MOSQUITO COMMUNITY INTERACTIONS IN EASTERN RED CEDAR ENCROACHED AREAS IN CENTRAL AND WESTERN OKLAHOMA

By

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Abstract: The most important mosquito-borne virus in the United States is West Nile Virus (WNV) with the highest incidence of neuroinvasive disease in the Central Plains region. While much is known regarding the epidemiology of WNV transmission, the influence of landscape on the 'nidus' of infection requires further study. One important regional change involves the woody plant encroachment (WPE) by eastern red cedar (ERC) (*Juniperus virginiana*) at 5-7 times faster than other regions. Changes produced by this invasive tree are occurring on an ecosystem level, providing productive habitats for specific disease vectors. While more mosquitoes are collected in ERC compared with other habitats, there are specific questions related to host-mosquito interactions as well as habitat usage by mosquitoes in ERC-encroached areas. To address these questions, two studies were developed: The first main study focused on the hosts that mosquitoes collected in ERC-encroached areas are feeding on. The second study focused on specific habitats within ERC-encroached areas that are preferred by specific mosquito species. In 2021, 3,187 mosquitoes were collected. 95 blood-fed mosquitoes of 14 different species were identified that had fed on 12 different hosts, the majority being white tailed deer, cattle, horses, and only one avian species. We identified blood-fed mosquitoes that had flown up to 1200m into ERC-encroached areas. Additionally, *Anaplasma platys* was detected in the blood of 5 mosquitoes that had fed on WTD. In 2022, 3,248 mosquitoes were collected of which 3 pools were infected with WNV. Significantly more *Ae. albopictus* were collected inside ERC-encroached areas compared with outside and nearby grassland while more *Ps. columbiae* were collected in grassland compared with any ERC habitat. While not significant at the model level, the abundance of *Cx. tarsalis*, *Cx. erraticus* and *An. punctipennis* was all significantly higher outside of ERC-encroached areas than in grassland traps with no difference between outside and inside ERC traps. Together, these studies identified important relationships between specific mosquito species and hosts in the vicinity of ERC-encroached areas. These aspects become important from a public health perspective when the focus shifts from rural to urban areas where ERC is encroaching in the region.

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CHAPTER I

REVIEW OF THE LITERATURE

The Great Plains Region

What is going on in the Great Plains?

The Great Plains region, also known as the Central Plains, is made up of twelve states, including Colorado, Iowa, Kansas, Minnesota, Nebraska, New Mexico, North Dakota, Oklahoma, South Dakota, Texas, and Wyoming. West Nile virus (WNV) is currently the most important mosquito-borne virus in the United States, and this region is known for having the highest cumulative incidence rate of WNV cases and West Nile virus neuroinvasive disease (WNND) (Lindsey et al. 2008; DeGroote and Sugumaran 2012; Curren 2018). WNV transmission can only occur when competent mosquito vectors feed on infected avian hosts within a particular habitat, also known as the 'nidus of infection' (Reisen 2010). Studies have shown that *Culex tarsalis* and *Culex pipiens* mosquitoes are the primary vectors of WNV in this region and have been known to feed on both avian and mammalian hosts during different times of the year (Lindsey et al. 2008; Thiemann et al. 2011).

The Great Plains as a whole has seen an increase in woody plant encroachment (WPE). However, the southern Great Plains (Kansas, New Mexico, Oklahoma, and Texas) has seen increase in WPE, due to the native invasive eastern red cedar trees (ERC), *Juniperus virginiana,* throughout its grasslands and shrublands (Zou et al.2018; Kaur et al. 2020). WPE has affected the new environments in both biotic and abiotic ways (temperature, moisture, windspeeds, etc.)

that can change the way that mosquitoes, birds, and mammals interact within the environment (Eldridge et al. 2011; DeGroote and Sugumaran 2012). It is hypothesized that these WPE areas are creating a small 'oases' for animals to rest, in areas where they otherwise would not, thus possibly be affecting the transmission cycle of WNV.

Importance of West Nile virus in the Great Plains

West Nile virus was first discovered in the United States in 1999 in New York and rapidly made its way west, reaching California by 2003 (Kramer et al. 2019). Since becoming endemic, there have been years where epidemics have broken out in certain areas of the U.S. These epidemics have caused severe neuroinvasive disease in 1-2% of infected individuals and caused encephalitis in children, immunocompromised, and elderly persons (Lindsey et al. 2008). Currently, WNV is hard to predict and detect, as it requires the mass collection of mosquitoes, extraction of RNA and the use of quantitative polymerase chain reaction (qPCR). In recent studies, data has suggested that the invasive ERC may be impacting the transmission rate of WNV in the southern Great Plains due to ERC's ability to drastically impact the ecosystem and provide new habitats for vectors and hosts (O'Brien and Reiskind 2013; Noden et al. 2021a, Maichak et al. 2022). If this indeed is the case, then the Great Plains may be at risk of greater WNND incidence rates in the case of another WNV epidemic.

West Nile Virus

Nidality of West Nile virus

In order for any mosquito-borne pathogen to be transmitted, it must have the necessary components, such as competent hosts, vectors, and disease agents. If all three components are present, then the 'nidus of infection' can occur in the environment (Reisen 2010). This involves both biotic and abiotic factors residing perfectly in an area to allow for pathogen transmission. WNV requires *Culex* mosquitoes to be in the ecosystem and infected avian hosts as well. Many avian species are capable of being reservoir hosts for WNV (Reisen et al. 2013; Komar et al.

2018). Mosquitoes like *Cx. tarsalis, Cx, pipiens, Cx. erraticus,* and *Cx. restuans* are enzootic vectors and continuously transmit the virus amongst birds, in some cases, birds of certain species will become ill and die, however, many will not show signs of disease (Molaei et al. 2006; Komar et al. 2018).

In most cases these vectors do not feed on humans or other mammals. However, in some cases, when these hosts enter the environment, *Culex* vectors will feed on them which may result in pathogen transmission to the host (Molaei et al. 2006; Kent et al. 2009; Thiemann et al. 2011; Reisen et al. 2013; Komar et al. 2018). However, mammals, including humans, are dead end hosts, meaning the virus does not replicate enough to infect mosquitoes that feed on the infected mammal host, thus ending the transmission cycle of WNV (David and Abraham 2016; Lustig et al. 2018; Knap et al. 2020).

For successful pathogen transmission, certain environmental factors must be present for the vector, host, and the virus. Factors such as climate, shade, wind speeds, landscapes and habitat availability can heavily impact the spread of WNV, especially in relation to the way these factors impact the vectors (Reisen 2010). The spread of ERC in the southern Great Plains has altered the environmental factors in this region by causing normally arid areas to develop areas of humidity that can serve as shelter for mosquitoes, birds, and mammals. These new resting areas allow for host-vector interactions that may otherwise not be present in the environment (Eldridge et al. 2011). Such an example has been observed for the Lone Star tick, *Amblyomma americanum*. This tick has used the conducive habitat created by ERC to spread west across the state of Oklahoma and establish in counties where it was not previously reported. These areas in western Oklahoma are dry and arid, typically unsuitable for *Am. americanum.* However, the ERC altered the ecosystem, providing habitat for the ticks in a region of extreme heat and dry weather (Noden and Dubie 2017). Based on this example of lone star ticks, we hypothesize that ERC may also be providing increased habitat for WNV-infected *Culex* mosquitoes in the Great Plains region (Noden et al. 2021a; Maichak et al. 2022).

Dead End and Reservoir Hosts of West Nile virus

West Nile virus is a zoonotic virus that has been known to reside in many different bird hosts, and it has also been detected in many species of mammalian hosts. WNV does not replicate in mammalian bodies enough for continued transmission of the virus back to a competent mosquito species, which results in a dead-end host ('accidental hosts') (Zeller and Schuffenecker 2004). WNV causes encephalitis and neuroinvasive disease to humans and equine species and can result in death if not treated properly. This is not always the case in humans and horses as many individuals will not show clinical signs of infection (Lindsey et al. 2008; DeGroote and Sugumaran 2012; Angenvoort et al. 2013). Avian species that are reservoir hosts of WNV are also known as 'amplifying' hosts; hosts in which virus is actively replicating quickly and in high concentrations (Thiemann et al. 2011). There has been some evidence to suggest that infected *Cx. tarsalis* mosquitoes have fed on American robin, European starling, house sparrow and mourning dove (Kent et al. 2009). The American robin, the house finch, the mallard, the Eurasian collared dove and the mourning dove tested positive for West Nile antibodies in seroprevalence microsphere immunoassays (Komar et al. 2018). A few other studies concluded that mosquitoes that had fed on European starling, house sparrow, as well as the other species previously listed tested positive for WNV. There is also evidence that there may be many more avian species influencing WNV transmission as *Cx. tarsalis* and *Cx. pipiens* have been found to feed on a wide variety of different avian species, however, none of these species have been found in mosquito bloodmeals that have also tested positive for WNV virus (Kilpatrick et al. 2006; Kent et al. 2009; Thiemann et al. 2011; Reisen et al. 2013; Komar et al. 2018). Understanding the species that are directly related and involved with the transmission cycle of WNV could help prevent the spread and may allow researchers to track the patterns of these birds more effectively in areas where they are found and more precisely predict where possible WNV epidemics may arise.

West Nile virus Disease System and Vectors

West Nile virus is a flavivirus currently detected across the United States. It is a ssRNA virus that is enveloped in the host membrane, which allows the virus to avoid the host immune system (Lindsey et al. 2008; Brinton 2013; David and Abraham 2016). Normally, in nature, this pathogen spreads from infected birds to competent mosquito vectors back into birds after mosquito transmission. In the U.S., 65 species of mosquitoes, 326 bird species, and over 30 non-avian hosts are reportedly involved with WNV transmission (Kilpatrick et al. 2006; Kent et al. 2009; Thiemann et al. 2011; Andreadis 2012; Reisen et al. 2013; David and Abraham 2016; Komar et al. 2018). There has also been evidence that transovarial transmission of the virus can occur from an infected female mosquito to her progeny. This only occurs if the infected female mosquito has a high viral titer (David and Abraham 2016). The genus *Culex* has been considered to be the main vector responsible for transmission to accidental or dead-end hosts, however, some studies have indicated other bridge vectors may be spreading WNV into mammalian hosts (DeGroote and Sugumaran 2012; David and Abraham 2016). Mosquitoes from the genus *Aedes* and *Anopheles* may be bridge vectors that are removing WNV from their normal bird-mosquito-bird cycle and transmitting the pathogen to human hosts, (DeGroote and Sugumaran 2012; David and Abraham 2016; Komar et al. 2018). Although most transmission of WNV occurs via an infected mosquito bite, there have been cases of the virus spreading through infected blood in blood transfusions, organ transplants, and breast feeding from infected mothers (Valiakos et al. 2013; Grinev et al. 2016). Such risk factors make it more difficult to prevent the spread of WNV, especially in situations where infected individuals are asymptomatic (Valiakos et al. 2013; David and Abraham 2016).

West Nile virus Strains and Lineages

Across the globe, WNV strains are grouped into 8 major lineages with some strains further divided into different genetic clusters and clades (Valiakos et al. 2013; Fares et al. 2021). Only two lineages have been identified to cause human and animal disease, lineage 1 and lineage

2. Lineage 1 is the most widespread across the globe and the most commonly found in the United States (Beasley et al. 2004; Davis and Abraham 2016). Lineage 1 is most commonly associated with WNDD in humans and rodents. This lineage has been reported in both hosts and vectors which indicates that there is not a mutation or change during the transmission of WNV (Beasley et al. 2004; Fall et al. 2017). Lineages 3-8 have only been identified in mosquito vectors (Fares et al. 2021). If these lineages are detected in the southern Great Plains, it would be important to characterize their occurrence in in *Cx. tarsalis* and *Cx pipiens* to determine which lineage they are spreading to humans and other mammals.

Vectors and Host Preference determined by blood-meal analysis

Culex tarsalis, Culex pipiens and Culex erraticus are the most common *Culex* species in the southern Great Plains which are also the main WNV vectors collected in ERC (O'Brien and Reiskind 2013; Noden et al. 2021a). These mosquito species feed on a variety of hosts, mostly avian but, mammals as well (Kilpatrick et al. 2006; Thiemann et al. 2011; Komar et al. 2018). The determination of host using blood-meal analysis of blood-engorged females has been instrumental in evaluating host preferences for WNV vectors. Using PCR primers developed for the cytochrome c oxidase I (COI) and cytochrome b (cytoB) gene has been used to identify the species of animals fed on by mosquitoes during periods when WNV rates increase (Kilpatrick et al. 2006; Thiemann et al. 2011; Komar et al. 2018). By analyzing bloodmeal contents for WNV vectors, there may be the possibility of identifying different bird species and other wildlife during different periods of the year. These patterns could help identify which birds are migrating through the area and how they may be impacting WNV. Several studies have also shown that migratory birds may be altering the spread of WNV throughout the United States, and this can become an issue to areas like the Great Plains since ERC can provide a resting place for infected birds in new areas, tying together the nidus of infection in ERC (Valiakos et al. 2012; Valiakos et al. 2013; David and Abraham 2016; Noden et al. 2021a). Evidence suggests that *Cx. tarsalis* will feed on

both avian and mammalian hosts during the summer months but tends to feed on avian hosts during winter months (Thiemann et al. 2011). This change of feeding behavior could be a result of migratory birds being more prevalent in areas with *Cx. tarsalis. Cx. tarsalis* will feed on some species more in winter months because other birds move out of the area due to migration (since some species were not found during winter month census).

In Colorado, blood-fed mosquitoes were collected from bird roosts and identified seven bird species with WNV-reactive antibodies from 738 blood meals. These species included the American robin, house finch, mourning dove, Eurasian collared-dove, mallard, fox, and horse (Komar et al. 2018). This study helps us understand that avian hosts are still the preference for *Cx. tarsalis*, although they will feed on other hosts. However, there is still a gap in our knowledge of how and when *Cx. tarsalis* interact with mammal hosts, especially when bird hosts are available in the area. Another study, also out of Colorado, reported that only 12.6%, 16.2%, and 23% of blood meals came from mammals and humans in three different months, June, July, and August, respectively (Kent et al. 2009).

A California-based study to determine host selection patterns of *Cx. tarsalis* using a COI gene assay between 1998 and 2002 reported that 76.6% of bloodmeals were from avian hosts, 18.1% were from mammalian hosts and 5.3% were from reptiles (Reisen et al. 2013). These results further demonstrate that WNV mainly infects bird species with an occasional accidental host. However, we aim to identify which avian species are being fed on within ERC by the main WNV vectors and which ones are more closely linked to WNV transmission in the southern Great Plains.

Spread of Eastern Red Cedar in the Great Plains

Eastern red cedar, along with other woody plants, is encroaching west across the southern Great Plains region due to the lack of prescribed fires (Eldridge et al. 2011; Kaur et al. 2020). This encroachment has changed savannas and open woodlands into shrublands and has created

drastic ecological changes in arid areas of the U.S. In general, encroachment is linked to general reduction of ecosystem functions (Eldridge et al. 2011). These encroachment areas are providing a fertile oasis, allowing shrubs to grow and further develop shrublands at the expense of the grassland. Spread of ERC has increased water use of plants, increased the risk for wildfire, altered native plant species richness in the area and had an overall effect on soil temperature and moisture due to decreased light and rain penetration (Zou et al. 2018; Kaur et al. 2020). These systems changes attract new bird species, but it has also decreased others like the grasshopper sparrow, dickcissel, western meadowlark and horned lark, among other birds (Coppedge et al. 2001; Chapman et al. 2004). This poses a new problem in the southern Great Plains region as birds serve as reservoir hosts for WNV, and these new bird species could be effective reservoirs for more WNV infections in this region, which is already considered higher than the rest of the nation (Kilpatrick et al. 2006; Lindsey et al. 2008; Eldridge et al. 2011; Thiemann et al. 2011; Komar et al. 2018).

