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Comparison of West Nile Virus infection rates in Culex tarsalis and exposure rate of horses in Montana using RT-PCR and ELISA

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Abstract

Horses are more susceptible to West Nile virus (WNV) than human beings. In addition, for many ranchers in Montana, horses are their livelihood. In Montana, the only risk assessment tool for WNV is mosquito surveying. Testing horses across the state for WNV may contribute to a better estimate of high-risk areas. Also, since the numbers of human and horse cases are similar on a yearly basis, testing horses may predict the human risk. In this study, *Culex tarsalis* infection rates were compared with horse exposure rates in Montana. Mosquitos were collected from 46 sites on a weekly or biweekly basis. Collected mosquitoes were sorted, homogenized, and run through an RNA-extraction. WNV in mosquitoes was detected using RT-PCR. Horse serum was collected from the Helena area and analyzed for NS1 and envelope IgM and IgG antibodies to determine WNV exposure and/or vaccination. Three counties had positive *Cx. tarsalis* pools for WNV; the state infection rate was 0.108%. Two unvaccinated horses had positive IgM WNV antibodies. However, due to borderline results and vaccination contamination a horse exposure rate could not be calculated to make a comparison with the mosquito vector infection rate. Since horse positives occurred in an area where no positive pools were found, horse surveillance may be critical for detecting WNV hot zones.

Introduction

Humans have a two to four percent fatality rate for West Nile virus (WNV) infection (Donadieu *et al.*, 2013), while horses have a 33% fatality rate (A CDC, 2014), which may be due to higher incidence of encephalitis and neurological damage in horses than humans (A CDC, 2014 and Donadieu *et al.,* 2013). With no effective treatment for WNV, the only preventive actions that can be taken are mosquito management and vaccination. Montana has become endemic for WNV; in 2013, 33 horses (USDA, 2013) and 38 humans were diagnosed with WNV (B CDC, 2013). Currently, Montana surveys several areas for WNV-vector mosquitoes to assess the risk of human exposure to the virus (Hokit *et al.,* 2013)*;* however, this technique has limitations and new techniques may need to be utilized for proper assessments. Surveillance of both mosquitoes and horses may better identify high, moderate, and even low risk areas, so that Montanans can take the proper preventative measures for their horses and themselves. For California, the Department of Food and Agriculture assesses the potential risk for WNV by testing dead birds, sentinel chickens, mosquito pools, horse and human samples and by monitoring horses that become infected (A CDC, 2014). Due to California's monitoring system, as of September 15, 2014 there were 10 horses infected with WNV in California for 2014 (A CDC, 2014). Accurate horse infection rates cannot be based on the number of horses diagnosed because some horses may not show clinical symptoms of WNV (A CDC, 2014). For instance, Newton (2013) tested horses in two counties in Montana and found two WNV-exposed horses with no clinical signs of WNV. Furthermore, since the numbers of horses infected is close to the number of humans

infected (USDA, 2013 and B CDC, 2013), an accurate infection rate for horses may also estimate the human risk.

West Nile Virus in Horses

WNV is a single stranded-RNA zoonotic flavivirus of the Japanese encephalitis virus complex (May, 2011; Castillo-Olivares *et al.*, 2011) and has become a worldwide problem affecting both humans and livestock. WNV is a mosquito-borne disease that exists in an enzootic cycle (Castillo-Olivares *et al.*, 2011). WNV-infected horses and humans do not produce enough viremia to infect another mosquito; as a result they are considered dead end hosts (Suen *et al.*, 2014). In Montana, there are two vector mosquitoes for WNV, *Culex tarsalis* and *Cx. pipiens* (Hokit *et al.*, 2013). These mosquitoes prefer to blood feed on avian sources (Bell, 2006).

Symptoms in horses range from flu-like to neurological disorders (A CDC, 2014). In severe cases, the virus causes inflammatory lesions and neurological damage (Donadieu *et al.*, 2013). The virus can target many important high-functioning areas of the brain including the medulla that can cause encephalitis (Donadieu *et al.*, 2013). In 2013, Montana had the third highest number of WNV infected horse cases in the United States (USDA, 2013). Certain stable conditions increase the rate of infection in horses. Rios *et al.* (2010) found that some of these factors were the construction material used for stables (wood or cement), whether or not a stable fan was used, the frequency of fan use, the presence of dead birds, and mosquito activity on the property (Rios *et al.*, 2010). Mosquito activity peaks between August and October, thus WNV horse cases are seasonal (Rios *et al.*, 2010).

The most effective preventive measure against WNV is to vaccinate horses. For instance, Gardner (2007) compared the infection rates between vaccinated and unvaccinated horses, and found that no cases of WNV were observed in vaccinated horses. Also, Seino *et al.* (2007) tested the efficiency of three different vaccines: an inactivated WNV vaccine (K-WN), a modified-live vaccine (CP-WN) and a live-chimera vaccine (WN-FV), and found that these three vaccines showed a 100% protection against WNV after 28 days and 56 days using IgM antibody Enzyme-Linked Immunosorbent Assays (ELISA) to test for immunity (Blitvich, 2002). Vaccinating every horse in Montana would be ideal; however, vaccines come in two doses, can cost up to $60 per horse, and must be given annually because antibodies diminish over time (Gardner, 2007). Therefore, the cost of vaccinating large numbers of horses hinders many ranchers from choosing this course of action. These yearly costs could add up based on the number of horses a rancher owns, so a better option is for ranchers to vaccinate when the risk is high in their area.

