Comparison of West Nile Virus exposure in horses and infection rates in the mosquito *Culex tarsalis* in Montana.

Honors Thesis

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Abstract

I compared infection rates of West Nile Virus in the vector *Culex tarsalis* and exposure of the virus via antibody presence in horses. This research was performed to determine which method is more efficient at detecting the virus, thus contributing to viral public risk assessment for the state of Montana. Mosquitoes and horse blood came from similar locations, including the Helena area and the Fort Belknap Indian Reservation. Mosquitoes were trapped throughout the summer using carbon dioxide baited CDC light traps and were homogenized before RNA was extracted and finally tested for WNV with RT-PCR. Horse sera were extracted in mid-September and tested for WNV antibodies using IgM ELISA, where positive results indicate viral exposure. Pools of mosquitoes that tested positive by RT-PCR were analyzed with an Excel program for infection rate calculations and compared to numbers of positive horse sera. Mosquito infection rates were higher than horses detected positive by ELISA, indicating that mosquito surveillance is a more efficient means of detecting viral presence.

Introduction

West Nile Virus (WNV) is a zoonotic flavivirus of the Japanese encephalitis virus complex (May, 2011) and is most commonly transmitted from ornithophilic mosquito vectors to a variety of bird species (Bell, 2006). In the US, infection rates are commonly surveyed by means of molecular and geographic detection protocols in mosquitoes and birds (Bell, 2006). However, as birds migrate in late summer, vectors may shift feeding behavior to mammalian hosts: most commonly humans and horses (Kilpatrick, 2006). Enzyme-Linked Immunosorbent Assays (ELISA) have been used to detect antibodies to WNV (Blitvich, 2002), raising a need for comparison of effectiveness in mammalian or mosquito surveillance of the virus (Powell, 2000).

West Nile Virus

West Nile Virus originated in Uganda, where it was first isolated in a local woman and has since become the most widely distributed flavivirus (Anderson, 1999). In addition to being favorable reservoirs of WNV, avian species are preferred blood meals by most *Culex* species (Bell, 2006). Introduced to the United States in New York in 1999, WNV and has since spread across the country (May, 2011). Vectors of the virus include members of the *Culex* genus; and specifically in Montana, *Culex tarsalis* is the most competent vector (May et al., 2011). *C. tarsalis* prefers avian blood meals; and as birds are competent hosts, WNV is sufficiently carried and geographically spread by birds (Bell, 2006).

West Nile Virus - Horses

Mammals are referred to as "dead-end hosts" becuase they do not develop sufficient viremia when infected with an arbovirus to transmit the virus to another vector (Calisher, 1994). One in five humans infected with WNV will develop a high fever, while less than one percent of those infected will develop fatal encephalitis or meningitis (B CDC, 2012). Horses, however, are typically more severely affected by infection, with about 30% of all cases leading to death and 17% of those that survive will suffer debilitating consequences (A CDC, 2012). In 2002, over 14,000 horses in the US were confirmed WNV positive and displayed a 30% mortality rate, and to date, there is no effective treatment protocol for an infected horse - leaving control of the disease to mosquito surveillance management and vaccination (Minke, 2004).

Due to the harsh penalties suffered by horses, the resulting large costs experienced by ranchers, and the increased risk of transmission with frequent movement of equines for various events, a WNV vaccine was developed to protect horses in high-risk areas (Powell, 2000; Minke, 2004). The current approved vaccine for equine use is an inactivated whole virus that requires two doses and provides 94% immunity 12 months post-vaccination (Minke, 2004). Immunity can be detected by IgM antibody ELISA testing of whole blood of horses (Blitvich, 2002).

While the disease has been prevalent in Montana in previous years, WNV outbreaks are difficult to predict and involve several factors including, but not limited to: temperature, geography, and surrounding variance in animal populations (Zou, 2007; Yiannakoulias, 2007). Typically, one dose of the vaccine costs 15-20 dollars (UVM 2002). Although the cost for one dose of the vaccine is fairly low, the cost may be up to

$60 dollars per horse (for both doses) including administration of the vaccine - so when ranchers raise numerous horses, paying for two doses per horse may become difficult (Gardner, 2007). However, in a comparison study of infection rates between vaccinated and unvaccinated horses in California, no vaccinated horse became diseased (Gardner, 2007). In addition, the cost of each clinically infected horse was about 45 times that of a two-dose round of WN vaccination, indicating that the benefits of paying to vaccinate outweigh the risks of deferring vaccination (Gardner, 2007). Antibodies to the virus wane over time, placing further importance on maintaining a yearly vaccination routine (Gardner, 2007).