Given these factors, the spread of ERC across the southern Great Plains could be a problem regarding WNV transmission, as it may be creating nidi of infections in once dry and arid areas. As previously stated, ERC has provided effective habitat to assist in the spread of the Lone star tick spread across arid areas of Oklahoma (Noden and Dubie 2017). It is, therefore, reasonable to consider the hypothesis that ERC is aiding in the spread of WNV in the southern Great Plains by provided habitat for mosquito vectors such as *Cx. tarsalis* and *Cx. pipiens.*

Current Status of WNV in ERC in the Great Plains

Mosquitoes in Eastern Red Cedar

In order to understand the risks of WNV in ERC in the Great Plains it is important to understand that the vectors of WNV are already present in these areas, and that mosquitoes prefer shady areas to rest in, similar to those offered by ERC (Sauer et al. 2021). A Kenya-based study demonstrated that mosquitoes choose plant resting sites which in turn, could be extending their

lives. This allowed for longer periods of feeding activity and increases the risk for arboviral disease transmission (Arum et al. 2016). Certain WNV-vector species, such as *Cx. tarsalis* and *Cx. pipiens* can fly long distances, ~2 km, in addition to ~200 m after having taken a blood meal (Ciota et al. 2012; Martínez-de la Puente et al. 2020). This may allow ERC-encroached areas to become an oasis where mosquitoes can rest and digest their blood meals. ERC alters ecosystems by causing changes in temperatures and humidity that benefit mosquitoes as mosquitoes will choose to rest in herb layers and deadwood to take shelter from high wind speeds, elevated temperatures, and rain fall (Sauer et al. 2021).

Since ERC provides shelter, it may be a place that *Culex* mosquitoes can take refuge in or search for hosts to feed on. Studies have shown that *Cx. tarsalis* and *Cx. pipiens* are found in ERC in the southern Great Plains, particularly in Oklahoma (O'Brien and Reiskind 2013; Noden et al. 2021a). The data also suggests that *Cx. tarsalis* is more likely to be found in grasslands or ERC than deciduous woodlands, suggesting that ERC may have an influence in host-vector interactions in the future and it may impact WNV transmission (O'Brien and Reiskind 2013). The *Culex* mosquitoes in ERC have already demonstrated that they are infected with WNV in late summer in both *Cx. tarsalis* and *Cx. pipiens* (Noden et al. 2021a).

Hosts in Eastern Red Cedar

Although indicated that WNV-infected *Culex* mosquitoes may be likely in ERC encroached areas, no studies have focused on the WNV reservoirs that these mosquitoes are feeding on (Noden et al. 2021a). There is a major gap in our understanding of the nidality of WNV in the great Plains region in regards to host-vector interactions in ERC. Studies indicated that avian species such as the American robin, blue jay, Carolina chickadee, cedar waxwing, dark-eyed junco, downy woodpecker, eastern bluebird, European starling, northern cardinal, northern mockingbird, white-breasted nuthatch and yellow-rumped warblers are present in ERC in different times of the year (Coppedge et al. 2001; Horncastle et al. 2004). Furthermore, some

mammalian-focused studies in ERC encroached areas have documented bobcat, deer mouse, fox, fulvous harvest mouse, golden mouse, hispid mouse, opossum, plains harvest mouse, prairie vole, racoon, southern flying squirrel, Texas mouse, white-footed mouse (Horncastle et al. 2004; Horncastle et al. 2005; Reddin and Krementz 2016). Based on these studies, the American robin, blue jay, eastern bluebird, European starling, northern cardinal, northern mockingbird, and yellow-rumped warbler are known reservoirs for WNV and the fox, opossum, and raccoon have been reported with WNV-reactive antibodies suggesting that these species have had WNV infections (Dietrich et al. 2005; Gleiser et al. 2007; Hill et al. 2010; Komar et al. 2018).
Since the American robin migrates to the south during the winter and rests in ERC, there is a possibility that early season mosquitoes may feed on the robin and contract WNV (Coppedge et al. 2001). If infected mosquitoes continue to feed on the birds found in ERC, even after the robin has migrated back to the north, there is a possibility for WNV transmission with the other avian species found in ERC during summer months especially since *Culex* mosquitoes typically follow a bird-mosquito-bird cycle. There are many avian species that are found in ERC that may be viable hosts to continue the cycle (Coppedge et al. 2001; Horncastle et al. 2004; Komar et al. 2018) and yet very few are actually documented as WNV vectors in this part of the U.S., therefore, it would benefit the scientific community for research to be conducted in this area, with a specific focus on host-vector interactions in ERC, especially taking a look at the bloodmeals of engorged mosquitoes and also determining if those blood-fed mosquitoes test positive for WNV.

WNV and ERC coming together in the southern Great Plains

Currently, West Nile virus is understudied in the southern Great Plains, specifically how WNV is affected by the encroachment of ERC and the habitats it provides for WNV vectors. In a recent study we saw that WNV was found in pools of *Cx. tarsalis* from western Oklahoma from ERC and deciduous areas and found in one pool of *Cx. pipiens* from central Oklahoma (Noden et al. 2021a). Also, there was another study done in the summer of 2020, where mosquitoes were

collected across western Oklahoma in areas of varying densities of ERC, that yielded one positive pool of WNV from *Cx. tarsalis* in Binger, Oklahoma (Maichak et al. 2022). However, it is also important to note that only *Cx. tarsalis* was tested for the pathogen and 2020 was also a low year for WNV detection across the United States (Arbonet 2023).

Where do we go next?

Our goal is to determine what host species are fed on by mosquitoes inhabiting ERC expansion areas in the southern Great Plains. We seek to determine the specific avian hosts on which mosquitoes in ERC are feeding. There is a large gap of knowledge on what hosts are present in ERC and how mosquitoes interact with them. Most WNV vectors are *Culex* mosquitoes, and they normally feed on birds (Kilpatrick et al. 2006; Thiemann et al. 2011; Reisen et al. 2013; Komar et al. 2018). However, it is unclear whether these vectors switch to mammalian hosts, or if these vectors are responsible for WNV transmission to mammals in this region. By evaluating blood meals, we will determine if avian hosts are transient, either early or late season, or if they are present throughout the whole season or whether host switching occurs. To complete this task, we will extract DNA from blood-fed mosquitoes and run PCR with primers that amplify the COI and cytoB genes in the samples. Positive DNA samples will be purified and sequenced. Sequences will be compared to known sequences using NCBI BLAST to identify the species of the host.

We also seek to determine WNV infection status from blood-fed mosquitoes in ERC expansion areas. Mosquitoes that are resting in ERC after taking blood meals may also be infected with WNV. These include primary species (*Cx. tarsalis* and *Cx. pipiens*) and secondary species (*Cx. erraticus* and *Cx. restuans*). Therefore, it is also necessary to test all blood-fed *Culex* mosquitoes found in ERC for the presence of WNV. This will involve extracting RNA from our blood-fed mosquitoes and testing them for WNV. If these blood-fed mosquitoes also test positive for WNV, we will be able to connect mosquito vectors to host species to WNV-vector status,

thereby confirming relationships not well studied in the region and possibly creating new relationships not yet studied. To accomplish this, we will collect legs from blood-fed mosquitoes at the time of processing and extract the RNA and test the extracted RNA for WNV using quantitative PCR (qPCR).

Lastly, we will evaluate the effect of ERC on the nidus of infection of WNV in the southern Great Plains. Our current thought is that ERC encroachment into the southern Great Plains is providing an "oasis" for mosquitoes, *Cx. tarsalis* and *Cx pipiens*, that fly long distances. In the same area where various hosts are finding important resources, these WNV vectors also find a place to rest and a blood meal in a normally inhospitable grassland (Reisen et al. 1995; Ciota et al. 2012; Martínez-de la Puente et al. 2020). Additionally, these ERC expansion areas may be forcing different mosquito species to feed on new hosts that may be competent WNV-species and may produce WNV-transmission in bridge vectors. Our goals will identify the mosquitoes present, the infection rates and the hosts available to those vectors. We will then combine all the data to form a better understanding of how WPE in the context of ERC expansion may be playing a role in the nidus of infection of WNV.

Woody plant encroachment has been addressed regarding its ability to alter an ecosystem's properties. However, we have not focused on the way arboviruses and their vectors may be moving throughout ERC encroached ecosystems in regard to WNV. Currently, there is no research that has addressed how WPE affects a whole-disease system. Future research must focus on the effects of ERC in the southern Great Plains on WNV hosts, vectors, and vector-host interactions to determine how it influences WNV transmission in itself. Therefore, the aims of this study were several fold:

1) Determine the hosts that mosquitoes resting in ERC encroached areas are feeding on and possibly link host-feeding to WNV prevalence.
2) Determine the impact of zone preferences of mosquito species within ERC-encroached areas, focusing on mosquito abundance, host-blood meals, and WNV prevalence.

CHAPTER II

WHO'S USING WHO? MOSQUITO BLOODMEAL IDENTIFICATION TO DISCOVER HOST-MOSQUITO INTERACTIONS IN EASTERN RED CEDAR

Introduction

Mosquitoes are the most important vectors of arboviruses globally (Gubler 2002). In the United States, the most common mosquito-borne virus is West Nile Virus (WNV) (Curren et al. 2018, Rosenberg et al. 2018), causing neurological damage that can result in death. Arriving in the United States in New York City in 1999 (Lanciotti et al. 1999), WNV rapidly spread throughout the country, completing the transverse in 2003 (Kramer et al. 2019). Although many mosquito species found in the US are able to transmit WNV in laboratory conditions (Turrell et al. 2005), the most efficient transmitters of this virus are mosquitoes in the genus *Culex* with the principal species *Cx. tarsalis* and *Cx. pipiens* (Kramer et al. 2019). The reservoir for WNV are birds and the normal transmission cycle occurs between birds and mosquitoes (Thiemann et al. 2011). Occasionally, a WNV-infected mosquito will feed on humans and horses, also known as dead-end hosts, because the virus only causes symptoms and never reaches a viremia high enough to infect a mosquito (Zeller and Schuffenecker 2004). There are many avian species thought to be reservoir hosts of WNV (Kent et al. 2009), however, there is very little knowledge regarding bird-mosquito interactions involving host preferences, habitat effects, and where mosquitoes actually encounter WNV-infected birds in a given landscape in ERC encroachment areas (Kilpatrick et al. 2006; Hamer et al. 2009; Kramer et al. 2019).

While there is a national gap in knowledge regarding this topic, there is a critical lack of knowledge regarding host-mosquito interactions in the Great Plains region.

The Great Plains region is known to have a higher prevalence of WNV, compared to other regions of the country, and it also has the most cases of West Nile neuroinvasive disease (WNND) (Lindsey et al. 2018). The Great Plains is also undergoing another phenomenon, woody plant encroachment (WPE) by many species, but eastern red cedar (ERC) (*Juniperus virginiana*) is the dominant encroacher as it is expanding 5-7 times faster in Kansas, Oklahoma, and Texas than any other region in the nation (Zou et al. 2018; Kaur et al. 2020). Spreading at $40km^2$ per year (Wang et al. 2018) and growing half a meter per year (Zou et al. 2018), this native invasive plant can turn a grassland into a cedar forest within 40 years (Briggs et al. 2002). As part of that expansion, ERC changes specific abiotic and biotic factors in the surrounding environment that impact the way other plant and animal species use the land around them (Eldridge et al. 2011). In addition to changing water content and soil properties, ERC encroachment causes animals to leave their native habitats and introduces new species into the area. This can mean that bird species in ERC may be completely different than species inhabiting grassland habitats (Coppedge et al. 2001; Horncastle et al. 2004). To date, there is a lack of information regarding avian and mammalian presence in ERC-encroached areas which may have important implications for the spread of WNV throughout the region.

Studies have demonstrated that ERC in the southern Great Plains provides habitat for various blood-sucking arthropods, including ticks (Noden and Dubie 2017; Noden et al. 2021b), horse flies (Sherrill 2019) and mosquitoes (O'Brien and Reiskind 2013; Noden et al. 2021a). In addition to collecting more potential WNV vectors (*Cx. tarsalis* and *Cx. pipiens*) in ERC encroached areas than deciduous forests and grasslands (O'Brien and Reiskind 2013; Noden et al. 2021a), this invasive plant is also the habitat choice of other mosquito species including *Cx. erraticus*, *Ps. columbiae*, and *An. quadrimaculatus* (Maichak et al. 2022). Additionally, studies have reported the propensity of WNV-infected *Cx. tarsalis* and *Cx. pipiens* to rest in ERC trees as

opposed to other habitats (Noden et al. 2021a, Maichak et al. 2022). While these studies have focused on the affinity of mosquitoes to ERC-encroached habitats, there is little knowledge on the mammalian and avian hosts that mosquitoes in ERC encroached areas are feeding on. This knowledge has direct importance to gaining a better understanding regarding the 'nidus of infection' (Reisen 2010) for the transmission of WNV to mosquitoes. To address this limited knowledge, the two main aims of this study were to determine 1) host identity and location that mosquitoes collected in ERC encroached areas are feeding on and 2) the prevalence of WNV of *Culex tarsalis* collected in ERC-encroached areas.

Material and Methods

Study Locations

The main portion of the study occurred in central Oklahoma with a one night sampling trip into western Oklahoma. Due to the main focus of the study on collecting blood-fed mosquitoes, we established trapping locations where resting (bucket) traps (Burkett-Cadena et al. 2011) could be left for the entire study period (Supplemental Figure 1). Sites in Payne County, Oklahoma, USA were chosen because of their accessibility and the concentration of mature ERC present. During the entirety of the sampling period, 15 resting traps were set up at 4 trapping locations: Highland Park (6 traps), the Sangre Ridge site (2 traps), the Briarcreek site (3 traps), and Whittenberg Park (4 traps) (Figure 1.1). Due to the success of the Briarcreek site, an additional resting trap was added on 2 July 2021 to collect more mosquitoes. The number of resting traps per trapping location varied due to location and available cedar trees.

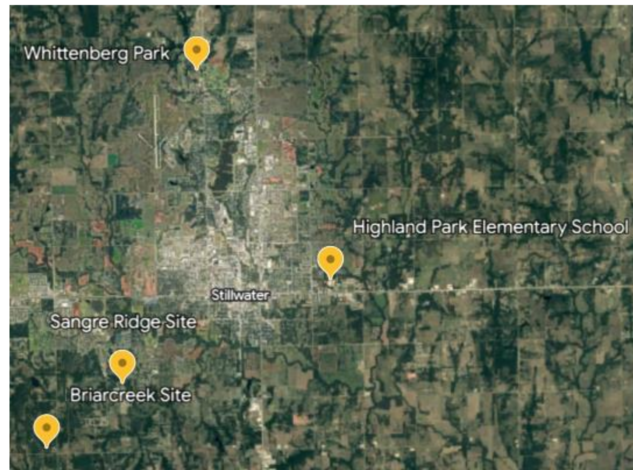

Figure 1.1. Payne County trapping locations with labels. Taken from Google Earth Pro.

