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West Nile Virus Presence and Blood-Feeding Behavior of *Culex tarsalis* in Wildlife Refuges and Management Areas in Montana

Honors Thesis
Carroll College Department of Natural Sciences
Helena, Montana

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April 12, 2011

This thesis for honors recognition has been approved for the Department of Natural Sciences by:

4-6-11

Dr. Sam Alvey, Director

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4/6/11

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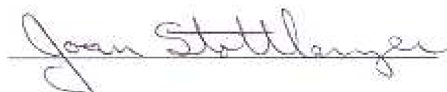
Date



4-06-11

Joan Stottlemyer, Reader

Date

Acknowledgements

I would like to thank Dr. Alvey, Dr. Geiger, and Dr. Hokit for their guidance and assistance throughout this project. I would also like to thank Dr. Shields for all of his advice, and Dr. Joan Stottlemyer for her comments. In addition I would like to thank Katie Semmens and Joe Maricelli for their assistance with virus and blood extractions, Billy McElroy and Brian Murphy for their time spent trapping blood-fed mosquitoes, and each member of the research team for his or her time spent sorting mosquitoes. This research would not have been possible without Dr. Greg Johnson and our many cooperators throughout the state. Finally, I would like to thank INBRE and the Murdock Charitable Trust for providing financial support for this project, and the Wiegand Undergraduate Research Lab and Carroll College for providing the laboratory space.

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Abstract

West Nile virus (WNV) has been documented across the state of Montana since 2002. Humans, other mammals, and birds have been affected by this virus in a heterogeneous manner throughout the state. Correlating the feeding patterns of *Culex tarsalis*, the principal WNV vector in the Western United States, with the presence of virus at Nine Pipe, Freezeout, Benton, and Bowdoin wildlife refuges and management areas allowed for analysis of the link between competent avian hosts and the presence of WNV. Further, identification of avian species present in *Cx. tarsalis* blood-meals allowed for the analysis of the correlation between relative abundance of avian species and their incidence in blood-meals. Presence of WNV RNA in mosquito pools was measured using RT-PCR and TaqMan assay. Mitochondrial cytochrome b and cytochrome c oxidase I gene sequence analysis of DNA extracted from individual blood-fed *Cx. tarsalis* mosquitoes was used for blood-meal identification by the comparison of sequences of both the cytochrome b and cytochrome oxidase I gene fragments with the GenBank DNA database. The combination of WNV detection and blood-meal analysis can be used to better understand the temporal relationship of viral presence or absence and seasonal feeding patterns of the WNV vector mosquito *Cx. tarsalis*.

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Introduction

West Nile virus (WNV), initially characterized in Uganda in 1937 (Centers for Disease Control, CDC 1, 2004) was first documented in the Eastern United States in 1999 (CDC 2, 2010). The virus spread rapidly across the country reaching Montana in 2002 (Sugumaran et al., 2009), and by 2004 WNV was present in all 48 contiguous United States (Brault, 2009). The virus has caused illness and death among human, bird, and livestock populations around the country; since its arrival there have been over 29,000 documented human cases (CDC 3, 2010) and 326 bird species have tested positive for WNV (CDC 4, 2009).

WNV is a positive-strand RNA virus of the family Flaviviridae (genus Flavivirus), a family that contains members such as Saint Lewis Encephalitis virus, which is also endemic to the United States, Japanese Encephalitis virus, and Yellow Fever virus (Savage et al., 2007). Like many emerging diseases, WNV is a zoonotic disease, an animal disease that can be transmitted to humans. WNV, the most geographically widespread of the arthropod-borne viruses (arbovirus), affects a small number of birds in a population, but is consistently maintained among the avian population and is thus sustained in an enzootic cycle (Swaddle and Calos, 2008). WNV is transmitted among competent bird hosts by mosquito vectors (Goddard et al., 2002; Kramer et al., 2008). A female mosquito must take a blood-meal in order for egg development to occur. Blood-fed females often seek dark resting places while they digest their blood-meals (Burkett-Cadena, 2009). Eggs reach maturity two to five days after blood feeding, and are subsequently laid (Johnson et al., 2009). Since mosquitoes have a lifespan of several weeks, an individual female is likely to complete several egg laying cycles (Johnson et

al., 2009). A new blood-meal is required for each batch of eggs (Johnson et al., 2009). If a competent mosquito feeds on a host infected with WNV, the virus amplifies in the insect's stomach and eventually becomes disseminated throughout the body. Once the infection becomes widespread, the virus enters the mosquito's salivary glands and can be transmitted to subsequent blood-meal sources (Johnson et al., 2009). Though blood-feeding mosquitoes are the main enzootic and epidemic hosts of WNV (Molaei, 2006), of the 174 species in the United States, only 64 species produce a high enough viremia, the level of virus present in the blood, to transmit WNV (American Mosquito Control Association, 2010; CDC 5, 2009).