Surveillance

Weidong (2008) suggests that there are inconsistencies in mosquito arbovirus screening that compromise its effectiveness in predicting public risk exposure, including overemphasis on random sampling, lack of expansion of surveillance only when infection rates begin to rise and the overuse of infection rates as a risk exposure tool. Too much emphasis on random sampling takes away from early detection when transmission centers are sporadic and infection rates are low (Weidong, 2008). Also, neglecting to shift to expansion of surveillance when infection rates begin to rise decreases opportunity to improve knowledge of range of transmission and its intensity (Weidong, 2008). Another problem with mosquito surveillance is the overuse of infection rates as a risk exposure tool, as it is imprecise to ignore factors such as human and mosquito population densities (Weidong, 2008).

Weidong (2008) presents several methods to correct error in WNV mosquito surveillance, including: targeted rather than random surveillance, variable size pooling in

calculating infection rates of higher transmission areas, and maximal likelihood estimation (rather than MIR) to estimate risk exposure alongside mosquito and human population abundances. Contrary to Weidong's (2008) suggestions, a large-scale equine serosurvey study performed in Iran determined that equine infection rates follow similar environmental and geographic patterns as mosquito infection rates (Ahmadnejad, 2011). Based on that observation, using minimal infection rates (MIR) for comparison of WNV infection in *C. tarsalis* and horses was used in the present study.

Maintaining up-to-date information on diseased horses could minimize overall disease and improve local prevention measures (Powell, 2000). Reporting of equine disease has also been limited, indicating a need to improve surveillance (Powell, 2000). Intensive trapping and RT-PCR analysis of mosquitoes showed low levels of virus despite having found fairly high infection rates in unvaccinated horses in the same area in a California study (Gardner, 2007). This indicates that WNV surveillance via ELISA testing of horses may be more effective than surveillance with RT-PCR detection in mosquitoes (Gardner, 2007). This also suggests validity in comparing infection rates of mosquitoes and viral exposure in horse sera. Limitations of mosquito surveillance and the possibility of improved WNV surveillance using serological detection in horses is the basis of my hypothesis.

I hypothesized that horses would have higher infection rates than mosquitoes for several reasons. Unvaccinated horse populations are at high risk for WNV infection (Gardner, 2007), and the amount of trapping necessary to detect the virus at similar levels would require more trapping, sorting, and virus detection than funding and students on the WNV project can achieve. In addition to numerous mosquito surveillance issues

(Weidong, 2008), it is difficult to detect high amounts of virus in hundreds of *C. tarsalis* pools due to the variability of many environmental factors (Zou, 2007).

Significance

In the present study, I compared WNV infection rates in *C. tarsalis* and WNV antibody presence in unvaccinated horses. The significance of this study could provide useful information for ranchers in Montana. Horses that do not undergo vaccination may be at a higher risk for contracting WNV (Gardner, 2007). While infected horses may or may not show symptoms, investigating viral exposure could provide ranchers with valuable information and may also act as preventative information for future vaccinations and thus future reduction in equine infection (Powell, 2000). This study could also provide information on the effectiveness of monitoring mosquitoes in surveillance of the virus; if horses display higher viral exposure, then it could be possible that antibody testing in horses is more efficient in predicting human risk exposure and tracking WNV in Montana.

Materials and Methods

Collection of Mosquito Samples

Mosquito trapping performed on public lands such as national forests required no permits and/or special regulations. Permits were required and obtained via refuge managers when trapping was performed on preserves like Bowdoin and Medicine Lake National Wildlife Refuges. Professors and students of the West Nile project at Carroll College trapped mosquitoes using CDC light traps baited with carbon dioxide (Ginsberg, 2010).

Mosquitoes were kept alive on ice or killed on dry ice until permanent storage in a -20°C freezer. Mosquitoes were sorted by species, separating female *C. tarsalis* for viral testing. All sorting was performed under microscopes and on ice to prevent RNA degradation.

