

Infection Rate of West Nile virus in *Culex tarsalis* at Medicine Lake Wildlife Refuge and
Ninepipe Wildlife Refuge in 2012

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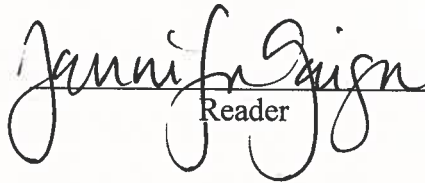
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Table of Content

Acknowledgements.....	2
Abstract.....	4
Introduction.....	5
Methods and Materials.....	11
Results.....	16
Tables and Figures.....	18
Discussion.....	20
Work Cited.....	24

Abstract

This study focused on the infection rate of West Nile virus of *Culex tarsalis* populations at Medicine Lake Wildlife Refuge (MLWR) and Ninepipe Wildlife Refuge (NWR). My research was done in order to determine if there was a statistical difference in the infection rate between NWR and MLWR via a bias corrected ML estimate. These locations were chosen because they were ideal locations for West Nile virus (WNV) and in the case of MLWR, past positive *C. tarsalis* pools. My study involved trapping mosquitoes, sorting out the *C. tarsalis*, and isolating WNV from mosquito homogenate. Once *C. tarsalis* had been sorted, they were homogenized and total RNA was extracted. This RNA was analyzed for WNV by using a TAQman assay RT PCR. My research indicated that there was no statistical difference in the relative infection rate between MLWR and NWR. Positive samples were found at Bowdoin, Fort Belknap, MLWR, and NWR as part of an active monitoring program for West Nile virus in Montana.

Introduction

West Nile virus

West Nile virus (WNV) belongs to a family of viruses known as the flavivirus group B (Calisher, 1994). It is an arbovirus from the family *Flaviviridae* (Marra *et al.*, 2006) and is related to Japanese Encephalitis, Yellow Fever, and Dengue 1 and 2 (Calisher, 1994). WNV is historically native to North Africa, Europe, the Middle East, and parts of Asia (Murgue *et al.*, 2002) but arrived in the United States in 1999 (Marra *et al.*, 2004). Birds are the primary host for the virus, however it has been isolated in mammals, reptiles, and amphibians as well (McLean, 2006). The primary vectors for WNV in the United States are mosquitoes of the *Culex* genus including *Culex tarsalis*, *Culex pipiens*, and *Culex quinquefasciatus* (Hayes *et al.*, 2005).

West Nile Virus in Humans

West Nile viral infection in humans can cause a wide spectrum of symptoms which range from mild to much more severe depending on the strain (Brault, 2009). Symptoms include fever, headache, tiredness, and rarely coma, tremors, convulsions, and paralysis (CDC A). There are at least two distinct lineages of WNV that cause infection, known as lineage 1 and lineage 2 (Petersen and Roehrig, 2001). Lineage 1 is the strain typically behind human and avian epidemics (Maklinson, 2002) and the predominant strain of WNV in the United States (Petersen and Roehrig, 2001). Lineage 2 viruses show a distribution primarily in Madagascar and South Africa (Petersen and Roehrig, 2001) and are not associated with significant human illness or avian mortality

while lineage 1 strains are associated with severe illness, virulence, and widespread encephalitis (Maklinson, 2002).

The majority of WNV infected humans are asymptomatic and only one in 150 cases develop into severe illness (CDC A). Typically 20% of cases are mild with fever, rash, headache, nausea, and vomiting (CDC A). Approximately 80% of cases show no symptoms at all and go unnoticed; however the elderly and young are at higher risk for showing symptoms (CDC B). The remaining cases of WNV show severe symptoms including fatigue, anorexia, insomnia, and blurred speech and symptoms typically associated with encephalopathy (Zdenek *et al.*, 2002). Symptoms typically appear between three and fourteen days after being exposed to the virus (CDC B).