To augment mosquito diversity and blood-fed mosquitoes, we set out CDC light traps (Bioquip, Rancho Dominguez, CA) for one-night collections on three (two in Payne County and one in western Oklahoma) occasions. The CDC traps were used without lights and baited with CO_2 (dry ice) to attract host-seeking mosquitoes (Supplemental Figure 2). Trapping locations were chosen for their proximity to grasslands or along naturally occurring corridors within the cedar trees. In Payne County, three CDC light traps were placed at each site where resting traps were already placed, resulting in 12 additional trap nights/mosquito collection. Trapping locations in western Oklahoma were chosen because of their accessibility, permission from property owners and their use in a previous study (Maichak et al. 2022). These locations consisted of one east of Watonga (Blaine County), one east of Okeene, near the Cimarron River (Kingfisher County), and the final site was south of Binger (Caddo County). During this one-night sampling visit, six CDC light traps with CO_2 were used in each trapping location, providing an additional 18 trap nights (Figure 1.2). Also, no resting traps were placed in western Oklahoma as only one sampling visit was made. Each site was digitally identified using Google Earth to identify areas that possibly contained ERC, and then visually confirmed to ensure the adequate amount of ERC was available for the study.

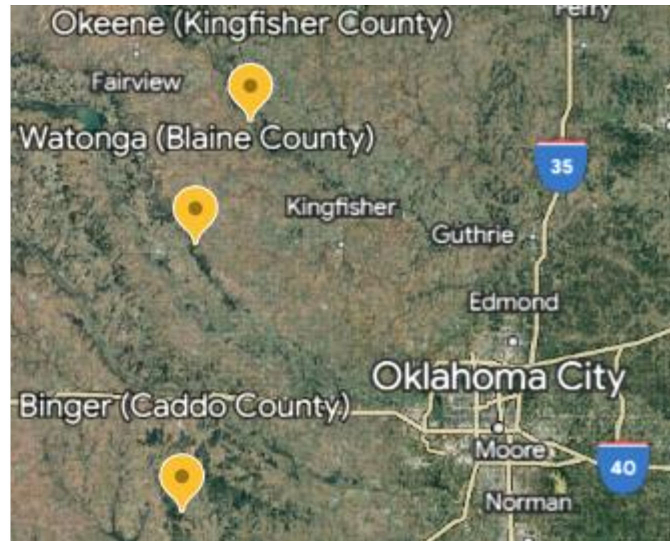

Figure 2.2. Western Oklahoma trapping locations with labels. Taken from Google Earth Pro.

Trapping Protocol

Collections of blood-fed mosquitoes began on 23 June 2021 and continued through 30 July 2021 with each site in Payne County visited 2-3 times per week, depending on the weather. A total of fifteen 32-gallon black garbage cans served as resting traps (Lowes) (Burkett-Cadena et al., 2011) and were placed inside ERC trees so that the opening was flush with the foliage of the tree. The resting traps were set out the day before collections began (22 June 2021). During each collection, the resting traps were aspirated with InsectZooka Field Aspirators (Bioquip, Rancho Dominguez, CA), including the foliage surrounding the trap to capture any mosquitoes that may have flown out while aspirating. All sites were aspirated before 10:00.

CDC light traps (Bioquip, Rancho Dominguez, CA) were placed one meter off the ground and about halfway between the trunk and the edge of the branch and baited with two pounds of dry ice in ERC encroached areas. Three CDC light traps per trapping location were set prior to 16:00 and left overnight and then picked up the following morning by 10:00. In Payne County, CDC traps were set out on the nights of 8 July 2021 and 23 July 2021. The distance between traps was 100m in an effort to ensure independent activity (Reiskind et al. 2017). In

western Oklahoma, CDC traps were set out on the night of 13 July 2021 in Blaine, Caddo, and Kingfisher counties. Six CDC traps were set out over 100m apart at each location.

Sample Sorting and Mosquito Identification

Mosquitoes were collected from resting traps in Insectzooka collection cups (Bioquip, Rancho Dominguez, CA), clear propylene cups with mesh aluminum netting at the bottom for air flow, and labelled with the date, trapping location, trap type, and trap number. Mosquitoes collected with CDC traps were kept in their collection containers and labelled with the same information. At the time of collection, CDC light trap containers were placed into a Whytner Portable Freezer (85 quart, Whynter, Brea, CA) to euthanize and preserve the mosquitoes during transport. Upon arriving to the lab, mosquitoes were immediately sorted by feeding status to prevent degradation of host DNA in the bloodmeal (Coulson et al. 1990). Mosquitoes containing bloodmeals were individually placed into 1.5mL clear microtubes and labelled with date, trapping location, trap type and trap number, genus and species and placed into the -80°C deep freezer until a later date for bloodmeal extraction (Egizi et al. 2018; Greenberg et al. 2013; Mann et al. 2020; Molaei et al. 2006, 2008; Thiemann et al. 2012). Nonblood-fed mosquitoes were placed into plastic snap cap vials (7-dram, Fisher Scientific, Hampton, NH) and labelled with the same information as blood-fed mosquitoes and stored at -20°C until they could be identified (Darsie and Ward 2005).

West Nile Virus Analysis

Only *Culex tarsalis* were tested for West Nile virus due to financial constraints. At the time of sorting, mosquitoes were pooled in groups of up to 25 individuals from the same date, location, and trap. Before processing, a master mix solution was made using phosphate-buffered saline (PBS) and 2x lysis buffer were combined in a 1:1 mixture (Applied Biosystems, Foster City, CA). Pooled mosquitoes were placed into appropriately labelled 2ml XXTuff reinforced microvials (Biospec) and 200µL of master mix was added to each pool (300µL for pools that

contained 8 mosquitoes or more) along with two 2.3mm stainless steel beads (Biospec Products, Bartlesville, OK). Tubes were placed into a Mini-Beadbeater-16 (Biospec Products, Bartlesville, OK) for two rounds of 2 minutes. Tops were checked between rounds to ensure effective sealing. Samples were then placed into a centrifuge (Microfuge 16, Beckman Coulter, Brea, CA) and spun at 6,000 rpm for 4 minutes. Supernatants were then extracted and placed into sterile, labelled 1.5mL tubes (VWR) and stored at -80°C.

Once extractions were completed, mosquito samples were kept on ice for total RNA extractions using the QIAmp Viral RNA Mini Kit (250) (Qiagen, Valencia, CA) and following the manufacturer's instructions. Samples were once again stored at -80°C until they were tested. Real-time RT-PCR was performed on RNA extracted from mosquito pools using a combination of QuantiTect Probe RT-PCR Kit (Qiagen), 25 pmol of WNV primers (Lanciotti et al. 2000), 3.25 pmol of the probe, and 10 µL of the RNA extracted from the mosquitoes for a total reaction volume of 25µL. A single cycle of 50°C for 30min (reverse transcription) and 95°C for 15 min (hot start), followed by 40 cycles of 94°C for 30s, 55°C for 1min, and 68°C for 1 min. Result reports were created after cycles were completed. Positive controls were graciously provided by Dr. Gabriel Hamer (Texas A & M).

Bloodmeal Identification

The process of bloodmeal determination began by removing the blood-engorged abdomen from the thorax of the mosquito. It was imperative to make sure that only the abdomen, containing the midgut, was used so that no other mosquito body parts would interfere with the PCR assay. Blood-fed mosquitoes were placed into a drop of PBS buffer on a glass microscope slide. In the event that the abdomen burst, the blood and PBS could be pipetted into tubes pre-filled with 200µL of PBS. After this process was repeated for all blood-fed mosquitoes, we followed the manufacturer's protocols from a DNeasy 96 Blood and Tissue Kit (Qiagen, Valencia, CA) to extract the DNA from the bloodmeals. In summary, 20µL of proteinase K and

200µL of Buffer AL were added to each tube and the mosquito abdomen was then pressed against the edge of the tube with the pipette tip to expulse the bloodmeal from the abdomen to be completely exposed to the solution (Thiemann et al., 2012). Samples were then vortexed and placed into an incubator set at 56°C for 2 hours on a rocking platform. Once samples were removed from the incubator, they were placed back on ice and 200µL of 100% ethanol was added to each sample and vortexed once again until the solution was homogenized. Samples were then transferred to DNeasy wash tubes and manufacturer's instructions were used with slight modification; we eluted the DNA from the column with 100µL Buffer AE instead of 200µL to concentrate the samples. DNA samples were stored at -20°C until PCR amplification.

Blood meal hosts were identified using PCR amplification and DNA sequencing using a fragment of either the vertebrate mitochondrial genes cytochrome oxidase 1 (COI) or cytochrome b (CytoB). Initially, all samples were screened with a COI primer cocktail consisting of forward primers VF1 t1, VF1d t1, and VF1i t1 and reverse primers VR1d t1, VR1 t1, and VR1i t1 were each mixed at a ratio of 1:1:2 (Ivanova et al. 2007). The PCR conditions were 94°C for 1 min, five cycles of 94°C for 30s, 50°C for 40s, and 72°C for 1 min, followed by 35 cycles of 94°C for 30s, 54°C for 40s, and 72°C for 1 min, with a final extension at 72°C for 10 min. The PCR samples were then run in a 2% agarose gel containing ethidium bromide in a 1x TBE buffer and visualized under ultraviolet light. All positive amplicons were extracted from the gels using the QIAquick Gel Extraction Kit (Qiagen), and resulting DNA was bidirectionally sequenced using the forward and reverse primers in an Applied Biosystems 3730 DNA analyzer at the Oklahoma State University Core Facility to identify the animal species. We compared each resulting sequence with GenBank submissions using default conditions on NCBI BLAST (blastn, NCBI, 2022) where the highest % sequence identity was used to determine species identity.

If the initial screening produced no useable sample, samples were rerun with CytoB primers focused on specific avian (Molaei et al. 2006), mammalian (Molaei et al. 2006), and reptilian/amphibian (Cupp et al. 2004) sequences. The first avian primer used was AV1F/R

primers with initial denaturation at 95°C for 5 min, followed by 36 cycles of 94°C for 30s, 60°C for 50s, and 72°C for 40s. The final 72°C extension was 5 min with a finished product of 508bp (Molaei et al. 2006). The second avian primer was AV2F/R with initial denaturation at 95°C for 5 min, followed by 35 cycles of 95°C for 60s, 58°C for 60s, and 72°C for 60s. The final 72°C extension was 5 min with a finished product of 515bp (Molaei et al. 2006). After screening with avian primers, two mammalian primer sets were used. The first mammalian primer set was MAMF/R1 with initial denaturation at 95°C for 5 min, followed by 35 cycles of 94°C for 30s, 55°C for 45s, and 72°C for 90s. The final 72°C extension was 5 min with a finished product of 772bp (Molaei et al. 2006). The second mammalian primer set was MAMF/R2 with initial denaturation at 95°C for 5 min, followed by 32 cycles of 95°C for 60s, 54°C for 60s, and 72°C for 60s. The final 72°C extension was 5 min with a finished product of 195bp (Molaei et al. 2006). The final primer set used was REPF/R with initial denaturation step at 95°C for 2 min, followed by 55 cycles at 94°C for 45s, 50°C for 50s, and 72°C for 1 min, and a final extension at 72°C for 7 min (Cupp et al. 2004). All positive amplicons used the same extraction process as COI amplicons for sequencing described above. If unique hosts were detected at a specific location, we investigated the area by foot or vehicle as well as asking the local residents to ascertain how far away the host may have been from the trap site where the mosquito was collected.

Results

Mosquito collection

Mosquito collections occurred at 45 sites at 7 trapping locations in areas of encroaching ERC across four counties. No trap failures occurred during the study period. Between 23 June and 30 July 2021, a total of 3,187 mosquitoes were collected, involving 248 trap nights (Table 1.1). A majority (2,264, 71.04%) of the mosquitoes were collected from one collection night (13 July 2021) in western Oklahoma with Okeene producing the most of the three sites (1,291, 57.0%). In Payne County, the most mosquitoes were collected at the Briarcreek site (443, 48%).

Of the species collected in Stillwater, most *Aedes trivittatus* (83%), *Psorophora cyanescens* (77%), *Ps. ferox* (83%) and *Ps. longipalpus* (81%) were collected at Highland Park while most anophelines (*Anopheles quadrimaculatus* (92.6%), *An. punctipennis* (74%)) and *Culex erraticus* (60%) were collected in Briarcreek. The majority (2,853, 89.52%) of mosquitoes were collected using the CO_2-baited CDC light traps with the rest collected in resting traps (334, 10.48%).

Of the medically important mosquitoes, the most common species collected was *Cx. tarsalis* (595) followed by *Ps. columbiae* (451), *An. quadrimaculatus* (220), *Cx. erraticus* (194), *Ae. albopictus* (149), *Cx. pipiens* (26) and *Cx. restuans* (7) (Table 1.1). These collections, however, differed by region. The majority of *Cx. tarsalis* (99%), *Ae. vexans* (94%), and *Ps. columbiae* (92%) were collected in western Oklahoma while the majority of *An. quadrimaculatus* (86%), *Ae. albopictus* (94%), and *Cx. erraticus* (94%) were collected in central Oklahoma.

West Nile Virus Results

Of the 32 pools of *Cx. tarsalis* that were tested for WNV, none tested positive for WNV (Fig 1.3).

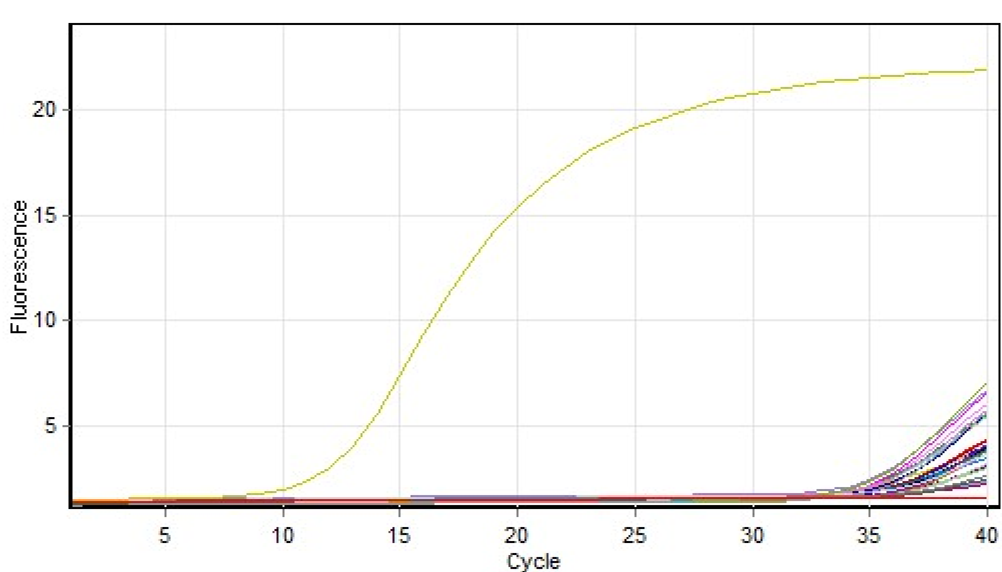

Figure 1.3 qPCR results from WNV analysis. No pools resulted in a positive result. The positive control WNV G-Block (yellow line) appeared in cycle 7.