In the Western United States, *Culex tarsalis* is the principal vector of WNV (Brault, 2009). *Cx. tarsalis* serves as a competent vector capable of carrying the virus among birds, and also as a bridge vector transmitting the virus from birds to humans, horses, or other dead-end hosts. Though susceptible to infection by WNV, these mammalian species are termed dead-end hosts because they do not produce high enough viremia levels to transmit WNV to a mosquito that bites following the initial infection (Kent et al. 2009). *Cx. tarsalis* has a consistent preference for avian blood-meals, but as the summer progresses, this species takes an increased number of blood-meals from mammals (Hak Lee et al., 2002). Paralleling the increase in mammalian blood-meals is an increase in the number of *Cx. tarsalis* in late July and August as a result of warmer temperatures (Tempelis et al., 1965). The change in feeding behavior is not due to a change in blood-meal preference, but rather is a result of increasing numbers of *Cx. tarsalis* and decreasing numbers of avian hosts (Kent et al., 2009). The change in total

number of birds results in avian species' playing varying roles in WNV transmission throughout the summer months (Kent et al., 2009).

In the Northwestern United States, the largest proportion of blood-meals in the early summer come from the American Robin (*Turdus migratorius*) and dove species, including Mourning Doves (*Zenaida macroura*) (Kent et al., 2009). These species are also the primary amplifying hosts for WNV in June and July (Kent et al., 2009). House Sparrows (*Passer domesticus*) join doves, and other passerines as the major blood-meal source at the end of the summer and also serve as the primary amplifying source for WNV in August (Kent et al., 2009). The number of blood-meals from doves increases through the summer, possibly as a result of nestlings which are more vulnerable to infection by WNV than are adult birds (Kent et al., 2009). Along with identifying these high-risk bird species, the study by Kent et al. (2009) also supports the idea that communally roosting birds, such as the House Sparrow, may be reservoirs for the virus and the source of infection for many mosquitoes. A mosquito that obtains the virus from an infected bird may spread the disease to other easily accessible members of the roost; this may have a significant impact on the transmission of WNV. Thus, the shift in feeding behavior from avian to mammalian species allows for the amplification of the virus among competent avian hosts in the early summer followed by virus transmittance to livestock and humans in the later summer months resulting in increased infection rates.

Despite their inability to transmit the virus, incidental hosts such as humans and livestock can be affected by WNV (CDC 6, 2006). Results of human infection depend on the level of virus present in the blood, and infection may also be dependent on genetic factors (Perelygin et al., 2002). The 20% of humans who are susceptible to the virus

experience symptoms such as headaches, fever, paralysis, and nerve damage (CDC 6, 2006). The effects of WNV on horses include fever and blindness, and result in death in roughly one third of infected individuals (CDC 7, 2010). Birds also experience symptoms of WNV infection including ataxia, inability to move, and often death (Johnson et al., 2010). In contrast to humans and horses, birds can serve as competent hosts for WNV (Molaei et al., 2006). Passerines including the Blue Jay (*Cyanocitta cristata*), Common Grackle (*Quiscalus quiscula*), House Finch (*Carpodacus mexicanus*), House Sparrow, and the American Crow (*Corvus brachyrhynchos*) are the most competent reservoir species for WNV under laboratory conditions (Komar, 2002). These avian species meet several qualifications for virus transmittance including the following: sufficient viremia, maintenance of blood virus levels for an adequate amount of time to infect an arthropod vector, and sufficient contact with competent mosquito vectors (Savage et al., 2007; Marra et al., 2004).

The distribution of WNV is heterogeneous throughout the state of Montana (CDC 8, 2010). The role that reservoir-competent birds play and extent to which these bird species influence virus distribution need to be examined in order to better understand WNV. In the present study, the feeding patterns of *Cx. tarsalis* were examined through blood-meal analysis by polymerase chain reaction (PCR) amplification of the cytochrome b (CYTB) and cytochrome c oxidase 1 (COI) genes. The CYTB gene has been applied to successfully identify organisms at the subspecies level (Molaei et al., 2006). Amplified DNA from blood-meals was sequenced and compared with the GenBank DNA database to identify the species of bird fed on by *Cx. tarsalis*. The most abundant birds across the state of Montana were compared with their incidence of identification in *Cx. tarsalis*