Surveillance

The number of WNV cases varies from year to year due to many factors. These factors include temperature, climate, geography, surrounding animal population distribution, and other biological factors such as host diversity and abundance, the age of the mosquito, and bird migration (Hokit *et al.,* 2013; Zou, 2007; Yiannakoulias, 2007); as a result, the prediction of infection rates is very difficult. Some states use mosquito surveillance as their main risk assessment (A CDC, 2013; Hokit *et al.*, 2013). However, there are many limitations to mosquito surveillance. For instance, Weidong (2008) found that mosquito surveillance is not an effective tool for predicting high risk areas and assessing public risk because of the overemphasis on random sampling, the lack of expanding surveys when there

is low risk, and the overuse of mosquito infection rates as a risk exposure tool. Random sampling prolongs early detection since it yields low infection rates because of the sporadic surveillance (Weidong, 2008). Also, mosquito surveyors rarely expands in range, as a result this decreases the opportunity to improve knowledge of transmission and intensity (Weidong, 2008). Additionally, mosquito surveillance overuses infection rates as a risk exposure tool because it ignores human populations' densities (Weidong, 2008). Also, since horses are not the main blood meals for vector mosquitoes, the mosquito infection rate is an overestimate for the potential risk to horses and humans (Bell, 2006). As a result, other methods for predicting the infection rate and the potential risks of WNV should be explored or added to mosquito surveillance.

Weidong (2008) argues that mosquito surveillance has many flaws, but he also presents several methods to correct for these issues. These include: targeted surveillance, variable size pooling in calculating infection rates of higher transmission areas and maximal likelihood estimation to estimate risk exposure alongside mosquito and human population abundances (Weidong, 2008). Another method for WNV surveillance may be horse surveillance. Powell (2000) suggests that maintaining information on WNV infected horses could minimize disease transmission and improve prevention measures. Also, a large-scale equine serosurvey study performed in Iran determined that the equine exposure rates are similar to environmental and geographical patterns of mosquito infection rates (Ahmadnejad, 2011), which suggests the use of minimal infection rate (MIR) as opposed to using maximal likelihood estimation as Weidong suggests. As a result, of Ahmadnejad's (2011) observation MIR was used in the current study.

Many countries and regions have started surveying horses for WNV in order to map transmission and establish high risk areas. For example, Berxholi *et al* (2013) tested 167 horses for WNV using ELISAs. Horse surveying could make up for some of the issues with mosquito surveillance as well as obtain a more accurate exposure rate. For instance, in California Gardner (2007) found fairly high exposure rates in unvaccinated horses using ELISA, while intense trapping and RT-PCR of mosquitos showed low levels of the virus. This may indicate a need for horse surveillance for WNV via ELISA in addition to RT-PCR detection in mosquitoes. Also, testing hundreds to thousands of *Cx. tarsalis* pools for WNV is tedious as well as difficult due to some environmental factors (Zou, 2007). Further investigation into horse surveillance is needed.

Current Study

Over the summer of 2014, the West Nile Research program at Carroll College tested *Cx. tarsalis* and *Cx. pipiens* for WNV to asses high risk areas at 46 sites across Montana (Hokit *et al.*,2013). The objective of the study was to test these collected vector mosquitoes for WNV using RT-PCR. Equine blood samples were collected from the Helena area and tested for WNV exposure using ELISA. A total of four ELISA tests were run on each equine blood serum sample to determine whether exposure occurred this year or previous years and whether or not the horse was vaccinated. The infection rates for *Cx. tarsalis* were then compared with the horse rates. Since horses are not the primary blood source (Fall et al., 2014), I hypothesized that the mosquito infection rate would be higher than the mammalian infection rate. I also hypothesized that horse and mosquito positives should coincide in the same area.

Method and Materials

Mosquito Collection

To trap mosquitoes, CDC light traps were baited with carbon dioxide using CO_2 tanks or dry ice (Ginsberg, 2011). A total of 46 collection sites (Figure 1**)** were set around Montana by Carroll College, four Tribal Colleges and Montana State University. Some of the regular trappings occurred on a weekly basis in the Helena area, Havre area and Medicine Lake National Wildlife Refuges and on a biweekly basis in the Bozeman, and Three Forks area.

Once trapped, the mosquitoes were put on ice and sent to Carroll College and stored in a -20°C freezer. Once dead, mosquitoes were sorted for female *Cx. tarsalis* and *Cx. pipiens*. The sorters used microscopes and kept the mosquitoes on ice to prevent RNA degradation. Samples were then returned to the -20ºC freezer until homogenization and viral testing. A total of 600 µL mosquito homogenate per sample was made. Three hundred microliters and sent to the Public Health Laboratory of the Montana Department of Public Health and Human Services (DPHHS) for WNV testing and the other 300 µL was retained at Carroll College for testing.

