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Prevalence And Distribution Of Ranavirus, Chytrid Fungus, And Helminths In North Dakota Amphibians

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PREVALENCE AND DISTRIBUTION OF *RANAVIRUS*, CHYTRID FUNGUS, AND
HELMINTHS IN NORTH DAKOTA AMPHIBIANS

by

Melanie Paige Firkins
Bachelor of Science, Iowa State University, 2012
Master of Science, University of North Dakota, 2015

A Thesis

Submitted to the Graduate Faculty

of the

University of North Dakota

in partial fulfillment of the requirements

for the degree of

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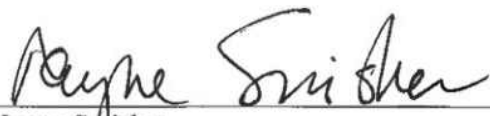
This thesis, submitted by Melanie P. Firkins in partial fulfillment of the requirements for the Degree of Master of Science from the University of North Dakota, has been read by the Faculty Advisory Committee under whom the work has been done and is hereby approved.

Dr. Robert A. Newman

Dr. Vasyl V. Tkach

Dr. Jefferson A. Vaughan

This thesis is being submitted by the appointed advisory committee as having met all of the requirements of the School of Graduate Studies at the University of North Dakota and is hereby approved.

Wayne Swisher
Dean of the School of Graduate Studies

November 30, 2015
Date

PERMISSION

Title   Prevalence and distribution of *Ranavirus*, chytrid fungus, and helminths in North Dakota amphibians.

Department   Biology

Degree   Master of Science

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Melanie Firkins
December 2015

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ABSTRACT

Worldwide reports of population declines and extinctions of amphibians have continued for at least two decades. Factors frequently reported in association with these declines include habitat loss, infectious diseases, and environmental degradation. Three sources of disease have been a concern for amphibians: chytrid fungus, *Ranavirus*, and some helminth parasites. Much of the existing reporting on disease results from anecdotal accounts associated with localized outbreaks, but understanding the scope and dynamics of infectious agents in host populations requires a regional perspective. My objectives were to estimate geographic distribution and prevalence of *Ranavirus*, chytrid fungus, and helminths in amphibians across the state of North Dakota. I also tested for associations between disease and helminth occurrence and general ecological factors. I sampled broadly across the state, including all major ecoregions and land use categories and obtained a total of 705 amphibians of six different species. I used real time PCR to detect *Ranavirus* and chytrid fungus infections. I also identified parasites as precisely as possible by morphological and molecular techniques. I found *Ranavirus* in 238 of 668 (35.6%) assayed amphibians. *Ranavirus* prevalence varied significantly by species, ecoregion, and land use. Infections were found broadly across North Dakota (55.9%), but exhibited a spatially structured distribution at a finer scale. *Ranavirus* was more frequently encountered in the Missouri Coteau ecoregion than other ecoregions. I found few occurrence of chytrid fungus (0.007%) and all infections were found in central North Dakota. In contrast, helminths were commonly found. The majority of amphibians were

infected with digeneans (60.3%), followed by nematodes (17.4%), but cestodes were much less frequent (2.8%). Parasite species varied intheir distribution across the state, with some showing a fine scale spatial dependency, indicating a patchy distribution. More complete surveys need to be designed to provide a more comprehensive understanding of parasite distributions and ecological associations.

CHAPTER I

AMPHIBIAN PATHOGENS AND PARASITES

INTRODUCTION

Amphibian extinctions and population declines have occurred worldwide and been attributed to a range of factors, including infectious diseases, habitat loss, ultraviolet radiation, overharvesting, invasive species and environmental degradation (Cushman 2006; Gray et al. 2009; Patz et al. 2000; Rachowicz et al. 2006; Rohr et al. 2011; Szuroczki et al. 2009). Among these, the most frequently implicated factors are habitat loss (Cushman 2006) and infectious diseases (Gray et al. 2009; Patz et al. 2000; Rachowicz et al. 2006). Amphibian diseases of concern include *Ranavirus*, chytrid fungus, and some helminth parasites. Moreover, none of these factors, including disease, are likely to act independently. For example, the probability of mortality of infected amphibians increases when the host is impaired from other causes such as habitat loss, unfavorable temperature, or agricultural runoff (Rohr et al. 2008; Szuroczki et al. 2009). Consequently, understanding the role of infectious agents in host population dynamics requires both estimate of the occurrence of those agents and of the ecological context in which they are found.

Amphibians require both aquatic and terrestrial habitats to complete their lifecycle exposing them to a wide range of environmental stressors. Amphibians are vulnerable to stress because of their small body size, limited mobility and hence limited ability to avoid or move away from unfavorable conditions, and permeable skin (Cushman 2006). Stressed amphibians

have a compromised immune system, increasing their susceptibility to disease and helminth infections (Davidson et al. 2002; Davidson and Knapp 2007; Green et al. 2002; Gray et al. 2009). Several studies provide evidence that the incidence of disease and helminth infection in amphibians is correlated with environmental stressors such as agricultural chemicals (Rohr et al. 2008), climate change (Paull et al. 2012), habitat loss and degradation, and altered landscape patterns (Greer and Collins 2008). Habitat loss can result in clustering of individuals and increased risk of disease and helminth transmission locally (Gray 2004; Greer and Collins 2008; Knutson et al. 1999; Kolozsvary and Swihart 1999). Fewer wetland sites across a landscape may also concentrate terrestrial life stages and increase the risk of disease and helminth transmission regionally (Johnson and Paull 2011).

In North Dakota, the predominance of agricultural land use and livestock ponds creates circumstances that may impose greater stress to amphibians inhabiting these landscapes. North Dakota also contains many prarie pothole wetlands that provide habitat to amphibians and other animal reservoirs for disease and helminths. The Missouri Coteau and Northern Glaciated Plains ecoregions have the highest densiy of wetlands (Figure 1) so I would expect disease and helminth occupancy to be highest in these ecoregions. Ecoregions with higher wetland density should increase opportunities for transmission because of habitat connectivity and increased amphibian movement and occupancy. In addition, two previous *Ranavirus* outbreaks have been reported in Nelson and Stutsman County, North Dakota resulting in mass mortality in larval wood frogs (*Lithobates sylvaticus*) and tiger salamanders (*Ambystoma tigrinum*) (Green et al. 2002). However, a systematic survey for amphibian disease has never been done in North Dakota and the available information on parasite distributions is limited to Nelson County and the Sheyenne National Grassland (Gustafson et al. 2013; Pulis et al. 2011). For these reasons, I will

survey North Dakota amphibians to provide information on disease and helminth prevalence and geographical distribution.
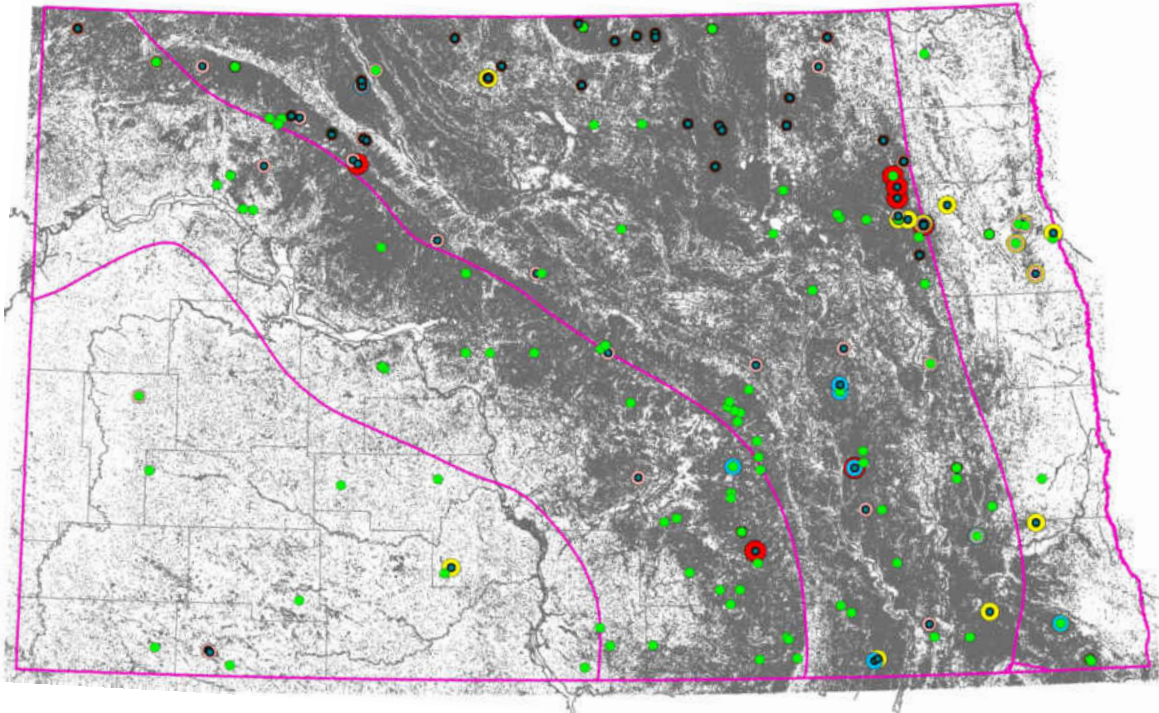
Figure 1. The geographic distribution of wetlands across North Dakota. The purple lines on the map demarcate ecoregions. The ecoregions going from east to west are the Northwestern Great Plains, Missouri Coteau, Northern Glaciated Plains and Red River Valley. The circles are color coded by amphibian species: Green: Northern leopard frog, Brown= Wood frog, Pink = Chorus frog, Blue = Great Plains Toad, Yellow = Canadian Toad, and Red= Tiger salamander.

The following sections discuss each of the major agents, beginning with microbial diseases and then helminths. For each major agent (*Ranavirus*, chytrid fungus, and helminths), I will discuss the taxonomy, geographic distribution, disease symptoms, transmission routes, and ecological factors that influence their occurrence and transmission.

Ranavirus

Ranavirus is in the family Iridoviridae, which are large double stranded DNA viruses consisting of an icosahedral shape (Chinchar 2002). *Ranavirus* infects amphibians, reptiles, and

osteichthyan fish (Gray et al. 2009). Currently six species of *Ranavirus* are recognized but only three infect amphibians: frog virus 3 (FV3), bohle iridovirus (BIV), and *Ambystoma tigrinum* virus (ATV) (Chinchar 2002). These strains differ in which amphibian species they infect and their geographic distribution. Researchers discovered FV3 can infect a variety of ectothermic hosts (Lesbarreres 2012), and can be found on every continent inhabited by amphibians (Duffus et al. 2015; Lesbarreres 2012). In contrast, BIV is found primarily in Australian anurans but a BIV-like virus was recently detected from boreal toads (*Anazyrus boreas*) at an aquarium in Iowa (Cheng et al. 2014). ATV causes infections in salamander populations and found primarily in western North America (Jancovich et al. 2005; Lesbarreres 2012).

Ranavirus strains replicate at a range of temperatures and synthesize proteins rapidly. FV3 replication occurs between 12°C and 32°C and protein synthesis occurs within hours of cell infection (Chinchar 2002). Cell death can occur in a matter of two hours following infection, by either necrosis or apoptosis (Gray et al. 2009). These symptoms usually occur in mid-to-late summer and involve late stage tadpoles and recent metamorphs (Green et al. 2002). Mortality is often sudden, resulting in more than 90% of larvae dead within several days (Green et al. 2002). These rapid mid-to late summer mortality events have been observed in wild amphibians located in Europe (Ariel et al. 2009, Kik et al. 2011), South and Central America (Fox et al. 2006; Stark et al. 2014), and Asia (Une et al. 2009). In contrast, bullfrogs (*Lithobates catesbeinanus*) in Japan and the American southeast do not show the same seasonality trend and have reported rapid declines in October (Hoverman et al. 2012; Une et al. 2009).

Researchers have suggested four hypotheses for the seasonality trend in *Ranavirus* infections during mid-to-late summer. One hypothesis is the pattern might be caused by detection bias because amphibian metamorphs are easier to observe when they move to shallow water to

complete metamorphosis (Green et al. 2002). Mortality events are often difficult to detect in remote locations especially in cryptic species and at times when humans are not usually active (Brunner et al. 2015). Secondly, the die-offs may reflect the disease dynamics following the introduction of *Ranavirus* earlier in the year. For example, sub-lethally exposed adults' return to wetlands to breed and transmit infections directly to larvae (Brunner et al. 2004). Third, hosts may become more susceptible to *Ranavirus* infections at certain development stages that coincide with summer months. Researchers discovered amphibians are more susceptible to *Ranavirus* during metamorphosis or close to metamorphosis (Green and Converse 2002; Greer et al. 2005; Speare and Smith 1992). Lastly, many researchers provide evidence that *Ranavirus* replication is temperature dependent (Altizer et al. 2013; Ariel et al. 2009; Echaubard et al. 2014; Rojas et al. 2005; Speare and Smith 1992).