blood-meals. To determine the role of these bird species in WNV presence and transmission, the most abundant bird species at four wildlife refuges in Montana—Benton and Freezeout lakes, Bowdoin, and Ninepipe—were compared to a list of bird species that have been killed by WNV (CDC 4, 2009). These locations were chosen for study because of their high density of birds and riparian habitat which is preferred by *Cx. tarsalis*. Viral detection was performed using real-time reverse transcription polymerase chain reaction (RT-PCR) and TaqMan assay. Due to the difficulty of trapping and detecting virus in live birds, a less direct but equally effective detection approach using pools of *Cx. tarsalis* was used. The hypothesis of this study is that the most abundant bird species will be the most commonly identified species in the blood-meals of *Cx. tarsalis*. This analysis will provide information about the feeding habits of *Cx. tarsalis* and the role that avian species play in virus transmission.

Materials and Methods

Mosquito Collection

Mosquitoes were collected from wildlife refuges across the state of Montana from June to September of 2009 and 2010. CDC light traps baited with CO_2 from tanks or dry ice were run from dusk until dawn one night each week. The next morning the mosquitoes were placed on ice and transported back to the lab where they were frozen at -20° C for two days; this resulted in death of the mosquito and preservation of the virus. Following freezing, mosquitoes were sorted on ice blocks to maintain the cold chain. Morphological characteristics as defined in the key by Darsie and Ward (2005) were used to isolate *Cx. tarsalis*.

The majority of the blood-fed *Cx. tarsalis* analyzed were found in the CDC light traps; however, another method of trapping was used and yielded several blood-fed *Cx. tarsalis*. The inside surfaces of fiber pots were spray painted black and placed around the wildlife refuges in groups of four, each pot opening toward a different direction. The pots were left in their positions for the duration of the summer and were checked one morning each week. Any mosquitoes found inside were collected using C-Cell MAG-LITE aspirator (BioQuip Products, Rancho Dominguez, CA, USA). These mosquitoes were placed on ice for transport back to the lab and subsequently frozen at -20°C.

RNA Extraction

Pools of 50 or fewer female *Cx. tarsalis* from a particular trap site and night were placed in 1.5 mL Lysing Matrix tubes with a ceramic bead (MP Biomedicals, Solon, OH, USA). For mosquito pools of less than 10 mosquitoes, 600 µL RNA Later (Qiagen,

Austin, TX, USA) and 300 µL of BA-1 homogenization buffer (Lanciotti et al., 2000) were added to the tube. Mosquito pools of 10 to 50 mosquitoes received 1000 µL of RNA Later and 500 µL of BA-1 homogenization buffer. The mosquito pools were then homogenized using a 115V FastPrep FP120 (Thermo Fisher Scientific Inc., Waltham, MA, USA), run at speed five for 30 seconds.

Following homogenization 300 µL of homogenate were removed and used for the RNA extraction protocol, while the remainder was stored at -80° C. RNA extractions from mosquitoes trapped during the summer of 2009 were performed according to the manufacturer's instructions for the QIAamp Fibrous Tissue RNA Kit (Qiagen Inc, Valencia, CA, USA). Mosquito pools from the summer of 2010 were extracted with a QIAcube (Qiagen, Austin, TX, USA) using the QIAamp Fibrous Tissue protocol and reagents (Qiagen Inc, Valencia, CA, USA). Extractions were performed by the QIAcube in order to standardize the extraction protocol and to decrease the chance of contamination. Upon completion of the extraction, the eluent was split into 10 µL aliquots which were stored at -80°C.

RNA was also extracted from 140 µL NATtrol WNV (ZeptoMetrix Corporation, Buffalo, NY, USA) following the manufacturer's directions included with the QIAamp Viral RNA Mini Kit (Qiagen Inc, Valencia, CA, USA). A 1:10 dilution of this extracted RNA was used in each group of PCR reactions as a positive control.

Real-time RT-PCR and TaqMan Assay

Real-time RT-PCR and TaqMan assay were used to detect the presence of WNV. Two primer sets and their corresponding probes were used. Each probe contained the FAM reporter dye at the 5' end and a TAMRA quencher at the 3' end (Lanciotti et al.,

2000; Table 1). The total reaction volume was 50 µL, and contained 5 µL of template RNA, 1 µL of 50 pmol of each the forward and reverse primers (Eurofins MWG Operon, Huntsville, AL, USA), 2 µL of 10 pmol probe (Eurofins MWG Operon, Huntsville, AL, USA), 1.25 µL MultiScribe and RNase Inhibitor Mix (Applied Biosystems Inc, Foster City, CA, USA), 25 µL TaqMan PCR Master Mix (Applied Biosystems Inc, Foster City, CA, USA), and 16 µL water. Each assay was run with a positive control of extracted NATtrol WNV RNA and a no-template control which contained water instead of template RNA. All samples and controls were run in duplicate. The RT-PCR conditions were 1 cycle at 48°C for 30 min, 1 cycle at 95°C for 10 min, and 55 cycles at 95°C for 15 sec followed by 60°C for 1 min.