Homogenization

Homogenization was performed as described by Lanciotti (2009) with a few alterations. *C. tarsalis* were grouped into pools of 50 or less in Lysing Matrix A tubes with a ceramic bead. Each tube was labeled with the number corresponding to the sample site number from which the *C. tarsalis* came. A volume of 500µL of homogenization buffer and 1000µL of Qiagen RNA Later were added to each lysing tube. The homogenization buffer was made up of 1M Tris buffer, M199, Anti-Anti, and 30% bovine serum albumin (Table 1). The tubes were then homogenized in a Fast Prep FP120 (ThermoSavant) bead beater at 5.5 oscillations per minute for 30 seconds. The homogenate was stored at -80°C until RNA extraction.

Table 1: Reagents Used for Homogenization

Reagents – Homogenization Buffer	Purpose	Reagents - Other	Purpose
30% Bovine Serum Albumin	Stabilize DNase	RNA Later	RNA stabilization
M199	pH detection		
Anti-Anti	Antibiotic		
1 M Tris Buffer	Consistent pH		

RNA Extraction

A QIAcube (Qiagen) was used for vector RNA extraction in a similar method to Lanciotti (2009). A volume of 300µL of homogenate was prepared for extraction with addition of 350µL lysing buffer RLT (Qiagen, 2006) and placed in the QIAcube. The QIAcube was loaded with proper reagents, rotor adapters, 1000µL pipette tips, spin columns, and collection tubes for the extracted RNA. QIAcube loading and protocol were performed based on the RNeasy fibrous tissue kit by Qiagen (2006). RNA was removed from spin columns with RNA free water and stored at -80°C until PCR amplification.

Mosquito Viral Detection

The RT-PCR West Nile Virus detection protocol by Lanciotti (2000) was used with several modifications. Twenty five µL total volume was used per well in a PCR

plate that included: 2.5µL of sample RNA and 22.5µL of Master Mix (Table 2). Plates were incubated in a BioRad IQ500 thermocycler with the following cycling times and temperatures: one cycle of 48°C for 30 minutes, one cycle of 95°C for ten minutes, and 55 cycles each of 95°C for 15 seconds and 60°C for one minute.

Table 2: RT-PCR Master Mix contents

Reagent	Amount (µL per well)
Taqman Master Mix	12.5
Taqman Multiscribe (enzyme)	0.625
RNase-free Water	7.38
Forward Primer (ENV, 3')	0.5
Reverse Primer (ENV, 3')	0.5
Probe (ENV, 3')	1

Horse Blood Collection

Veterinarians in the Helena area and on the Fort Belknap Indian Reservation performed extraction of horse blood in September of 2013. About 5-10 mL of blood was taken from each animal. Blood samples were centrifuged and separated from whole blood, then kept on dry ice for transport to an -80°C freezer until ELISA testing.

Detection of WNV Antibodies in Horse Sera

The West Nile Virus Antibody Test Kit, ELISA (USDA licensed) from InBios was used (A InBios, 2013). The kit detects IgM antibodies in equine serum to West Nile Virus derived recombinant antigen. ELISA plates were read on a Finstruments microplate reader at absorbance 450 nanometers.

Comparison of Infection Rates

Calculation of infection rates of female *C. tarsalis* was performed using an Excel program by Biggerstaff (2009). I used Minimal Infection Rate (MIR) to calculate infection rates for *C. tarsalis* statewide and by county. MIR assumes that each positive pool only contained one positive mosquito. The program is able to account for skewing within the data or inconsistencies between numbers of mosquito pools. Infection rates were then compared to numbers of positive horse sera samples.

Results

The collective mosquito data for the summer of 2013 are compiled in Appendix 1, and infection rate data are compiled by counties of trapping sites in the state of Montana (Table 3). The average infection rate statewide was 2.5%.

Table 3: Infection rates and collective mosquito pool information.

County	Num Individuals	Num *C. tarsalis*	Num Pools	Num Pos Pools	Lower Limit	Upper Limit	Infection Rate
Statewide	258,215	10,749	135	27	1.54	3.41	2.48
Blaine	2012	677	16	2	0.00	2.37	0.99
Custer	315	105	3	0	0.23	9.08	0.00
Gallatin	833	833	8	0	0.23	9.08	0.00
Granite	5	5	1	0	0.23	9.08	0.00
Hill	3243	777	22	6	0.37	3.33	1.85
Jefferson	63	63	2	0	0.23	9.08	0.00
Lewis & Clark	1212	667	33	1	0.00	2.44	0.83
Musselshell	18	18	3	0	0.23	9.08	0.00
Phillips	5002	1,622	45	7	0.36	2.44	1.40
*Powder River	653	304	7	2	0.00	7.30	3.06
Prairie	149	95	3	2	0.00	31.90	13.42
*Richland	72	72	3	1	0.00	40.92	13.89
Sheridan	33809	3,005	56	16	0.24	0.71	0.47
Teton	11396	1,885	42	1	0.00	0.26	0.09
**Valley	50	3	1	1	0.00	58.81	20.00
*Wheatland	1317	487	19	3	0.00	4.85	2.28
*Wibaux	357	127	3	0	0.23	9.08	0.00