History

West Nile virus was first isolated in 1937 in a Ugandan woman (Smithburn *et al.*, 1940). The virus was introduced to the United States in 1999 (Marra *et al.*, 2004). The first signs of a problem became apparent when the American Crow (*Corvus brachyrhynchos*) began to die off in large numbers in Queens, New York (Marra *et al.*, 2004). Soon afterwards, humans began going to the hospital with symptoms similar to St. Louis encephalitis. Subsequent testing in September 1999 found that the responsible agent was West Nile virus. This led to the first reported cases of West Nile virus in the western hemisphere. The speed at which the virus spread was concerning (Brault *et al.*, 2009). By 2004, nearly every state in the lower 48 had reported a human case of WNV (Marra *et al.*, 2004).

In 1999, at the end of the first infection cycle, 62 severe cases of WNV in humans had occurred with two deaths (Pollock 2008). By 2007, WNV had been associated with 3,510 cases of human illness and over 100 deaths in the United States alone (Soverow *et al.*, 2009). In an attempt to monitor the spread and prevalence of West Nile virus, the Center for Disease Control (CDC) created a visual map that enables individuals to monitor incidents of human infection nationwide (Gubler *et al.*, 2003).

Vector-Host Interactions

The typical method of transmission of WNV is via the bird-mosquito cycle, with humans and most mammals representing dead end hosts (Marra *et al.*, 2001). This means that infection in animals outside of bird populations are not a part of WNV's typical life cycle. Birds represent a significant reservoir for the virus due to variable host competency (McLean, 2006). In the United States the American Crow is a major host species for bird transmission and this bird population in New York City began to decline in 1999 (Marra *et al.*, 2001). Despite a nearly 100% death rate in crows, some evidence suggests that American Crow mortality is beginning to decline (Reed *et al.*, 2009). This indicates that some species that are susceptible to West Nile viral infection can develop some resistance to the virus.

The American Crow represents a major indicator of predicting epidemics because of their high susceptibility to WNV (McLean, 2006). However, as species begin to develop resistance, health officials will not be able to rely on American Crow mortality as an indicator of potential WNV epidemics. The implications for research are significant. If researchers can no longer rely on the American Crow for predicting

epidemics, another method must be developed for detection. Other species of birds may be helpful. However, isolation of WNV from mosquito tissue offers a reliable method.

WNV has since been identified in several other North American bird species such as Mourning Doves (*Zenaida macroura*), Northern Cardinals (*Cardinalis cardinalis*), and House Finches (*Carpodacus mexicanus*) (Loss *et al.*, 2009) along with American Robins (*Turdus migratorius*) (Bradley *et al.*, 2008) which have a high susceptibility and are known for migrating out from urbanization which can contribute to WNV spread (Bradley *et al.*, 2008). Several of these susceptible species have been identified in Montana, specifically Mourning Doves, House Finches, and American Robins.

Bird infection of WNV involves several mosquito species and depends a great deal on both the host and whether or not the virus is transmitted via a suitable vector such as ticks or mosquitoes (Kilpatrick *et al.*, 2006). A study by Reisen *et al.* (2005) found that WNV often infects Mourning Doves (*Zenaida macroura*) but does not kill them. This suggests chronic infection is present in Mourning Doves and could serve as a method by which WNV overwinters in Montana. Reisen *et al.* (2006) suggests three mechanisms which can allow mosquitoes harboring WNV to successfully overwinter. The first mechanism involves continued enzootic transmission to hosts that are present during the winter. The second mechanism uses vertical transmission from parent to offspring. The third mechanism is chronic infection in birds in which the infection lasts longer than usual and allows for continued transmission at the end of winter (Reisen *et al.*, 2006).

The most common vector for transmitting the virus in the United States is the mosquito family Culicidae. However, it is believed that WNV also can be transmitted via ticks, fleas, and louse flies (Blackburn *et al.*, 1990). Even within the *Culicidae*, WNV is known to have variable vector competency because in some species it is not spread to the salivary glands (Sardelis, 2001). The *Culex* genus plays the largest role in spreading WNV in the United States (Hayes *et al.*, 2005). According to a California study, this genus has shown variable competency in various species (Goddard *et al.*, 2002). However, in the north central areas of North America, WNV is primarily transmitted via *Culex tarsalis* (Brault *et al.*, 2009).