A sample was considered positive if the cycle threshold (ct) value of 10,000 relative fluorescence units (RFU) was reached at or before 35 cycles. A sample was considered negative if neither duplicate attained the ct value of 10,000 RFU. If the sample reached 10,000 RFU after 35 cycles, or if only one of the duplicates reached the ct value, it was rerun.

Table 1. Primer sets and probes used in the RT-PCR and TaqMan assay

Set Name	Probe Sequence (5'-3')	Primer Sequence (5'-3')
WN3'NC forward	TCAGCGATCTCTCCACCAAAG	CAGACCACGCTACGGCG
WN3'NC reverse		CTAGGGCCGCGTGGG
WNENV forward	TGCCCGACCATGGGAGAAGCTC	TCAGCGATCTCTCCACCAAAG
WNENV reverse		GGGTCAGCACGTTTGTCATTG

DNA Extraction

The abdomens of blood-fed *Cx. tarsalis* were removed under a dissecting microscope using a sterile razor blade and microscope slide. The abdomens were placed in 2 mL round bottom-tubes and homogenized in DNA-zol BD solution (Molecular Research Center, Cincinnati, OH, USA) using flame-sterilized glass rods. After homogenization the DNA was isolated using the DNA-zol BD protocol (Molecular Research Center, Cincinnati, OH, USA) according to the manufacturer's recommendation with the following exceptions: 4 µL Polyacryl Carrier (Molecular Research Center, Cincinnati, OH, USA) were used in the initial lysis step, and all volumes, excluding the water in the DNA solubilization step, were halved. The extracted DNA was stored at -20° C.

Blood-meal Analysis

The blood-meal source was determined using polymerase chain reaction (PCR) amplification of two genes, mitochondrial cytochrome c oxidase 1 (COI) and cytochrome b (CYTB). Amplification of the 648 base pair COI fragment required a forward primer cocktail of 50 pmol concentrations of VF1 t1, VFid t1, and VF1i t1, and a reverse cocktail VR1 t1, VRid t1, and VR1i t1 (Ivanova et al., 2007), mixed at a 1:1:2 ratio respectively (Kent et al., 2009) (Table 2). The total reaction volume was 50 µL, consisting of 8 µL template DNA, 0.5 µL of each of the forward and reverse primer cocktails, 25 µL GoTaq Colorless Master Mix (Promega, Madison, WI, USA), and brought to volume with water. Thermocycling conditions were 1 cycle at 94°C for 1 min; 5 cycles at 94°C for 30 sec, 50°C for 40 sec, 72°C for 1 min; 35 cycles at 94°C for 30 sec,

54°C for 40 sec, 72°C for 1 min; and 72°C for 10 min (Ivanova et al., 2007). If sufficient amplification was not achieved the sample was rerun with 9 µL of template and 40 cycles instead of 35.

A 50 µL reaction volume was also used to amplify the 508 base pair avian a (CYTB) fragment. The reaction mix consisted of 2 µL template DNA, 1 µL of 50 pmol of each the forward and reverse primers (Table 2), 25 µL GoTaq Colorless Master Mix (Promega, Madison, WI, USA), and brought to volume with water. Thermocycling conditions from Molaei et al. (2006) were modified to 1 cycle at 95°C for 5 min; 36 cycles at 94°C for 30 sec, 55°C for 50 sec, 72°C for 40 sec; and 72°C for 5 min. If sufficient amplification was not achieved 3 µL of template DNA were used, and the number of cycles was increased from 36 to 41.

The DNA product from both COI and CYTB PCR reactions was visualized on a 1.2% agarose gel containing ethidium bromide with a 100-bp ladder (Promega, Madison, WI, USA). Following amplification and visualization, 10 µL of ExoSAP-IT (USB Corporation, Cleveland, OH, USA) were added to the reaction and placed in the thermocycler for 1 cycle of 37°C for 15 min and 80°C for 15 min. Following the cleanup, the amplified product was sequenced by Macrogen in South Korea. The sequences were compared with the GenBank DNA database to identify the blood-meal source.