* Estimation of total mosquito counts is lower than the true number trapped due to documentation error.

***C. tarsalis* pool number was increased to 50 from 3 to obtain a more conservative MIR.

A positive ELISA sample requires an ISR (WNR Antigen/ NC Antigen) ratio of greater than three, and a negative sample is indicated by a ratio less than two. Each sera sample was tested in duplicate and averaged to obtain results (Table 4). Of the 63 tested samples, two positive results yield a 0.03% exposure rate.

Table 4: Horse sera ELISA results.

County	Number Horses Tested	Number Positive
Lewis & Clark	29	0
Blaine	34	2

Discussion

The goal of this study was to compare infection rates of the WNV vector *C. tarsalis* with viral exposure in horse sera and I hypothesized that exposure in horses would be higher than the infection rate in mosquitoes. I reject my hypothesis: mosquitoes displayed a 2.5% statewide infection rate and surveyed horses showed a 0.03% exposure rate.

Minimal infection rate (MIR) calculations are commonly used as a risk exposure surveillance mechanism (Macedo, 2010). The number of positive pools from the summer of 2013 was higher than in previous summers (Maricelli, 2012), which indicates human and horse exposures were also higher (Macedo, 2010). Importantly, horses are commonly reared in the same areas where *C. tarsalis* flourish and are surveyed, e.g.: farm land (including those of higher temperature) and urban/suburban environments (Chevalier et al., 2010). This suggests that it is likely to find horses in areas where WNV

was detected in mosquitoes.

It appears that higher numbers of *C. tarsalis* led to detection of virus, regardless of infection rate. Notable exceptions are Prairie, Richland, and Valley counties that had relatively low numbers of *C. tarsalis*, which resulted in larger infection rates. The total number of *C. tarsalis* in Valley County was increased from three to 50 in order to obtain a more conservative MIR, however, as the only pool tested was positive, MIR was high at 20%. This value was excluded from the statewide average prediction to prevent skewing. In addition, the higher infection rates from Prairie, Richland, and Valley counties are inaccurate predictions of human risk exposure. Due to small sample sizes, a rise in infection rate cannot be correlated to a higher risk exposure (Weidong, 2008), highlighting an issue in mosquito surveillance that is helpful in determining presence of the virus but is not reliable in human exposure prediction.

Interestingly, in previous years, Hokit et al. (2013) found no correlation between *C. tarsalis* population size and MIR. This suggests that this summer's loosely correlated increased population size and MIR was not likely due to chance, and a more likely conclusion is that increase in virus was more heavily dependent on environmental factors. All surveyed counties, with the exception of Granite County, were east of the continental divide (Figure 1), which maintains a higher average temperature than west of the divide (Hokit et al., 2013). Higher summer temperatures promote viral amplification as displayed by Figure 2 (Zou, 2007; Yiannakoulias, 2007), where much of the higher average temperature area in the state overlaps with WNV positive counties (shown in yellow).