Culex tarsalis has shown preferential feeding towards birds in the order *Passeriformes* (Kent *et al.*, 2009). As of 2012, there were approximately 250 species of birds susceptible to WNV infection (UNSG, 2012). However, when avian populations begin to decline and mosquito populations increase towards the end of summer the *Culex* genus tends towards opportunistic feeding on humans, horses, and other animals (Kent *et al.*, 2009).

Tracking WNV

Global Information Systems (GIS) have been used to track the prevalence of WNV and identify the geographical spread of the virus (Sugumaran *et al.* 2009). GIS also helps to identify areas which consistently test positive for WNV in order to implement measures of control on populations of *Culex tarsalis* (Sugumaran *et al.* 2009) by providing a map of at-risk areas. Sugumaran (*et al.*, 2009) also determined, through use of geographical models, that areas with high precipitation have a higher risk for WNV

and represent a significant predictor of prevalence. One study found that when a hot spot for WNV became subject to drought researchers saw a marked decrease in the total number mosquitoes (Johnson *et al.*, 2010). However research indicates that WNV continues to be present despite the decrease in *Culex tarsalis* population sizes (Johnson *et al.*, 2010). Dohm *et al.* (2002) found that increases in temperature in the later summer months contributed to high infectivity in mosquitoes.

MLWR has been a WNV hot spot in the past due to higher temperatures during the summer particularly at Pelican point, due to a prevalence of migratory birds which settle there to nest. In contrast NPWR is west of the continental divide in Montana, but has many similar environmental conditions (NOAA, 2012) to MLWLR, supports populations of *C. tarsalis*, and is home to large numbers of migratory birds. However, no detections of WNV in mosquitoes have occurred west of the Continental Divide in the state to date. My null hypothesis is that there will be no significant difference between the prevalence of WNV at MLWR and NWR. The hypothesis of my research is that there will be statistical differences in infectivity of WNV in mosquito populations at Medicine Lake Wildlife Refuge (MLWLR) compared to Nine Pipe Wildlife Refuge (NPWR).

Methods and Materials

Mosquito collection sites

Mosquitoes were collected from two primary locations; Medicine Lake Wildlife Refuge (MLWR) and Ninepipe Wildlife Refuge (NWR). NWR is located in the northwestern part of Montana and is flanked to the east and west by mountain ranges. MLWR is located in northeastern Montana and is largely flat farmland. Within the MLWR three sites were sampled, the first Medicine Lake Headquarters (ML HQ, GPSUTM Coordinates 13 E 0540623 N 5370152) had direct access to water. The vegetation at the site included some sparse tree coverage and an abundance of grass approximately 3 feet tall. The second site was Medicine Lake pelican point (ML PP, E0546225 N 5368540) because of its proximity to the Medicine Lake pelican nesting grounds. Pelicans are a major host species for WNV. There was heavy tree coverage and dense understory foliage approximately 4-5 feet high and direct access to water. The third site was ML Homestead (E 0531582 N 5360086) and was located on the site of the road in a runoff ditch. The area had poor soil drainage but the site was primarily dry due to little rainfall. There was little to no tree coverage at ML Homestead and no obvious standing water.

Four collection sites were located in the NWR. Ninepipe 1 (11 E 714997 N 5259180) was located next to a single tree with tall grass approximately 5 feet tall. The site was located next to a pond and had several cattails surrounding the area. Ninepipe 2 (E 715480 N 5256933) was located near marshlands with no tree coverage. There was extensive foliage comprised primarily of tall grass and cattails. Ninepipe 3 (E 715916 N

5258453) was located in dense foliage and extensive tree coverage. Ninepipe 4 (E 718835 N 5258574) had less grass but large tree coverage and was located close to water. Figure 1 shows the relative locations of the study areas and sample location.

Mosquito collection

Mosquitoes were collected using CDC light traps. These traps use CO₂ to attract mosquitoes which are then pulled into the collection net using a fan. They are set out at approximately 6-7 p.m. and left overnight. The traps are turned off and the mosquitoes are collected at approximately 7-8 a.m. Typically, two traps were used at each site and the CO₂ was generated by hanging a cooler filled with solid CO₂ with holes drilled on the bottom in order to supplement the trap. The other trap was connected to compressed CO₂ and allowed to run at 15 pbar. (VDOH 2008)

Mosquito Sorting

Mosquitoes were sorted under microscopes in order to separate out the female *Culex tarsalis*. The non *Culex tarsalis* and males were counted and placed in large test tubes and stored at -20° C. The female *Culex tarsalis* were counted and stored in a freezer at -20° C until they were ready for homogenization. Females were sorted from the males because female mosquitoes are the only sex requiring a blood feed to reproduce.