  Ranavirus infections do not always result in mortality. Duffus et al. (2015) discovered no notable disease symptoms or mortality in larval and post metamorphic amphibians exposed to *Ranavirus* (Duffus et al. 2015). One explanation for these results is insufficient sampling spanning multiple years to detect mortality events (Gray et al. 2015). Even so, sub-lethal *Ranavirus* infections can still impact fitness-related traits (Echaubard et al. 2010). Examples of symptoms in both tadpoles and adults include lordosis, erratic swimming, lethargy, skin hemorrhages and ulcerations (Gray et al. 2009). Juveniles and adults can also experience red swollen legs and irregular areas of erythema (red) on the outside of internal organs (Gray et al. 2009). In fatal cases, adults and larvae contain hemorrhages in internal organs especially the kidneys, reproductive organs, and the liver (Gray et al. 2009). These skin hemorrhages are a significant source of *Ranavirus* that can be transmitted to susceptible hosts (Cunningham et al.

2007; Pearman et al. 2004). *Ranavirus* infections may also cause anorexia and symptoms include an empty gastrointestinal tract and an enlarged gallbladder (Gray et al. 2009).

Ranavirus transmission and host reservoirs

Amphibians contract this disease through multiple transmission routes including direct contact with an infected individual or water and sediment (Gray et al. 2009). Researchers speculate cannibalism is the most common transmission pathway in amphibians and reptiles (Crump 1983; Gray et al. 2009; Harp and Petranka 2006; Polis and Myers 1985). Currently, there are no published studies on *Ranavirus* transmission rates or dynamics in wild populations. The majority of research focuses on transmission routes but ignore host behavior, host density and contact rates in transmission dynamics (Brunner et al. 2015).

Amphibians are the primary reservoir for *Ranavirus* because individuals can be sub-lethally exposed and be a source of infection to other individuals through intraspecific interactions (Brunner et al. 2004; Gray et al. 2007; Miller et al. 2009; Pearman et al. 2004). Other non-amphibian reservoirs are abundant and widespread because *Ranavirus* infects a diverse suite of ectothermic vertebrates (Duffus et al. 2015). Researchers have isolated FV3-like viruses from many species of fishes with little to no mortality observed (Bang-Jensen et al. 2009; Brenes et al. 2014; Chinchar and Waltzek 2014; Gobbo et al. 2010; Gray et al. 2009; Mao et al. 1999; Moody and Owens 1994; Prasankok et al. 2005; Waltzek et al. 2014). In addition, Iridioviruses sharing more than 96% of the major capsid protein (MCP) gene for *Ranavirus* have been detected in many species of reptiles and reports continue to increase in the USA (Allender et al. 2006, 2012; De Voe et al. 2004; Johnson et al. 2008; Marschang et al. 1999; Marschang et al. 2005; Zhao et al. 2007). In Europe and Asia, both FV3 and BIV are found in reptiles (Duffus et al. 2015;

Huang et al. 2009). These animal reservoirs are a continued source of infections in amphibian populations (Gray et al. 2009).

Several experimental studies demonstrated *Ranavirus* transmission among various ectothermic classes (Bayley 2013; Mao et al. 1999; Picco et al. 2010; Schock et al. 2008). Brenes (2014) discovered infected gray tree frog larvae could transmit *Ranavirus* to unexposed red- eared sliders (*Trachemys scripta*) with a 30% infection rate. The infected turtles and mosquito fish (*Gambusia affinis*) could transmit *Ranavirus* to unexposed gray tree frog larvae with 50% and 10% infection rates respectively (Brenes 2014). This study resulted in nearly all amphibians dying, but infected turtles and fish persisted without mortality (Brenes 2014). Other studies have shown unsuccessful interclass transmission including brown tree snakes (*Boiga irregularis*), common green tree snakes (*Dendrelaphis punctualatus*) and keelback snakes (*Tropidonophis mairii*) (Ariel 1997). However, BIV was re-isolated from one of the brown tree snakes four weeks after inoculation (Ariel 1997). Researchers are just beginning to understand the host range of *Ranavirus* and continued surveillance is critical to evaluate the extent of transmission between ectothermic vertebrates.

Little information is known about the persistence of *Ranavirus* in the natural environment, but persistence is directly affected by temperature and precipitation (Adams et al. 2010; Collins et al. 2004; Jancovich et al. 1997). Brunner (2007) found moist soil inoculated with ATV causes 87% mortality in larval salamanders, but didn't cause any infections when allowed to dry for four days and rehydrated. Other researchers found *Ranavirus* could persist in the soil for 30-48 days (Nazir et al. 2012), and withstand freezing temperatures for long periods of time in frozen carcasses providing a source for infections after thawing (Bollinger et al. 1999; Langdon 1989).

Variation in *Ranavirus* susceptibility and life expectancy

Amphibians vary in susceptibility and life expectancy after infection because of differences between life stage (Brunner et al. 2015), species (Echaubard et al. 2014; Hoverman et al. 2011), individual genetic factors (Brunner and Collins, 2009) and dosage at exposure (Gray et al. 2009). Amphibians are most susceptible to FV3 infections during larval or early metamorph stages of development and mortality usually occurs (Brunner et al. 2015). Adults can survive with sub-lethal infections because they have a more competent immune system (Cunningham et al. 2007; Echaubard et al. 2014; Gray et al. 2009). However, Brunner (2004) found that metamorph tiger salamanders were more likely to die than larvae, but only a single clutch was used for this experiment.

Amphibians differ in susceptibility to *Ranavirus* even for the same viral strain (Echaubard et al. 2014; Hoverman et al. 2011). Certain taxonomic families are more susceptible on average than others, perhaps because of phylogenetic relatedness, life history, and their ecology (Brunner et al. 2015; Hoverman et al. 2011). Each *Ranavirus* strain tends to be superior at infecting animals within the taxonomic class from which they were isolated. Therefore, fish and reptiles are less susceptible to ATV and FV3-like *Ranavirus* than amphibians (Allender et al. 2013; Brenes et al. 2014; Jancovich et al. 2001; Picco et al. 2010).

Because of species' differences in susceptibility to infection, community composition has variable impacts on disease dynamics. Certain amphibian species can amplify pathogen transmission; consequently the order in which species are exposed can alter outcomes of disease outbreaks (Paul et al. 2012). Researchers observed greater community-level mortality when wood frog tadpoles were first exposed to *Ranavirus*, compared to exposing larvae of upland chorus frog (*Pseudacris feriarum*) or spotted salamander (*Ambystoma maculatum*) first (Brenes

2013). Not surprisingly, greater mortality was observed in communities composed of highly susceptible species (Brenes 2013).

At the individual level, genetic differences can also affect *Ranavirus* susceptibility (Brunner et al. 2005; Brunner and Collins 2009; Echaubard et al. 2014; Pearman et al. 2004; Pearman and Garner 2005; Schock et al. 2008). The host's genotype influences the rate of infection, timing of symptoms, and mortality (Brunner et al. 2004; Brunner et al. 2005). Brunner et al. (2005) observed different clutches of tiger salamanders and found variable outcomes in the early stages of infection because of individual genetic differences.

The route of transmission can result in high or low dosage of virions and consequently impact how long individuals survive after infection (Gray et al. 2009). Ingestion of *Ranavirus* infected tissue results in a higher dosage and rapid mortality (Gray et al. 2009; Harp and Petranka et al. 2006; Pearman et al. 2004). Indirect transmission including the exposure to *Ranavirus* contaminated water results in a low dosage and has variable effects on life expectancy (Harp and Petranka et al. 2006; Pearman et al. 2004).

Abiotic and biotic impacts on *Ranavirus* occurrence

Amphibians routinely endure many abiotic and biotic stressors that can increase susceptibility and transmission of *Ranavirus* including metamorphosis, cold-water temperatures, exposure to predators, and resource limitation (Garner 2009; Gray et al. 2009). The immune response at metamorphosis is weaker than all other developmental stages to facilitate the development of organs. This results in a period of high pathogen susceptibility and increased transmission rates (Gray et al. 2009). After the tail is reabsorbed, juvenile immunity increases until adulthood (Gantress et al. 2003; Rollins-Smith 1998).

Temperature can affect an amphibian's ability to mount an effective immune response. In general, higher temperatures induce a better antibody response and lower temperatures lead to immunosuppression (Gray et al. 2009). A field study in Tennessee found American bullfrog (*Lithobates catesbeianus*) tadpoles collected in winter were 7.7-fold more likely to be infected than in summer (Gray et al. 2007). Colder water temperatures do not always increase susceptibility. A study compared the symptoms of infected northern leopard frog (*Lithobates pipiens*) and wood frog (*Lithobates sylvaticus*) tadpoles while exposed to colder water temperatures. Wood frogs showed no change in symptoms after exposure to cold temperatures but devastating effects were observed in northern leopard frog tadpoles (Echaubard et al. 2014). Wood frogs are particularly adapted to colder climates, which may explain these results (Echaubard et al. 2014). In summary, temperature can influence *Ranavirus* epidemics, but this likely depends on the host species (Echaubard et al. 2014).

Several studies demonstrate frequent or prolonged exposure to predators can increase susceptibility to infection (Glennemeier and Rot-Nikcevic et al. 2005). The threat of predation can alter the production of the stress hormone corticosterone in tadpoles lowering their immunocompetence (Fraker et al. 2009). Kerby et al. (2011) observed *Ranavirus* prevalence and mortality to increase after larval tiger salamanders were exposed to chemical cues from predators. In contrast, Haislip et al. (2012) found no effect of predator cues on mortality or infection in a similar set of experiments with four species of larval anurans (*Lithobates clamitans, Lithobates sylvaticus, Pseudacris ferrirum,* and *Hyla chrysoscelis*) and two predator species (*Anax sp* and *Belostoma flumineum*). These results suggest the effects of predator exposure on immunocompetence varies between individuals and species likely depending on dosage and individual fitness at time of infection.

Human encroachment has also lead to higher occurrences of *Ranavirus* worldwide (Gray et al. 2009; Rohr et al. 2008). Several studies provide evidence that *Ranavirus* occurrence is associated with agricultural chemicals (Davidson et al. 2002; Davidson and Knapp 2007; Gray et al. 2009; Green et al. 2002; Kerby and Storfer 2009; Rohr et al. 2008). Aquatic systems can receive agricultural chemicals by direct application, terrestrial runoff, or windborne drift (Davidson et al. 2002). Numerous studies provide evidence that pesticides promote *Ranavirus* emergence by suppressing the immunological response in amphibians (Davidson et al. 2002; Davidson and Knapp 2007; Gray et al. 2009; Green et al. 2002; Kerby and Storfer 2009; Kerby et al. 2011). Researchers discovered larval tiger salamanders had reduced peripheral leukocyte counts and increased susceptibility to ATV infection when exposed to atrazine (Forson and Storfer 2006).

Humans can also potentially spread *Ranavirus* to other locations by transporting contaminated objects including boots, fishing, camping and research gear, farm equipment, and boats to uninfected wetlands (Gray et al. 2009). North Dakota has abundant shallow water bodies providing excellent habitat for waterfowl hunting, fishing, trapping and camping. These recreational activities provide opportunities for translocation of contaminated objects to unexposed wetlands. With the ease of human travel, it is difficult to prevent contacts to unexposed wetlands even across long distances (Martel et al. 2014).

Another human activity, international trade and transport in livestock, wildlife and animal products also promotes the spread and transmission of disease (Fevre et al. 2006). A study found 8.5% of amphibians collected from food marketers were infected with chytrid fungus and *Ranavirus* (Schloegel et al. 2009). These infections could be transmitted to wild populations by the release of contaminated water into drains, the release of live bullfrogs, or inappropriate

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disposal of amphibian skin (Fevre et al. 2006; Schloegel et al. 2009). Despite the worldwide recognition of risks associated with wildlife translocation, new diseases continue to emerge as a result of wildlife trading (Fevre et al. 2006).

Treatment options for *Ranavirus*

Ranavirus treatment options include antiviral drugs (Allender et al. 2012), heat therapy (Allender et al. 2013; Ariel et al. 2009; Bayley et al. 2013), vaccines (Caipang et al. 2006; Zhang et al. 2012) and the application of aquatic microbes to wetlands. These treatments are mostly applicable to captive populations. Guanine analogue antiviral drugs have successfully treated chelonian infections in captive populations (Allender et al. 2012). Heat therapy treatments vary in effectiveness between host species and viral strains and continued research is needed (Allender et al. 2013; Ariel et al. 2009; Bayley et al. 2013). DNA vaccine treatments have primarily focused on fish species within the aquaculture industry (Caipang et al. 2006; Zhang et al. 2012). These vaccine treatments have been successful in fish populations and some even reported immunity against infections (Zhang et al. 2012). Aquatic microbes can also reduce *Ranavirus* persistence in wetlands by consuming *Ranavirus* particles (Johnson and Brunner 2014). Aquatic microbes have shown to reduce viral counts in water within one day (Johnson and Brunner 2014).

Chytrid Fungus

Batrachochytrium dendrobatidis (Bd) is a chytrid fungus of the phylum Chytridiomycota and order Rhizophydiales. Within true fungi, this is the only phylum to reproduce with motile spores called zoospores (James et al. 2006) and the only order to parasitize vertebrates (James et al. 2006). There is a substantial amount of Bd evolutionary complexity with dramatic differences

among isolates and among genomic regions in chromosomal copy number and patterns of heterozygosity (Rosenblum et al. 2013).