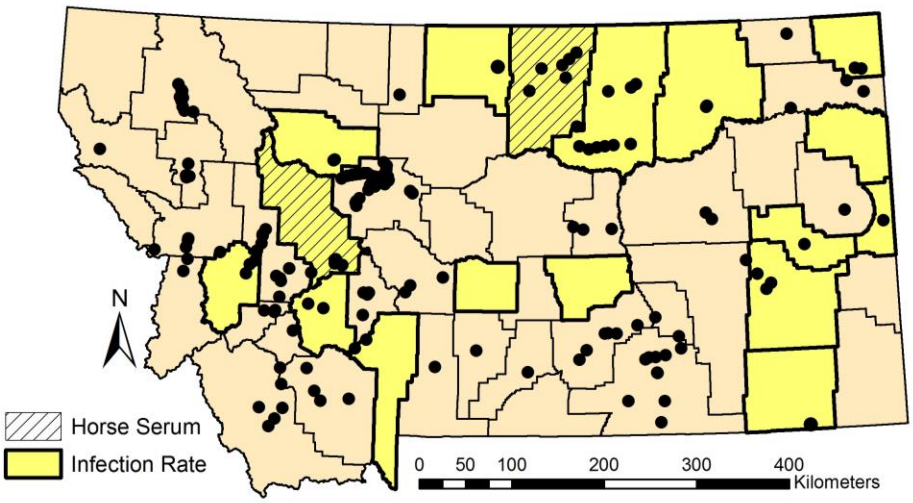

Figure 1: Counties surveyed for mosquito and horse WNV presence.

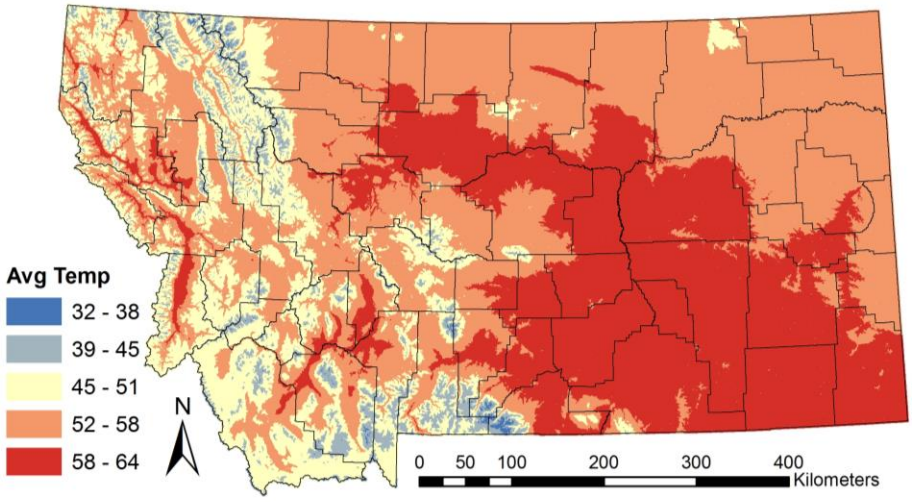

Figure 2: Average yearly temperatures in the state of Montana.

With only two positive results, these data suggest that WNV surveillance in horse sera is not more efficient than mosquito surveillance. However, in comparison to the large number of trapped and RT-PCR tested mosquitoes, the sample size of horse sera is miniscule and has only been surveyed in two counties. In a serosurvey for WNV IgG

antibodies performed in France, 305 of 906 tested horses were positive, or 34% (Durand et al., 2005). Only 23 of those 305 IgG positive horses tested IgM positive (Durand et al., 2005). In addition, only nine of 249 seropositive horses of 1,054 tested horses total were positive for IgM antibodies in a similar survey in Iran (Ahmadnejad et al, 2011). This suggests that increasing sample size would likely increase the horses testing positive, but also that IgM antibodies may not be the most effective means of surveillance for the virus in horses. As IgM indicates recent exposure, it may have a narrower time window than the longer-lasting IgG antibody (Ahmadnejad et al., 2011).

Screening of IgM rather than IgG antibodies may have resulted in an underrepresentation of positive horses in this study. However, in order to determine which factor, IgM serosurveillance or small sample size, is the main factor contributing to a low number of positive horses, I suggest future studies first expand in sample size. Sampled horse blood for this study was drawn in September of 2013 after several warm summer months, allowing for statewide viral amplification (Zou, 2007; Yiannakoulias, 2007). This, then, would indicate recent exposure in sampled horses and thus, IgM antibody presence (Ahmadnejad et al., 2011).

The CDC reported 38 total human WNV cases for the summer of 2013 in Montana (D CDC, 2013). This suggests that viral surveillance in horses could be a useful tool in estimating human risk exposure, as the number of human cases shares a low number with the proportion of exposed horses tested in this study, however, based on my initial results, horse surveillance cannot be declared as more efficient than mosquito surveillance based on these data. In future studies, more insight may be gained by expanding horse surveillance in Montana. Increasing the numbers of horses and counties,

and targeting counties that commonly see viral activity, would provide more information on the usefulness of equine surveillance to determine human risk exposure.

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Appendix 1: Collective mosquito data from the summer of 2013.