Regression and Spearman Analysis

The relative abundance of the bird species detected in the blood-meals was determined from the Montana Natural Heritage Program's Montana Bird Distribution QQLL database by dividing the total number of records of each bird species by the total

record of all bird species classified as migratory summer breeders and year round residents. Regression analysis was performed to determine if there was a significant correlation between the relative abundance and the incidence of identification in blood-meals (Table 3). This tests the hypothesis that if mosquitoes are not exhibiting a preference for bird species, there will be an increased number of blood-meals from the most common bird species in Montana. A Spearman rank analysis was also performed to analyze the data that were not normally distributed (Table 4). The bird species prevalence across Montana was ranked from most to least abundant, and then the bird species identified in the blood-meals were ranked from most to least commonly identified. The values obtained were compared with the Spearman rank analysis statistic table.

Table 2. Primer sets used to amplify the COI and CYTB genes

Set Name	Sequence (5'-3')
VF1_t1	TGTAAAACGACGGCCAGTTCTCAACCAACCACAAAGACATTGG
VR1_t1	CAGGAAACAGCTATGACTAGACTTCTGGGTGGCCAAAGAATCA
VF1d_t1	TGTAAAACGACGGCCAGTTCTCAACCAACCACAARGAYATYGG
VR1d_t1	CAGGAAACAGCTATGACTAGACTTCTGGGTGGCCRAARAAYCA
VF1i_t1	TGTAAAACGACGGCCAGTTCTCAACCAACCAIAAIGAIATIGG
VR1i_t1	CAGGAAACAGCTATGACTAGACTTCTGGGTGICCIAAIAAICA
avian a forward	GACTGTGACAAAATCCCNTTCCA
avian a reverse	GGTCTTCATCTYHGGYTTACAAGAC

Results

In the summer of 2009, 204 trap nights with CDC light traps yielded 7,220 *Cx. tarsalis*. Of these, 12 were engorged and their blood-meal source was used to determine high-risk bird species. Of the 22 locations surveyed (Table 5), seven samples from three sites tested positive for WNV (Table 7).

During the summer of 2010, 174 trap nights yielded 4,953 *Cx. tarsalis*, five of which were engorged and used for blood-meal analysis. Of the 21 locations surveyed (Table 6), two samples from the same location tested positive for WNV (Table 7). A map of Montana detailing the results of counties tested for WNV is provided (Fig. 1). All sites testing positive for WNV had a ct value that was less than 35.

Regression analysis of the relative abundance of bird species present and the incidence of bird species identification in blood-meals resulted in a p-value of 0.78 (Table 8). As this value is much greater than 0.05, the null hypothesis that there is no correlation between the relative abundance and the incidence of bird species detected in blood-meals is accepted. Because regression analysis is based on the assumption that the data set exhibits a normal distribution, a Spearman rank analysis was also performed. The Spearman rank value obtained was 0.10. The critical value for a sample size of eight is 0.74; thus, it can be concluded that there was no significant correlation between the relative abundance of a species and its number of appearances in the blood-meals (Fig. 2).

The sample size of blood-fed *Cx. tarsalis* was small; only a combined 17 individuals were trapped during July and August of 2009 and 2010 (Table 9). The highest percentage of blood-meals came from the House Sparrow (five), followed by the

Black-crowned Night Heron (two), and the American Robin (two). No mosquitoes analyzed had mammalian-derived blood-meal sources during the month of July; however, during the month of August three mosquitoes fed on mammalian species.

Table 3. Data used for regression analysis

Bird	Number of Records	Number of Records of Bird Species /Total Records of all Birds * 1000 = Relative Abundance	Number of records of bird species/total records of bird species detected in blood-meals * 10	Proportion of blood-meals including species = Blood-Meal Incidence
House Sparrow	1547	2.505	0.2943	0.3571
American Robin	21641	35.04	4.117	0.1429
Black-crowned Night Heron	252	0.4081	0.04795	0.1429
Western Meadowlark	23474	38.01	4.466	0.07143
European Starling	3563	5.770	0.6779	0.07143
Bobolink	861	1.394	0.1638	0.07143
Sora Rail	681	1.103	0.1296	0.07143
Ruddy Duck	538	0.8712	0.1024	0.07143
Total	52557			

Table 4. Data for Spearman rank analysis

Bird Species	Rank of Records vs. Total Records	Rank of Records vs Blood-Meal Species	No. of Incidence in Blood-Meal	Blood-Meal Rank	Difference	d-Squared
House Sparrow	4	4	5	1	3	9
American Robin	2	2	2	2.5	-0.5	0.25
Black-Crowned Night Heron	8	8	2	2.5	5.5	30.25
Western Meadowlark	1	1	1	6	-5	25
European Starling	3	3	1	6	-3	9
Bobolink	5	5	1	6	-1	1
Sora Rail	6	6	1	6	0	0
Ruddy Duck	7	7	1	6	1	1
Total			14			75.5