The evolutionary diversity of Bd has led to many strategies for evading the host's immune system (Fites et al. 2013). A common strategy is to cause inhibition of host immunity, impair lymphocyte proliferation and induce apoptosis (Fites et al. 2013). After Bd has successfully invaded a host, mortality can occur especially in post metamorphic individuals and tadpoles (Kilpatrick et al. 2010; Stuart et al. 2004). Tadpoles also tend to develop slower to metamorphosis and lose sections of keratinized mouthparts, reducing grazing efficiency and food intake (Kilpatrick et al. 2010; Parris and Baud 2004; Parris and Cornelium 2004). Metamorph and adult symptoms include increased keratinized cells, fusing of keratin layers and reduced body size (Garner et al. 2009; Kilpatrick et al. 2010). The increased fusing of keratin layers and cells cause the permeable layers to thicken interfering with osmoregulation across the skin (Kilpatrick et al. 2010).

Infection also alters the consistency of some amphibian species calling probability with the lowest occurring in winter and highest in summer (Roznik et al. 2015a). Uninfected frogs were relatively consistent in calling across seasons (Roznik et al. 2015a). Infected frogs in poor condition were up to 40% less likely to call than uninfected frogs, whereas infected frogs in good condition were up to 30% more likely to call than uninfected frogs (Roznik et al. 2015a). These results suggest infected male reproductive success is related to body condition as well as infection status.

Linking Bd to population declines is difficult (Rohr et al. 2008) because of its variable effects on amphibian mortality (Briggs et al. 2005; Kilpatrick et al. 2010; Retallick et al. 2004) and the overall lack of long-term studies done to monitor impacts on amphibian populations.

However, one study observed two populations of infected boreal toads (*Anayrus boreas*) for 6 years to estimate survival probability and population growth rate in the Rocky Mountains (Pilliod et al. 2010). Toads infected with Bd had lower average annual survival than uninfected individuals (Pilliod et al. 2010) and diseased populations declined by 5-7% (Pilliod et al. 2010).

Chytrid fungus transmission and host reservoirs

The Bd transmission pathway is complex and involves both free-living and parasitic life stages. In the free-living stage, the zoospores are motile and feed on dead decaying matter (Rosenblum et al. 2008). In the infective stage, zoospores must infect a host to encyst and develop a reproductive thallus (Rosenblum et al. 2008). The thallus contains a sporangium that produces and releases zoospores (Rosenblum et al. 2008). Amphibians can contract Bd by direct contact with the motile zoospore during territorial and breeding encounters or by exposure to infected water or sediment (Kilpatrick et al. 2010). It takes approximately four days for one Bd replication cycle (Longcore et al. 1999). Infected animals can shed zoospores between 24-220 days before dying (Berger et al. 2005). Zoospores can also swim for up to 24 hours covering 2cm distances in still media (Berger et al. 2005; Longcore et al. 1999; Piotrowski et al. 2004). Zoospores can also grow and reproduce in a broad range of temperatures (4-25°C) and pH (4-8) and even withstand freezing to some degree (Gleason et al. 2008). Optimal zoospore growth occurs between 17-25°C and 6-7 pH (Woodhams et al. 2008).

Chytrid fungus infects ectothermic vertebrates and has a broad host range infecting hundreds of species (Kilpatrick et al. 2010; Olson et al. 2013; Valencia-Aguillar et al. 2015). In waterfowl, zoospores can adhere, survive and even proliferation on toe scales (Garmyn et al. 2012). Field surveys in Louisiana and Colorado also revealed 29% prevalence of zoosporangia in crayfish gastrointestinal tracts (McMahon et al. 2013). Crayfish maintained infection for at least

12 weeks and could transmit Bd to amphibians (McMahon et al. 2013). Crayfish also showed a seasonal infection intensity and prevalence pattern that closely resembled local amphibians, further indicating their importance as animal reservoirs (Brannelly et al. 2015).

Variation in chytrid fungus susceptibility and life expectancy

Amphibians vary in susceptibility and life expectancy after Bd infections (Rosenblum et al. 2010) depending on the strain of Bd (Berger et al. 2005), life stage of amphibian (Ardipradja 2001; Berger et al. 1999; Berger et al. 2005; Lamirande and Nicols 2002; Voordouw et al. 2010; Woodhams et al. 2003), amphibian species (Ardipradja 2001; Berger et al. 1999; Lamirande and Nicols 2002; Woodhams et al. 2003), and variation in skin maintenance (Ohmer et al. 2015). Researchers compared three different isolates of Bd infecting juvenile *Litoria caerulea* and discovered time until death was significantly different among the infected groups ($\chi = 30.5$, df = 2, $p < 0.001$) because of differences in virulence between strains (Berger et al. 2005). All *Litoria caerulea* frogs died after infection from Bd isolates of all three strains (Berger et al. 2005). The sporangia size and expressed proteins also differed between strains, which may affect host survival and account for differences in virulence (Fisher et al. 2009).

Immunological differences between amphibian life stages (tadpole, metamorph, juvenile, and adult) also result in variable susceptibility (Ardipradja 2001; Berger et al. 1999; Berger et al. 2005; Lamirande and Nicols 2002; Voordouw et al. 2010; Woodhams et al. 2003). In general, tadpoles are more resistant to infection than post metamorphic individuals because they have reduced amounts of keratin (only in their mouthparts), requiring a higher dosage for mortality (Berger et al. 1999; Bradley et al. 2002; Kilpatrick et al. 2010). Larvae do show variable sensitivities to Bd (Blaustein et al. 2005) and infected larvae have not been observed in mass mortality events even though Bd causes the loss of tooth rows and jaw sheaths (Berger et al.

1999). Researchers found Bd to accrue in a dose dependent manner during the larval stage and expressed at or soon after metamorphosis (Garner et al. 2009). Metamorph and adult amphibians contain greater amounts of keratin resulting in increased susceptibility to Bd infections (Blaustein et al. 2005; Hanlon and Parris 2013; Kilpatric et al. 2010). Several studies demonstrated frequent metamorph and adult mortality when infected with Bd, while tadpoles appeared to remain healthy (Berger et al. 1999; Bradley et al. 2002; Kilpatrick et al. 2010; Scheele et al. 2010).

The innate immune defenses, antimicrobial skin peptides and anti-fungal bacteria vary among different species of amphibians resulting in differential risks to infections (Becker et al. 2015; Gervasi et al. 2014; Harris et al. 2006; Woodhams et al. 2007) and mortality rates (Ardipradja 2001; Berger et al. 1999; Lamirande and Nicols 2002; Woodhams et al. 2003). For example, in *Pseudacris regilla* infection load increased over time and 16% of the population tended to display weaker bacteria killing responses than unexposed control animals (Gervasi et al. 2014). In contrast, Bd-exposed *Lithobates cascadae* experienced a decrease in infection load over time and no mortality was observed (Gervasi et al. 2014). *Lithobats cascadae* also showed stronger bacterial killing responses including an elevated number of neutrophils in the blood when compared to control animals (Gervasi et al. 2014). These results suggest variation in immunological responses may contribute to different patterns in survival and infection load between amphibian species (Gervasi et al. 2014). Skin peptides and anti-fungal bacteria can also increase survival or decrease weight loss in infected amphibians (Harris et al. 2009). Antimicrobial peptides are not present until approximately 12 weeks post metamorphosis and geographically distinct populations produce different types of peptides (Holden et al. 2015).

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The variation in skin turnover via routine sloughing between individuals could also account for differences in susceptibility to Bd (Ohmer et al. 2014). Sloughing is thought to play a role in immune defense by removing Bd skin-associated microbes. Researchers discovered sloughing does not affect Bd load on the ventral skin surface and does not alter the progression of Bd (Ohmer et al. 2014).

Abiotic and biotic impacts on Chytrid Fungus occurrence

Many abiotic factors influence Bd infection in amphibians, including elevation and temperature. Higher elevations are often associated with Bd occurrence because temperatures are favorable for Bd growth and survival (Roznik and Alford 2015b; Sapsford et al. 2013). Amphibian body temperatures are also within favorable temperatures for Bd growth year round at high elevation sites but only in winter at low elevation sites (Roznik and Alford 2015b). A different study found similar results, researchers sampled adult common mist frogs (*Litoria rheocola*) at six sites: two at high (>400m) elevations, two sites at low elevations connected to high elevation streams, and two sites at low elevations not connected with streams (Sapsford et al. 2013). The prevalence of Bd was highest in winter at high elevation sites and declined to lower levels at low elevation sites connected to high elevation streams and reached near zero at low elevation sites not connected to any streams (Sapsford et al. 2013).

Temperature plays a major role in determining Bd infection dynamics because of the ectothermic nature of amphibians and temperature dependency of Bd growth and reproduction (Olson et al. 2013; Piotrowski et al. 2004; Rohr et al. 2011; Woodhams et al. 2008). Temperatures between 17-25°C are optimal for Bd growth, and temperatures higher than 29°C or below 0°C are lethal (Piotrowski et al. 2004). Higher temperatures increase the maturation rate of the zoosporangium but decrease the number of zoospores produced per zoosporangium

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(Woodhams et al. 2008). Several studies have demonstrated Bd prevalence to drop dramatically in warm-water temperatures and increase in cold-water temperatures (Fernandez-Beaskoetxea et al. 2015; Forrest and Schlaepfer 2011; Kilpatrick et al. 2010; Whitfield et al. 2012). Researchers found with each increase in degree of temperature, Bd detection decreased by 8.8% (Olson et al. 2013). A different study found no effect of temperature on survival times of *Anaxyrus boreas* toads held at 12° C and 23° C (Carey et al. 2006). These results may reflect the interaction between temperature dependent growth of Bd and the host immune response, which may also be temperature dependent (Kilpatrick et al. 2010).

Biotic factors including amphibian behavior (Roznik and Alford 2015b), surrounding land use (Saenz et al. 2015), agricultural runoff (Gahl et al. 2011; Gaietto et al. 2014; Hanlon and Parris 2013; Paetow et al. 2012), animal and human dispersal (Johnson and Speare 2005; Kilpatrick et al. 2010; Kolby et al. 2015b) and the pet trade (Winters et al. 2014) can increase transmission and spread of infections. The behavior and ecology of amphibian species provides information for predicting and managing the impacts of Bd (Roznik and Alford 2015b). During winter, frogs move shorter distances than summer, and spent less time in vegetation and more time in water, which increases zoospore exposure (Roznik and Alford 2015b). The combination of reduced movement, prolonged zoospore exposure, and ideal water temperature for Bd survival increases the probability for infection in winter.

The land use around a wetland contributes to significant differences in Bd infection rates (Saenz et al. 2015). Saenz et al. (2015) compared Bd occurrence rates in *Pseudacris crucifer* at both urban and forested breeding sites. They found the occurrence of Bd was dramatically lower at urban sites (19.5%) compared to forested sites (62.9%) even with the same latitude, altitude, and season of sites sampled. Urban locations likely had lower Bd occurrence because of warmer

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temperatures, lower population densities and species richness than forested locations (Saenz et al. 2015). Another study found similar results in yellow bellied toads (*Bombina variegate*) with higher Bd occurrence in ponds surrounded by forest cover because of cool and wet conditions favorable for Bd growth and survival (Scheele et al. 2015).

Wetlands surrounded by agriculture can also receive chemical runoff that affects Bd infection intensity and prevalence (Gahl et al. 2011; Gaietto et al. 2014; Hanlon and Parris 2013; Paetow et al. 2012). The effects of pesticide exposure on Bd infection intensity depend on the infected species, timing of pesticide exposure (tadpole or metamorph), and the particular pesticide treatment applied (Buck et al. 2015; Gaietto et al. 2014). In wood frog populations, exposure to sub-lethal amounts of glyphosate-based herbicide and two strains of Bd did not significantly alter growth or time to metamorphosis (Gahl et al. 2011). Other studies found pesticides combined with Bd exposure affected overall amphibian body size at metamorphosis and survival (Gaietto et al. 2014; Hanlon and Parris 2013; Paetow et al. 2012).

The downstream flow of water and sand and possibly bird, human, amphibian or other animal movement increases Bd transmission and dispersal (Kilpatrick et al. 2010). Bd can survive up to three months in sterile moist river sand and grow on sterile bird feathers (Johnson and Speare, 2005). Strong wind and rainfall also assists in Bd dispersal confirmed by quantitative PCR (Kolby et al. 2015a). Researchers found homozygous boreal toads (*Anaxyrus boreas*) had lower probabilities of infection than heterozygous toads, which is usually indicative of dispersal and gene flow (Addis et al. 2015). In a different study, researchers tested both amphibians and their leaf perches for Bd presence and the pathogen was detected on 76.1% of leaves where a Bd positive frog had rested (Kolby et al. 2015b). High prevalence of infection (88.5%) in

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metamorphs and frequent shedding of Bd residue on foliage demonstrates a pathway for Bd dispersal between aquatic and terrestrial habitats (Kolby et al. 2015b).