County	Location	Sample #	Date Sampled	Est. Total # Mosquitoes	# *Culex tarsalis*	Date Tested	# Pools Tested	# Positive Pools
L&C	Police Academy	822	6/17/13	563	1		1	0
L&C	Lake Helena	823	6/18/13	3000	3		1	0
L&C	Regulating Reservoir	824	6/19/13	2000	2		1	0
Hill	Havre	825	6/19/13	3800	5	6/28/13	1	0
L&C	Police Academy	826	6/24/13	149	5	6/28/13	1	0
L&C	Lake Helena	827	6/25/13	437	2	6/28/13	1	0
L&C	Regulating Reservoir	828	6/26/13	2020	2	6/28/13	1	0
Teton	Freezeout #1	829	6/25/13	333	30	6/28/13	1	0
Blaine	Liz McClain's Farm	830	6/24/13	54,000	0	N/A	0	0
Phillips	Bowdoin Visitor Center	831	6/24/13	7,500	15		1	0
Phillips	Bowdoin Visitor Center	832	6/24/13	18,500	1	6/28/13	1	0
Hill	Havre	833	6/24/13	3,200	2	6/28/13	1	0
L&C	Police Academy	834	7/1/13	200	4	7/9/13	1	0
L&C	Lake Helena	835	7/1/13	950	0	N/A	0	0
L&C	Regulating Reservoir	836	7/1/13	8,657	2	7/9/13	1	0
Teton	Freezeout #1	837	7/1/13	2,113	86	7/9/13	2	0
Teton	Freezeout #2	838	7/1/13	1700	20	7/9/13	1	0
Phillips	Pelican Island	839	7/2/13	4,550	15	7/9/13	1	0
Blaine	Liz McClain's Farm	840	7/2/13	25,000	0	N/A	0	0
Phillips	Bowdoin Visitor Center	841	7/2/13	73	0	N/A	0	0
Hill	Havre	842	7/2/13	2,351	65	7/12/13	2	0
Blaine	Liz McClain's Farm	843	7/9/13	1,580	20	7/12/13	1	0
Phillips	Bowdoin Visitor Center	844	7/9/13	999	78	7/12/13	2	0
Teton	Freezeout #1	845	7/9/13	314	29	7/12/13	1	0
Teton	Freezeout #2	846	7/9/13	521	170	7/12/13	4	0
Musselshell	Lavina	847	5/27/13	9	0	N/A	0	0
Musselshell	Lavina	848	6/6/13	67	1	7/12/13	1	0
Musselshell	Lavina	849	6/12/13	122	0	N/A	0	0
Gallatin	Three Forks	850	6/24/13	332	21	7/12/13	1	0
Powder River	Biddle	851	7/1/13	202	20	7/12/13	1	0
Gallatin	Three Forks	852	7/1/13	205	66	7/12/13	2	0