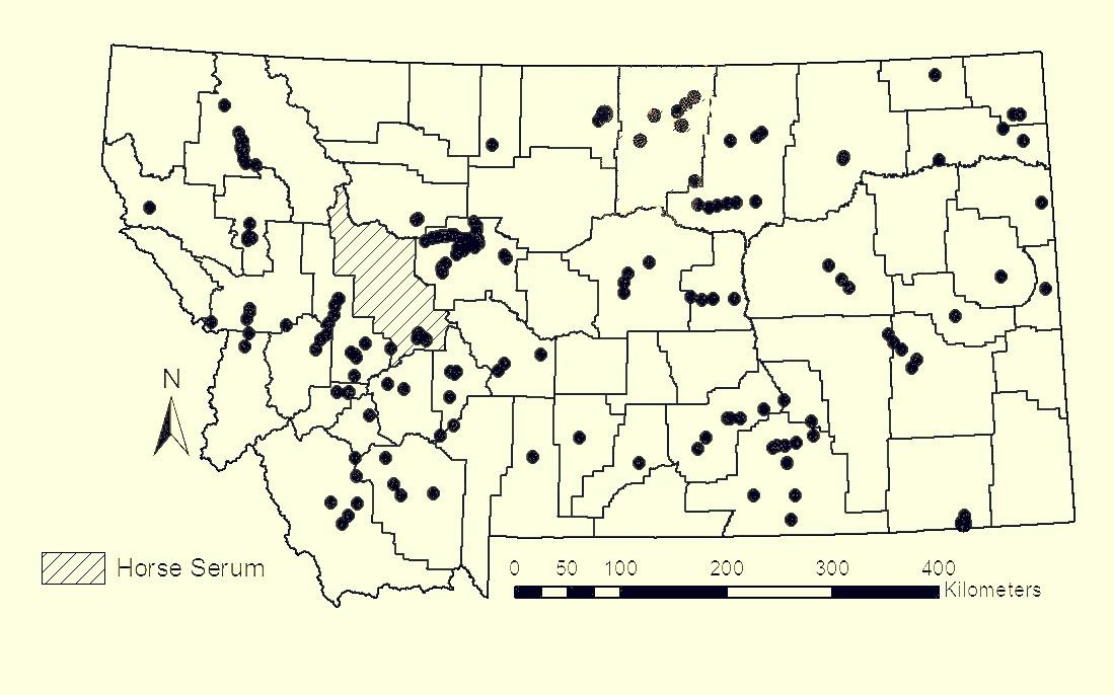

Figure 1 The mosquito sites collected in Montana for the past 5 years and the area where horse samples collected for this study.

Homogenization

The *Cx. tarsalis* samples were pooled into Lysing Matrix A tubes with ceramic beads and labeled with the number to the corresponding site. No more than 50 *Cx. tarsalis* were grouped into each lysing tube. Homogenization followed Lanciotti's (2000) protocol. A thousand microliters of Qiagen RNA Later and 500µL of RLT lysing buffer were added to each lysing tube. If a site had less than 10 *Cx. tarsalis*, then 600µL of Qiagen RNA Later and 300µL of RLT lysing buffer were added to each lysing tube. The lysing tubes were then placed in a Fast Prep FP120 (ThermoSavant) bead beater and homogenized at 4.5 oscillations per minute for 45 seconds. Homogenates were then stored at -80ºC until used for RNA extractions.

RNA Extraction

RNA extraction followed the method described by Lanciotti (2000), using a QIAcube (Qiagen). Six hundred microliters of homogenate was prepared and placed in the QIAcube as described in the RNeasy fibrous tissue kit (Qiagen, 2006). After the extraction, the collection tubes were stored at -80ºC until RT-PCR was performed for the viral test.

RT-PCR

Lanciotti's (2000) protocol for RT-PCR West Nile Virus detection was used. A total volume of 25µL made up of: 2.5µL of RNA and 22.5µL of Master Mix (Table 1) was placed in each well of the PCR plate. The prepared plates were then transferred to a BioRad IQ500 thermocycler where they were incubated under the following conditions: one cycle of 48ºC for 30 minutes, one cycle of 95ºC for 10 minutes and 55 cycles of 95ºC for 15 seconds followed by 60ºC for one minute.

Table 1 Master Mix components for RT-PCR

Reagents	Amount (µL per well)
Taqman Master Mix	12.5
RNase-free water	7.38
ENV/ 3' Probe	1
Taqman Multiscirbe (enzyme)	0.625
ENV/ 3' Forward Primer	0.5
ENV/ 3' Reverse Primer	0.5

Horse Blood Sample Collection

Veterinarians in the Helena area drew 10mls of blood from 68 horses during September and October of 2014 (Figure 1**)**. Each sample was centrifuged to separate the serum from the whole blood. Sera were transported on dry ice and stored at -80ºC to preserve antibodies until testing.

ELISA

The equine sera were tested for WNV antibodies using ELISA kits from Alpha Diagnostic International. Four ELISA kits were run on each sample: two for IgG WNV antibodies and two for IgM WNV antibodies following the Alpha Diagnostic International (2014) procedure. The two different antibody types were used to test when the horse was exposed to WNV. If a positive test occurred on the IgM, then the horse had been exposed or vaccinated with WNV this summer. If a positive test occurred on the IgG, then the horse had been exposed or vaccinated a year or more ago. The two different tests for each antibody were used to distinguish whether the horse's WNV antibodies were generated from exposure or vaccination. One test looked for the antibodies for NS1 WNV protein and the other for the envelope protein. A positive for envelope protein antibodies in the horse identified that the horses was exposed to WNV and a positive for NS1 protein antibodies identified that the horse was vaccinated. A horse was considered positive if the optical density of the sample was greater than two; it was considered negative if it was less than one and borderline if it was between one and two.

Comparing the Infection and Exposure Rates

Infection rates for female *Cx.tarsalis* were calculated using an Excel program created by Biggerstaff (2009). To prevent skewing problems such as inconsistencies between

numbers of mosquitos in each pool, Minimal Infection Rate (MIR) was used to calculate infection rates by county and for the whole state. MIR assumes that only one positive mosquito is within one positive pool (Biggerstaff, 2009). For horse samples, exposure rates were computed based on the number of positive horse sera samples. These two rates were then compared.

Results

The mosquito data for both *Cx. tarsalis* and *Cx. pipiens* from the summer 2014 collection are compiled in Appendix one and two. No pools of *Cx. pipiens* were positive for WNV. The infection rate for Montana was 1.08 per 1,000 individual mosquitoes (Table 2) based on 46 sites tested. Three counties, Big Horn, Blaine and Wibaux, had positive pools and their infection rates were calculated (Table 2). All the other counties with no positive pools had an infection rate of zero.

Table 2 Infection rates for positive pools from counties and the state.

County	Num. Individuals	Num. *Cx. tarsalis*	Num. Pools	Num. Pos. Pools	Lower Limit	Upper Limit	Infection Rate
Statewide	*142,804	4,934	185	6	0.22	1.95	1.08
Big Horn	7,220	83	5	1	0.00	35.52	12.05
Blaine	7,051	966	24	4	0.08	7.08	3.58
Wibaux	N/A	232	7	1	0.00	11.50	3.89

*****This is an estimated number of individuals.
******Infection rates were scaled per 1,000 individual mosquitoes.

Forty six percent of the horse samples were positive for IgM WNV antibodies and 4.5% were positive for IgG WNV antibodies (Table 4).Also, 46% of the horse sample had

borderline results for the IgM antibody and 74% for the IgG (Table 4). Two horses that were unvaccinated had positive WNV IgM antibodies with borderline IgG antibodies (Table 5). Of the vaccinated and unknown horses, 27 had positive IgM WNV antibodies (Table 5). However, the positive controls for the ELISA tests were inconsistent and did not give clear positives on all the tests (Table 3).

