were terminated at 168 hr post-infection, however, the WNV titers in co-infected and solely-infected cultures were the same. These findings indicate possible interactions between CXFV and WNV in C6/36 cells. *Culex* flavivirus is associated with *Culex* spp. mosquitoes in nature and therefore experimental results from C6/36 (*Aedes albopictus*) cells may not be biologically relevant. It would be beneficial to conduct additional co-infection experiments with a *Culex* cell line, exploring different MOIs and lag times between primary and secondary infections.

Vector competence experiments were also performed to determine the effects of persistent infection with CXFV on infection, dissemination, and transmission rates for WNV in *Cx. pipiens.* The only differences detected between the two colonies, after receiving a WNV infectious blood meal, were the dissemination rate and WNV titers at 7dpi, which were significantly higher for the CPIA colony mosquitoes. These differences, however, disappeared at 14dpi. It is important to consider the fact that the two *Cx. pipiens* colonies are from different geographic locations, so there could be factors other than co-infection with CXFV that affect vector competence for WNV, including genetic differences. If the differences detected by our study represent competitive suppression of WNV by CXFV, this could be an important factor in determining the extrinsic incubation period (EIP), which is defined as the period of time from ingestion of an infectious blood meal to the time of transmission capability (Black and Moore, 2005). It is possible that prior infection with CXFV suppresses replication of WNV in *Cx. pipiens* mosquitoes, resulting in an increased EIP, and therefore decreased vectorial capacity.

The studies described in this dissertation underline the importance of combining field surveillance activities with laboratory experiments to provide a more comprehensive

understanding of the dynamics of vector-borne disease systems. In Chapter II, entomological field data (*Cx. tarsalis* abundance and the vector index for infected females) was found to be strongly associated with human WNV disease cases, with lag times of 4-7 wks and 1-2 wks, respectively. This information can be utilized by vector control agencies to make informed decisions about when to initiate mosquito control activities, such as adulticiding. Chapter III describes the detection and seasonal infection rates for two insect-specific flaviviruses in *Culex* spp. mosquitoes in northern Colorado. This is the first description of insect-specific flaviviruses in Colorado. Knowledge of heterologous flaviviruses co-circulating in primary vector species for WNV is valuable information, as co-infections may alter vector competence. Laboratory experiments described in Chapter IV explored the transmission dynamics of CXFV in a naturally infected colony and also the potential effects of CXFV infection on vector competence for WNV. Vertical transmission appears to be the primary mechanism for viral persistence in the colony and results from vector competence experiments indicate possible suppression of WNV replication by persistent CXFV infection. These findings are important as insect-specific flavivirus transmission dynamics have not been described yet. Further studies are needed to more thoroughly investigate putative interactions between insect-specific flaviviruses with arboviruses, like WNV. A better understanding of complex vector-borne disease systems will lead to better predictive models for epidemic risk to humans.

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