Humans also play an important role in the dispersal of Bd. Fungal characteristics such as habitat flexibility, environmental persistence, and multiple reproductive modes promotes Bd dispersal by humans (Fisher et al. 2012). Humans can transport Bd to other locations by fomites and intercontinental trade of *Lithobates catesbeianus* (Fisher et al. 2012). The introduction of pre-exposed *L. catesbeianus* populations to other native amphibian populations is a common route of transmission (Rachowicz et al. 2006). For example in Brazil, *L. catesbeianus* is regularly farmed for human consumption since the 1930's and five farms in Sao Paulo and Para confirmed 78.5% Bd prevalence (Schloegel et al. 2009). Zoos and pet stores may also facilitate the transmission of Bd into the local amphibian community (Winters et al. 2014) by disposing of dead or alive amphibians into wild habitats (Goka et al. 2009).

Treatment options for chytrid fungus

There are several treatment options for Bd including antifungal drugs and heat therapy (Woodhams et al. 2012). When considering applying antifungal products to wetlands, it is important to consider the toxicity and effects to non-target animals (Heard et al. 2014; Stockwell et al. 2015a; Woodhams et al. 2012). Some commercial antifungal products include, Itraconazole, Mandipropamid, SteriplantN, and PIP Pond Plus. Itraconazole fungicide has shown to be extremely toxic in metamorphic and adult frogs, even at low concentrations (Woodhams et al. 2012). Sodium chloride is a natural fungicide used to decrease Bd prevalence and increase survival in field conditions (Stockwell et al. 2015b). Heat therapy is another treatment option that exposes amphibians at 35°C for 24 hours to reduce Bd growth (Woodhams et al. 2012). The common midwife toad (*Alytes obstetricans)* cleared infection when exposed to temperatures

higher than 2°C for 5 days (Geiger et al. 2011). Antifungal drugs and heat therapy are not always effective treatments therefore, a single cure all treatment is not realistic for infected amphibians (Woodhams et al. 2012).

Researchers' also found increasing the *Daphnia* population in wetlands to consume zoospores is a promising treatment option (Buck et al. 2015; Searle et al. 2013). Researchers found high densities of *D. magna* decreased the amount of Bd detected in water, leading to a reduction in the proportion of tadpoles that became infected (Searle et al. 2013). *D. dentifera*, also reduced the amount of Bd in water, but did not effect tadpole infection (Searle et al. 2013). The effect of *Daphnia* predation also varies within species, algal concentration, and *Daphnia* density (Searle et al. 2013).

Helminths

Helminths are parasitic worms that require nourishment from a host to survive (Roberts, Janovy, and Nadler 2013). Helminths can be divided into 3 groups: 1) platyhelminth*s* (flatworms) that consist of trematoda (flukes) and cestoda (tapeworms), 2) acanthocephala (thorny-headed worms), and 3) nematoda (roundworms) (Roberts, Janovy, and Nadler 2013). Digeneans and nematodes are discussed in detail here because they are commonly found in North Dakota amphibians.

Digenea

Digenea is a subclass of the class Trematoda and parasitizes all classes of vertebrates, especially marine fishes, and can inhabit nearly every organ. Most Digenea are dorso-ventrally flattened and appear oval in shape and contain an oral and ventral sucker to maintain position within the host. Most digeneans except schistosomes are hermaphroditic, and some are capable of self-fertilization (Roberts, Janovy, and Nadler 2013).

Digenea need at least two hosts to complete development. The typical life cycle (Figure 2) begins when an infective host defecates eggs into a wetland. These eggs develop into ciliated, free-swimming larvae called miracidium. The miracidium penetrates the first intermediate host, usually a snail. At the time of penetration or soon after, the larvae metamorphose into a mother sporocyst or a simple saclike structure. Within the mother sporocyst, a number of embryos develop asexually into rediae possessing a pharynx and a gut (alternatively, daughter sporocysts develop in some digeneans). Additional embryos develop within rediae and become cercariae. The cercariae are shed from the snail and penetrate the skin of the second intermediate host, often an amphibian and develop into metacercariae. These metacercariae require consumption by a definitive host to reproduce and restart the lifecycle (Roberts, Janovy, and Nadler 2013). The specific life cycles of digenean taxa found in North Dakota amphibians are shown in Table 35.
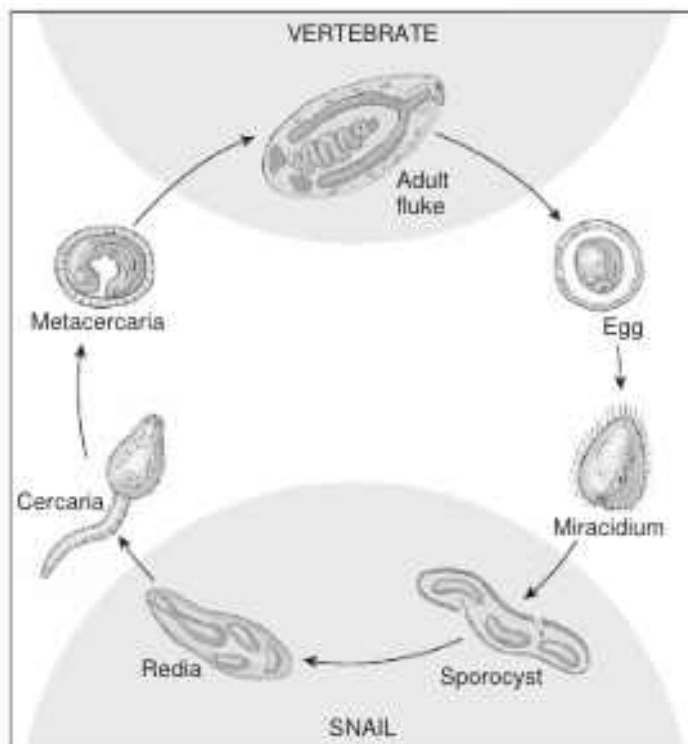
Figure 2. A generalized lifecycle of digeneans**.** Many variations do occur. Drawing by William Ober and Claire Garrison (Roberts, Janovy, Nadler 2013)

Some digeneans including *Ribeiroia ondatrae* and *Echinostoma trivolvis* can reduce the fitness and cause severe health problems to amphibians. *Ribeiroia ondatrae* can cause deformities such as extra or missing limbs, misshaped eyes and tails, skin lesions, and whole body deformities (Blaustein and Johnson 2003). These deformities compromise the ability of amphibians to find mates, forage, and call (Blaustein and Johnson 2003). *Echinostoma trivolvis* can cause edema, growth inhibition, compromised renal function, and mortality especially in tadpoles, which ultimately decrease amphibian recruitment rates (Beasley et al. 2005; Holland et al. 2007; Scotthoefer et al. 2003; Szuroczki et al. 2009; Toledo et al. 2007). In addition, researchers observed high frequencies of *Echinostoma* cercariae shedding at the most vulnerable stage of tadpole development increasing probability of transmission (Holland et al. 2007).

Cestoda

Tapeworms or cestodes are not commonly found in North Dakota amphibians. Cestodes contain a strobilia with a linear series of proglottids storing reproductive organs. Usually each proglottid has a complete set of both male and female reproductive organs but a few species are dioecious. Tapeworms have a head or scolex equipped with a variety of holdfast organs that maintain their position in the gut. Scolices may bear suckers, grooves, hooks, spines, glands, tentacles, or a combination. Cestodes lack a digestive system and must absorb all required substances through their external covering (Roberts, Janovy, and Nadler 2013).

Tapeworms also exhibit indirect life cycles that begin when a gravid proglottid releases eggs into the intestine. The eggs exit the host with the feces and develop into an oncosphere that enters the same or different host by ingestion. The oncosphere develops into a metacestode usually with a scolex. The metacestode can inhabit anywhere but the intestine within a host but must travel to the intestine to develop into an adult tapeworm. An amphibian can become

infected with cestodes by ingesting eggs or metacestodes from the environment or from intermediate host (Roberts, Janovy, and Nadler 2013). The specific life cycles of cestode taxa found in North Dakota amphibians are shown in Table 35.

Nematoda

A typical nematode is bilaterally symmetrical, elongated, and tapered at both ends. Nematodes have non-segmented integument and a body cavity called a pseudocoel. The digestive system is usually complete, with a mouth at the anterior end and an anus at the posterior end. Most nematodes are dioecious and show sexual dimorphism. However, some species are hermaphroditic, and others parthenogenetic. Nematodes constitute the most abundant multicellular animal on earth (Roberts, Janovy, and Nadler 2013). In such a large and diverse phylum details of development and life history differ greatly among various groups of nematodes. For these reasons, I will discuss *Rhabdias,* a common genus of nematodes in amphibians that parasitize their lungs.

Rhabdias has a direct life cycle that infects amphibians and squamate reptiles. Their lifecycle begins when free-living adults in the soil lay eggs that can give rise to either free-living or parasitic forms. The mechanism that determines if an embryo is free- living or parasitic is unclear. The eggs develop and undergo three molts represented as (L1,L2,L3) into a rhabditiform juvenile. The non-parasitic juveniles (L1 and L2) feed on bacteria and other inhabitants in the soil. The filariform or parasitic (L3) juveniles undergo developmental arrest until they can find a suitable host. When a suitable host is found, the filariform juvenile enters a host by ingestion or by penetrating the skin. Blood transports the juveniles to the lungs where they molt into (L4) adult protandrous hermaphrodites that ultimately develop as females. The resulting shelled zygotes pass up the trachea of the host and are then swallowed, differentiating into an embryo

along the way. The eggs hatch in the intestines and the rhabditiform juveniles exit with the feces and molt into adults where they mate and lay more eggs (Langford et al. 2009; Roberts and Janovy 1998; Roberts, Janovy, and Nadler 2013). The specific life cycle characteristics of nematode taxa found in North Dakota amphibians are shown in Table 35.

Rhabdias can cause several health problems in amphibians including decreased growth rates (Goater et al.1993; Kelehear et al. 2011), compromised lung function (Goater et al. 1993; Goater and Vandenbos 1997) and burst performance (Goater et al.1993). *Rhabdias pseudosphaerocephala* and *Rhabdias bufonis* can decrease growth rates in toads (Goater et al. 1993), which maybe the result from decreased appetite, increased investment in the immune system, consumption of resources by parasites, or a combination along with additional factors (Kelehear et al. 2011). Amphibian body size is directly related to performance, therefore *Rhabdias* infections can impact fitness related traits (Goater et al. 1993). In general, *Rhabdias* occupies a significant portion of the lung cavity, which may impair the mechanical functioning of the lung, damage the lung lining and cause blockages of blood vessels (Goater et al. 1993). These symptoms can impede the chorusing ability of amphibians, which has important implications on reproductive success (Goater and Vandenbos 1997). *Rhabdias* infections can also affect burst performance by inferring with oxygen consumption or other aspects of lung function (Goater et al. 1993).

Ecological factors influencing helminth occurrence

There are many factors affecting parasite transmission and occurrence in amphibian hosts. Several studies demonstrate amphibian exposure to pesticide runoff can result in immunosuppression (Carey and Bryant 1995; Carey et al. 2003; Christin et al. 2003, 2004; Gendron et al. 2003; Kiesecker 2002; Rohr et al. 2008). High concentrations of pesticides have

also shown to accelerate the migration of *Rhabdias ranae* leading to the establishment of twice as many worms in the lungs of juvenile northern leopard frogs (*Lithobates pipiens*) (Gendron et al. 2003). Atrazine runoff and eutrophication increase algae production in wetlands and therefore increase the biomass of the first intermediate host, a snail (Johnson et al. 2007; Rohr et al. 2008). A larger snail can harbor more parasites, potentially increasing parasitic load and the likelihood of infection in the second intermediate host, amphibians (Johnson et al. 2007; Rohr et al. 2008; Szuroczki et al. 2009).

Atrazine exposure also negatively impacts parasites by reducing survival and infectivity (Koprivnikar et al. 2006; Pietrock and Marcogliese 2003). Atrazine causes significant reduction in *E. trivolvis* cercaria survival but only at concentrations greater than commonly found in aquatic ecosystems (Rohr et al. 2008). None of the pesticides including atrazine, glyphosate, carbaryl, and malathion significantly enhanced *E. trivolvis* virulence, decreased tadpole survival, or reduced snail survival, growth or fecundity (Rohr et al. 2008).

Human altered landscapes in general can affect the abundance and species richness of amphibian parasites (Beasley et al. 2005; McKenzie 2007; Skelly et al. 2005). Researchers found parasite abundance and species richness in the host *Lithobates vaillanti* to be significantly higher in pasture habitat (McKenzie 2007). Other researchers found the infra-community parasite richness to increase throughout the season, and be more pronounced in agricultural wetlands (King et al. 2008). A different study found mean infra-community parasite species richness to be negatively correlated with urban and agricultural areas (King et al. 2007). These variable results suggest multiple factors promoting parasite species richness such as habitat quality and habitat accessibility for hosts (King et al. 2007).

Habitat fragmentation and isolation can also impair amphibians' access to wetlands and decrease overall wetland use (Green 2005; Semlitsch and Bodie 2003). Decreased forest cover, intensely farmed areas and increased road density also restricts amphibian movement between wetlands (Houlahan and Findlay 2003; Lomen and Lardner 2006). Therefore, human disturbances can contribute to reduced amphibian species diversity and abundance in wetlands, which significantly limits opportunities for parasite transmission (King et al. 2007).