Table 3 Control results of ELISA tests.

	IgM		
	IgM Pos	IgM Borderline	IgM Neg
Positive Control	1	1	1
Negative Control	0	0	3
	IgG		
	IgG Pos	IgG Borderline	IgG Neg
Positive Control	0	4	0
Negative Control	0	0	4

Table 4 IgG and IgM ELISA results, with the percents of the total number of samples for each antibody.

	IgM		
	IgM Pos	IgM Borderline	IgM Neg
Vaccinated	7(10%)	12(18%)	1(1.5%)
Unvaccinated	2(3%)	10(15%)	1(1.5%)
Unknown	22(32%)	9(13%)	4(6%)
	IgG		
	IgG Pos	IgG Borderline	IgG Neg
Vaccinated	1(1.5%)	12(18%)	6(9%)
Unvaccinated	1(1.5%)	8(11%)	4(6%)
Unknown	1(1.5%)	31(45.5%)	4(6%)

Table 5 IgG and IgM results for horses sampled in Helena, MT.

	IgM pos, IgG pos	IgM pos, IgG B	IgM pos, IgG neg	IgM neg, IgG pos	IgM neg, IgG B	IgM neg, IgG neg	IgM B, IgG pos	IgM B, IgG B	IgM B, IgG neg	Total
Vaccinated	1	6	0	0	1	0	0	6	6	20
Unvaccinated	0	2	0	0	1	0	1	5	4	13
Unknown	1	19	2	0	2	2	0	9	0	35
Total	2	27	2	0	4	2	1	20	10	68

*B represents the borderline values

Discussion

The objective of this study was to test *Cx. tarsalis* and horses for WNV. I hypothesized that the *Cx. tarsalis* infection rate would be higher than the horse exposure rate. This hypothesis can neither be rejected nor accepted because a horse infection rate could not be calculated due to borderline results. Three counties in Montana had positive WNV pools of mosquitoes (Table 2), and the statewide infection rate was 1.08 per 1,000 individuals. However, the ELISA tests yielded unclear results due to vaccine contamination with multiple antigens and an exposure rate could not be determined. I also, hypothesized that mosquito and horse WNV positives would coincide in the same location. This hypothesis can be rejected as no positive mosquito pools and two positives horses were found in the Helena area. Since horse positives occurred in an area where no positive pools were found, horse surveillance may be critical for detecting WNV in lower and moderate risk areas.

Conclusions

The statewide infection rate was lower than in 2013 (Newton, 2013), but higher than 2012 (Maricelli, 2012). The mosquito populations collected during these three years vary with 2012 having the fewest. Weidong (2008) found that a correlation between infection rate and risk of exposure cannot be seen in small sample sizes. Previous studies have also found

with WNV and thus a larger amount of negatives would be expected in the unvaccinated horses and unknown horses. Further analysis of the ELISA test itself may be needed to establish sensitivity

For all the ELISA tests, only one of the positive controls had an optical density greater than two, a strong positive reading (Table 3). For the IgM ELISA tests, one positive control was positive another borderline and the last negative (Table 3). These readings indicate an inconsistency with the procedure and that the readings overall may not be valid. A lower reading from the positives could indicate an over washing. With over washing, however, the sample readings would be lower and thus more horse samples would be negative. For the IgG ELISA tests, all of the positive controls were consistent and had borderline results (Table 3). This may indicate why there was a higher percent of borderline results than positive samples in the IgG ELISA tests. Out of 68 horse samples only two horses had negative readings for both IgG and IgM (Table 5). This value seems low; it was expected that more negative readings would be seen from the unvaccinated horses since the exposure rate was low in the area. The IgG had a consistent reading for the positive control and had a higher percentage of negative readings (Table 4). This indicates that the plates were consistently washed. However, the percentage of borderline and positive reads for the IgG were still higher than expected and thus these ELISA tests may not be as reliable.

In Maraghi's *et al.* study on Toxoplasma in Iran, IgM and IgG ELISA tests were used to identify anti-Toxoplasma antibodies; their tests had 13% borderline readings for IgG and 14% borderline readings for IgM (2013). In another study, looking at the effectiveness of IgG and IgM ELISAs on identifying WNV infected patients, they found that IgG ELISAs had a sensitivity of 97.6% and a specificity of 92.1%, the IgM ELISA's had a 99.3% sensitivity

and specificity but also had 15 false positives (Hogrefe *et al.*, 2004). In the same study, they had 7% of their samples with borderline readings for IgG and 20% for IgM (Hogrefe *et al.*, 2004). Lastly, Sueur *et al.* also looked at the sensitivity of IgG and IgM ELISA on 54-kDa protein and found that IgG and IgM antibodies had a sensitivity of 66.7% and a specificity of 79.2% when compared to PCR detection (2005). The ELISA test is a non- invasive method for testing horses for WNV. However, because of the false positives and the borderline results, it may not be the most accurate test.

For further studies, I would suggest retesting a number, if not all of the 68 samples and to use an automatic washer to eliminate human error in inconsistent washing. Due to the contamination of the vaccine generated antibodies in the exposure test, a different ELISA kit may need to be run in order to calculate an infection rate for horses. However, overall this study showed that horse surveillance may be needed as in addition to mosquito surveillance since positives for WNV occurred with no positive mosquito pool detected in that area.

no correlation between *Cx. tarsalis* population size and MIR (Neweton, 2013; Marcelli, 2012; and Hokit *etal.;* 2013). As a result, the changes in the infection rate between the years are because of environmental factors. In the previous summer, Montana had a higher average temperature (Newton, 2013) than 2014. The infection rate shows a correlation with a higher average temperature (Hokit *et al.* 2013, and Newton, 2013).