Table 1.1: Total numbers of each mosquito species by trap type collected between June and July 2021 across seven trapping locations in central and western Oklahoma. HighlandP: Highland Park Elementary School, SangreR: Sangre Ridge, Whittenberg: Whittenberg Park.

Mosquitoes	**Location**									**Trap Type**		
Species	Stillwater, OK				**Total**	Western Oklahoma			**Total**	CDC	Bucket	**Total**
	Briarcreek	HighlandP	SangreR	Whittenberg		Binger	Okeene	Watonga				
Ae. albopictus	65	52	5	18	**140**	6	1	2	**9**	142	7	**149**
Ae. atlanticus	0	2	0	0	**2**	0	0	0	**0**	1	1	**2**
Ae. c. mathesoni	0	1	0	0	**1**	0	0	0	**0**	0	1	**1**
Ae. canadensis	0	7	1	0	**8**	0	0	0	**0**	0	8	**8**
Ae. epactius	1	0	0	0	**1**	1	0	0	**1**	1	1	**2**
Ae. sollicitans	0	0	0	1	**1**	0	5	33	**38**	38	1	**39**
Ae. triseriatus	7	6	0	1	**14**	3	0	0	**3**	12	5	**17**
Ae. trivitattus	27	144	0	2	**173**	150	113	3	**266**	439	0	**439**
Ae. vexans	5	4	0	16	**25**	71	254	38	**363**	378	10	**388**
An. perplexens	0	0	0	1	**1**	0	0	0	**0**	0	1	**1**
An. quadrimaculatus	176	4	9	1	**190**	0	23	7	**30**	30	190	**220**
An. punctipennis	26	4	1	4	**35**	2	1	11	**14**	16	33	**49**
Cx. erraticus	110	40	13	20	**183**	3	4	4	**11**	157	37	**194**
Cx. nigripalpus	2	1	0	0	**3**	0	0	0	**0**	0	3	**3**
Cx. pipiens	8	10	1	4	**23**	2	1	0	**3**	8	18	**26**
Cx. restuans	0	1	0	0	**1**	1	5	0	**6**	7	0	**7**
Cx. salinarius	2	1	0	3	**6**	4	7	81	**92**	98	0	**98**
Cx. tarsalis	3	1	1	2	**7**	154	306	128	**588**	593	2	**595**
Ps. ciliata	0	1	0	0	**1**	1	24	3	**28**	29	0	**29**
Ps. columbiae	2	7	6	21	**36**	16	265	134	**415**	446	5	**451**
Ps. cyanescens	3	20	3	0	**26**	7	131	1	**139**	160	5	**165**
Ps. discolor	0	0	0	0	**0**	17	33	79	**129**	129	0	**129**
Ps. ferox	3	25	1	1	**30**	3	11	0	**14**	38	6	**44**
Ps. longipalpus	3	13	0	0	**16**	7	107	1	**115**	131	0	**131**
Unknown	0	2	0	0	**2**	2	0	0	**2**	4	0	**4**
Total	443	346	41	95	**925**	450	1291	525	**2266**	2857	334	**3191**

Bloodmeal Identification

A total of 132 blood-fed mosquitoes were collected during the study period (Table 2). The majority (83, 62.9%) were collected using the resting traps with the remaining (49, 37.1%) collected using CO_2-baited CDC light traps. The highest collected species (69, 52.27%) of blood-fed mosquitoes was *An. quadrimaculatus*. Of the 132 blood-fed mosquitoes, majority (95, 71.97%) were collected in the Stillwater area with all *An. quadrimaculatus* collected in the Stillwater area (Table 1.2). Four of the blood-fed mosquitoes were considered unknowns as they could not be identified both visually or molecularly.

We confirmed the host specificity for 95 (72%) of the 132 blood-fed mosquitoes collected (Table 1.3). Of 12 different hosts detected, most blood meals involved white-tailed deer (WTD) (32; 33.7%) followed by cow (31; 32.6%) and horse (13; 13.7%). Table 1.3 includes all animal species that were identified with the mosquito species that fed on the host. By using different assays, we identified that 5 (5.3%) mosquitoes had fed on WTD which were infected with *Anaplasma platys*. These included *An. quadrimaculatus* (3) and *Ps. columbiae* (1) collected in Stillwater and an *Ae. vexans* (1) collected in the Okeene site.

Mosquitoes	Location									Trap Type		
Species	Stillwater, OK				Total	Western Oklahoma			Total	CDC	Bucket	Total
	Briarcreek	HighlandP	SangreR	Whittenberg		Binger	Okeene	Watonga				
Ae. albopictus	1	0	0	0	**1**	0	0	0	**0**	1	0	**1**
Ae. sollicitans	0	0	0	0	**0**	0	0	5	**5**	5	0	**5**
Ae. triseriatus	0	0	0	0	**0**	0	1	0	**1**	1	0	**1**
Ae. trivitattus	0	0	0	0	**0**	4	1	0	**5**	5	0	**5**
Ae. vexans	0	0	0	0	**0**	1	2	0	**3**	3	0	**3**
An. quadrimaculatus	61	2	6	0	**69**	0	0	0	**0**	0	69	**69**
An. punctipennis	3	0	0	0	**3**	0	0	0	**0**	0	3	**3**
Cx. erraticus	8	2	0	0	**10**	0	0	0	**0**	2	8	**10**
Cx. nigripalpus	2	0	0	0	**2**	0	0	0	**0**	0	2	**2**
Cx. pipiens	1	0	0	1	**2**	0	0	0	**0**	1	1	**2**
Ps. columbiae	0	0	0	5	**5**	2	0	11	**13**	18	0	**18**
Ps. cyanescens	0	0	0	0	**0**	0	4	0	**4**	4	0	**4**
Ps. discolor	0	0	0	0	**0**	2	0	2	**4**	4	0	**4**
Ps. ferox	0	1	0	0	**1**	0	0	0	**0**	1	0	**1**
Unknown	0	2	0	0	**2**	2	0	0	**2**	4	0	**4**
Total	76	7	6	6	**95**	11	8	18	**37**	49	83	**132**

Table 1.2. Total number of blood-fed mosquitoes collected between June and July 2021 at seven trapping locations in central and western Oklahoma.

Mosquito Species	Total no.	Cow	Horse	Alpaca	WTD	Human	Sheep	Raccoon	Opossum	Armadillo	Cat	Rabbit	Mourning dove
Ae. albopictus	**1**	-	-	-	-	1	-	-	-	-	-	-	-
Ae. sollicitans	**5**	3	-	-	1	-	-	-	-	-	-	-	-
Ae. triseriatus	**1**	-	-	-	1	-	-	-	-	-	-	-	-
Ae. trivitattus	**5**	-	-	-	3	-	-	-	-	-	-	2	-
Ae. vexans	**3**	-	-	-	3	-	-	-	-	-	-	-	-
An. punctipennis	**3**	-	-	-	2	-	1	-	-	-	-	-	-
An. quadrimaculatus	**69**	15	11	1	13	1	5	-	-	-	-	1	-
Cx. erraticus	**10**	1	1	-	4	-	-	-	1	-	1	1	-
Cx. nigripalpus	**2**	-	-	-	2	-	-	-	-	-	-	-	-
Cx. pipiens	**2**	-	-	-	1	-	-	-	-	-	-	-	1
Ps. columbiae	**18**	8	1	-	1	-	-	-	-	-	-	-	-
Ps. cyanescens	**4**	2	-	-	-	-	-	-	-	-	-	-	-
Ps. discolor	**4**	2	-	-	1	-	-	-	-	-	-	-	-
Ps. ferox	**1**	-	-	-	-	-	-	-	-	-	-	1	-
Unknown	**4**	-	-	-	-	-	-	1	-	1	-	-	-
Total	**132**	31	13	1	32	2	6	1	1	1	1	5	1

Table 1.3. Total number of blood-fed mosquitoes that fed on specific hosts between June and July 2021 in trapping locations in central and western Oklahoma.

Flight Distance Analysis

In the process of identifying bloodmeals, we recognized that some hosts were uniquely placed in reference to the trapping locations in Stillwater. Here, we only considered mosquitoes that had fed on animals that could be physically identified in a relatively fixed position and were not transient hosts. At the Briarcreek site, we identified *An. quadrimaculatus* (3) that had flown varying distances from their original feeding site on sheep (357m), cattle (357m), and alpaca (1200m) to a specific resting trap, all most likely involving flight down a stretch of paved road (Supplemental Figure 3a,b). At Whittenberg Park, we identified a *Ps. columbiae* (1) that had flown a minimum of 590m from their original feeding site on cattle across a residential area into the encroached area where the resting trap was located (Supplemental Figure 4). At the Sangre Ridge site, we identified *An. quadrimaculatus* (1) that flew a minimum of 500m, down a residential road containing 22 houses, after having fed on a horse (Supplemental Figure 5). At the Highland Park site, a *Cx. erraticus* (1) fed on a horse which was housed almost 200m away through an industrial business (Supplemental Figure 6).

Discussion

Our study demonstrates that ERC is providing habitat for different species of mosquitoes after feeding on a variety of hosts in semi-urban and rural areas of the U.S. Great Plains. The main hosts fed on were WTD, cows, and horses. A majority of blood-fed species collected are considered grassland mosquitoes (*An. quadrimaculatus*, *Ps. columbiae,* and *Cx. erraticus*) (Reiskind et al. 2017) and yet they were collected in traps placed within ERC. This demonstrates that mosquitoes may be flying into ERC to feed on hosts that are resting in those areas or after having taken a bloodmeal from a host outside the ERC, as suggested by the flight distance analysis, for a variety of reasons. One reason could involve the special microclimate that ERC provides, which includes alterations in temperatures, wind speeds, and humidity (Garner and Steinberger 1989; Caterina et al. 2013; Acharya et al. 2018; Wang et al. 2018). Another reason is

that these blood-fed grassland mosquitoes may fly into ERC to find a nectar source or to rest before oviposition (Reisen 2010; O'Brien and Reiskind 2013; Reiskind et al. 2017).

Mosquitoes in our study areas had fed on 14 different species of animals, totaling 95 probable hosts. In central Oklahoma, various mosquito species had fed on 10 different hosts (WTD, cow, sheep, horse, human, mourning dove, rabbit, cat, opossum, and alpaca). The majority of mosquitoes fed on WTD and cows that were present at most collection locations. Animals such as alpaca, cow, horses, sheep, and WTD were visually identified as being present in the area. When evaluating host-feeding relationships, it is important to note that cows and horses are typically found in fixed locations (pasture or fenced in area). This is especially the case in the semi-urban environments in the central Oklahoma region. Deer, however, are more transient, likely passing through the area or resting in cedar thickets during the spring and summer (Gee et al. 2011; Masters and Stewart 2017) and are likely convenience fed on by mosquitoes. Most of the host-feeding choices in this study have been reported by others. The *Aedes, Anopheles,* and *Psorophora* species collected are known to feed primarily on mammals with an occasional avian species (Whitehead 1951; Edman 1971; Kuntz et al. 1982; Robertson et al. 1993; Meece et al. 2005; Molaei et al. 2006, 2008, 2009; Tuten et al. 2012). Most *Culex* species reportedly feed predominantly on birds with an occasional mammal species with *Culex erraticus* and *Cx. nigripalpus* being opportunistic feeders (Robertson et al. 1993, Molaei et al. 2007; Savage et al. 2007; Tuten et al. 2012; Mann et al. 2020; Ber et al. 2021; Hancock and Camp 2022). While most host-feeding relationships were already reported, we detected 6 unique combinations: *Ae. trivitattus* and a rabbit, *An. quadrimaculatus* and an alpaca and sheep, *Ps. columbiae* and a WTD, and *Ps. cyanescens* and a cow, *Ps. discolor* and WTD.

These six novel host-mosquito relationships occurred with mosquito species that prefer grassland habitat (Reiskind et al. 2017; Maichak et al. 2022). In fact, the majority (78%) of the blood-fed mosquitoes collected in ERC-encroached areas in semi-urban locations in central Oklahoma prefer grassland habitats (Reiskind et al. 2017; Maichak et al. 2022). There is a

question, then, as to why mosquito species that prefer grassland habitat were collected inside ERC once they had fed on a host. There are various reasons why mosquitoes are found in various habitats, including nutrition (available nectar sources), feeding (available hosts) or reproduction (available oviposition sites) (Reisen 2010; O'Brien and Reiskind 2013; Reiskind et al. 2017). The probable reason that blood-fed grassland species were collected in our traps within ERC encroached areas was probably due to the presence of oviposition sites in the vicinity. In fact, ~90 meters from our resting traps at Briarcreek was a natural spring on the property where different mosquito species were developing [Henriquez, unpublished data] and the majority of our blood-fed mosquitoes were collected in resting traps.

In addition to new mosquito host interactions, we identified several mosquito species that flew further post-blood meal than has been previously reported. While studies have identified that mosquitoes can fly for long distances to find a blood meal (Bailey et al. 1965; Reisen and Lothrop 1995; Reiskind et al. 2017), there is limited literature on how far they can fly after taking a blood meal. Our study sites were unique in that particular animals were housed around some of our semi-urban trapping locations. If we collected a mosquito that had fed on that animal at that particular location, we could determine the minimum distance the mosquito had flown from the host to the resting trap (Supplemental figures). For example, one blood-fed *An. quadrimaculatus* was collected with alpaca blood. The only site in the neighborhood with alpaca was over 1200 meters down a long-paved road so we could assume she had flown from the alpaca site to our resting trap. Distances of over 500m were determined at other locations with different species of mosquitoes. These long post-blood meal flight distances are not normally reported in the literature. While a zoo study in Germany revealed a blood-fed *An. maculipennis* had flown 770m (Heym et al. 2019), most studies report blood-fed mosquitoes flying distances of up to 360m with most flying under 130m (Tuten et al. 2012; Heym et al. 2019; Martínez-de La Puente et al. 2020; Hernandez-Colina et al. 2021). We know that long post-bloodmeal flights are possible as blood-fed *Ae. sollicitans* were reported to have flown over 3000m from their cattle host (Schaefer and

Steelman 1969). However, there are few studies which have evaluated post-bloodmeal behaviors in any mosquito species. In the case of our semi-urban locations, there are many questions about where mosquitoes are originating, how they find their hosts, why they flew so far to our resting traps, and where they would go to oviposit after digestion.

Our study is the first to report the presence of *Anaplasma platys* in WTD in Oklahoma. We identified five mosquitoes from three different species, *An. quadrimaculatus* (3), *Ps. columbiae* (1) and *Ae. vexans* (1) which had fed on WTD and also were positive for *Anaplasma platys*. This indicated that the pathogen was in the blood of WTD as mosquitoes are not vectors of this tick-borne pathogen. While this is the first report of this pathogen in WTD in Oklahoma, *Anaplasma platys* has been reported from WTD in Alabama and Texas (Rankins et al. 2017; Yu et al. 2020). *A. platys* is transmitted by *Rhipicephalus sanguineus* ticks to WTD and dogs and is a significant canine pathogen in this region (Little et al. 2021). This finding becomes more important as more studies report the possible zoonotic risk of *A. platys* in humans (Maggi et al. 2013; Arraga-Alvarado et al. 2014; Breitschwerdt et al. 2014).