Hill	Havre	853	7/9/13	263	22	7/12/13	1	0
L&C	Lake Helena	854	7/10/13	184	2	7/12/13	1	0
L&C	Police Academy	855	7/10/13	552	0	N/A	0	0
L&C	Regulating Reservoir	856	7/10/13	1,174	8	7/12/13	1	0
L&C	Police Academy	857	7/15/13	490	40	7/12/13	1	0
L&C	Lake Helena	858	7/15/13	28	1	7/12/13	1	0
L&C	Regulating Reservoir	859	7/15/13	998	23	7/12/13	1	0
Teton	Freezeout #1	860	7/16/13	21	17	7/12/13	1	0
Teton	Freezeout #2	861	7/16/13	515	308	7/12/13	7	0
Blaine	Liz McClain's Farm	862	7/17/13	718	138	7/12/13	3	0
Phillips	Bowdoin Pelican Island	863	7/17/13	80	48	7/12/13	1	0
Phillips	Bowdoin Visitor Center	864	7/17/13	256	42	7/12/13	1	0
L&C	Police Academy	865	7/22/13	110	7	7/26/13	1	0
L&C	Lake Helena	866	7/22/13	423	21	7/26/13	1	0
L&C	Regulating Reservoir	867	7/22/13	1,755	32	7/26/13	1	0
Phillips	Waters	874	7/10/13	595	231	7/26/13	5	0
Musselshell	Lake Mason	875	7/10/13	32	6	7/26/13	1	0
Musselshell	Lake Mason	876	7/17/13	28	11	7/26/13	1	0
Phillips	Buffalo Jump Flats	877	7/10/13	493	59	7/26/13	2	0
Wheatland	Lehfeldt	878	6/26/13	34	2	7/26/13	1	0
Wheatland	Lehfeldt	879	7/2/13	199	3	7/26/13	1	0
Wheatland	Lehfeldt	880	7/10/13	92	5	7/26/13	1	0
Wheatland	Lehfeldt	881	7/17/13	47	0	N/A	0	0
Phillips	Buffalo Jump Creek	882	7/10/13	3000	0	N/A	0	0
Prairie	Terry	883	7/16/13	68	54	7/26/13	2	2
Wibaux	Wibaux	884	7/18/13	125	119	7/26/13	3	0
Wheatland	Musselshell River	885	7/10/13	121	53	7/29/13	2	0
Wheatland	Musselshell River	886	7/17/13	37	11	7/29/13	1	0
Powder River	Biddle	887	7/16/13	174	143	7/29/13	2	0
Gallatin	Three Forks	888	7/8/13	221	97	7/29/13	2	0
Gallatin	Three Forks	889	7/14/13	165	167	7/29/13	4	0
Richland	Sidney	890	7/14/13	253	37	7/29/13	1	0
Gallatin	Three Forks	891	7/21/13	388	245	7/29/13	5	0
Phillips	Bowdoin Visitor Center	892	7/23/13	685	219	7/29/13	5	0
Blaine	Liz McClain's Farm	893	7/23/13	510	74	7/29/13	2	0
Teton	Freezeout #1	894	7/24/13	833	652	7/29/13	10	0
Teton	Freezeout #2	895	7/24/13	467	238	7/29/13	5	0
Phillips	Waters	896	7/22/13	665	41	7/31/13	1	0
Phillips	Buffalo Jump Creek	897	7/22/13	170	18	7/31/13	1	0

Phillips	Buffalo Jump Flats	898	7/22/13	205	21	7/31/13	1	0
Sheridan	Bridgerman Point	899	7/23/13	1,900	1,018	7/31/13	19	4
Sheridan	Bridgerman Point	900	7/24/13	574	536	8/1/13	10	8
Sheridan	Bridgerman Point	901	7/25/13	24	21	8/1/13	1	0
Sheridan	Medicine Lake HQ	902	7/23/13	52	34	8/1/13	1	0
Sheridan	Medicine Lake HQ	903	7/24/13	1,000	603	8/1/13	12	1
Sheridan	Medicine Lake HQ	904	7/25/13	584	316	8/1/13	1	0
L&C	Police Academy	905	7/29/13	126	41	8/1/13	1	0
L&C	Lake Helena	906	7/29/13	246	16	8/1/13	1	0
L&C	Regulating Reservoir	907	7/29/13	1,480	66	8/1/13	2	0
Phillips	Bowdoin Visitor Center	909	7/30/13	1,026	245	8/1/13	5	3
Phillips	Bowdoin Pelican Island	910	7/30/13	369	156	8/1/13	2	1
Blaine	Liz McClain's Farm	911	7/30/13	852	205	8/1/13	4	1
Teton	Freezeout #1	912	7/31/13	138	75	8/1/13	2	1
Teton	Freezeout #2	913	7/31/13	230	72	8/1/13	2	0
Hill	Havre	914	7/29/13	360	82	8/1/13	2	2
Jefferson	Bison Creek	917	8/3/13	9	1	8/8/13	1	0
Jefferson	Boulder	918	8/3/13	146	62	8/8/13	2	0
L&C	Police Academy	919	8/5/13	187	31	8/8/13	1	0
L&C	Lake Helena	920	8/5/13	70	13	8/8/13	1	0
L&C	Regulating Reservoir	921	8/5/13	749	90	8/8/13	3	0
Prairie	Terry	922	7/29/13	45	41	8/8/13	1	0
Powder River	Biddle	923	7/28/13	62	38	8/8/13	1	0
Richland	Sidney	924	7/28/13	47	9	8/8/13	1	0
Phillips	Buffalo Jump Creek	925	7/10/13	3,000	75	8/8/13	2	0
Gallatin	Three Forks	926	7/28/13	325	119	8/8/13	3	0
Wheatland	Lehfeldt	927	7/24/13	36	1	8/8/13	1	0
Wheatland	Musselshell River	928	7/24/13	294	87	8/8/13	2	0
Wheatland	Lehfeldt	929	7/30/13	59	2	8/8/13	1	0
Wheatland	Musselshell River	930	7/30/13	61	25	8/16/13	1	0
Phillips	Bowdoin Pelican Island	931	8/5/13	800	53	8/16/13	2	0
Philips	Bowdoin Visitor Center	932	8/5/13	1,278	66	8/16/13	2	0
Teton	Freezeout #1	933	8/6/13	156	35	8/16/13	1	0
Teton	Freezeout #2	934	8/6/13	2,572	100	8/16/13	2	0
Custer	Miles City	935	8/7/13	798	105	8/16/13	3	0
Blaine	Liz McClain's Farm	936	8/7/13	655	100	8/16/13	2	1
Not recorded	Not recorded	937	8/7/13	303	199	8/16/13	4	0
Hill	Havre	938	8/6/13	633	253	8/16/13	6	1
Sheridan	Bridgerman Point	948	8/6/13	191	152	8/17/13	4	1