The ELISA tests overall had unclear results. Over half the samples had one or more borderline results. Twenty seven horses were positive for one or more of the antibodies, but they could not be concluded as exposed or vaccinated due the absence of a vaccination history. Only one out of the 20 known vaccinated horses was positive for both IgG and IgM antibodies. One sample was negative for IgM and borderline for IgG antibodies. Lastly, two unvaccinated horses were positive for IgM and borderline IgG antibodies. (Table 5)

The large number of borderline samples may indicate the vaccines carry multiple antigens, our procedure needs refining, or even the ELISA test itself is inconsistent. For vaccinated horses and possibly some without records, the inconsistent data may be due to contamination of the vaccine with the exposure tests. Little was known about which parts of the virus were used to make the vaccines; it is likely the vaccines contained the virus envelope and/or NS1 protein. If the vaccines used both parts of the virus, then the antibodies would appear in both tests for exposure and for vaccination. If contamination was present in the vaccines, horses would not be exposed to equal quantities of antigen leading to an inconsistent and ambiguous data set within vaccinated horses. Many of the positives and borderline results could have been due to inconsistent washing. Due to the high percentage of borderlines and a low number of negative samples, this data overall would not be considered reliable. All blood samples were performed on healthy animals not demonstrating infection

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Appendix 1

Table 3 The sites and samples where *Cx. tarsalis* were collected and tested from in Montana.

County	Location	Date Sampled	Sample Number	Estimated No. of Non-target Spp	No. of Cx tarsalis	MTPHL Specimen ID	WNV Result	SLE/WEE Results
Beaverhead	Glen	6/30/2014	1073	687	1	887818	ND	ND
Jefferson	Boulder Cem	6/30/2014	1074	3000	0			
Jefferson	Boulder Fairground	6/30/2014	1075	832	3	887819	ND	ND
Silver Bow	Butte Central	6/30/2014	1076	46	3	887820	ND	ND
Beaverhead	Salmon	6/30/2014	1077	1187	4	887821	ND	ND
Lewis and Clark	Police Academy	6/30/2014	1078	354	1	887822	ND	ND
Lewis and Clark	Regulating Reservoir	6/30/2014	1079	1410	14	887823	ND	ND
Jefferson	Boulder Fairground	7/7/2014	1080	699	34	887843	ND	ND
Silver Bow	Butte Central	7/7/2014	1081	144	31	887844	ND	ND
Beaverhead	Glen	7/7/2014	1082	769	14	887845	ND	ND
Lewis and Clark	Police Academy	7/7/2014	1083	381	6	887846	ND	ND
Lewis and Clark	Regulating Reservoir	7/7/2014	1084	8600	0	887829	ND	ND
Lake	Nine Pipes 1	6/30/2014	1090	668	5	887847	ND	ND
Lake	Nine Pipes 2	6/30/2014	1091	279	13	887848	ND	ND
Lake	Nine Pipes 1	7/7/2014	1092	168	36	887849	ND	ND
Lake	Nine Pipes 2	7/7/2014	1093	117	10	887850	ND	ND
Powell	Avon	7/7/2014	1094	791	5	887851	ND	ND
Granite	Bearmouth	7/7/2014	1095	1500	9	887852	ND	ND
Missoula	Council Grove	7/7/2014	1096	2066	58	887853	ND	ND
Blaine	Liz's Farm	7/7/2014	1097	1358	7	887855	ND	ND
Hill	Havre	7/7/2014	1098			887857	ND	ND
Wibaux	Wibaux	7/6/2014	1099	NA	33	887833	ND	ND
Gallatin	Three Forks	6/22/2014	1100	NA	23	887839	ND	ND
Ravalli	Stevensville	7/12/2014	1101	1804	16	887858	ND	ND
Lewis and Clark	Police Academy	7/14/2014	1102	959	21	887859	ND	ND
Lewis and Clark	Regulating Reservoir	7/14/2014	1103	28,000	1	887860	ND	ND
Beaverhead	Glen	7/14/2014	1104	258	16	887861	ND	ND
Jefferson	Boulder Fairgrounds	7/14/2014	1105	30	3	887862	ND	ND
Silver Bow	Butte Central	7/14/2014	1106	102	42	887863	ND	ND

Blaine	Liz's Farm	7/14/2014	1107a	1661	120	887864	ND	ND
			1107b			887865	ND	ND
			1107c			887866	ND	ND
Lake	Nine Pipes 1	7/14/2014	1108	66	18	887867	ND	ND
Lake	Nine Pipes 2	7/14/2014	1109	59	15	887868	ND	ND
Hill	Havre	7/14/2014	1110	231	20	887869	ND	ND
Flathead	Caroline	7/13/2014	1111	129	8	887870	ND	ND
Flathead	Sanders	7/12/2014	1112	1678	12	887871	ND	ND
Flathead	Trumble Creek Rd	7/13/2014	1113	573	5	887872	ND	ND
Broadwater	Canyon Ferry 1	7/9/2014	1114a	NA	75	887873	ND	ND
			1114b			887874	ND	ND
			1114c			887875	ND	ND
			1114d		0	887876	ND	ND
Gallatin	Three Forks	7/6/2014	1115a	NA	104	887877	ND	ND
			1115b		0	887878	ND	ND
			1115c			887879	ND	ND
Lewis and Clark	Police Academy	7/21/2014	1116	953	49	887894	ND	ND
Lewis and Clark	Regulating Reservoir	7/21/2014	1117	34,200	2	887895	ND	ND
Beaverhead	Glen	7/21/2014	1118	485	22	887896	ND	ND
Silver Bow	Butte Central	7/21/2014	1119A	97	50	887897	ND	ND
Silver Bow	Butte Central	7/21/2014	1119B		47	887898	ND	ND
Jefferson	Boulder Fairgrounds	7/21/2014	1120	153	26	887899	ND	ND
Blaine	Liz's Farm	7/21/2014	1121	558	22	887900	ND	ND
Hill	Havre	7/21/2014	1122	73	13	887926	ND	ND
Yellowstone	Indian Creek Rd.	7/21/2014	1123	5	1	887927	ND	ND
Prairie	Terry	7/16/2014	1124A		50	887901	ND	ND
			1124B		50	887902	ND	ND
			1124C		13	887903	ND	ND
Powder river	Biddle	7/15/2014	1125		36	887904	ND	ND
Gallatin	Three Forks	7/14/2014	1126A		50	887905	ND	ND
			1126B		50	887907	ND	ND
			1126D		50	887906	ND	ND
			1126E		50	887908	ND	ND
			1126G		50	887909	ND	ND
			1126H		50	887910	ND	ND
			1126I		46	887915	ND	ND
			1126J		30	887916	ND	ND