Preparation for Homogenization

Mosquitoes were assigned a number according to the site they were found at and the date of collection. Between 1-50 *Culex tarsalis* from one site were placed in a MP lysing matrix A tubes along with a ceramic bead (MP Biomedical). The caps were

marked if the sample contained less than 10 mosquitoes along with the sample number because a slightly different amount of reagents were required (see below). The samples were then taken to a Purifier® Inflow Class 2 Biological Safety Cabinet (Labconco, Kansas) under BSL II conditions.

Homogenization

To each sample with 10 or more mosquitoes, 1000ul of RNA later and 500 ul of homogenization buffer was added while with samples with less than 10 mosquitoes, 600 ul of RNA later and 300 ul of homogenization buffer was added. The samples were placed in a FastPrep FP120 (ThermoSavant, Massachusetts) at speed 5.5 for 30-45 seconds in order to liquefy the mosquitoes. The samples were removed and half of the homogenate was placed in numbered micro-centrifuge tubes to later be tested by Laboratory Services at the State of Montana Department of Health and Human Services (DPHHS). Both the DPHHS samples and the samples for extraction were stored at -80° C in order to preserve RNA integrity.

RNA extraction

Two methods were used to extract the RNA from the homogenate. One method used a machine which automated the procedure while the other relied on manually adding the reagents. Before the extraction procedure the samples were prepared by removing 300 µl of the sample and placing it in a separate micro-centrifuge tube which was appropriately labeled. To these newly labeled tubes, 300 µl of Buffer RLT was added to each sample. At this point in the procedure the samples were often loaded into a QIACUBE which automated the process described below. If a QIACUBE was not available

the extraction was done by hand. If the extraction was done by hand, an aliquot of 10 μ l of proteinase K and 290 μ l of RNase free water was mixed and added into each sample. The samples were then allowed to incubate for 10 min. at room temperature. After incubation, the samples were centrifuged in order to pellet the particulate matter. The supernatant was removed and placed in a micro centrifuge tube while 400 μ l of 100% ethanol was added to each sample and mixed via pipetting. The sample was taken and 700 μ l was transferred to a spin column and spun at 10,000 rpm for 15 seconds. The supernatant collected at the bottom of the spin column was discarded. The remainder of the sample was added to the spin column and then centrifuged again with the supernatant discarded. To each column 350 μ l of Buffer RW1 was added and spun in the centrifuge with the supernatant again being discarded. This was followed by 10 μ l of DNase 1 mixed with 70 μ l of Buffer RDD and added directly to each spin column. The samples were allowed to incubate at room temperature for 15 minutes. After incubation 350 μ l of Buffer RW1 was again added to the spin column and spun. The supernatant was then discarded. Afterwards, 500 μ l of Buffer RPE was added to the spin columns and the spin columns were again spun. This process was repeated once more with the supernatant being discarded after each spin. Finally, 40 μ l of RNase free water was added directly to the spin column and centrifuged at 10,000 RPM for one minute. The supernatant was then collected and transferred to appropriately labeled micro centrifuge tubes. These tubes were then stored in a -80° C freezer until ready for Real Time Polymerase Chain Reaction (RT PCR) (Qiagen RNeasy® Fibrous Tissue Handbook 2006).

RT PCR

Mosquito samples were analyzed for WNV by a RT-PCR and Taqman Assay using the same primers, solution concentrations, and run time protocol as Lanciotti *et al.* (2000). Each sample was analyzed in four different PCR wells using two different primers, designated WNV and 3', in order to prevent false positives. The wells of the PCR plate were loaded in a separate sterile hood in order to prevent potential contamination. If one or more sample was positive then the sample was rerun separately in order to confirm the positive. The sample was run using a iQ5™ (BioRad).