Table 5. 2009 Sampling locations

County	Location
Blaine	Fort Belknap
Broadwater	Toston
Broadwater	Canyon Ferry Site 1
Broadwater	Canyon Ferry Site 2
Custer	Miles City
Dawson	Glendive
Flathead	2115 HWY 2
Flathead	Forest Acres
Flathead	Bigfork
Lake	Ninepipe Site 1
Lake	Ninepipe Site 2
Lewis and Clark	Lake Helena
Lewis and Clark	Scratch Gravel
Lewis and Clark	Carroll College
Missoula	Ft. Missoula
Powell	Helmville
Phillips	Bowdoin Site 1
Phillips	Bowdoin Site 2
Ravalli	Florence
Sheridan	Medicine Lake
Teton	Freezeout Site 1
Teton	Freezeout Site 2

Table 6. 2010 Sampling Locations

County	Location
Blaine	Fort Belknap
Broadwater	Canyon Ferry Site 2
Broadwater	Canyon Ferry Site 3
Broadwater	Toston
Deer Lodge	Upper Clark Fork River: Kohrs Bend
Deer Lodge	Upper Clark Fork River: Rock Creek Cattle Road
Deer Lodge	Upper Clark Fork River: Sager Lane
Deer Lodge	Warm Springs at hwy. 48
Flathead	Mountain View
Flathead	Forest Acres
Flathead	Bigfork
Lake	Ninepipe Site 1
Lake	Ninepipe SIte 2
Missoula	Ft. Missoula
Phillips	Bowdoin Site 1
Phillips	Bowdoin Site 2
Powell	Helmville
Powell	Little Blackfoot
Sheridan	Medicine Lake
Teton	Freezeout Site 1
Teton	Freezeout Site 2

Table 7. Locations testing positive for WNV

Location	County	Date	Number of Mosquitoes	Number of *Cx. tarsalis* tested
Medicine Lake Refuge	Sheridan	7/20/2009	N/A	200
Bowdoin Site 2	Phillips	7/23/2009	1042	200
Medicine Lake Big Island	Sheridan	7/23/2009	N/A	200
Fort Belknap	Blaine	7/27/2009	1033	200
Bowdoin Site 2	Phillips	7/30/2009	1035	95
Fort Belknap	Blaine	8/11/2009	2500	200
Bowdoin Site 1	Phillips	8/13/2009	2000	11
Medicine Lake BP	Sheridan	8/6/2010	N/A	650
Medicine Lake BP	Sheridan	8/11/2010	N/A	400

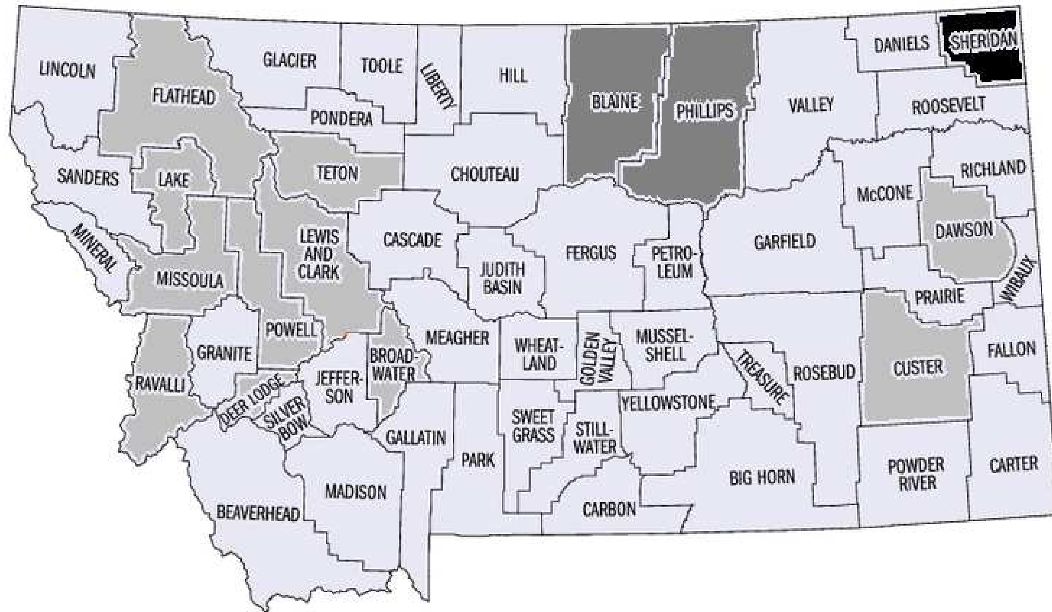

Figure 1. Map of Montana showing WNV detection results

- Counties Surveyed
- Counties Not Surveyed
- Counties Testing Positive for WNV in 2009
- Counties Testing Positive for WNV in 2009 and 2010