Wibaux	Wibaux	7/21/2014	1127A		50	887911	DETECTED	ND
			1127B		50	887912	ND	ND
			1127C		50	887913	ND	ND
			1127D		25	887914	ND	ND
Lake	Nine Pipes 1	7/22/2014	1128A	505	50	887928	ND	ND
			1128B		32	887929	ND	ND
Lake	Nine Pipes 2	7/22/2014	1129	53	12	887930	ND	ND
Lewis and Clark	Regulating Reservoir	7/28/2014	1130	17,500	1	887931	ND	ND
Lewis and Clark	Police Academy	7/28/2014	1131	449	24	887932	ND	ND
Powell	Gold Creek	7/28/2014	1132	1071	24	887933	ND	ND
Granite	Drummond	7/28/2014	1133	1042	49	887934	ND	ND
Missoula	Council Grove	7/28/2014	1134	326	3	887935	ND	ND
Ravalli	Poker Joe	7/28/2014	1135	570	1	887936	ND	ND
Lake	Nine Pipes 2	7/28/2014	1136	68	7	887937	ND	ND
Blaine	Liz's Farm	7/28/2014	1137A	514	50	887938	ND	ND
			1137B		50	887939	ND	ND
			1137C		7	887940	ND	ND
Beaverhead	Glen	7/29/2014	1138	80	15	887941	ND	ND
Jefferson	Boulder Fairgrounds	7/29/2014	1139	440	16	887942	ND	ND
Silver Bow	Butte Central	7/29/2014	1140	106	56	887943	ND	ND
Hill	Havre	7/29/2014	1141A	257	50	887944	ND	ND
			1141B		50	887945	ND	ND
			1141C		23	887946	ND	ND
Prairie	Terry	7/28/2014	1142A	297	50	887947	ND	ND
			1142B		50	887948	ND	ND
			1142C		50	887949	ND	ND
			1142D		47	887950	ND	ND
Powder River	Biddle	7/28/2014	1143	16	11	887951	ND	ND
Gallatin	Clarkson	7/20/2014	1144A	305	50	887952	ND	ND
			1144B		50	887953	ND	ND
			1144C		36	887954	ND	ND
Gallatin	Parks	7/20/2014	1145A		50	887955	ND	ND
			1145B		50	887956	ND	ND
Gallatin	Wilcox	7/20/2014	1146	19	18	887957	ND	ND
Gallatin	Dewey	7/20/2014	1147		42	887958	ND	ND
Gallatin	Augney	7/20/2014	1148	53	37	887959	ND	ND
Gallatin	Three Forks	7/20/2014	1149A		50	887960	ND	ND

			1149B		50	887961	ND	ND
			1149C		36	887962	ND	ND
Gallatin	Dewey	7/27/2014	1150		41	887963	ND	ND
Gallatin	Augney	7/27/2014	1151		44	887964	ND	ND
Gallatin	Parks	7/27/2014	1152		50	887965	ND	ND
Gallatin	Three Forks	7/27/2014	1153		50	887966	ND	ND
Gallatin	Wilcox	7/27/2014	1154		28	887967	ND	ND
Lake	RMH	7/29/2014	1155	232	42	887968	ND	ND
			1155A			887985	ND	ND
Custer	Miles City	7/28/2014	1156A	216	50	887986	ND	ND
			1156B		15	887987	ND	ND
Lewis and Clark	Police Academy	8/4/2014	1157A	621	22	887988	ND	ND
Lewis and Clark	Regulating Reservoir	8/4/2014	1158	5620	1	887989	ND	ND
Jefferson	Boulder Fairground	8/4/2014	1159	112	32	887990	ND	ND
Silver Bow	Butte Central	8/4/2014	1160	42	10	887991	ND	ND
Beaverhead	Glen	8/4/2014	1161	26	6	887992	ND	ND
Blaine	Liz's Farm	7/30/2014	1162A	500	50	887993	ND	ND
			1162B		50	887994	ND	ND
			1162C		36	887995	ND	ND
Lake	Nine Pipes 1	8/4/2014	1163	556	24	887996	ND	ND
Lake	Nine Pipes 2	8/4/2014	1164	66	4	887997	ND	ND
Lake	RMH	8/4/2014	1165	49	5	887998	ND	ND
Hill	Havre/ Buffalo Jump	8/4/2014	1166A			992019	ND	
			1166B			992020	ND	
			1166C			992021	ND	
Wibaux	Wibaux	8/3/2014	1167A		22	887999	ND	ND
Gallatin	Augney	8/4/2014	1168		24	992000	ND	ND
Gallatin	City	8/4/2014	1169A		29	992001	ND	ND
Gallatin	Clarkson	8/4/2014	1170A		50	992002	ND	ND
			1170B		7	992003	ND	ND
Gallatin	Dewey	8/3/2014	1171A		25	992004	ND	ND
Gallatin	Parks	8/3/2014	1172A		50	992005	ND	ND
			1172B		33	992006	ND	ND
Gallatin	Wilcox	8/3/2014	1173		14	992007	ND	ND
Lake	RMH	8/5/2014	1174	97	4	992022	ND	
Flathead	Nucleus	8/5/2014	1175	180	0			
Flathead	Lowes	8/5/2014	1176	350	10	992023	ND	
Flathead	Amdahl	8/5/2014	1177	946	0			