The conductivity and amount of dissolved organic carbon in a wetland can also significantly affect parasite community composition (King et al. 2007). Dissolved organic carbon is positively associated with community species richness and abundance of most parasite species (King et al. 2007). High concentrations of dissolved organic carbon can cause shifts in invertebrate community structure (Wissel et al. 2003) affecting the transmission of parasite species that use invertebrates as second intermediate hosts (King et al. 2007).

Host characteristics such as body size, habitat preference, feeding behavior, sex, species, and life stage can also influence parasite occurrence. A larger body size can support more parasite species than smaller individuals (Grutter 1998; Holmes and Price 1986). However, comparing the relationship of helminth diversity and body size is difficult because host diet and habitat are confounded with body size (McAlpine 1997). Host habitat preference is another important factor contributing to helminth composition in hosts. Amphibians that prefer a terrestrial habitat usually have a helminth fauna dominated by nematodes (Fransdsen 1974; Kuc and Sulgostowska 1988). Parasite occurrence is also often skewed between sexes because there are physiological, morphological, and behavioral differences (Alexander and Stimson 1988; Bundy 1988; Zuk 1990). Several studies found high testosterone levels can cause immunosuppression in males (Folstad and Karter 1992; Grossman 1985). Other surveys found

inconsistent differences between sexes (Alexander and Stimson 1988; Poulin et al. 1996; Zuk 1990). Parasite infection rates also vary by amphibian species and life stage with early developmental stages experiencing higher observed mortality (Holland 2010).

Alternative hosts can alter amphibian and parasite interactions. In particular, a study demonstrated that alternative hosts and predators help mediate *Ribeiroia ondatrae* infections in the host *Pseudacris regilla* (Orlofske et al. 2012). Mollusks, zooplankton, fish, larval insects, and newts removed 62-93% of infectious stages in *Pseudacris regilla* (Orlofske et al. 2012). In addition, damselfly nymphs and newt larvae reduced infections in *P. regilla* tadpoles by approximately 50% (Orlofske et al. 2012). These results suggest communities with multiple species can reduce infection load and pathology in sensitive hosts (Johnson et al. 2008).

Global temperature patterns are changing and play an important role in the development and persistence of both parasites and amphibians (Paull et al. 2012). Researchers' discovered higher temperatures enhanced *R. ondatrae* penetration but reduced establishment and survival outside of the host (Paull et al. 2012). Researchers also observed a peak in *R. ondatrae* induced limb deformities at 20°C with 63% prevalence and a decrease at 26°C with 12% prevalence (Paull et al. 2012). Warmer temperatures also accelerated metamorphosis but decrease the length and mass of amphibians at metamorphosis (Paull et al. 2012).

OBJECTIVES

In the following chapters (2-4), I will discuss the prevalence and geographic distribution of *Ranavirus* (Ch.2), chytrid fungus (Ch. 3), and helminth parasites (Ch.4) in North Dakota amphibians.

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CHAPTER II

PREVALENCE AND GEOGRAPHIC DISTRIBUTION OF *RANAVIRUS* IN NORTH DAKOTA AMPHIBIANS

ABSTRACT

Amphibian population declines and species extinctions have occurred worldwide, including some attributed to *Ranavirus* infections. Two known *Ranavirus* outbreaks have occurred in Nelson and Stutsman County, North Dakota resulting in mass mortality in larval wood frogs and tiger salamanders, but no statewide survey has ever been done. In this study, I estimated the geographic distribution and prevalence of *Ranavirus*-infected amphibians across North Dakota. I collected amphibians broadly across the state, sampling all major ecoregions and land use categories. Six species of amphibians were represented, including northern leopard frogs (*Lithobates pipiens*), wood frogs (*Lithobates sylvaticus*), boreal chorus frogs (*Pseudacris maculata*), Canadian toads (*Anaxyrus hemiophrys*), Great Plains toad (*Anaxyrus cognatus*), and tiger salamanders (*Ambystoma mavortium*). I used real time PCR to detect *Ranavirus* infections and found *Ranavirus* in 238 of 668 (35.6%) assayed amphibians. *Ranavirus* prevalence varied significantly by species, ecoregion, and land use. Infections were found broadly across North Dakota but exhibited a spatially structured distribution at a finer scale. *Ranavirus* was more frequently encountered in the Missouri Coteau ecoregion than other ecoregions. *Ranavirus* was sufficiently common to indicate a need for routine surveillance across the state to detect disease outbreaks and mortality events.

INTRODUCTION

Infectious diseases have contributed to mass mortality and species extinction events in amphibians for at least two decades (Cushman 2006; Gray et al. 2009; Rachowicz 2005; Rohr et al. 2011; Szuroczki and Richardson 2009). *Ranavirus*, one of two particularly important amphibian diseases, has been associated with amphibian mortality in 25 countries and at least 105 species across 18 families (Duffus et al. 2015). The number of reported mortality events are still likely to be underestimated because many species are cryptic, and mortality events are not easily observed because of rapid removal by predators and fast decomposition (Brunner et al. 2015). Disease outbreaks reduce population size, alter population structure and increase risk of local extinction in both common and rare species (Brunner et al. 2004; Collins et al. 1988; Cunningham et al. 1996; Cunningham et al. 2007; Daszak et al. 1999; Green et al. 2002; Greer et al. 2005; Schock and Bollinger 2005). *Ranavirus* has previously been detected in North Dakota in Nelson and Stutsman County, both resulting in mass mortality in larval wood frogs and tiger salamanders (Green et al. 2002). Given the importance of disease surveillance in amphibian populations, I estimated the prevalence and geographic distribution of *Ranavirus* in North Dakota. I also tested for associations between *Ranavirus* occurrence and ecological features.

North Dakota is an ideal location for disease occurrence because of its combination of ecological circumstances that interact with disease transmission and host susceptibility. Several landscape characteristics increase the probability of *Ranavirus* occurrence, including livestock accessible ponds (Gray et al. 2007; Greer and Collins 2008; Hoverman et al. 2012), pesticide runoff into wetlands (Davidson et al. 2002; Davidson and Knapp 2007; Gray et al. 2009; Green et al. 2002), and extensive agricultural land use, which combine to reduce habitat availability and increase exposure to environmental stressors (Gray 2015; Greer and Collins 2008; Knutson et al.

1999; Kolozsvary and Swihart 1999). North Dakota's climate extreme can also affect the host's ability to mount an effective immune response (Gray et al. 2009; Echaubard et al. 2014). In general, temperature can influence *Ranavirus* epidemics but this effect likely depends on the host and strain of *Ranavirus* (Echaubard et al. 2014).

Currently six strains of *Ranavirus* are recognized but only three infect amphibians: frog virus 3 (FV3), bohle iridovirus (BIV), and *Ambystoma tigrinum* virus (ATV) (Chinchar 2002). Researchers discovered FV3 infects a variety of ectothermic hosts (Lesbarreres 2012) and can be found on every continent that amphibians inhabit (Duffus et al. 2015; Lesbarreres 2012). In contrast, BIV is found primarily in Australian anurans but a BIV-like virus was recently detected from boreal toads (*Anazyrus boreas*) at an aquarium in Iowa (Cheng et al. 2014). ATV causes infections in salamander populations and found primarily in western North America (Jancovich et al. 2005; Lesbarreres 2012). Because the diagnostic assay I used is not strain-specific, I will not attempt to distinguish stains, but assume that FV3 is most likely the strain encountered in the present study.

OBJECTIVES AND HYPOTHESES

Based on *Ranavirus* outbreaks reported in the literature, I tested for associations between *Ranavirus* occurrence in North Dakota and several ecological factors. It should be noted that these factors, if they affect *Ranavirus* occurrence at all, most likely do not act independently and may even be confounded with each other. Consequently, estimating the independent contributions of each may be challenging or even not feasible in this study.

<u>Objectives</u>

(1). Estimate the geographic distribution of *Ranavirus* in North Dakota.

Hypothesis 1: *Ranavirus* occurrence will vary across the state because of different ecological conditions. For example, *Ranavirus* might be more common in ecoregions with lower temperatures and higher percentage of wetlands because these are favorable conditions for *Ranavirus* environmental persistence and transmission. Several studies have shown colder temperatures increase disease susceptibility and severity (Echaubard et al. 2014; Gray et al. 2007). However, decreased precipitation can result in droughts that reduce the overall habitat area and volume available to amphibians, resulting in clustering of individuals and increased contact rates (Greer and Collins 2008). The ecoregions in North Dakota differ in climate, wetland distribution and biotic communities and provide a starting point for analysis of geographic distribution and its causes.

(2). Estimate the prevalence of *Ranavirus* in North Dakota amphibians.

Hypothesis 2A: *Ranavirus* prevalence will vary among host species because certain amphibian taxonomic families are more susceptible on average than others. Researchers suggest amphibian taxa differ in susceptibility to *Ranavirus* because of phylogenetic relatedness, life history, and their ecology (Brunner et al. 2015; Hoverman et al. 2011).

Hypothesis 2B: Land use will influence the frequency of *Ranavirus* infections. The prevalence of infections will increase in wetlands surrounded by cropland. Several studies provide evidence that *Ranavirus* occurrence is correlated with agricultural chemicals (Davidson et al. 2002; Davidson and Knapp 2007; Gray et al. 2009; Green et al. 2002; Kerby and Storfer 2009; Rohr et al. 2008). Pesticide exposure in amphibians promotes *Ranavirus* emergence by suppressing the immunological response in amphibians (Davidson et al. 2002; Davidson and Knapp 2007; Gray et al. 2009; Green et al. 2002; Kerby and Storfer 2009; Kerby et al. 2011).

Hypothesis 2C: Wetlands at close proximity to each other are more likely to contain infected hosts because of increased habitat connectivity and amphibian movement leading to increased contact rates. *Ranavirus* is transmitted by direct contact with infected hosts or water and sediment (Gray et al. 2009) therefore increased proximity to other wetlands is likely to result in increased transmission.

Hypothesis 2D: Wetlands with smaller length and area should also have higher *Ranavirus* occurrence because amphibians have less available space to breed and find resources, thereby increasing the probability of contacting infected environment or other amphibians. However, larger ponds have the potential to support larger populations of amphibians, which also result in increased contact rates.

Hypothesis 2E: *Ranavirus* prevalence should be higher in the Missouri Coteau and Northern Glaciated Plains ecoregions because they contain many prairie pothole wetlands that provide habitats for amphibians and other animal reservoirs (Fig. 1). These wetlands are located fairly close to one another allowing for amphibian movement between wetlands. Increased amphibian movement should also increase opportunities for *Ranavirus* transmission in the surrounding area. In contrast, a lower percentage of wetlands might restrict and concentrate hosts to fewer wetlands increasing the chances of transmission, if an infected host is nearby (Greer and Collins 2008).

METHODS

Study Design

The main objective was to estimate the geographic extent of infections across a portion of the northern plains in a wide variety of habitats. To achieve this, I sampled amphibians broadly across the state of North Dakota using a stratified sampling design. The four level III ecoregions

(Omernik 1987) were the primary sampling strata, with the four general categories of land use

within ecoregion as secondary strata. The routes for collecting trips were selected to cover as

much of the state as possible, but were constrained by accessibility of wetlands, and incorporating

as much of the active season as possible. Routes were generally driven east to west and collection

trips focused on the southern, central, or northern tiers of the state. Wetlands were selected based

on availability (sites with water apparent) and accessibility (proximity to roads that were passable

under prevailing conditions) and to include a gradient of land uses. I specifically sought out

wetlands that were in or adjacent to croplands, pasture, grasslands (including hayland, CRP-type

land, or anything that resembled currently untilled, ungrazed land), and woodland. Sample

locations (N=171) are indicated in Figure 3. Each symbol on the map represents a location where

I collected amphibians.

Figure 3. Sampling locations from which amphibians were collected in 2013 and 2014 (combined). Symbols are color-coded by amphibian species and differ in size only to permit visibility of overlapping symbols. Only sites where I collected at least one species are included. Lines on the map demarcate county boundaries or ecoregions, with ecoregions labeled along the lower margin of the map. Ecoregion abbreviations: NWGP = Northwestern Great Plains, MC = Missouri Coteau, NGP = northern glaciated plains, RRV = Red River Valley.

Amphibian collection efforts spanned two field seasons, 2013 and 2014. The start of each field season depended on the weather and phenology of each amphibian species. In the early field season (April-May), collection focused on adult breeding amphibians. In the mid field season (June-July), I searched for tadpoles and adults and in the late season (August-September), meta-morphs and juveniles were primarily collected along with any additional adults found. The 2013 field season did not begin until late May because of extended snowfall leading to late thawing of wetlands resulting in limited time for collection of early breeders including the boreal chorus frog and the wood frog. I attempted to include all amphibian species and life stages in collection but did not encounter the gray tree frog (*Hyla versicolor)*, American toad (*Anaxyrus americanus*), spadefoot toad (*Spea bombifrons*), and the woodhouse's toad (*Anaxyrus woodhousei*) and collected very few tadpoles.