Sheridan	Medicine Lake HQ	949	8/6/13	1,315	113	8/17/13	3	0
Granite	Flint Creek	950	8/7/13	457	5	8/17/13	1	0
Phillips	Waters	951	8/5/13	1,568	34	8/17/13	2	1
Sheridan	Bridgerman Point	952	8/7/13	112	95	8/17/13	2	1
Sheridan	Medicine Lake HQ	953	8/6/13	372	121	8/17/13	3	1
Wheatland	Lehfeldt	974	8/6/13	42	2	8/19/13	1	1
Wheatland	Musselshell River	975	8/6/13	258	0	N/A	0	0
Gallatin	Three Forks	976	8/4/13	214	50	8/19/13	1	0
L&C	Regulating Reservoir	977	8/12/13	1,369	102	8/19/13	3	1
L&C	Lake Helena	978	8/12/13	456	28	8/19/13	1	0
Phillips	Bowdoin Visitor Center	979	8/13/13	2,975	17		1	0
Phillips	Bowdoin Pelican Island	980	8/13/13	2,521	19		1	0
Teton	Freezeout #1	981	8/13/13	229	11	8/19/13	1	0
Teton	Freezeout #2	982	8/13/13	267	27		1	0
Hill	Havre	989	8/12/13	1,732	209	8/19/13	5	2
L&C	Regulating Reservoir	996	8/19/13	1,437	95		2	0
L&C	Lake Helena	997	8/19/13	705	19		1	0
Teton	Freezeout #1	998	8/20/13	755	15		1	0
Teton	Freezeout #2	999	8/20/13	0	0	N/A	0	0
Phillips	Bowdoin Pelican Island	1000	8/20/13	3,090	8		1	1
Phillips	Bowdoin Visitor Center	1001	8/20/13	Not recorded	Not recorded		Not recorded	3
Blaine	Liz McClain's Farm	1002	8/20/13	27,467	135		3	0
Richland	Sidney	1003	8/12/13		26		1	1
Wheatland	Lehfeldt	1004	8/13/13		4		1	1
Wheatland	Musselshell River	1005	8/13/13		93		2	1
Powder River	Biddle	1006	8/11/13		103		3	2
Wibaux	Wibaux	1007	8/13/13		8		1	0
Gallatin	Three Forks	1008	8/11/13		68		2	0
Hill	Havre	1009	8/19/13	2,200	30		1	0
Phillips	Buffalo Jump Creek	1022	8/19/13	2,962	79		2	0
Phillips	Buffalo Jump Flats	1023	8/19/13	2,848	30		1	0
Blaine	Liz McClain's Farm	1056	8/27/13	5,000	5	10/12/13	1	0
Phillips	Bowdoin Visitor Center	1057	8/27/13	2,265	10	10/12/13	1	0
Phillips	Bowdoin Pelican Island	1058	8/27/13	1,015	42	10/12/13	1	1

Hill	Havre	1059	8/26/13	1,451	109	10/12/13	3	1
L&C	Regulating Reservoir	1060	8/29/13	4,360	0	N/A	0	0
L&C	Lake Helena	1061	8/29/13	365	11	10/12/13	1	0
Valley	Glasgow	1062		4,350	3	10/12/13	1	1

SIGNATURE PAGE

This thesis for honors recognition has been approved for the Department of Biology / Natural Science.

Director — Date: 4/22/14

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