Flathead	Lawrence Park	8/5/2014	1178	239	4	992024	ND	
Ravalli	Poker Joe	8/11/2014	1179	794	3	992025	ND	
Missoula	Council Grove	8/11/2014	1180	296	8	992026	ND	
Granite	Drummond	8/11/2014	1181	262	10	992027	ND	
Powell	Gold Creek	8/11/2014	1182	904	11	992028	ND	
Yellowstone	East Riverfront	8/11/2014	1183	183	13	992029	ND	
Yellowstone	West Riverfront	8/11/2014	1184	573	2	992030	ND	
Big Horn	Lodge Grass	7/2/2014	1185	6585	3	992031	ND	
Big Horn	Wyola	8/5/2014	1186	224	1	992032	ND	
Big Horn	Lodge Grass	8/5/2014	1187	183	23	992033	ND	
Big Horn	Pryor	8/5/2014	1188	65	9	992034	ND	
Big Horn	Ft Smith	8/5/2014	1189	163	47	992035	DETECTED	
Lewis and Clark	Police Academy	8/12/2014	1190	109	0			
Lewis and Clark	Regulating Reservoir	8/12/2014	1191	7000	8	992036	ND	
Blaine	Liz's Farm	8/11/2014	1192A	1593	426	992037	ND	
			1192B			992038	DETECTED	
			1192C			992039	ND	
			1192D			992040	ND	
			1192E			992041	ND	
			1192F			992042	ND	
			1192G			992043	ND	
			1192H			992044	ND	
			1192I			992045	ND	
Lake	RH site	8/11/2014	1193	124	9	992046	ND	
Lake	RHM	8/11/2014	1194	162	22	992047	ND	
Lake	Nine Pipes 1	8/11/2014	1195	148	16	992048	ND	
Lake	Nine Pipes 2	8/11/2014	1196	134	8	992049	ND	
Prairie	Terry	8/11/2014	1197A		337	992050	ND	
			1197B			992051	ND	
			1197C			992052	ND	
			1197D			992053	ND	
			1197E			992054	ND	
			1197F			992055	ND	
			1197G			992056	ND	
Hill	Havre	8/13/2014	1198A	812	50	992095	ND	ND
			1198B		37	992070	ND	ND

Jefferson	Boulder Fairground	8/18/2014	1199	36	0			
Silver Bow	Butte Central	8/18/2014	1200	9	1	992071	ND	ND
Beaverhead	Glen	8/18/2014	1201	375	6	992072	ND	ND
Lewis and Clark	Police Academy	8/20/2014	1202	230	1	992073	ND	ND
Lewis and Clark	Reg Reservoir	8/20/2014	1203	3015	1	992074	ND	ND
Hill	Havre	8/20/2014	1204	124	3	992075	ND	ND
Blaine	Liz's Farm	8/18/2014	1205	730	39	992076	DETECTED	ND
Gallatin	Dewey	8/10/2014	1206		23	992077	ND	ND
Gallatin	Three Forks	8/10/2014	1207		12	992079	ND	ND
Gallatin	Clarkston	8/10/2014	1208		46	992080	ND	ND
Gallatin	Augney	8/10/2014	1209		5	992081	ND	ND
Gallatin	Parks	8/10/2014	1210		11	992082	ND	ND
Gallatin	Wilcox	8/10/2014	1211		11	992083	ND	ND
Lewis and Clark	Police Academy	8/26/2014	1212	25	0			
Lewis and Clark	Reg Reservoir	8/26/2014	1213	551	1	992096	ND	ND
Ravalli	Poker Joe	8/25/2014	1214	0	0			
Granite	Drummond	8/25/2014	1215	149	0			
Powell	Gold Creek	8/25/2014	1216	137	0			
Missoula	Council Grove	8/25/2014	1217	30	0			
Blaine	Liz's Farm	8/26/2014	1218A	137	109	992097	ND	ND
Blaine	Liz's Farm	8/26/2014	1218B	137	109	992098	ND	ND
Blaine	Liz's Farm	8/26/2014	1218C	137	109	992099	DETECTED	ND
Hill	Havre	8/27/2014	1219	406	48	992100	ND	ND
Gallatin	Augney	8/17/2014	1220		4	992101	ND	ND
Gallatin	Three Forks	8/17/2014	1221		14	992102	ND	ND
Gallatin	Clarkston	8/17/2014	1222		16	992103	ND	ND
Gallatin	Dewey	8/17/2014	1223		16	992104	ND	ND
Gallatin	Parks	8/17/2014	1224		59	992105	ND	ND
Gallatin	Wilcox	8/17/2014	1225		3	992106	ND	ND
Wibaux	Wibaux	8/19/2014	1226		2	992107	ND	ND

Appendix 2

Table 4: The sites and samples where *Cx. pipiens* were collected and tested from in Montana

County	Location	Date Sampled	Sample Number	No. of Cx pipiens	MTPHL Specimen ID	WNV Results	SLE/ WEE Results	Date Tested
Beaverhead	Glen	6/30/2014	1073	0				
Jefferson	Boulder Cem	6/30/2014	1074	0				
Jefferson	Boulder Fairgrounds	6/30/2014	1075	0				
Silver Bow	Butte Central	6/30/2014	1076	0				
Beaverhead	Salmon	6/30/2014	1077	0				
Lewis and Clark	Police Academy	6/30/2014	1078	0				
Lewis and Clark	Regulating Reservoir	6/30/2014	1079	0				
Jefferson	Boulder Fairgrounds	7/7/2014	1080	2				
Silver Bow	Butte Central	7/7/2014	1081	0				
Beaverhead	Glen	7/7/2014	1082	0				
Lewis and Clark	Police Academy	7/7/2014	1083	0				
Lewis and Clark	Regulating Reservoir	7/7/2014	1084	0				
Lake	Nine Pipes I	6/30/2014	1090	0				
Lake	Nine Pipes II	6/30/2014	1091	0				
Lake	Nine Pipes I	7/7/2014	1092	0				
Lake	Nine Pipes II	7/7/2014	1093	0				
Powell	Avon	7/7/2014	1094	0				
Granite	Bearmouth	7/7/2014	1095	1				
Missoula	Council Grove	7/7/2014	1096	0				
Blaine	Liz's Farm	7/7/2014	1097	0				
Hill	Havre	7/7/2014	1098	0				
Wibaux	Wibaux	7/6/2014	1099	0				
Gallatin	Three Forks	6/22/2014	1100	0				
Ravalli	Stevensville	7/12/2014	1101	0				
Lewis and Clark	Police Academy	7/14/2014	1102	0				
Lewis and Clark	Regulating Reservoir	7/14/2014	1103	0				
Beaverhead	Glen	7/14/2014	1104	0				
Jefferson	Boulder Fairgrounds	7/14/2014	1105	0				
Silver Bow	Butte Central	7/14/2014	1106	0				
Blaine	Liz's Farm	7/14/2014	1107	0				
Lake	Nine Pipes I	7/14/2014	1108	0				
Lake	Nine Pipes II	7/14/2014	1109	0				