Field Protocol

I relied on chance encounters to collect amphibians while I walked around the perimeter and waded through wetlands. This was the most efficient method because it limited the time needed at individual wetlands and allowed inclusion of more sites. This method of sampling is subject to substantial bias in terms of which amphibian species were found. Some amphibian species have limited geographic ranges and specific habitat preferences that reduce the likelihood for encounters. For example, the plains spadefoot toad spends most of its time buried underground, limiting our ability to detect them. In addition, the gray tree frog is only found in the eastern margin of the state and is highly arboreal except during larval stages of development. Another species, the tiger salamander *Ambystoma mavortium*, is unlikely to be encountered by this method and is best sampled using traps or seines. Hence, any inference related to this host species will be limited.

The field protocol from James Cook University was followed to avoid cross contamination in the field and maintain field hygiene when handling amphibians (Speare et al. 2004). After amphibians were collected, each individual was assigned a numerical ID for future reference along with a photograph to confirm species identification and placed in a numbered container. In each container, I placed a wet paper towel in with the adults, juveniles and metamorphs to avoid desiccation. Tadpoles were kept in sufficient water so they remained submerged during transport. The containers with each individual amphibian were then stored in a cooler with ice to avoid over-heating. I recorded the snout-vent length of each amphibian, GPS coordinate of the location, and environmental characteristics such as wind speed, humidity, temperature, vegetation structure, and land use, and also took a picture of the location for future reference.

Necropsy Protocol

Amphibians collected for necropsy focused on males and abundant species to avoid demographic and population impacts. Five of each species at each location were collected for necropsy and euthanized following an IACUC approved protocol (#1305-2). The blood, liver, spleen and skin swab were also collected from each amphibian for DNA extraction and stored in 95% ethanol at -20°C. The spleen and sometimes liver tissue where later used for *Ranavirus* detection. During dissection, the mouth, urinary bladder, body cavity, lungs, kidneys, liver, intestines, and leg muscles were examined for helminths using a dissecting scope (Ch. 4). I also made blood smears of each necropsied amphibian to later search for blood parasites.

DNA extraction

I extracted DNA from spleen tissue following a modification of Tkach and Pawlowski (1999) protocol. The first step was to place a small piece of ethanol-fixed spleen into a 1.5ml

micro-centrifuge tube and add enough ultrapure water to cover the tissue. Next, the spleen and ultrapure water mixture were placed on a rocker platform for 20 minutes. I repeated this procedure twice making sure to discard and replace the ultrapure water between washes. After discarding the second wash, 60µl of ultrapure water was added to the micro-centrifuge tube for sonication. The spleen was then broken apart by sonication using a UP100H compact ultrasonic processor (Hielscher USA, Inc., Ringwood, NJ) at 80-100% for 20 seconds. After sonication, the spleen and ultrapure water should be a homogenous mixture. Next, 250µl of extraction buffer containing guanidine thiocyanate is added to each spleen mixture and the remaining steps follow Tkach and Pawlowski's (1999) protocol.

Real-time PCR

A modified version of the real-time PCR protocol (Forson and Storfer, 2006) was used to identify *Ranavirus* infections. A 70 base-pair region of the major capsid protein (MCP) of *Ambystoma tigrinum* virus (ATV) was amplified using the primers (5' ACA CCA CCG CCC AAA AGT AC 3') and (5' CCG TTC ATG ATG CGG ATA ATG 3') (Brunner et al, 2004). Each PCR reaction included approximately 100ng/µl of DNA template, 7.5µl SYBR green master mix, 900ng/µl of each forward and reverse primers and enough ultrapure water for the reaction total to be 15µl. I ran the PCR reactions on CFX96 Real Time system for 40 cycles: 95°C denaturing (5sec), 60°C annealing (30sec), and melt curve 72°C to 88.5°C at increments of 5 sec. A synthetic gene block of the major capsid protein (5'-TACGGTAGACTGACCAACGCCAGCCTTAACGTCACCCTGTCCGCTGAGGCCACCACG GCCGCCGCAGGAGGTGGAGGTAACAACTCTGGGTACACCACCGCCCAAAAGTACGC CCTCATCGTTCTGGCCATCAACCACAACATTATCCGCATCATGAACGGCTCGATGGG ATTCCCAATCTTGTAAAGAGTATTTTTCAGCGCAAAGTCTTTTCCGTCATGGGTCCTC

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CATGATGGAAATAAA-3') was used as the positive control. I included the positive and negative controls during each run with the amphibian samples to make comparisons and detect any contamination. If the *Ranavirus* assay was positive, I confirmed the result with the 250nmol fluorescent taqman probe (5' FAM-CCT CAT CGT TCT GGC CAT CAA CCA C-31ABkFQ 3') (Forson and Storfer, 2006). In these reactions, I used 100ng of DNA, 900ng/µl of the forward and reverse primers, 7.5µl of SsoAdvanced™ Universal Probes Supermix (works optimal for our CFX96 Real Time system), and enough ultrapure water for the reaction total to be 15µl. For these reactions, I set the Bio Rad CFX96 Real time system for 40 cycles: 95°C initial step (2min 30sec), 95°C denaturing (10sec) and 54°C annealing (30sec).

After verifying all positive infections with the suggested fluorescent probe, I randomly chose positive samples from each real-time PCR plate to verify by sequencing. I selected a 228bp gene fragment of the major capsid protein using the forward primer (5'- GTC GGC TCC AAT TAC ACC -3') and reverse primer (5' CAG GCT GAG GGC ATA AGA GT -3'). In each sequencing reaction, I included 2µl of BigDye sequencing buffer, 1.5µl of each primer, 1-2µl of PCR template (depending on the strength of the PCR product), and 1µl of BigDye. I ran all sequences on a Bio rad T100 thermal cycler for 25 cycles: 96°C for 15 seconds, 50°C for 5 seconds, 60°C for 4 minutes. All samples were run on an ABI Prism 3100 automated capillary sequencer.

Data analysis

To summarize the occurrence of *Ranavirus* in amphibians across the state, I calculated the prevalence (percent of infected individuals within a sample) and occupancy (percent of locations containing infected individuals) for each ecoregion and land use. I also tested for

statistical differences in *Ranavirus* prevalence among amphibian species, ecoregions, and land use categories using exact χ^2 tests (StatXact version 9.0, Cytel, Inc. 2010).

I used ArcGIS version 10.2 (ESRI 2014) to visualize the geographic distribution of *Ranavirus* and to estimate the wetland neighborhood around sample locations. I created buffers of 250 m and 500 m around each sample location to estimate wetland density, the percent cover of wetlands identified in the National Wetlands Inventory database within buffers. Because wetland densities at these scales were highly correlated, I retained only one for further analysis. I also estimated wetland density as the number of wetlands within each buffer.

To determine if locations with *Ranavirus* infected amphibians were spatially dependent, I estimated spatial autocorrelation in relation to distance separating sample locations. Distances were estimated from GPS coordinates using the software Passage 2.1 (Rosenburg et al. 2011). Significance of autocorrelations was determined using a permutation test with1000 permutations.

Spatial autocorrelation analysis suggested that the occurrence of *Ranavirus* exhibited some spatial structure. Specifically, infections exhibited a positive autocorrelation at distances less than 40km, suggesting a patchy spatial structure. I used Moran's eigenvector-based maps (Legendre and Legendre 2012) to generate a series of factors reflecting different components of the underlying spatial structure to incorporate into regression models. Spatial factors in this approach are derived based purely on the geographic arrangement of sampling locations. This method allows for more complex aspects of spatial structure to be represented, rather than simply distance along linear axes (UTM coordinates or latitude and longitude) for each location. For example, some components might appear as simple gradients, whereas more complex aspects of spatial structure would appear as different patterns of patchiness, perhaps differing in scale or spatial arrangement. These spatial factors can then be used as predictor variables in regression

models to test for similarities between response variables (e.g., disease occurrence) and spatial patterns. During the initial spatial factor selection process, I looked at each factor individually and selected those that appeared to have at least some correlation with *Ranavirus* occurrence. I used program SAM (Spatial Analysis in Macroecology version 4, Rangel et al. 2010) to estimate and conduct initial screening of spatial factors using a minimum separation threshold of 10 km between sites.

To test for associations between *Ranavirus* occurrence and the combined influence of all ecological and spatial predictor variables (Table 1), I constructed generalized linear models with various combinations of predictors. Because presence-absence is a binary response variable, I fit logistic regression models in R version 3.1.2 (R Core 2013). Model selection was based on a best subsets approach (Hosmer and Lemeshow 2000), with AICc as the criterion for comparing models (Burnham and Anderson 2002). The model selection process allowed us to identify factors that have the strongest association with *Ranavirus* occurrence. Because multiple models had a ΔAICc < 2, I used multi-model inference to generate a model averaged estimate compiled from all the component models for each predictor variable for *Ranavirus* occurrence. I specified ΔAIC of less than two (Burnham and Anderson, 2002) for model averaging, and estimated the contribution of all variables included in these models to the prediction of *Ranavirus* occurrence.

Table 1. The name and definition of predictor variables used in statistical models.

Predictor variables	Definition
Ecoregion	Level 3 ecoregion classification Bailey (1980) including Northwestern Great Plains (43-NWGP), Northwestern Glaciated Plains including the Missouri Coteau (42-NGP), Northern Glaciated Plain (46-NGP), and Lake Agassiz Plain, primarily the Red River Valley (48-RRV).
Land use	The four major types of land use include, from most to least disrupted by human activity, cropland, pasture, grassland, and woodland.
Length	Longest linear dimension of wetland.
Area	Wetland area.
Spatial factors	see text.
PcWet500	The percentage of wetlands within a 500 meter buffer extending from the center of each wetlands.
Nwetlands500	The number of wetlands within the 500 meter buffer.

RESULTS

I collected a total of 705 amphibians from the 2013 and 2014 field seasons combined. The sample size of each species is broken down by life stage during both collecting years in Tables 2 and 3. The northern leopard frog was the most abundant species collected during both years. Tiger salamanders had the smallest overall sample size because I did not use collecting methods suitable for that species.

Table 2. Amphibians collected during the 2013 field season from 68 different sites.

Amphibian species	Adult	Juvenile	Total
Barred tiger salamander (*Ambystoma mavortium*)	6	2	8
Canadian toad (*Anaxyrus hemiophrys*)	1	8	9
Great Plains toad (*Anaxyrus cognatus*)	1	6	7
Boreal chorus frog (*Pseudacris maculata*)	11	5	16
Northern leopard frog (*Lithobates pipiens*)	54	120	174
Wood frog (*Lithobates sylvaticus*)	14	35	49
Total			263

Table 3. Amphibians collected during the 2014 field season from 103 different sites.

Amphibian species	Adult	Juvenile	Tadpole	Total
Barred tiger salamander (*Ambystoma mavortium)*	4	2	NA	6
Canadian toad (*Anaxyrus hemiophrys*)	11	25	0	36
Great Plains toad (*Anaxyrus cognatus*)	13	8	1	22
Boreal chorus frog (*Pseudacris maculata*)	81	8	0	89
Northern leopard frog (*Lithobates pipiens*)	171	54	4	229
Wood frog (*Lithobates sylvaticus*)	44	16	0	60
Total				442

Objective 1: Estimate the geographic distribution of *Ranavirus* in North Dakota

Ranavirus infections were common and found broadly across the state (Figure 4). *Ranavirus* was detected in at least one amphibian specimen at 55.9% of sample locations (Table 4) and found at significantly more sites in the Missouri Coteau ecoregion (76.7%) than other areas when pooled across all amphibian species. Land use was not significantly associated with *Ranavirus* occupancy (Table 5).
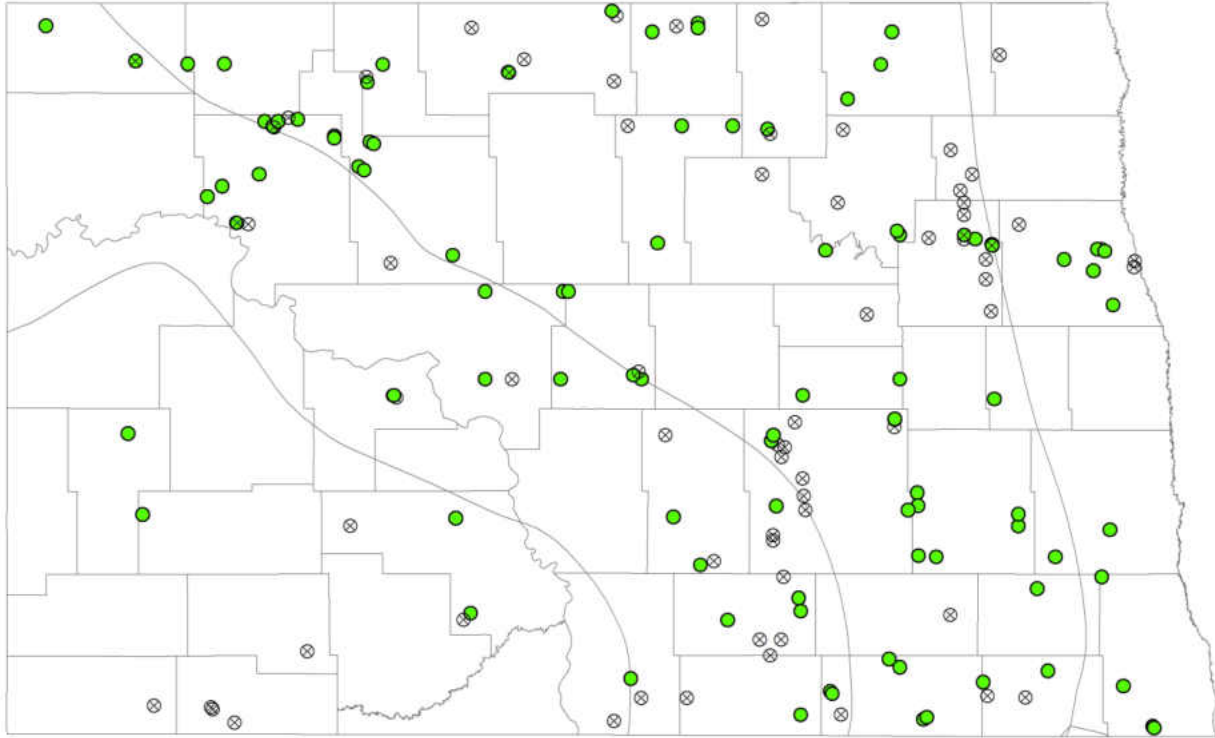
Figure 4. Geographic occurrence of *Ranavirus* infections across all amphibian species. Green circles represent infected amphibians, open circled "X"s indicate amphibian specimens testing negative. Lines illustrate county boundaries and ecoregions as in Figure 3 (from west to east: NWGP, MC, NGP, RRV).