In addition to ecological and behavioral results, we noted a difference in blood-fed mosquitoes collected by different trapping methods. Of 248 trap nights, 212 nights involved resting traps and 36 nights involved CO_2 baited CDC light traps. Yet, 37% (49/132) of the blood-fed mosquitoes were collected on one of three nights in which CDC light traps were used. CDC light traps are typically used to attract host-seeking mosquitoes and attract a higher species diversity (O'Brien and Reiskind 2013; Reiskind et al. 2017) while various types of resting traps are used to collect blood-fed mosquitoes (Burkett-Cadena et al. 2008, 2011; Kent et al. 2009). While our resting traps in the semi-urban locations, which were monitored almost daily, produced the majority of our blood-fed mosquitoes, the CDC light traps used in locations where we were unable to monitor daily, also collected a high percentage of blood-fed mosquitoes. Similar to a California based study where resting, suction and CO_2 traps were used and 54.72% of the blood-

fed mosquitoes were collected by CDC light traps (Reisen et al. 2013), this study highlights that certain traps work differently depending on location and sampling regimens.

As in every study, there were limitations that we made a conscious effort to overcome. The first limitation involved host identifications that could not be made with the methods used. Of the 132 dissected and classified as blood-fed, we confirmed 95 mosquitoes. This is common in bloodmeal studies as reported by others (Molaei et al. 2008; Kent et al. 2009; Reisen et al. 2013; Tuten et al. 2012). As it is not possible to know how long the mosquitoes had been engorged before collection, we believe that the bloodmeals were too degraded by midgut enzymes to be useful for amplification of DNA. Another related issue is the dark abdomen coloration of some species, especially those in the genus *Psorophora*, which makes it difficult to distinguish whether its contents are blood or nectar. To improve our chances of host identification, we used many known primer sets and, in some instances, this was useful as at least one of the primer sets provided usable product which could be sequenced. Another limitation encountered involved the lack of identifying any WNV in *Cx. tarsalis*, especially in the blood-fed mosquitoes. While there were only 22 human cases and 2 deaths in Oklahoma in 2021 (Arbonet 2023), we may have detected WNV in other species with additional funding. A third limitation was the lack of avian blood meals in our collection. One of our overall goals was to connect the use of ERC by specific avian hosts which are known as reservoirs for WNV. As such, we used the traps and assays that others have used, but we were not successful in collecting mosquitoes that had fed on birds or WNV-infected mosquitoes. The cessation of funding at the end of July 2021 meant that we were not able to collect *Culex* mosquitoes at the time of year (August-November) when they were most likely to be infected with WNV (Noden et al. 2021). Future studies are needed which focus on these months of highest transmission risk.

In conclusion, given that the majority of blood-fed mosquitoes using ERC in semi-urban areas had fed on WTD or cattle, and the fact that many of these mosquitoes are able to fly long distances just after taking a blood meal, up to 1200 meters, there is a potential threat posed for

certain pathogens of major importance, even ones that are not currently present in the United States, such as Rift Valley Fever (RVF). RVF threats in urban/suburban areas surrounded by wildlife are plausible in the United States due to the presence of reservoir hosts such as WTD which roam freely in urban areas (Wilson et al. 2018). Based on studies, we know that many US-based mosquito species are susceptible to RVF infections (Turell et al. 2010) and some of these species' feed on deer and livestock as reported in Hartman et al. (2019). If mosquitoes feed on these infected animals, then come into contact with humans, the potential for an outbreak could occur. As such, these studies involving mosquito usage of habitats in semi-urban areas are critical for public health planning for mosquito-borne diseases outbreaks.

CHAPTER III

EASTERN RED CEDAR HABITAT USAGE BY MOSQUITOES IN REGARD TO ABUNDANCE, HOST INTERACTION, AND WEST NILE VIRUS

Introduction

Mosquitoes are the most important vectors of arboviruses globally (Gubler 2002). In the United States, the most important mosquito-borne virus is West Nile Virus (WNV) (Curren et al. 2018, Rosenberg et al. 2018), causing neurological damage that can result in death. Arriving in the United States in New York City in 1999 (Lanciotti et al. 1999), WNV rapidly spread throughout the country, completing the transverse in 2003 (Kramer et al. 2019). In the years since that first trans-national outbreak, WNV has impacted regions differently with the highest incidence occurring in the Great Plains region (Colorado, Iowa, Kansas, Minnesota, Nebraska, New Mexico, North Dakota, Oklahoma, South Dakota, Texas, and Wyoming) (Lindsey et al. 2008; Curren et al. 2018). The region also has the most cases of West Nile neuroinvasive disease (WNND) (Lindsey et al. 2008).

The impact of WNV on the Great Plains is significant but there are important questions related to habitat and landscape which need to be addressed to better understand the impact of this arbovirus on the region. One aspect which may be contributing is the higher rate of woody plant encroachment (WPE) occurring in the Great Plains due to the invasion of eastern red cedar

(ERC) (*Juniperus virginiana*) (Zou et al. 2018). Spreading at 40km^2 per year (Wang et al. 2018) and growing half a meter per year (Zou et al. 2018), this native invasive plant can transform a grassland into a forest within 40 years (Briggs et al. 2002). As part of that expansion, ERC changes specific abiotic and biotic factors in the surrounding environment that impact the way other plant and animal species use the land (Eldridge et al. 2011). In addition to changing water content and soil properties, ERC encroachment causes animals to leave their native habitats and introduces new species into the area. As the abiotic and biotic changes occur, the expansion of ERC has also provided habitat for certain blood-sucking arthropods such as ticks (Noden and Dubie 2017), horse flies (Sherrill 2019), and mosquitoes (O' Brien and Reiskind 2013; Noden et al. 2021a; Maichak et al. 2022) to thrive and expand. The habitats created by ERC encroachment may, thus, provide the ingredients needed for the 'nidus' of infection for WNV (bird host, viral pathogen, and competent mosquito vectors) to occur (Reisen 2010). There is a need, however, to further explore the relationships between these key components within the environmental context of ERC expansion in the region.

The majority of studies to date have focused on studying the abundance and diversity of mosquito species within ERC-encroached areas in Oklahoma. The habitat affinity of specific species has been determined with some choosing ERC habitats in comparison to deciduous forests and grasslands (O' Brien and Reiskind 2013; Reiskind et al. 2017; Noden et al. 2021a). One study further assessed how abundance of different mosquito species varied with increasing concentrations of ERC (Maichak et al. 2022). While much has been learned, there is a need to continue defining how different mosquito species are using different zones within ERC-encroached areas. To further this knowledge, we collected mosquitoes in different zones within the same ERC-encroached areas for the entire collection season. Through this study, three hypotheses were tested: 1) Abundance of mosquito species in ERC encroached areas differs seasonally and regionally in Oklahoma; 2) Mosquito species utilize specific zones within an ERC-encroached area; and 3) These different zones influenced the host-feeding and WNV

prevalence in the main four vector species in Oklahoma (*Cx. tarsalis*, *Cx. pipiens*, *Cx. erraticus*, and *Cx. restuans*). Together, the results from this study provides insights into how mosquito species utilize ERC habitats and may provide important links to understand WNV transmission in the southern Great Plains.

Material and Methods

Study Locations

This study was conducted in two regions of Oklahoma where eastern redcedar (ERC) is expanding (Wang et al. 2018). Mosquito collections occurred in 120 trapping sites in 8 trapping locations within five different counties: three in western Oklahoma and two in central Oklahoma. In western OK, we used three previous sites in Blaine (east of Watonga), Kingfisher (east of Okeene), and Caddo (south of Binger) counties (Maichak et al. 2022) and worked with state park personnel to establish a new site in Ft. Cobb State Park (Caddo county). In central OK, we used three previous sites (Highland Park, OSU research range, OSU Carl Blackwell) in Payne County (Noden et al. 2021a; Maichak et al. 2022) and worked with a landowner to establish a site in Noble County (Figure 2.1 and 2.2).

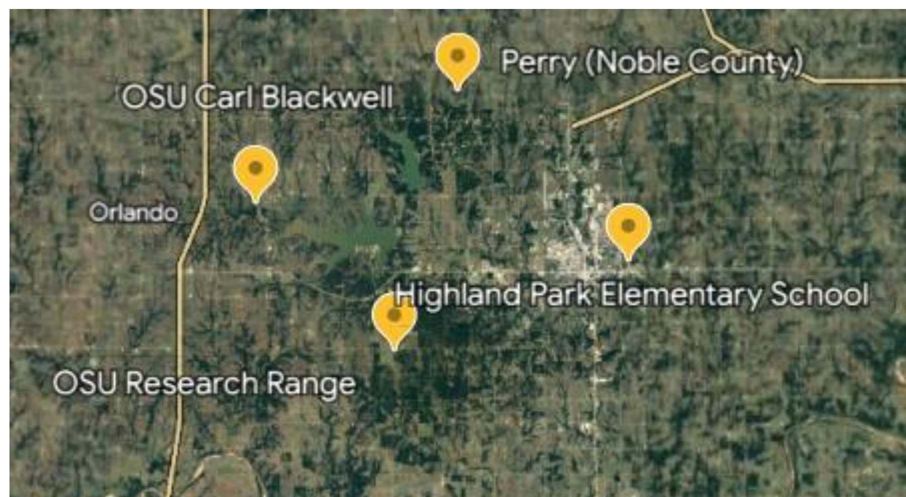

Figure 2.1. Stillwater trapping locations with labels. Taken from Google Earth Pro.

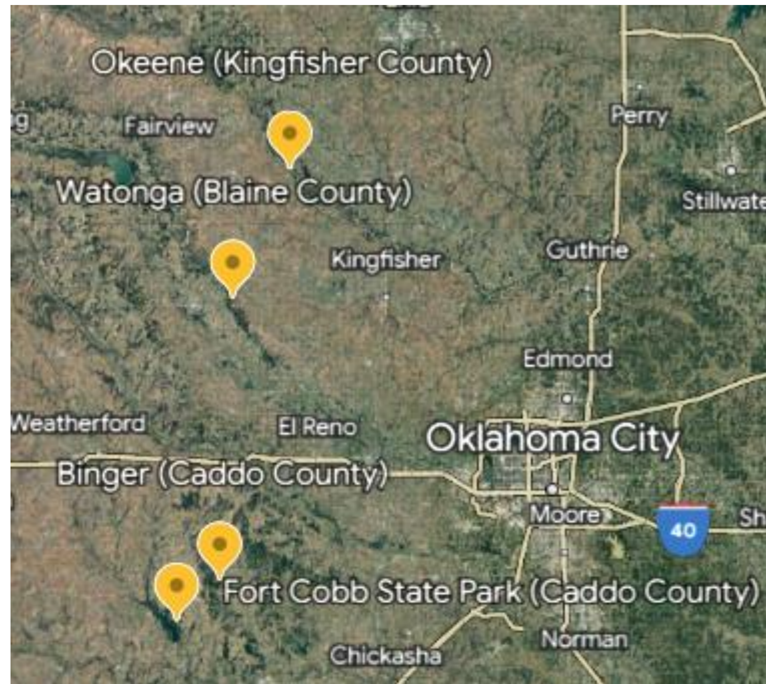

Figure 2.2. Western Oklahoma trapping locations with labels. Taken from Google Earth Pro.

To fulfil the main objective of the study, we chose sites of 40-60% ERC encroachment with average tree heights above 3 meters. Each collection location (county site) had five trapping sites within ERC-encroached landscapes with the objective to collect mosquitoes at each location in three different areas in the ERC habitat. Two trapping sites (labelled 'inner') were chosen over 50m apart inside dense concentrations of ERC along naturally occurring open corridors among the trees. Two other trapping sites were chosen over 50m apart along the outer edges of ERC (labelled 'outer') that also bordered grassland habitat; traps were hung from the outside ERC branches of the tree bordering the grassland.. The fifth trapping site was in the middle of a grassland area, 150 meters from any ERC trees, which served as the comparison site for mosquito species outside of the ERC systems. These arrangements ensured the independence of trapping events between the different habitats (Reiskind et al. 2017).

Trapping Protocol

Collections for mosquitoes began 6 June 2022 through 1 November 2022. The trapping season was divided into two parts (6 June – 12 August / 15 August – 1 November) for academic

reasons when collections shifted to weekends only. Three different trap types were used to collect different groups of mosquitoes: 1) 32-gallon black garbage cans (Lowes) were used as resting traps (Burkett-Cadena et al. 2008, 2011) to collect resting and blood-fed mosquitoes, 2) 9"x 9" fiber pots (Greenhouse Megastore) (Kent et al. 2009) spray painted black on the inside (Supplemental Figure 1), were also used to collect resting and blood-fed mosquitoes (Supplemental Figure 7), and 3) CDC Light Traps baited with CO_2 (dry ice) were used to collect host seeking female mosquitoes (Supplemental Figure 2). Lights were removed to reduce non-target insect collection. Each trapping site in a collection location contained one 32-gallon trash can, one fiber pot, and one CDC Light trap. This meant that each sampling area contained 3 traps x 5 trapping sites = 15 traps x 8 locations = total 120 trapping sites for the study. Resting traps were placed inside the lower branches of ERC trees with the opening facing the outside. The garbage can opening was covered with the corresponding lid with a 3-inch diameter hole cut into the center so mosquitoes could enter but have difficulty exiting. CDC light traps and their corresponding buckets with dry ice were tied directly to the tree branches, 1.5 meters off the ground, while the traps were attached to a T-pole pushed into the ground in the grassland sites, adjusted to be the same height as CDC light traps placed in ERC.

During the early season collection period (6 June until 12 August 2022), sampling occurred at each location (2 locations in western and 2 locations in central Oklahoma) for two nights every other week. CDC traps were set up around 14:00 and the resting traps and surrounding the foliage were aspirated with an InsectZooka Field Aspirator (Bioquip, Rancho Dominguez, CA) to capture any mosquitoes resting inside or outside the traps. Because it was two-day collection, CDC traps were collected prior to 09:00 the next morning then set back up around 14:00 to repeat the process again the following morning. Aspiration occurred each time the traps were manipulated to augment the chances for collecting blood-fed mosquitoes over the two days of collection. During the later season collection period (15 August until 1 November 2022), to continue the biweekly protocol, collections were reduced to one single collection night

at sampling locations every other weekend. Local Stillwater sites were visited during the week, typically arriving to the site around 15:00 and CDC Light traps were set out and then collected the following morning around 07:00. At the beginning of August, all collections were stopped for two weeks due to dangerously high temperatures (>105°F), low relative humidity (<20%) and virtually no rainfall (Weather underground, 2022). Due to the high temperatures and low humidity that would kill resting mosquitoes in the garbage cans and the loss of the research assistant for academic reasons, all resting trap aspiration was halted for the rest of the collection period.

Throughout the sampling season there were a few instances when unexpected circumstances occurred that resulted in failure to collect mosquitoes. On 6 June 2022, lack of dry ice produced only one night of CDC traps for Okeene and Watonga. On 3 August 2022, all resting traps were destroyed at the Watonga site due to the removal of ERC to accommodate power lines. On 4-5 August 2022, a CDC light trap malfunctioned in grassland site, due to unnoticed motor issues. On 10 August 2022, the outer ERC CDC trap at Lake Carl Blackwell was not placed due to battery failure. On 20 August 2022, two traps failed at Highland Park: one was the inner ERC CDC trap at Site 1 with a broken net upon inspection the morning of retrieval and the grass CDC trap had a non-functioning battery. On 24 September 2022, the inner CDC trap at Site 2 at Fort Cobb failed due to a rodent chewing wires of the trap.