Hill	Havre	7/14/2014	1110	0				
Lake	Caroline	7/13/2014	1111	0				
Lake	Sanders	7/12/2014	1112	0				
Lake	Trumble Creek Rd	7/13/2014	1113	0				
Broadwater	Canyon Ferry 1	7/9/2014	1114	0				
Gallatin	Three Forks	7/6/2014	1115	0				
Lake	Nine Pipes I	7/22/2014	1128A	0				
Lake	Nine Pipes II	7/22/2014	1129	0				
Lewis and Clark	Regulating Reservoir	7/28/2014	1130	0				
Lewis and Clark	Police Academy	7/28/2014	1131	26	887982	ND	ND	8/1/2014
Powell	Gold Creek	7/28/2014	1132	0				
Granite	Drummond	7/28/2014	1133	2	887983	ND	ND	8/1/2014
Missoula	Council Grove	7/28/2014	1134	1	887984	ND	ND	8/1/2014
Ravalli	Poker Joe	7/28/2014	1135	0				
Lake	Nine Pipes II	7/28/2014	1136	0				
Blaine	Liz's Farm	7/28/2014	1137	0				
Beaverhead	Glen	7/29/2014	1138	0				
Jefferson	Boulder Fairgrounds	7/29/2014	1139	0				
Silver Bow	Butte Central	7/29/2014	1140	0				
Hill	Havre	7/29/2014	1141	0				
Lake	RMH	7/29/2014	1155B	20	992017	ND	ND	8/8/2014
Custer	Miles City	7/28/2014	1156	0				
Lewis and Clark	Police Academy	8/4/2014	1157B	9	992018	ND	ND	8/8/2014
Lewis and Clark	Regulating Reservoir	8/4/2014	1158	0				
Jefferson	Boulder Fairgrounds	8/4/2014	1159	0				
Silver Bow	Butte Central	8/4/2014	1160	0				
Beaverhead	Glen	8/4/2014	1161	0				
Blaine	Liz's Farm	7/30/2014	1162	0				
Lake	Nine Pipes I	8/4/2014	1163	0				
Lake	Nine Pipes II	8/4/2014	1164	0				
Lake	RMH	8/4/2014	1165	0				
Lake	RMH	8/5/2014	1174	0				
Flathead	Nucleus	8/5/2014	1175	0				
Flathead	Lowes	8/5/2014	1176	0				
Flathead	Amdahl	8/5/2014	1177	0				
Flathead	Lawrence Park	8/5/2014	1178	0				
Ravalli	Poker Joe	8/11/2014	1179	0				
Missoula	Council Grove	8/11/2014	1180	0				

Granite	Drummond	8/11/2014	1181	3	992064	ND		8/14/2014
Powell	Gold Creek	8/11/2014	1182	2	992065	ND		8/14/2014
Yellowstone	East Riverfront	8/11/2014	1183	0				
Yellowstone	West Riverfront	8/11/2014	1184	0				
Big Horn	Lodge Grass	7/2/2014	1185	0				
Big Horn	Wyola	8/5/2014	1186	0				
Big Horn	Lodge Grass	8/5/2014	1187	0				
Big Horn	Pryor	8/5/2014	1188	1				
Big Horn	Ft Smith	8/5/2014	1189	0	992066	ND		8/14/2014
Lewis and Clark	Police Academy	8/12/2014	1190	0				
Lewis and Clark	Regulating Reservoir	8/12/2014	1191	1	992067	ND		8/14/2014
Blaine	Liz's Farm	8/11/2014	1192	1	992068	ND		8/14/2014
Lake	RH site	8/11/2014	1193	0				
Lake	RHM	8/11/2014	1194	0				
Lake	Nine Pipes I	8/11/2014	1195	0				
Lake	Nine Pipes II	8/11/2014	1196	0				
Prairie	Terry	8/11/2014	1197	0				
Lewis and Clark	Police Academy	8/26/2014	1212	0				
Lewis and Clark	Regulating Reservoir	8/26/2014	1213	1	992114	ND	ND	8/29/2014
Ravalli	Poker Joe	8/25/2014	1214	0				
Granite	Drummond	8/25/2014	1215	0				
Powell	Gold Creek	8/25/2014	1216	1	992115	ND	ND	8/29/2014
Missoula	Council Grove	8/25/2014	1217	0				
Blaine	Liz's Farm	8/25/2014	1218	2	992116	ND	ND	8/29/2014
Hill	Havre	8/27/2014	1219	35	992110	ND	ND	8/29/2014
Gallatin	Augney	8/17/2014	1220	0				
Gallatin	Three Forks	8/17/2014	1221	0				
Gallatin	Clarkston	8/17/2014	1222	0				
Gallatin	Dewey	8/17/2014	1223	0				
Gallatin	Parks	8/17/2014	1224	0				
Gallatin	Wilcox	8/17/2014	1225	0				
Wibaux	Wibaux	8/19/2014	1226	0				