Data Analysis

Culex tarsalis pools were homogenized and analyzed in order to determine if WNV was present in the pool. This information was catalogued and statistically analyzed in order to determine the relative infection rate of WNV in *Culex tarsalis* at the two study areas. The infection rate calculated in this research was an informative index used to monitor viral activity rather than a percent of total population exposed to a particular pathogen.

Culex tarsalis populations were then analyzed with the Microsoft® Excel 2007 PooledInfRate version 4.0 add-in (Biggerstaff, 2012). This software is used to calculate reliable infection rate estimates without assumptions taken while calculating minimum infection rate. (CDC B)

Results

Mosquito Populations

At NWR 19,034 mosquitoes were collected from the four sites. Ninepipe 1 and 4 showed a much higher prevalence of mosquitoes compared to sites 2 and 3. The proportion of *C. tarsalis* in relation to total mosquito population was slightly higher at Ninepipe 2 and 3; however at Ninepipe 4 more total *C. tarsalis* were found (Table 1). At MLWR 2,875 mosquitoes were collected from three sites. At Medicine Lake Homestead, more total mosquitoes were collected than the other two sites combined. *C. tarsalis* prevalence was relatively low however and Medicine Lake Pelican Point represented more than two thirds of the total *C. tarsalis*. Medicine Lake Pelican Point and HQ had the highest percent of *C. tarsalis* (Table 2).

WNV Infection Rate

This study assessed 176 collections comprising 5,420 *C. tarsalis* between the dates of June 16 and September 1 of 2012. The 176 collections were separated into 185 pools of *C. tarsalis* ranging from 1 to 50 *C. tarsalis*. Positive samples for West Nile virus (WNV) were found at Liz's farm, Bowdoin, Medicine Lake Wildlife Refuge (MLWR), and Ninepipe Wildlife Refuge (NWR) in Montana (Figure 2). These positives were found at seven sites comprised of two Medicine Lake sites, two Bowdoin sites, one Liz's farm site, one Miles City site, and one Ninepipe site.

At NWR 49 pools comprising of 1,070 *C. tarsalis* were tested for the presence of WNV. One sample was positive. The pooled infection rate for NWR was 0.92 (Table 3).

The 95% confidence interval places the lower and upper limits at 0.05 and 4.45, respectively.

At MLWR 33 pools comprising of 669 *C. tarsalis* were tested for the presence of WNV. Four of the 33 pools were positive. The pooled infection rate for MLWR was 6.33 (Table 3). The 95% confidence interval places the lower and upper limits at 2.10 and 15.24, respectively. The upper limit of the NWR overlaps lower limit of the MLWR (Table 3). This indicates that there is no statistical difference in the WNV infection rate at the two sites.

Tables and Figures

Table 1. Mosquito totals and proportion of total that was *C. tarsalis* at Ninepipe Wildlife Refuge

	Total	<i>C. tarsalis</i>	% <i>C.</i>
	Mosquitoes		<i>tarsalis</i>
Ninepipe 1	8438	190	2%
Ninepipe 2	1778	170	10%
Ninepipe 3	1744	296	17%
Ninepipe 4	7074	400	6%
Total	19034	1070	6%
Average	4758.5	264	

Table 2. Mosquito totals and proportion of total that was *C. tarsalis* at Medicine Lake Wildlife Refuge

	Total	<i>C. tarsalis</i>	% <i>C.</i>
	Mosquitoes		<i>tarsalis</i>
Medicine Lake HQ	282	132	47%
Medicine Lake Pelican	577	377	65%
Point			
Medicine Lake	2016	158	8%
Homestead			
Total	2875	669	23%
Average	958	222	