Table 8. Results of regression analysis

	Coefficients	Standard Error	t Stat	P-value	Lower 95%	Upper 95%	Lower 95.0%	Upper 95.0%
Intercept	0.1327	0.04607	2.880	0.02804	0.01996	0.2454	0.01996	0.2454
X Variable 1	-0.0007239	0.002500	-0.2895	0.7818	-0.006841	0.005393	-0.006841	0.005393

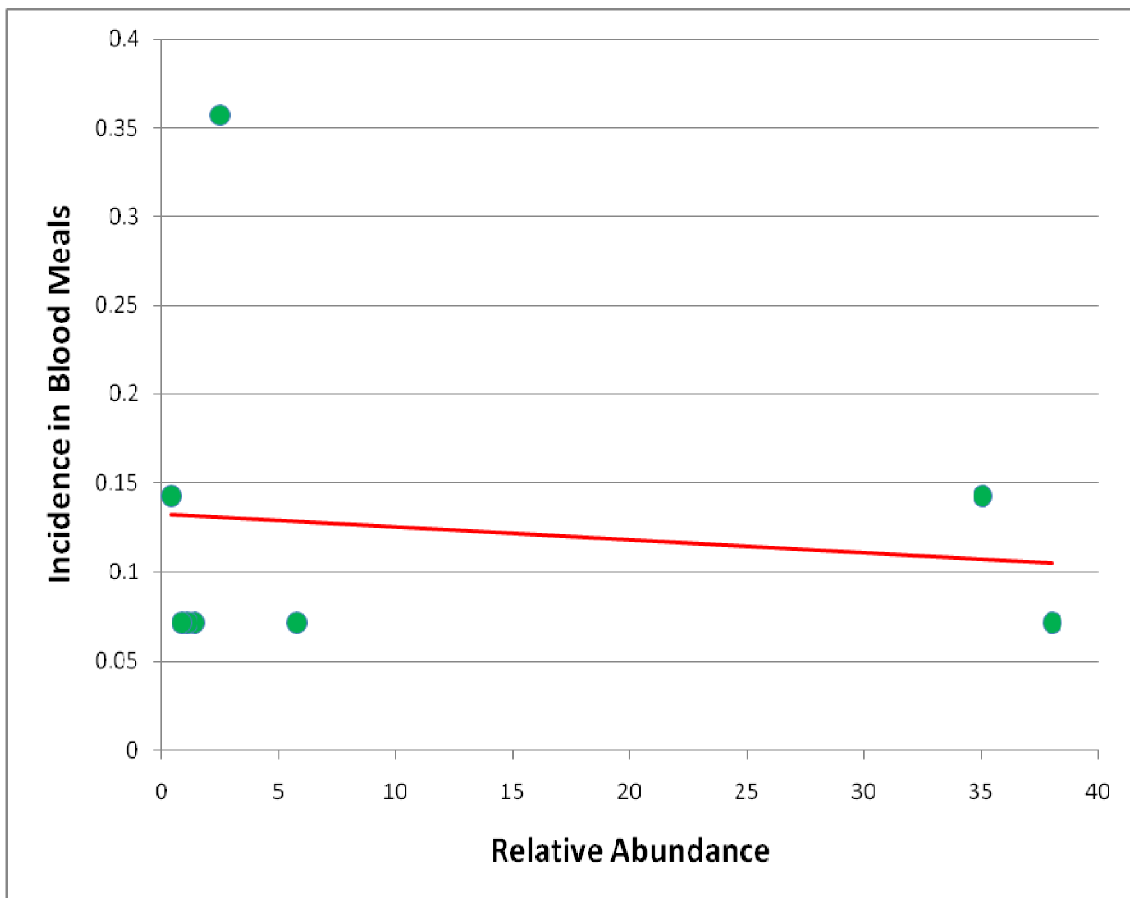

Figure 2. Results from regression analysis comparing the relative abundance of bird species present and the incidence of bird species identified in blood-meals