Sample Sorting and Mosquito Identification

Mosquitoes were collected from resting traps in Insectzooka collection cups (Bioquip, Rancho Dominguez, CA), clear propylene cups with mesh aluminum netting at the bottom for air flow, and labelled with the date, site location, trap type, trap designation (inner, outer, or grass) and trap number. Mosquitoes collected with CDC traps were kept in their collection containers and labelled with the same information. At the time of collection, CDC light trap containers were placed into a Whytner Portable Freezer (85 quart, Whynter, Brea, CA) to euthanize and preserve

the mosquitoes during transport. Upon arriving to the lab, mosquitoes were immediately sorted by feeding status to prevent degradation of host DNA in the bloodmeal (Coulson et al. 1990). Mosquitoes containing bloodmeals were individually placed into 1.5mL clear microtubes and labelled with date, collection location, trap type, trap designation, genus and species and placed into the -80°C freezer until a later date for bloodmeal extraction (Egizi et al. 2018; Greenberg et al. 2013; Mann et al. 2020; Molaei et al. 2006, 2008; Thiemann et al. 2012). Nonblood-fed mosquitoes were placed into plastic snap cap vials (7-dram, Fisher Scientific, Hampton, NH) and labelled with the same information as blood-fed mosquitoes and stored at -20°C until they could be identified (Darsie and Ward 2005).

West Nile Virus Analysis

We extracted RNA from four mosquito species commonly associated with the transmission of West Nile virus in the region: *Culex erraticus, Cx. pipiens, Cx. restuans,* and *Cx. tarsalis.* At the time of sorting, mosquitoes were pooled in groups of up to 25 individuals from the same date, site, and trap. Before processing, a master mix solution was made using phosphate-buffered saline (PBS) and 2x lysis buffer were combined in a 1:1 mixture (Applied Biosystems, Foster City, CA). Pooled mosquitoes were placed into appropriately labelled 2ml XXTuff reinforced microvials (Biospec) and 200µL of master mix was added to each pool (300µL for pools that contained 8 mosquitoes or more) along with two 2.3mm stainless steel beads (Biospec Products, Bartlesville, OK). Tubes were placed into a Mini-Beadbeater-16 (Biospec Products, Bartlesville, OK) for two rounds of 2 minutes. Tops were checked between rounds to ensure effective sealing. Samples were then placed into a centrifuge (Microfuge 16, Beckman Coulter, Brea, CA) and spun at 6,000 rpm for 4 minutes. Supernatants were then extracted and placed into sterile, labelled 1.5mL tubes (VWR) and stored at -80°C.

Once extractions were completed, mosquito samples were kept on ice for total RNA extractions using the QIAmp Viral RNA Mini Kit (250) (Qiagen, Valencia, CA) and following

the manufacturer's instructions. Samples were once again stored at -80°C until they were tested. Real-time RT-PCR was performed on RNA extracted from mosquito pools using a combination of QuantiTect Probe RT-PCR Kit (Qiagen), 25 pmol of WNV primers (Lanciotti et al. 2000), 3.25 pmol of the probe, and 10 µL of the RNA extracted from the mosquitoes for a total reaction volume of 25µL. A single cycle of 50°C for 30min (reverse transcription) and 95°C for 15 min (hot start), followed by 40 cycles of 94°C for 30s, 55°C for 1min, and 68°C for 1 min. Result reports were created after cycles were completed. Positive controls were graciously provided by Dr. Gabriel Hamer (Texas A & M). All qPCR positive samples were confirmed using the primers to amplify, followed by extraction and sequencing of positive amplicons from 2% agarose gels then confirmed by comparing sequences with published WNV sequences in NCBI.

Bloodmeal Identification

The process of bloodmeal determination began by removing the blood-engorged abdomen from the thorax of the mosquito. It was imperative to make sure that only the abdomen, containing the midgut, was used so that no other mosquito body parts would interfere with the PCR assay. Blood-fed mosquitoes were placed into a drop of PBS buffer on a glass microscope slide. In the event that the abdomen burst, the blood and PBS could be pipetted into tubes pre-filled with 200µL of phosphate-buffered saline (PBS). After this process was repeated for all blood-fed mosquitoes, we followed the manufacturer's protocols from a DNeasy 96 Blood and Tissue Kit (Qiagen, Valencia, CA) to extract the DNA from the bloodmeals. In summary, 20µL of proteinase K and 200µL of Buffer AL were added to each tube and the mosquito abdomen was then pressed against the edge of the tube with the pipette tip to expulse the bloodmeal from the abdomen to be completely exposed to the solution (Thiemann et al., 2012). Samples were then vortexed and placed into an incubator set at 56°C for 2 hours on a rocking platform. Once samples were removed from the incubator, they were placed back on ice and 200µL of 100% ethanol was added to each sample and vortexed once again until the solution was homogenized.

Samples were then transferred to DNeasy wash tubes and manufacturer's instructions were used with slight modification; we eluted the DNA from the column with 100µL Buffer AE instead of 200µL to concentrate the samples. DNA samples were stored at -20°C until PCR amplification.

Blood meal hosts were identified using PCR amplification and DNA sequencing using a fragment of either the vertebrate mitochondrial genes cytochrome oxidase 1 (COI) or cytochrome b (CytoB). Initially, all samples were screened with a COI primer cocktail consisting of forward primers VF1 t1, VF1d t1, and VF1i t1 and reverse primers VR1d t1, VR1 t1, and VR1i t1 were each mixed at a ratio of 1:1:2 (Ivanova et al. 2007). The PCR conditions were 94°C for 1 min, five cycles of 94°C for 30s, 50°C for 40s, and 72°C for 1 min, followed by 35 cycles of 94°C for 30s, 54°C for 40s, and 72°C for 1 min, with a final extension at 72°C for 10 min. The PCR samples were then run in a 2% agarose gel containing ethidium bromide in a 1x TBE buffer and visualized under ultraviolet light. All positive amplicons were extracted from the gels using the QIAquick Gel Extraction Kit (Qiagen), and resulting DNA was bidirectionally sequenced using the forward and reverse primers in an Applied Biosystems 3730 DNA analyzer at the Oklahoma State University Core Facility to identify the animal species. We compared each resulting sequence with GenBank submissions using default conditions on NCBI BLAST (blastn, NCBI, 2022) where the highest % sequence identity was used to determine species identity.

If the initial screening produced no useable sample, samples were rerun with CytoB primers focused on specific avian (Molaei et al. 2006), mammalian (Molaei et al. 2006), and reptilian/amphibian (Cupp et al. 2004) sequences. The first avian primer used was AV1F/R primers with initial denaturation at 95°C for 5 min, followed by 36 cycles of 94°C for 30s, 60°C for 50s, and 72°C for 40s. The final 72°C extension was 5 min with a finished product of 508bp (Molaei et al. 2006). The second avian primer was AV2F/R with initial denaturation at 95°C for 5 min, followed by 35 cycles of 95°C for 60s, 58°C for 60s, and 72°C for 60s. The final 72°C extension was 5 min with a finished product of 515bp (Molaei et al. 2006). After screening with avian primers, two mammalian primer sets were used. The first mammalian primer set was

MAMF/R1 with initial denaturation at 95℃ for 5 min, followed by 35 cycles of 94℃ for 30s, 55℃ for 45s, and 72℃ for 90s. The final 72℃ extension was 5 min with a finished product of 772bp (Molaei et al. 2006). The second mammalian primer set was MAMF/R2 with initial denaturation at 95℃ for 5 min, followed by 32 cycles of 95℃ for 60s, 54℃ for 60s, and 72℃ for 60s. The final 72℃ extension was 5 min with a finished product of 195bp (Molaei et al. 2006). The final primer set used was REPF/R with initial denaturation step at 95°C for 2 min, followed by 55 cycles at 94°C for 45s, 50°C for 50s, and 72°C for 1 min, and a final extension at 72°C for 7 min (Cupp et al. 2004). All positive amplicons used the same extraction process as COI amplicons for sequencing.

Statistical Analysis

For this study, we tested three hypotheses. The first focused on differences in abundance of mosquitoes by season or region. The second hypothesis focused on relationships of mosquito species abundance in different habitats within ERC-encroached areas. The third focused on how these different habitats influenced the host-feeding and WNV prevalence in the main four vector species in Oklahoma (*Cx. tarsalis*, *Cx. pipiens*, *Cx. erraticus*, and *Cx. restuans*).

To evaluate these hypotheses, we tabulated the bi-weekly collections of each species by region (central or western), season (early or late), trapping location, habitat (grassland, outside trap, inside trap), and collection date. Only mosquito species where over 100 individuals were collected were used in the analysis, except for *An. punctipennis* (72) and *Cx. pipiens* (33) (important WNV species). Next, the raw mosquito species counts were divided by number of trap nights per collection period (early or late) to control for differences in sampling efforts and a square root transformation was performed to normalize the data. We began by comparing abundance of individual species with region and season (hypothesis 1) using one way ANOVA. Here we also compared means (SE) of species abundance by habitat types using one way ANOVA. Next, we used the linear Mixed Model (SAS JMP Pro 15.2 (SAS Institute, Cary, NC,

USA) analysis to test the hypothesis (hypothesis 2) that the abundance of mosquito species differs across habitat type. Response variables were mean abundances of each mosquito species at each site and fixed effects were habitat type (grassland, outside trap, inside trap), season (June to August/Sept to Nov) and region (central vs western Oklahoma) with site of collection as the random effect. For each analysis, the distribution of data, as visualized using quantile-residual plots and prediction profiler plots, suggested that transformed response variables met the assumption regarding normality. As part of each species analysis, post-hoc testing included pairwise comparisons to determine specific differences between group means. Due to low numbers of blood-fed and WNV-infected mosquitoes, we were unable to analyze the data related to hypothesis 3.

Results

Mosquito Collections

Between 6 June and 1 November 2022, we collected 3,248 mosquitoes involving 27 species over 1,210 trapping nights. Of the total collected, 1,598 (49.20%) mosquitoes, consisting of 24 species, were collected from sites in central Oklahoma with Highland Park being the most productive (742, 22.84%). The remaining 1,650 (50.80%) mosquitoes, consisting of 24 species, were collected in western Oklahoma with Watonga being the most productive (531, 16.35%). All mosquitoes were collected by CDC Light traps, except for one from a resting trap.   Abundance of mosquitoes by habitat within encroached ERC differed by species and region (Table 2.1). The CDC traps placed inside ERC-encroached areas collected 1,389 (42.76%) mosquitoes, the CDC traps placed on the outside of ERC-encroached areas collected 1,182 (36.39%) mosquitoes, the CDC traps placed in the grassland collected 676 (20.81%) mosquitoes. Only one mosquito was collected in a bucket trap placed on the inside of an ERC-encroached area. No mosquitoes were collected from bucket traps placed on the outside of ERC or grassland sites, and no mosquitoes were collected with any of the fiber pots placed at any site.

Focusing on medically important mosquito species, we collected *Aedes albopictus* (175, 5.39%), *Ae. sollicitans* (232, 7.14%), *Ae. triseriatus* (21, 0.65%), *Ae. vexans* (164, 5.05%), *Anopheles quadrimaculatus* (32, 0.99%), *Culex erraticus* (421, 12.96%), *Cx. pipiens* (33, 1.02%), *Cx. restuans* (147, 4.53%), and *Cx. tarsalis* (1079, 33.22%) (Table 2.1).

Table 2.1. Total numbers of mosquitoes collected at 8 locations in central and western Oklahoma between 6 June and 1 November 2022. OSU Research Range: O.R.R., Highland Park Elementary School: HighlandP, OSU Carl Blackwell: O.C.B., Fort Cobb State Park: Ft Cobb

Mosquitoes	**Location**								
Species	Central Oklahoma				Western Oklahoma				Total
	O.R.R.	HighlandP	O.C.B	Perry	Binger	Ft Cobb	Okeene	Watonga	
Aedes albopictus	1	146	0	16	2	2	5	3	175
Ae. atlanticus	0	1	0	0	0	0	0	0	1
Ae. canadensis	0	0	0	0	1	0	1	3	5
Ae. epactius	0	1	0	2	7	2	0	2	14
Ae. hendersoni	1	0	0	0	0	0	0	0	1
Ae. sollicitans	1	1	0	1	3	2	60	164	232
Ae. triseriatus	1	15	0	3	0	0	1	1	21
Ae. trivitattus	5	19	44	17	13	1	2	1	102
Ae. vexans	1	13	33	2	2	8	105	0	164
Ae. zoosophus	0	0	0	0	0	2	0	1	3
Anopheles perplexens	0	0	0	0	0	0	0	2	2
An. pseudopunctipennis	0	0	0	0	0	0	1	0	1
An. punctipennis	3	0	1	1	0	1	0	66	72
An. quadrimaculatus	1	5	14	6	0	2	3	1	32
Culex erraticus	33	79	82	147	6	4	5	65	421
Cx. pipiens	0	29	1	0	0	2	0	1	33
Cx. restuans	0	136	5	1	1	3	1	0	147
Cx. salinarius	0	0	1	0	0	1	1	0	3
Cx. tarsalis	103	235	57	72	51	302	67	192	1079
Culiseta inornata	5	2	11	5	9	7	12	5	56
Psorophora ciliata	0	1	0	1	3	0	0	1	6
Ps. columbiae	9	10	32	5	0	24	34	12	126
Ps. cyanescens	2	5	57	5	231	85	1	3	389
Ps. discolor	10	1	7	20	10	18	8	6	80
Ps. ferox	13	8	0	0	0	0	0	0	21
Ps. longipalpus	12	35	3	1	1	1	0	0	53
Uranotaenia sapphirina	0	0	0	1	0	0	0	0	1
Unknown	1	0	0	0	2	2	1	2	8
Total	202	742	348	306	342	469	308	531	3248

In central Oklahoma, we collected more *Ae. albopictus* (163, 93.14%), *Ae. trivitattus* (85, 83.33%), *An. quadrimaculatus* (26, 81.25%), *Cx. erraticus* (341, 81%), *Cx. pipiens* (30, 90.90%), *Cx. restuans* (142, 96.59%), *and Ps. longipalpus* (51, 96.23%) compared with western Oklahoma. *Ps. ferox* (21) was only collected in central Oklahoma. In western Oklahoma, we collected more *Ae. sollicitans* (229, 98.70%), *Ae. vexans* (115, 70.12%), *Cx. tarsalis* (612, 56.72%), and *Ps. cyanescens* (320, 82.26%) (Table 2.1).

West Nile Virus Results

We tested 279 pools of four species of *Culex* mosquitoes: *Culex erraticus* (95), *Cx. pipiens* (10), *Cx, restuans* (29), and *Cx. tarsalis* (145). Of these, three (1.1%) tested positive for WNV. One pool consisted of one *Culex tarsalis* collected on 3 September 2022 from a CDC light trap placed on the outside of an ERC-encroached areas at the western Okeene site (Figure 2.3). Another pool consisted of three *Culex erraticus* collected on 27 September 2022 from a CDC light trap placed on the outside of an ERC-encroached areas at the central Highland Park site (Figure 2.4). The third pool consisted of 8 *Culex tarsalis* collected on 27 September 2022 from a CDC light trap placed in the grassland at the central Highland Park site (Figure 2.5).