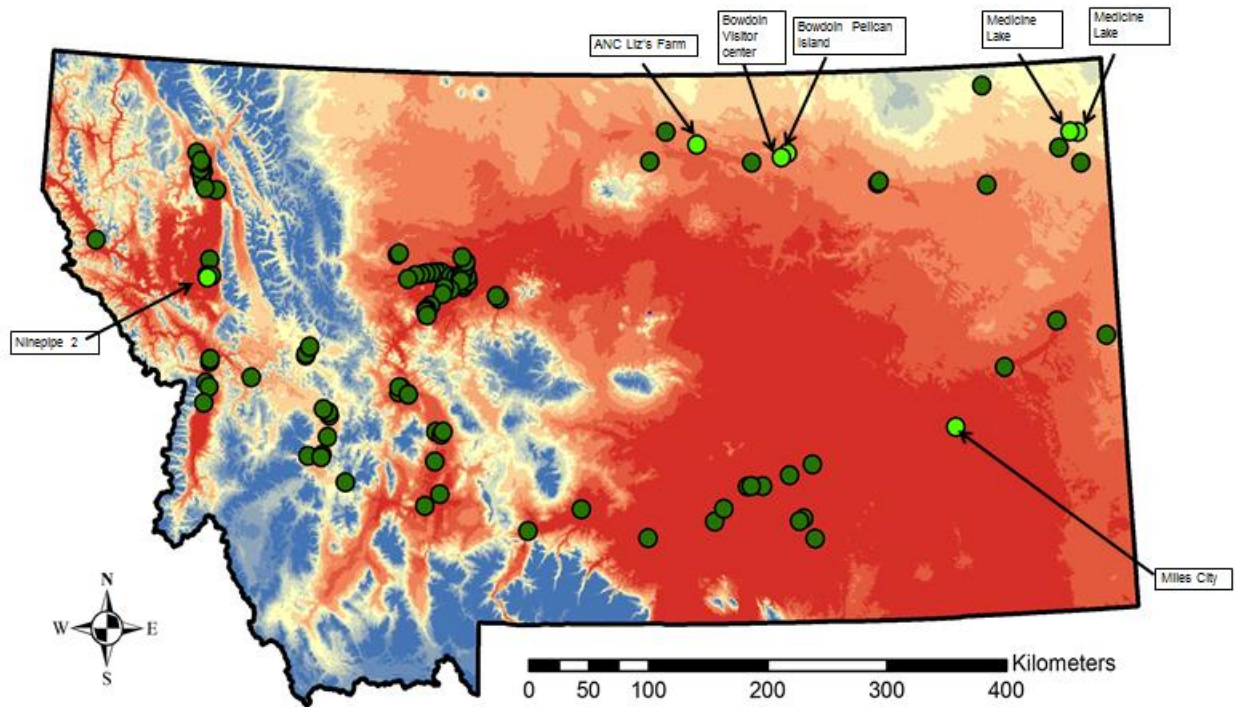


Figure 1. Map of Montana showing the average summer temperatures of the area with an overlay of sampled areas. Areas highlighted with light green indicate where *Culex tarsalis* tested positive for West Nile Virus. Red areas indicate locations with high average temperatures while blue indicates area with lower average temperature.

Table 3. West Nile virus infection rates in *Culex tarsalis*.

Infection Rate	Lower Limit	Upper Limit	Scale	Num Pools	Num Pos Pools	Num Individuals
Ninepipe						
0.92	0.05	4.45	1,000	49	1	1070
Medicine lake						
6.33	2.10	15.24	1,000	33	4	669

Discussion

My results suggest that there is no significant difference in the WNV infection rate in *Culex tarsalis* of NWR versus MLWR. The fact that there is no statistical difference between MLWR and NWR could be because of several factors. Our sample sizes were relatively small and a larger sample size would have helped to reduce the upper and lower limits of the pooled infection rate calculations. Weather also played a role in the study because low temperatures and high winds during the visit to MLWR from 7/23/12-7/26/12 made trapping difficult and reduced the effectiveness of our CDC light traps.

The detection of WNV at NWR is of interest due to the fact it is the first detection in *Culex tarsalis* at this location (Marcelli 2012). There are two likely explanations of this result. The first is that WNV, while present at NWR, may have maintained extremely low levels of viral activity which made detection difficult. The other possibility is that WNV migrated to NWR and is new to the location.

Grant Hokit (2012) has suggested that *Culex tarsalis* trapping is highly heterogeneous and that biased trappings should be done east of the continental divide in Montana due to a lack of detection of WNV west of the divide. This research supports this conclusion despite the detection of WNV at NWR because a majority of the detections of WNV in Montana have been found primarily in the eastern side of the state. This suggests that a majority of funding for WNV detection should be used to focus on the eastern side of the state while maintaining some detection in the western part of Montana.