Table 4. Number (N) and percentage of sites where *Ranavirus* was found in at least one amphibian specimen of any species in each ecoregion.

Ecoregion (N sites)	N amphibian specimens	N sites with *Ranavirus* detection	Percent of sites with *Ranavirus* detection (occupancy)
NWGP (19)	87	7	36.8
NGP (79)	317	37	46.8
MC (43)	140	33	76.7
RRV (29)	160	18	62.1
Total (170)	704	95	55.9
Chi^2 (3 df)		13.46	
P (exact, 2-sided)		0.0037	

Table 5. Number (N) and percentage of sites where *Ranavirus* was found in at least one amphibian specimen of any species in each land use category.

Land use (N sites)	N amphibian specimens	N sites with *Ranavirus* detection	Percent of sites with *Ranavirus* detection (occupancy)
Cropland (104)	428	60	57.7
Pasture (12)	162	7	58.3
Grassland (43)	66	21	48.8
Woodland (11)	51	7	63.6
Total (170)	707	95	55.9
Chi^2 (3 df)		1.53	
P (exact, sided)		0.689	

Objective 2: Estimate the prevalence of *Ranavirus* in North Dakota amphibians

I detected *Ranavirus* in 238 out of 668 assayed amphibians, resulting in an overall prevalence of 35.6%. *Ranavirus* prevalence varied significantly among amphibian species (Table 6, χ^2 = 47.69, df = 5, p < 0.0001). In particular, *Anaxyrus cognatus, Anaxyrus hemiophrys*, and *Pseudacris maculata* had higher prevalence than other species.

Table 6. *Ranavirus* infections by amphibian species.

Amphibian species	Sample size	Prevalence (%)
Barred tiger salamander (*Ambystoma mavortium)*	14	21.4
Great Plains toad *(Anaxyrus cognatus)*	29	58.6
Canadian toad *(Anaxyrus hemiophrys)*	45	57.8
Northern leopard frog (*Lithobates pipiens)*	367	26.1
Wood frog (*Lithobates sylvaticus)*	109	36.6
Boreal chorus frog (*Pseudacris maculata)*	103	54.3

Pooling all amphibian species, *Ranavirus* prevalence varied significantly across ecoregions (Figure 5). The overall prevalence combined across all amphibian species for the NWGP was 17.6%, MC 52.6%, NGP 29.2%, and RRV 44.6%. *Ranavirus* prevalence was higher in the MC and RRV than in the NGP or NWGP ecoregions. Because species were not equally

represented across ecoregions and there is a possibility of unequal susceptibility to infection, I also tested geographic patterns separately for the two species with largest sample size and broadest geographic sampling distribution. Infection prevalence still varied significantly across ecoregions for leopard frogs ($\chi^2 = 44.9$, p < 0.0001) and chorus frogs ($\chi^2 = 9.1$, p < 0.028).
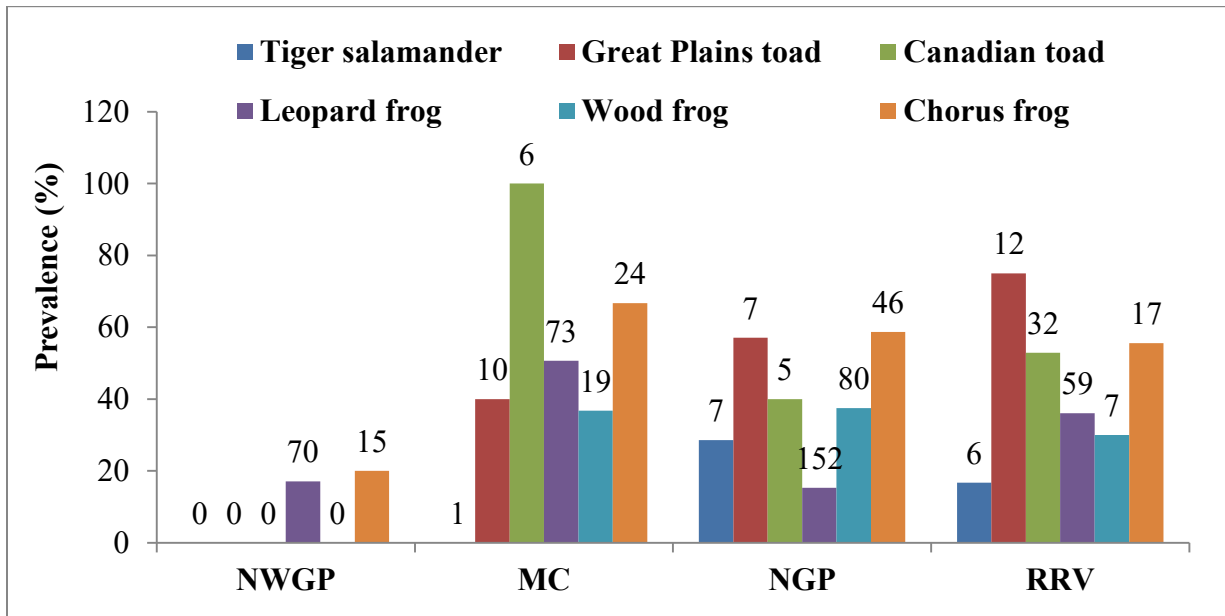
Figure 5. *Ranavirus* prevalence by amphibian species and ecoregion. The numbers above the bars represent sample size.

Ranavirus prevalence also varied significantly among locations in relation to predominant land use (Figure 6) pooled across all amphibian species. The prevalence across all amphibian species for cropland was 39.2%, pasture 30.6%, grassland 26.2%, and woodland 39.2%. In contrast to the overall pattern, for the species with the largest sample size, the northern leopard frog and boreal chorus frog infection rates did not differ significantly among land use categories ($\chi^2 = 3.33$, df = 2, p = 0.189 and $\chi^2 = 4.36$, df = 2, p = 0.113 respectively). Therefore, across all amphibian species, land use was significant but the effect varied among species. Woodland sites were excluded from analyses because of low sample size for these species.
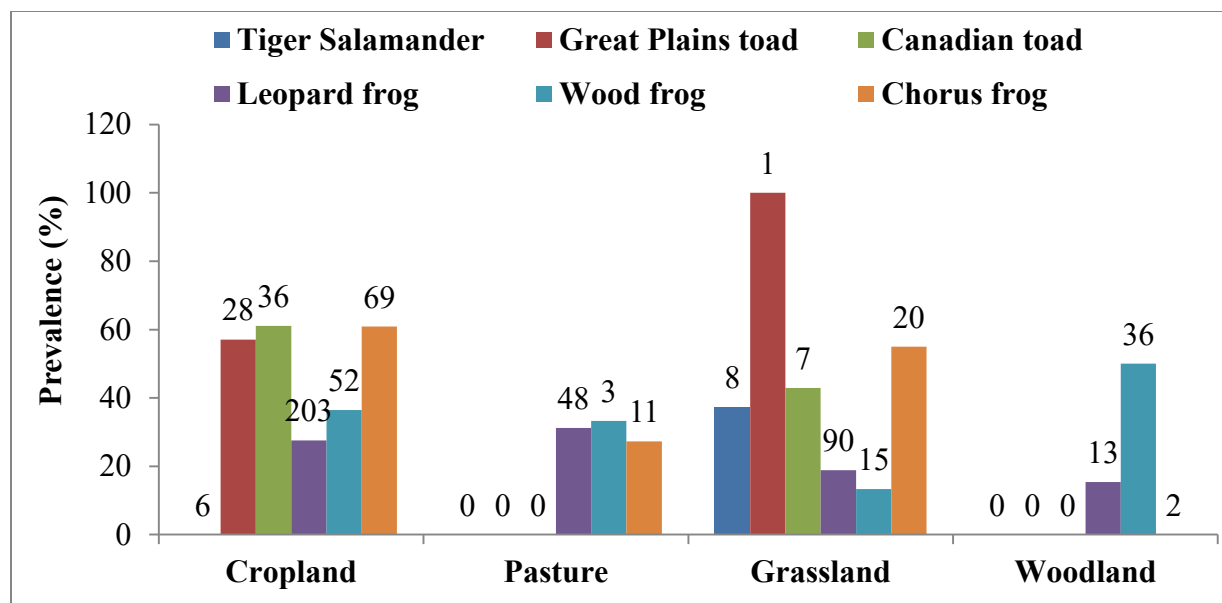
Figure 6. *Ranavirus* prevalence by amphibian species and predominant land use. The numbers above the bars represent sample size.

In addition to broad-scale patterns across the state, I tested for purely spatial effects that might be caused by underlying environmental influences or demographic linkages among sites affecting disease transmission rates. These linkages could be caused by amphibian dispersal or movement among sites by organisms serving as vectors of disease. The environment could also be similar among wetlands located closer together resulting in similar values for infection. In an analysis based on combined detections across all amphibian host species, I found evidence for spatial autocorrelation (non-independence in disease occurrence) among wetlands closer than about 40 km, but not at greater distances, indicating a moderately patchy distribution of *Ranavirus* infections on this scale (Fig. 7). In contrast, in an analysis based only on *Ranavirus* detections in northern leopard frogs, I found no evidence for significant spatial autocorrelation even at a fine scale (Fig. 8).
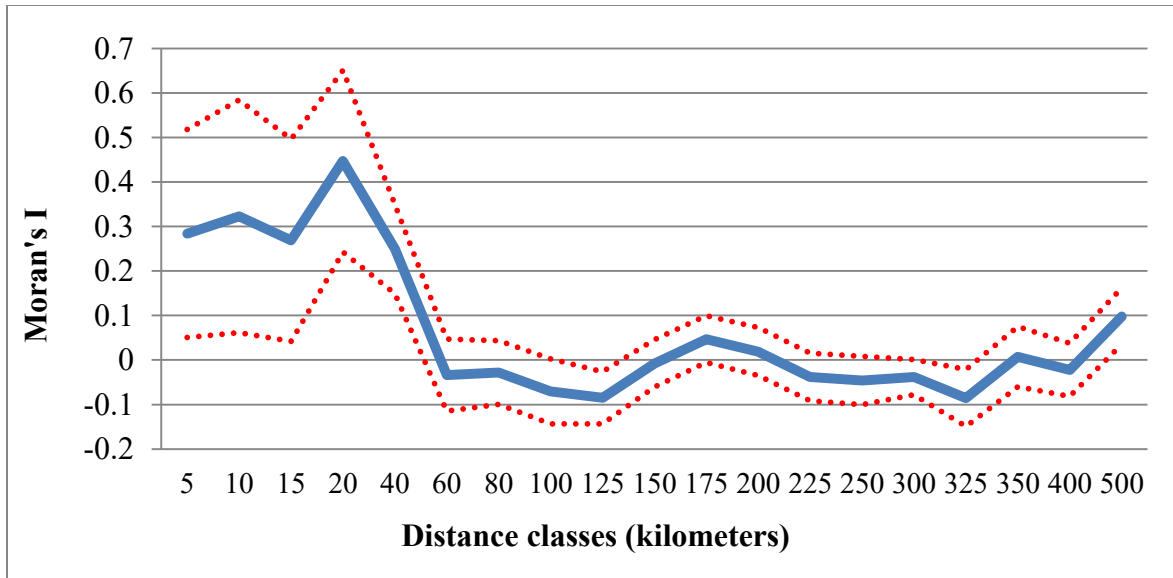
Figure 7. Spatial autocorrelation among sites separated by indicated distance (lag) classes, for *Ranavirus* presence/absence pooled across all amphibian species from 171 sites. Moran's I (blue) was used as the measure of autocorrelation; 95% confidence envelope is illustrated in red (dashed lines). The number of pairs of points for each distance class ranged from 55-72 at 5-15km to over 1000 at distances beyond that.
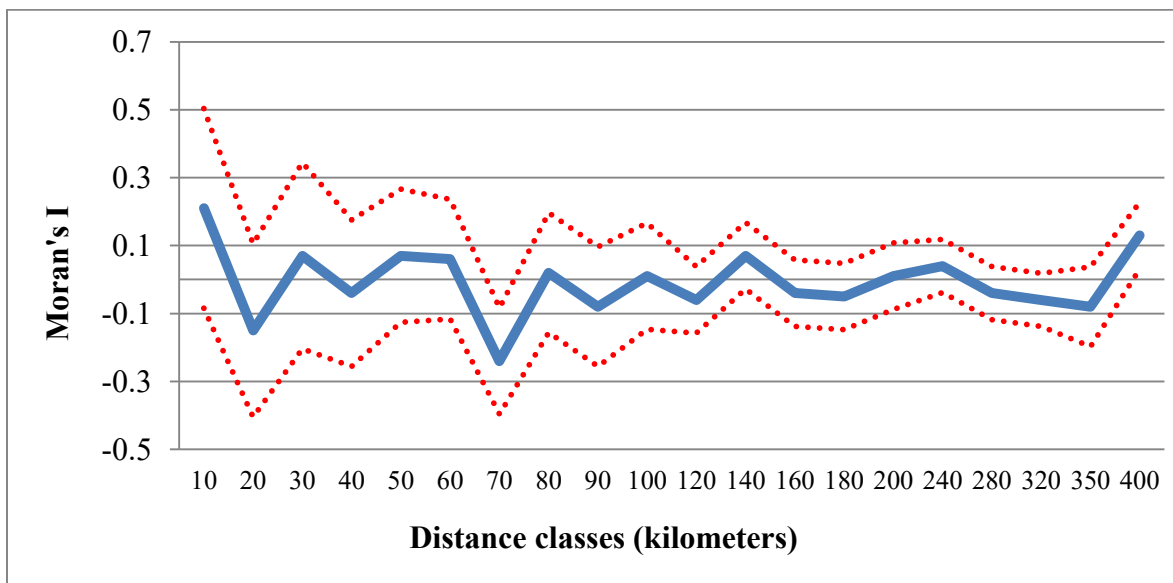
Figure 8. Spatial autocorrelation among sites separated by indicated distance (lag) classes, for *Ranavirus* presence/absence for northern leopard frogs tested from 105 sites. Moran's I (blue) was used as the measure of autocorrelation; 95% confidence envelope is illustrated in red (dashed lines). The number of pairs of points for each distance class ranged from 55-72 at 5-15km to over 1000 at distances beyond that.