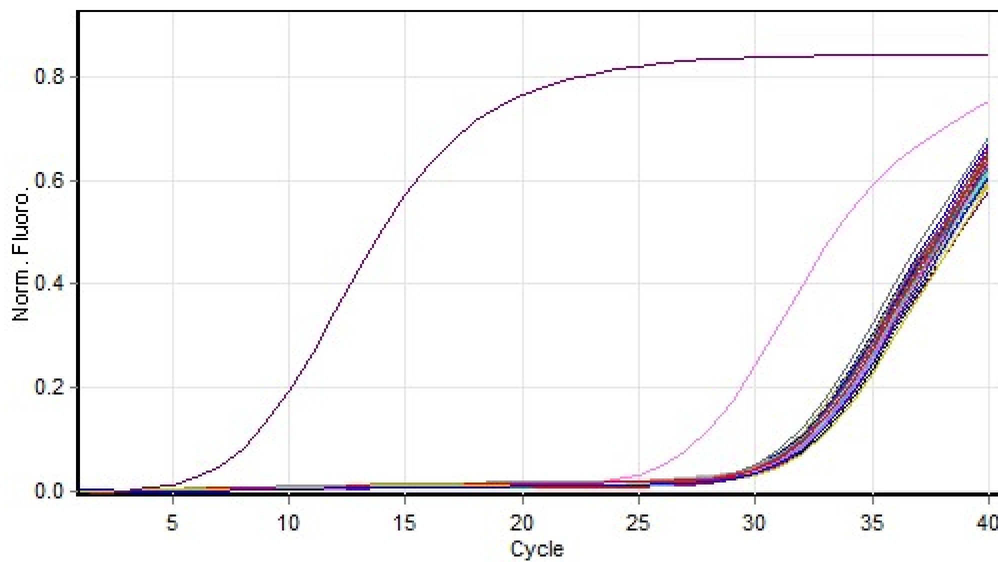

Figure 2.3. qPCR results from WNV analysis. One pool from this run resulted positive for WNV (light purple line) appeared in cycle 24. The positive control WNV G-Block (dark purple line) appeared in cycle 7. This pool contained one *Cx. tarsalis* from Okeene.

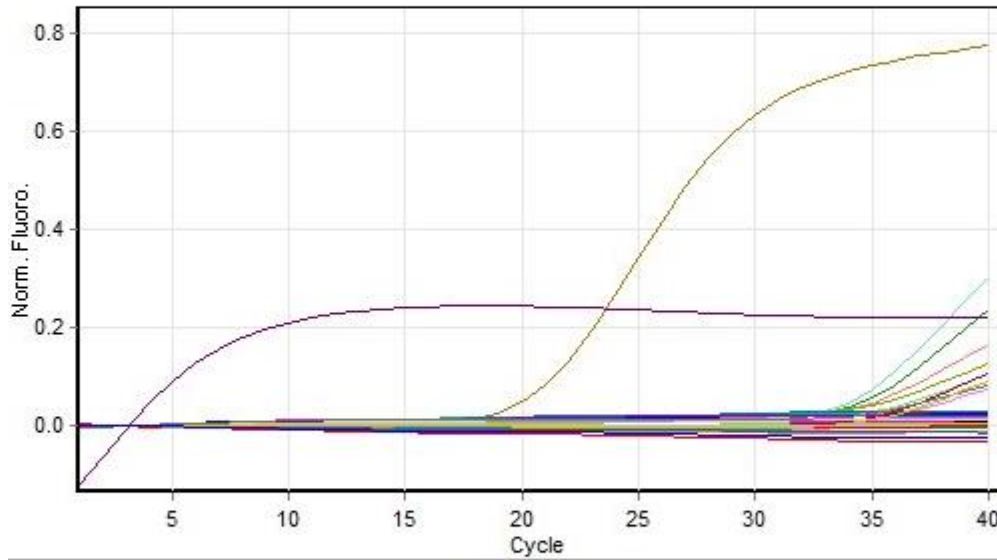

Figure 2.4. qPCR results from WNV analysis. One pool from this run resulted positive for WNV (gold line) appeared in cycle 19. The positive control WNV G-Block (dark purple line) appeared in cycle 3. This pool contained three *Cx. erraticus* from Highland Park.

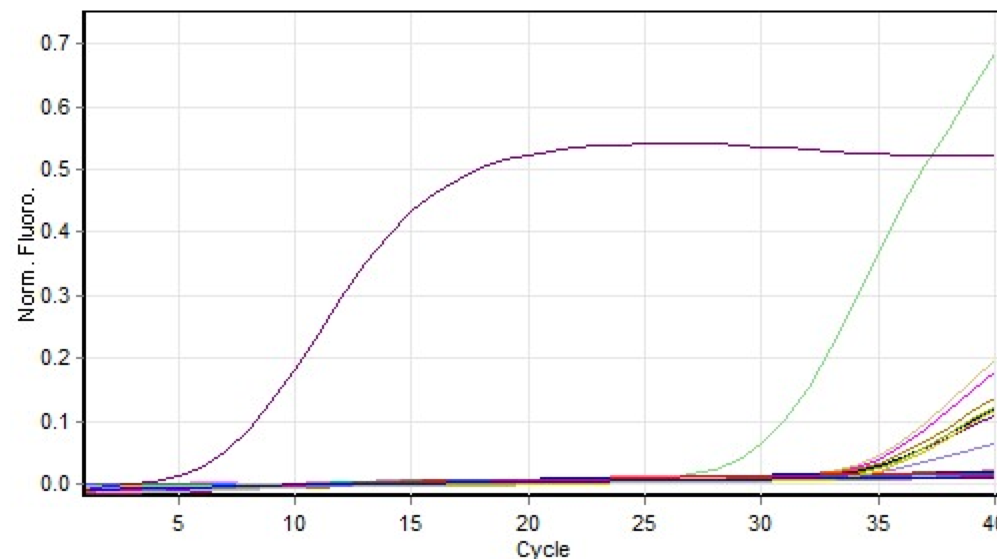

Figure 2.5. qPCR results from WNV analysis. One pool from this run resulted positive for WNV (light green) appeared in cycle 27. The positive control WNV G-Block (dark purple line) appeared in cycle 4. This pool contained eight *Cx.tarsalis* from Highland Park.

Bloodmeal Identification

A total of 52 blood-fed mosquitoes were collected during the study period (Table 2.2), 32 (61.54%) in western Oklahoma and 20 (38.46%) in central Oklahoma. All blood-fed mosquitoes were collected using CDC light traps. The majority were collected in traps set up in grassland areas (20, 38.46%), followed by traps set up on the outside (19, 36.54%) and inside (13, 25%) of ERC-encroached sites. Most of the blood-fed mosquitoes collected were *Ae. sollicitans* (18, 34.62%) followed by *Cx. erraticus* (11, 21.15%), *Ps. columbiae, Ps. cyanescens,* and *Ps. discolor* (5, 9.62%). Only one blood-fed mosquito was *Cx. tarsalis* (Table 2).

Table 2.2. Total number of blood-fed mosquitoes collected between 6 June and 1 November 2022 in 8 locations in central and western Oklahoma.

Mosquitoes	**Location**								
Species	Central Oklahoma				Western Oklahoma				Total
	O.R.R.	HighlandP	O.C.B.	Perry	Binger	Ft Cobb	Okeene	Watonga	
Ae. sollicitans	0	0	0	0	0	0	8	10	**18**
An. punctipennis	0	0	0	0	0	0	0	2	**2**
Cx. erraticus	2	0	1	5	0	2	0	1	**11**
Cx. restuans	0	1	0	0	0	0	0	0	**1**
Cx. tarsalis	0	0	0	0	0	1	0	0	**1**
Cs. inornata	0	0	1	0	0	0	0	0	**1**
Ps. columbiae	0	2	0	0	0	1	2	0	**5**
Ps. cyanescens	0	0	1	0	3	1	0	0	**5**
Ps. discolor	1	0	2	1	0	1	0	0	**5**
Ps. ferox	1	1	0	0	0	0	0	0	**2**
Ps. longipalpus	0	1	0	0	0	0	0	0	**1**
Total	4	5	5	6	3	6	10	13	**52**

Host-specificity was determined for 38 (73.08%) of the blood-fed mosquitoes collected (Table 2.3). Eleven mosquito species fed on 5 animal species with the primary hosts consisting of white-tailed deer (WTD) (21, 55.26%) and cow (14, 36.84%) followed by rabbit (1, 2.63%), donkey (1, 2.63%), and goat (1, 2.63%). We identified 6 (15.8%) mosquitoes that had fed on WTD which were infected with an *Anaplasma* sp. reported from human samples (4) or *Anaplasma odocoilei* (2). These samples were detected as *Anaplasma* spp. using the *COI* primer set and confirmed to species by 16S primers. Two *Psorophora cyanescens* mosquitoes collected

in Binger (6/14/22) and Fort Cobb (6/15/22) had blood containing *Anaplasma odocoilei.* An *Anaplasma* sp. reported in a human was detected in the bloodmeal from a *Ps. ferox* collected at Highland Park (6/10/22), a *Cx. erraticus* collected in Perry, OK (8/12/22), a *Cx. erraticus* collected in Watonga, OK (9/3/22), and a *Ps. columbiae* collected in Fort Cobb, OK (9/10/22).

Table 2.3. Total number of blood-fed mosquitoes that fed on specific hosts between June and November 2022 in trapping locations in central and western Oklahoma.

Mosquito Species	Total no.	Cow	WTD	Rabbit	Donkey	Goat
Ae. sollicitans	**18**	10	4	-	-	-
An. punctipennis	**2**	-	-	-	-	-
Cx. erraticus	**11**	2	7	-	-	1
Cx. restuans	**1**	-	-	-	-	-
Cx. tarsalis	**1**	-	1	-	-	-
Cs. inornata	**1**	-	1	-	-	-
Ps. columbiae	**5**	-	1	-	-	-
Ps. cyanescens	**5**	1	4	-	-	-
Ps. discolor	**5**	1	2	-	1	-
Ps. ferox	**2**	-	1	-	-	-
Ps. longipalpus	**1**	-	-	1	-	-
Total	**52**	14	21	1	1	1

Mosquito collections with encroached areas

Mosquito abundance varied between trap placements within encroached areas (Supplemental Table 1). Based on collections, we observed that, more *Ae. sollicitans* (155, 66.81%*), Ps. columbiae* (61, 48.41%), *and Ps. cyanescens* (165, 42.42%) were collected in grassland, more *Ae. vexans* (113, 68.90), *Cx. pipiens* (17, 53.13%), *Cs. inornata* (22, 39.29%), and *Ps. discolor* (35, 43.75%) in traps on the outside of ERC encroached areas, and more *Ae. albopictus* (107, 61.14), *Cx. erraticus* (184, 43.99%), *Cx. tarsalis* (573, 53.10%), and *Ps. longipalpus* (42, 79.24%) on the inside of ERC encroached areas.

Collections of mosquito species were dependent on season and region. In general, several *Aedes* species and one *Psorophora* species were collected more in the early part of the season (June-August) and most of the important *Culex* species were collected more in the later

part of the season (late-August-November) (Table 2.4). Regional collections did not separate by genus as did the seasonal collections (Table 2.4). Higher abundance of *Ae. albopictus*, *Ae. trivitattus*, *Cx. erraticus*, *Cx. pipiens*, and *Cx. restuans* were all collected in central Oklahoma while higher numbers of *Ae. sollicitans* and *An. punctipennis* were collected in western Oklahoma. In this study, *Cx. tarsalis*, *Ae. vexans* and *Ps. columbiae* did not differ by region (Table 2.4).

Table 2.4. ANOVA results comparing mean abundance of mosquitoes collected by season (June-Aug/Sept-Nov) and region (central/western)

Mosquito Species	Season				Region			
	F	df	P		F	df	P	
Ae. albopictus	2.62	1	0.1065		21.6	1	**<0001**	central
Ae. sollicitans	5.91	1	**0.0157**	early	8.77	1	**0.0033**	western
Ae. trivitattus	13.73	1	**0.0003**	early	6.89	1	**0.0091**	central
Ae. vexans	4.77	1	**0.0298**	early	0.001	1	0.975	
An. puncticpennis	0.68	1	0.4087		6.49	1	**0.0114**	western
Cx. erraticus	4.85	1	**0.0284**	late	19.58	1	**<0.0001**	central
Cx. pipiens	3.83	1	0.0513	late	3.69	1	0.0557	central
Cx. restuans	15.89	1	**<0.0001**	late	8.66	1	**0.0035**	central
Cx. tarsalis	74.5	1	**<0.0001**	late	0.91	1	0.3406	
Ps. columbiae	0.24	1	0.6227		0.08	1	0.7748	
Ps. cyanescens	4.34	1	**0.038**	early	2.88	1	0.0906	

Abundance of *Ae. albopictus* was significantly influenced by ERC habitat (Table 2.5) with more collected in traps on the outside of ERC-encroached areas when compared with grassland traps (Estimate: -0.155; 95% U/L -0.221, -0.090; P = <0.0001) and more collected on the inside of ERC-encroached areas than on the outside (Estimate: 0.130; 95% U/L 0.065, 0.195; P = <0.0001). Abundance of *Ps. columbiae* was also significantly influenced by ERC habitat (Table

2.5) with relationships that were opposite that of *Ae. albopictus*. More *Ps. columbiae* were collected in grassland traps than in those on the outside of ERC-encroached areas (Estimate: 0.097; 95% U/L 0.012, 0.182; P = 0.0253) and more *Ps. columbiae* were collected in traps on the outside of ERC-encroached areas than on the inside (Estimate: -0.088; 95% U/L -0.172, -0.003; P = 0.0422). While the overall models for habitat for *Cx. tarsalis* and *Cx. erraticus* were nearly significant, there were significant fixed effects in the parameter estimates. The abundance of the two *Culex* species, *Cx. tarsalis* (Estimate: -0.197; 95% U/L -0.369, -0.026; P = 0.0241), *Cx. erraticus* (Estimate: -0.152; 95% U/L -0.277, -0.028; P = 0.0168) and *An. punctipennis* (Estimate: -0.056; 95% U/L -0.106, -0.007; P = 0.0266) was all significantly higher in traps on the outside of ERC-encroached areas than in grassland traps with no difference between outside and inside ERC traps.

Table 2.5. Mixed model analysis of mosquito species collected by different eastern redcedar encroachment habitats, season and region for 2022.