Studies on *Culex tarsalis* indicate that this species is relatively sensitive to environmental conditions. Lungstrom (1954) found that mating in *Culex tarsalis* ceased in cool, humid environments. This suggests that periods of cool temperature can cause major decreases in the *Culex tarsalis* populations. In addition, Lungstrom (1954) found that feeding during the day was uncommon except during periods of cloudy weather. This is consistent with our methodology of trapping at night.

Johnson *et al.* (2010) found high levels of *Culex tarsalis* during the summer of 2007 but relatively low numbers in the summer of 2006 at MLWR. Oddly, mosquito surveys done by Marcelli (2012) found high numbers of *Culex tarsalis* numbers during the summer of 2011 while this study found low numbers of *Culex tarsalis* during the summer of 2012 at MLWR. The differences between this study and Marcelli (2012) in relation to *Culex tarsalis* numbers at MLWR are likely due to differences in summer precipitation. Between July and August of 2011 while Marcelli was collecting mosquitoes, MLWR received 5.43 inches of precipitation. In contrast during the months of July and August 2012 while collection for this study was taking place, MLWR received 2.76 inches of precipitation (NOAA). This lack of rain would have made *Culex tarsalis* breeding grounds scarce in comparison with previous years. When this is compared to Johnson *et. al* (2010) there is an interesting population dynamic occurring at MLWR. High numbers of *C. tarsalis* are likely due to summers of high precipitation along with high temperature which provides an abundance of breeding grounds for *Culex tarsalis*; however high precipitation and summer temperatures did not lead to an abundance of

WNV infected *Culex tarsalis* at MLWR. Despite the study done by Marcelli (2012) the percent *Culex tarsalis* at NWR remained relatively stable between his study and mine.

Trapping done by Lungstrom (1954) found that the ideal months for collecting *Culex tarsalis* were during July and August. In addition, Lungstrom found that populations of *Culex tarsalis* can vary a great deal. This problem of population levels continually being in flux and our irregular visits to MLWR may have been detrimental to consistent collecting. Lungstrom (1954) further found that *Culex tarsalis* reproduction is most successful in slightly alkaline water with high algae content, very similar to the waters found at both NWR and MLWR.

In this study there was no significant difference between the infection rate at NWR and MLWR. Studies done at MLWR this year correlate with the information presented in Johnson (*et. al* 2010) A study by Joseph Marcelli (2012) found similar data to this research with infection rates with overlapping confidence intervals with the 2007 data in Johnson *et. al.* (2010) study and my 2012 data for MLWR. This indicates that although a large difference exists between the population of *Culex tarsalis* of 2007, 2011, and 2012 at MLWR, there is not a significant difference between the infection rates. A study in 2001 by Bernard (*et al.*) found the infection rate in four species of mosquitoes to be between 3.42 and 11.42 during the summer of 2000. This fit extremely well with the infection rates calculated in my study with overlapping confidence intervals for both NWR and MLWR. A study conducted by Hokit *et al.* (2012) found that infection rates in *Culex tarsalis* gathered throughout Montana over the last 8 years appear to show strong variations in WNV infection rate from year to year. Hokit's

research has other important implications for this study relating to infection rate and relative *Culex tarsalis* abundance. Over 8 years of collecting data Hokit found no correlation between the relative abundance of *Culex tarsalis* and the minimum infection rate. Infection rate consistency appears to vary widely as research seems to suggest *Culex tarsalis* population size and infection rates are inconsistent with one another.

This information has important implications for this study. It suggests that although *Culex tarsalis* numbers are high in some years, WNV may be present at relatively low numbers of mosquitoes making it difficult to detect. This leads me to suggest that relative infection rate and *Culex tarsalis* abundance are likely independent of one another and that population has little influence on WNV infection rate within a given population and vice versa.

Further research must be done on *Culex tarsalis* in order to better understand the interplay that occurs between mosquito and virus. Information regarding environmental factors and their influence on the transmittance and receptivity of *Culex tarsalis* to WNV needs to be discovered in order to provide more concrete evidence to support this independence between *Culex tarsalis* population size and WNV infection rate.

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