Table 9. Results of analysis of blood-meals obtained from *Cx. tarsalis*

Location	Date	Avian Species Scientific Name	Avian Species Common Name
Bowdoin Site 1	7/30/09	*Dolichonyx oryzivorus*	Bobolink
Bowdoin Site 1	8/6/09	*Passer domesticus*	House Sparrow
Bowdoin Site 1	8/13/09	*Oxyura jamaicensis*	Ruddy Duck
Bowdoin Site 2	7/16/09	*Turdus migratorius*	American Robin
Bowdoin Site 2	7/30/10	*Turdus migratorius*	American Robin
Bowdoin Site 2	8/12/09	*Sturnella neglecta*	Western Meadowlark
Fort Belknap	7/7/10	*Passer domesticus*	House Sparrow
Fort Belknap	7/7/10	*Passer domesticus*	House Sparrow
Fort Belknap	8/3/10	*Equus caballus*	Horse
Fort Belknap	8/3/10	*Equus caballus*	Horse
Freezeout Site 1	8/6/09	*Porzana carolina*	Sora Rail
Freezeout Site 1	8/6/09	*Nycticorax nycticorax*	Black-crowned Night Heron
Freezeout Site 1	8/6/09	*Passer domesticus*	House Sparrow
Freezeout Site 1	8/6/09	*Nycticorax nycticorax*	Black-crowned Night Heron
Lake Helena	7/27/09	*Sturnus vulgaris*	European Starling
Lake Helena	7/27/09	*Passer domesticus*	House Sparrow
Lake Helena	8/12/09	*Bos taurus*	Domestic Cow

Discussion

During the summer of 2009, three locations (Blaine, Phillips, and Sheridan counties) tested positive for WNV, and during the summer of 2010 only one site (Sheridan County) tested positive for WNV. In the summer of 2009, five human cases were reported across the state of Montana, none of which were reported in the counties where we detected the virus. As of October 2010, no human cases of WNV were reported in Montana (USGS, 2010). These numbers show a dramatic drop from 2007 when the state reported 202 human cases spread among 35 counties (USGS, 2010). The low number of human cases and low levels of WNV detected in this study may be due to low temperatures and high amounts of rain during the summers of 2009 and 2010 as increased cases of WNV may result from years with wet spring months and hot, dry summer months (Marra et al., 2004; and Table 10). During summers with high temperatures and little rainfall, the availability of water is limited and bird species may be forced into closer proximity, providing increased chances of virus transmission (Epstein, 2001). The development of WNV within the mosquito is also increased at higher temperatures, resulting in the mosquito's maintaining higher virus levels for a longer period of time (Epstein, 2001). These two factors, absent during the years of the present study, may have impacted the low levels of virus detected.

Both the regression and Spearman rank analysis showed no significant correlation between the relative abundance of bird species and their presence in blood meals. Tempelis et al. (1965) found a correlation between the abundance of species present at a sampling site and the presence of blood of those species in *Cx. tarsalis* blood-meals. Bird species that provide blood-meals to mosquitoes are often among the most common at a

sample site; however, this correlation may not always occur (Apperson et al., 2004). Alternatively, mosquitoes may exhibit a host preference. The data obtained in the current study may not discriminate between these two possibilities due to the low number of mosquitoes trapped.

In the present study, the highest number of blood-meals in July came from the House Sparrow and American Robin, but in August from the Black-crowned Night Heron and mammalian species. Passerine birds are the most competent hosts for WNV (Komar et al., 2003) and may be important species in WNV transmission (Molaei et al. 2006). The presence of mammalian blood-meals in August may represent a switch in feeding patterns from birds to mammals. This feeding pattern may allow the virus to amplify among competent avian hosts early in the summer followed by infection of mammalian species in the later summer months (Tempelis et al., 1967). The present study did not establish a significant correlation between the relative abundance of a particular bird species and its incidence of detection in blood-meals. However, with increased sampling coupled with analysis of the many factors affecting WNV distribution and transmission, a more accurate description of *Cx. tarsalis* feeding behavior may be established which will be useful in predicting and containing WNV.

In summary, to better understand the ecology of WNV, we used real-time RT-PCR to detect virus, analyzed blood-meals from engorged *Cx. tarsalis*, and determined relative abundances of bird species across the state of Montana. Increased knowledge of factors affecting WNV transmission allows for prediction of WNV outbreaks and may determine high-risk areas. The information obtained about the ecology of WNV could be relevant to other zoonotic diseases. The number of trap nights for both CDC light traps

and fiber pots could be increased to obtain more blood-fed *Cx. tarsalis* individuals. Continued surveillance of the trapping sites of this study should be maintained, and new sites added to produce a more complete risk-assessment model for WNV in Montana.

Table 10. Temperature and precipitation values for Montana

Year	Average T (June-August) (°F)*	Average Precipitation (June-August) (inches)*
2007	71.4	7.20
2009	66.5	5.06
2010	57.5	8.24

*Values obtained from National Oceanic and Atmospheric Administration

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