	Habitat			Region			Season		
	F ratio	DF	Prob	F ratio	DF	Prob	F ratio	DF	Prob
Ae. albopictus	12.8	2, 290	**<0.0001**	1.2	1, 6	0.3159	5.18	1, 290	**0.0236**
Ae. sollicitans	1.84	2, 290	0.1608	2.38	1, 6	0.1739	5.39	1, 290	**0.0209**
Ae. trivitattus	1.48	2, 290	0.2283	7.82	1, 6	**0.0350**	8.76	1, 293	**0.0033**
An. puncticpennis	2.63	2, 290	0.0735	0.72	1, 6	0.4302	2.09	1, 290	0.1489
Cx. erraticus	2.95	2, 290	0.0539	6.62	1, 6	0.4302	0.002	1, 291	0.9624
Cx. pipiens	2.18	2, 290	0.1146	0.84	1, 6	0.4302	9.27	1, 291	**0.0025**
Cx. restuans	1.19	2, 290	0.2815	1.16	1, 6	0.4302	8.91	1, 290	**0.0031**
Cx. tarsalis	2.86	2, 290	0.0587	0.22	1, 6	0.4302	129.8	1, 292	**<0.0001**
Ps. columbiae	3.08	2, 290	**0.0473**	0.07	1, 6	0.8014	1.23	1, 292	0.2690
Ps. cyanescens	0.46	2, 290	0.6329	1.15	1, 6	0.3245	2.38	1, 291	0.1238

Discussion

Our study demonstrates that specific habitats within ERC-encroached areas are being utilized by different species of host-seeking mosquitoes. We tested three hypotheses to determine different aspects of mosquito communities in Oklahoma. In addition to confirming seasonal and regional relationships, we identified higher usage of *Ae. albopictus* in areas inside ERC encroached habitats as compared with those on the outside of ERC encroached habitats or grassland. We also confirmed that *Ps. columbiae* prefer grassland over both inside and outside ERC habitats. While not significant at the level of the model, *Cx. tarsalis*, *Cx. erraticus*, and *An. punctipennis* trended towards preferring the outside of ERC encroached areas when compared with grassland. These relationships of different species to differing habitats within ERC encroached areas have important ramifications for public health and require consideration in any mosquito mitigation plan.

Aedes albopictus is an important mosquito vector worldwide for Dengue and Chikungunya viruses (Benedict et al. 2007, Garcia-Rejon et al. 2021) and canine heartworm in the United States (Fikrig and Harrington 2021). It is a species associated with various factors involving vegetation in urban and suburban habitats (Faraji et al. 2014, Little et al. 2017, Sanders et al. 2020). A recent Oklahoma-based study, focused on habitat preferences of host-seeking mosquitoes in rural areas, identified a preference of *Ae. albopictus* for increasing concentrations of ERC compared with grassland habitat (Maichak et al. 2022). The current study expanded this observation, focusing on specific zones within ERC encroached areas that *Ae. albopictus* are most likely to be collected instead of ERC at a wider landscape level. We found that, in addition to being more likely to choose ERC encroached areas, *Ae. albopictus* are significantly likely to be collected inside encroached areas compared with the outside. This type of choice is what we might expect of a mosquito species that is opportunistic, actively searching for and feeding on many different species of mammals and birds (Fikrig and Harrington 2021). While this study focused mainly on rural areas, this finding has important ramifications for urban and suburban

areas where ERC encroachment is also occurring in the southern Great Plains (Engle et al. 2008; Zou et al. 2018). Given the importance of this vector in potential arboviral outbreaks, very little is known regarding the involvement of vegetation, especially ERC, in providing habitat for *Ae. albopictus* in urban areas in the Great Plains (Sanders et al. 2020). Further urban-focused studies are needed to identify whether *Ae. albopictus* specifically prefer ERC or whether they are drawn to any tree type during host foraging (OBrien and Reiskind 2013; Noden et al. 2021).

This study further defined how other species utilize ERC-encroached areas. Other studies have demonstrated that *Ps. columbiae* significantly choose grassland sites (O'Brien and Reiskind 2013, Maichak et al. 2022), this study also demonstrated that within ERC-encroached systems, *Ps. columbiae* prefers to be outside the encroached areas rather than on the inside. A known nuisance feeder on cattle and horses (Kuntz et al. 1982), not much is known about the behaviors and host-preferences of this floodwater mosquito species. In the current study, *Cx. tarsalis* and *Cx. erraticus* were more likely to be collected in outer parts of ERC-encroached areas than inside or in grassland. While only a trend observed in the current study, this preference for ERC has been documented in other studies which have focused on general vegetation habitats in western (Noden et al. 2021a) and central (O'Brien and Reiskind 2013) Oklahoma. Both of these species are strong fliers, able to fly several kilometers in a night (Reisen and Lothrop 1995), which may make it more likely to be attracted to trees in the landscape that could provide refuge and, potentially, hosts for bloodmeals (O'Brien and Reiskind 2013). Because of the significance of these species in WNV transmission, it is important to continue defining how they utilize habitats in areas where they are most active such as in the Great Plains.

The collection of mosquitoes throughout an entire season provided the opportunity to establish regional and seasonal relationships not well-defined in previous studies (Noden et al. 2021a, Maichak et al. 2022). Because the same sites were sampled biweekly from June until November, we defined *Ae. sollicitans*, *Ae. trivitattus*, *Ae. vexans* and *Ps. cyanescens* as peaking early in the season (June-August) and the *Culex* species (*Cx. erraticus*, *Cx. pipiens*, *Cx. restuans*,

and *Cx. tarsalis*) as peaking later (Sept-November). The abundance of *An. punctipennis* and *Ps. columbiae* did not differ by season. On a regional scale, some species were more abundant in the central region (*Ae. albopictus*, *Ae. trivittatus*, *Cx. erraticus*, *Cx. pipiens*, and *Cx. restuans*) while *Ae. sollicitans* and *An. punctipennis* were significantly more abundant in western Oklahoma. Other species, including *Cx. tarsalis* and *Ps. columbiae*, did not differ by region. Seasonal and regional comparisons are important for mosquito surveillance initiatives to ensure that valuable resources are only expended in areas and during times of peak activity to track WNV infections in a given region. These types of results are also important in assisting the identification of mosquitoes so that those without much experience can know which species are more likely when and where in a given state.

Due to low numbers, we were not able to assess the effect of habitat preference on WNV-infections or blood-feeding (hypothesis 3). However, we did identify three pools of *Culex* mosquitoes that were positive for WNV. Two WNV-positive pools for two different *Culex* species were collected from two different trapping sites (grassland and outside ERC) in the same trapping location on the same date. This suggests that mosquitoes may have fed on one or more WNV-infected birds in the location in similar time periods. While intuitive that such relationships may occur, there is evidence that different *Culex* mosquitoes will feed on the same species of birds when roosting (Komar et al. 2018).

In addition to WNV, we identified nine mosquito species that had fed on five mammal species. Many of the host-mosquito interactions observed have already been described (Whitehead 1951; Kuntz et al. 1982; Irby and Apperson 1988; Molaei et al. 2008; Tuten et al. 2012; Mann et al. 2020) with the majority having fed on either WTD or cattle as these were most prevalent mammals. We did, however, identify two new combinations not yet described: *Ps. longipalpus* and a rabbit, *Cx. erraticus* and a goat. In addition to new host-mosquito interactions, we detected two different *Anaplasma* sp. in the bloodmeals from WTD. In earlier study, we identified *Anaplasma platys* in bloodmeals from WTD (Henriquez, unpublished data), but this

study identified *An. odocoilei* and an *Anaplasma* sp. reported from a human (Noden et al. 2022). The two *A. odocoilei* samples were collected in the same species, *Ps. cyanescens*, in the same county on two sequential days of sampling. The human *Anaplasma* sp. were collected in three different species of mosquito in both central and western Oklahoma, all from different sampling locations. To date, no studies have reported *A. odocoilei* in Oklahoma, but it has been reported in the southeastern US (Tate et al. 2013) as well as Mexico (Ojeda-Chi et al. 2019). Given the increasing canine infections with *A. platys* in the region (Little et al. 2021), the detection of different species of *Anaplasma* in WTD using mosquito bloodmeals indicates that more research is needed to evaluate the role of WTD in the epidemiology of *Anaplasma* in the southern Great Plains.

As with all studies, our study had some limitations that we worked to mitigate. First, our sampling was limited by the academic calendar which required shifting our weekly collections from two nights to one night to continue on weekends. While potentially limiting, this change allowed biweekly sampling to capture the peak period of activity for all *Culex* mosquitoes in the region (Noden et al. 2021a). Secondly, the extremely high temperatures, low humidity, and lack of rain throughout most of the season reduced mosquito numbers across the region and meant that we had to stop collections for two weeks in early August. While enough mosquitoes were collected for analysis, we are mindful that numbers may have been different in a year with more rainfall and higher humidity. Another limiting aspect of this study was the lack of *Culex* sp, that had fed on birds and the limited numbers of WNV-infected mosquitoes. In 2022, we specifically expanded and extended our sampling protocols to encompass the period of greater activity for *Culex* mosquitoes (August-October). However, this increased work effort did not produce bird-fed mosquitoes. The presence of WNV-infected *Culex* from three trap night collections indicates that WNV-infected birds are being fed upon but, to date, our trapping protocols are not able to collect mosquitoes that have freshly fed on birds.

In conclusion, when analyzing differences in mosquito community abundance within ERC encroachment habitats, we identified mosquito species that utilize ERC differently. While *Ps. columbiae* is most present in grassland habitats, *Aedes albopictus* was most present inside ERC-encroached areas, further defining the relationship with this important arbovirus vector and this native invasive tree (Maichak et al. 2022). The importance of this finding needs to be followed up in urban areas where ERC encroachment is also occurring. While *Cx. erraticus* and *Cx. tarsalis* appear to be more abundant on the outside of ERC-encroached areas, additional research is needed to define this aspect together with WNV prevalence and host availability. Together, these findings build on the conclusions of previous studies to identify the public health importance of ERC expansion in the Great Plains region.

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APPENDICES

Figure A.1. Resting trap, 32-gallon garbage can, placed flush with ERC.

Figure A.2. CDC Light trap, without light, baited with two pounds of dry ice.

Figure A.3. Distance between site location of particular animals and the resting trap in which the blood-fed mosquito was collected at the collection location. A) Briar Creek – west alpaca site; B) Briar Creek – eastern cattle and sheep sites

Figure A.4. Distance between site location of cattle and the resting trap in which the blood-fed mosquito was collected at Whittenberg Park.

Figure A.5. Distance between site location of horse and the resting trap in which the blood-fed mosquito was collected at Sangre Ridge location.

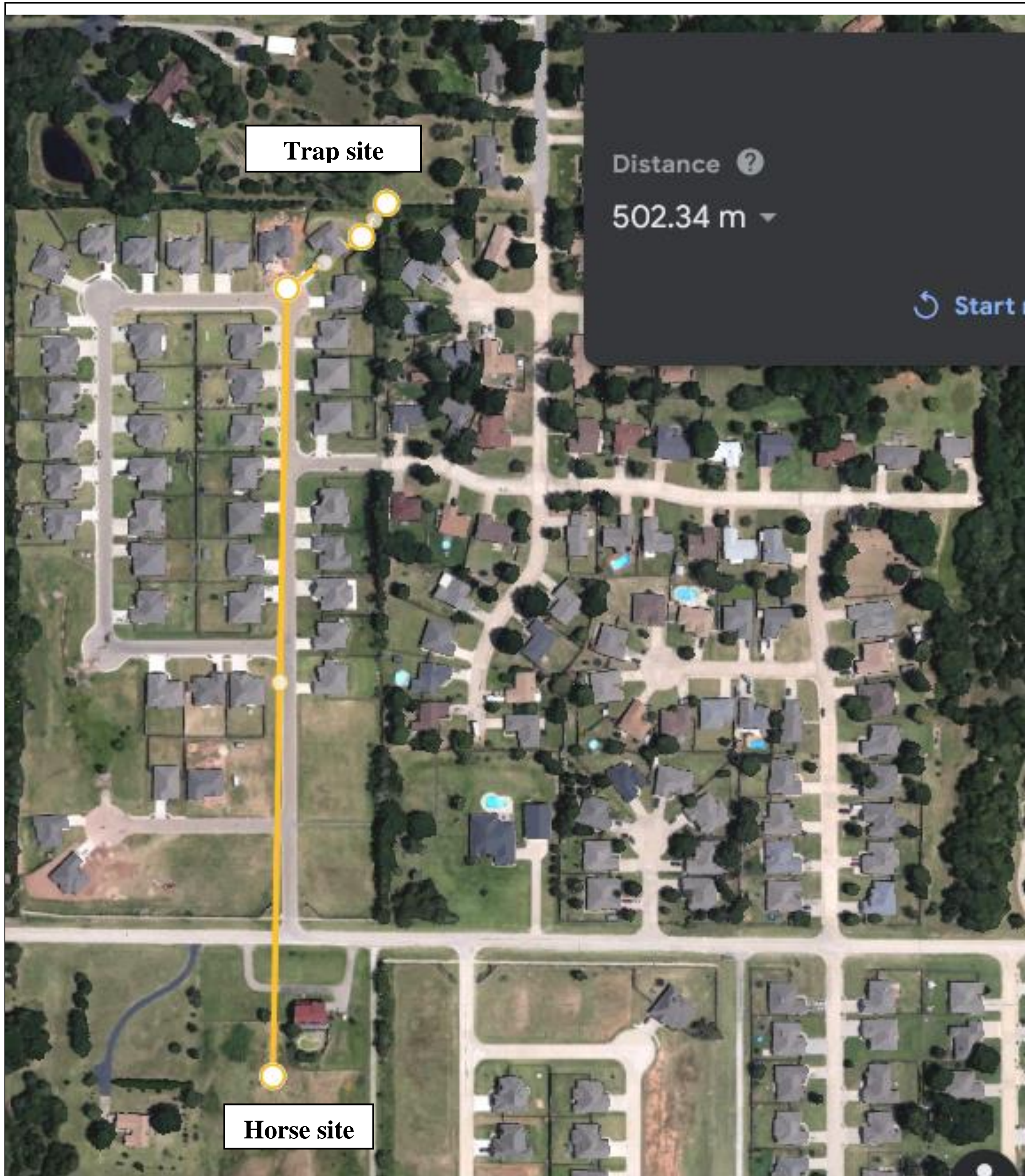

Figure A.6. Distance between site location of horse and the resting trap in which the blood-fed mosquito was collected at Highland Park location.

Figure A.7. Resting trap, fiber pot, placed within ERC.

	Grass CDC		Inside CDC		Outside CDC	
Ae. albopictus	0.06 ± 0.05	B	1.06 ± 0.31	A	0.61 ± 0.22	AB
Ae. sollicitans	1.57 ± 0.97	A	0.19 ± 0.08	A	0.57 ± 0.50	A
Ae. trivitattus	0.10 ± 0.07	A	0.46 ± 0.17	A	0.46 ± 0.21	A
An. puncticpennis	0.02 ± 0.02	A	0.50 ± 0.31	A	0.19 ± 0.08	A
Cx. erraticus	0.62 ± 0.32	B	1.82 ± 0.42	A	1.74 ± 0.49	AB
Cx. pipiens	0.00 ± 0.00	A	0.16 ± 0.09	A	0.17 ± 0.10	A
Cx. restuans	0.08 ± 0.05	A	0.68 ± 0.44	A	0.68 ± 0.40	A
Cx. tarsalis	1.27 ± 0.48	B	5.67 ± 1.85	A	3.76 ± 1.28	AB
Ps. columbiae	0.62 ± 0.29	A	0.18 ± 0.08	A	0.47 ± 0.23	A
Ps. cyanescens	1.67 ± 1.04	A	1.28 ± 0.79	A	0.94 ± 0.43	A

Table A.1. ANOVA results for mean (SE) abundance of mosquito species by habitat.

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