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Combined effects of geographic and ecological factors on *Ranavirus* occurrence

After discovering *Ranavirus* was positively autocorrelated at distances within 40km, I used Moran's I eigenvector maps and retained eight spatial factors for further analysis. Although included in the top models based on ΔAICc < 2, suggesting that the spatial structure exists on multiple scales, only spatial factor 18 was a significant individual variable in relation to *Ranavirus* occurrence (Table 8). Spatial factor 18 corresponds to a broad scale pattern contrasting the central portion of the sampled area with the eastern and western portions (Fig. 9). To clarify the interpretation of this spatial factor, if a response variable of interest, such as *Ranavirus* occurrence, corresponds to a purely spatial factor, which are derived solely from map coordinates of sample sites and hence their spatial arrangement, then that response variable must exhibit some degree of parallelism in its spatial pattern. In other words, if *Ranavirus* occupancy is correlated with spatial factor 18, then occupancy must be higher in the central portion of the region and lower in the eastern and western portions. Testing for associations with spatial factors derived by this method provides the potential for discovery of spatial patterns more complex than simple east-west or north-south trends, as would be the case if latitude and longitude were used as predictor variables (Lengendre and Legendre 2012).
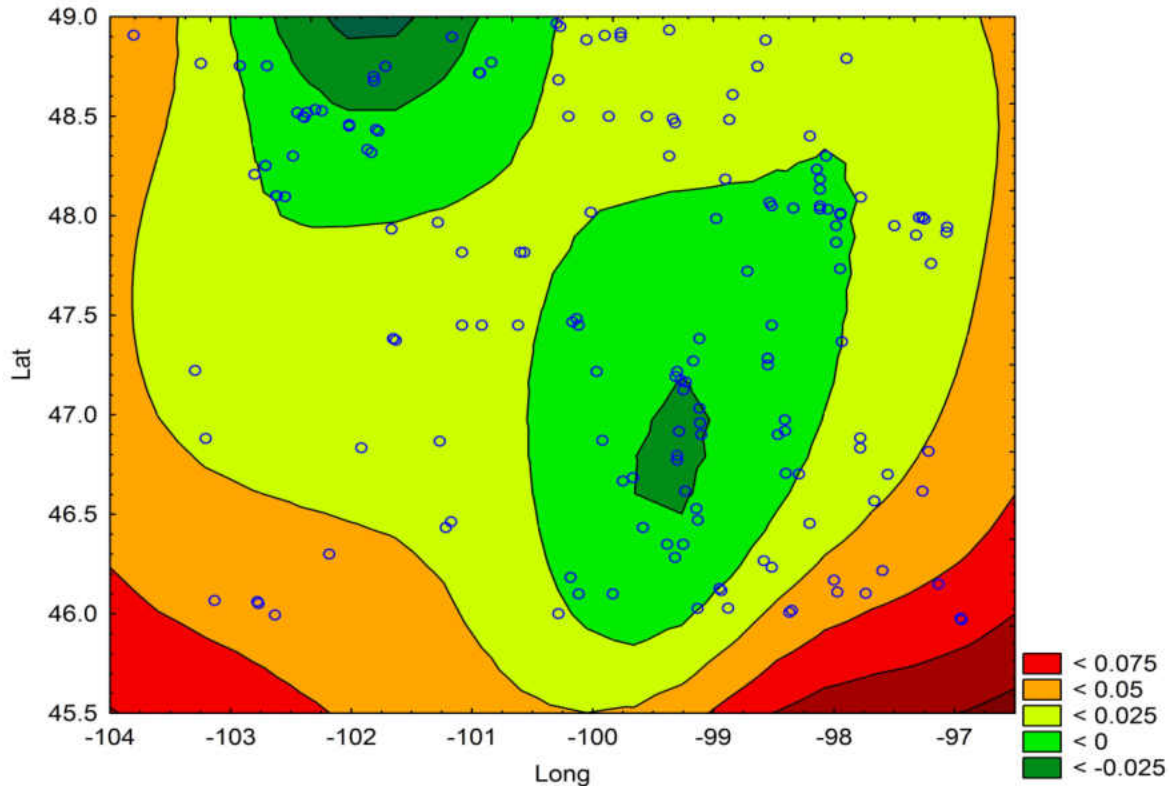
Figure 9. Contour plot of spatial factor 18 derived from coordinates of sample locations. The circles correspond to sampled locations. See text for explanation.

I used logistic regression models of *Ranavirus* presence/absence across all amphibian species and sites (n = 171 with complete information available). For model selection I included ecoregion, land use, wetland area, wetland length, the eight spatial factors, the number of wetlands within 500m (Nwetlands500) and the percent wetland coverage within 500m (Pcwet500). The top models with ΔAICc < 2 included ecoregion, Nwetlands500, and the eight spatial variables (Table 7). The top models are nearly indistinguishable from each other, in terms of the similar but small portion of the variation in *Ranavirus* occurrence they explain (based on residual deviance versus null deviance). They also identify similar sets of predictor variables. To synthesize the information content of the top models, I used multi-model inference to obtain a model-averaged estimate generated from the top component models for each predictor variable (Table 8). Among individual predictor variables, only ecoregion and specifically the Missouri

Coteau and SF18 were statistically significant (but bear in mind that model selection results are interpreted only with respect to the ensemble of included variables). Ecoregion and spatial effects are consistent with those identified above in the direct comparisons of prevalence (Fig. 5).

Table 7. Top models (AICc < 2) for *Ranavirus* occurrence pooled across all amphibians. The term codes for the variable are a=Ecoregion, b=Nwetlands500, c=SF12, d=SF14, e=SF15, f=SF18, g=SF22, h=SF27, i=SF31, j=SF5. Null deviance = 218.8.

Model	Variable	Residual Deviance	df	AICc	delta	weight
1	cdefghij	194.66	9	213.78	0	0.18
2	a defghi	193.10	10	214.47	0.70	0.12
3	cdefghi	197.78	8	214.67	0.89	0.11
4	a efghi	195.66	9	214.78	1.00	0.11
5	cdefgh j	198.17	8	215.06	1.28	0.09
6	a cdefghi	191.60	11	215.26	1.48	0.08
7	defghij	198.42	8	215.31	1.53	0.08
8	bcdefghij	194.02	10	215.40	1.62	0.08
9	a defghij	191.77	11	215.43	1.66	0.08
10	c efghij	198.78	8	215.66	1.89	0.07

Table 8. Model averaged coefficients of *Ranavirus* occurrence across all amphibian specimens using an ΔAIC < 2 criterion. The variable codes are given in Table 7.

Variable	Estimate	Standard Error	P
Intercept	0.06897	0.28540	0.810
c = SF12	-4.16275	2.59076	0.111
d = SF14	-5.67437	3.38158	0.096
e = SF15	-11.57740	8.51360	0.177
f = SF18	**9.74179**	**3.46306**	**0.005**
g = SF22	7.74945	4.70948	0.102
h = SF27	8.32305	5.02936	0.100
i = SF31	9.19136	6.48496	0.159
j = SF5	3.92459	2.68412	0.147
Ecoregion [MC]	**1.00037**	**0.47787**	**0.038**
Ecoregion [RRV]	0.71574	0.50224	0.157
Ecoregion[NWGP]	-0.36845	0.58777	0.534
Nwetlands500	0.01283	0.01626	0.433

Northern leopard frogs comprised the majority of collected amphibians; consequently I also ran a logistic regression with the same ecological variables using just the *Ranavirus* results for northern leopard frogs. The top models with ΔAICc < 2 included the same variables in models with all amphibians. Again, the top models explained a small portion of the variation in *Ranavirus* occurrence (Table 9) and included similar sets of predictor variables. I used multi-model inference to generate a model- averaged estimate of each predictor variable (Table 10). The Missouri Coteau ecoregion was the only significant predictor for *Ranavirus* occurrence in northern leopard frogs.

Table 9. Top models (AICc < 2) for *Ranavirus* occurrence in northern leopard frogs. The term codes for the variables are a= Ecoregion, b= Nwetlands500, c=SF13, d= SF18, e=SF19, f= SF2, g= SF21, h=SF4, i= SF6, j= SF8. Null deviance = 139.2.

Model	Variable	Residual Deviance	df	AICc	Delta	Weight
1	cde g i	114.09	6	126.94	0	0.10
2	cdefg i	111.96	7	127.12	0.17	0.09
3	cde ghi	112.04	7	127.19	0.25	0.08
4	cdefghi	109.90	8	127.40	0.46	0.08
5	cde g ij	112.37	7	127.53	0.58	0.07
6	cdefg ij	110.22	8	127.72	0.78	0.07
7	cde ghij	110.24	8	127.74	0.79	0.06
8	cdefghij	108.07	9	127.97	1.02	0.06
9	cde g	117.42	5	128.03	1.08	0.06
10	cdefg	115.30	6	128.16	1.22	0.05
11	cde gh	115.36	6	128.21	1.27	0.05
12	cdefgh	113.23	7	128.38	1.44	0.05
13	bcdefg i	111.0	8	128.60	1.66	0.05
14	bcde g i	113.57	7	128.72	1.78	0.04
15	a cde g	111.43	8	128.93	1.98	0.04

Table 10. Model averaged coefficients for *Ranavirus* occurrence in northern leopard frogs using an ΔAIC < 2 criterion. The term codes are given in Table 10.

Variable	Estimate	Standard Error	P
Intercept	-5.379e-01	3.525e-01	0.1308
c = SF13	8.696e+00	6.196e+00	0.1657
d = SF18	-1.662e+01	9.312e+00	0.0779
e = SF19	-3.969e+01	2.149e+01	0.0679
g = SF21	-1.016e+03	1.994e+03	0.6147
i = SF6	-5.041e+00	3.384e+00	0.1410
f = SF2	3.329e+00	2.443e+00	0.1784
h = SF4	-3.474e+00	2.711e+00	0.2057
j = SF8	4.453e+00	4.044e+00	0.2768
Nwetlands500	-2.053e-02	2.522e-02	0.4213
Ecoregion [MC]	**1.301e+00**	**6.263e-01**	**0.0403**
Ecoregion [RRV]	1.230e+00	6.802e-01	0.0742
Ecoregion [NWGP]	6.160e-01	6.648e-01	0.3601

DISCUSSION

I detected a high prevalence (35.6%) of *Ranavirus* in internal organ tissues, demonstrating that *Ranavirus* infection is common across amphibians of North Dakota. Previously researchers reported *Ranavirus* die-offs in three Canadian provinces and over 20 states in the USA (Bollinger et al. 1999; Green et al. 2002, Carey et al. 2003, Greer et al. 2005, Jancovich et al. 2005). *Ranavirus*-infected amphibians did not show any symptoms of disease upon collection. Diseased animals probably suffered high mortality rates and disappeared quickly, challenging our ability to detect disease occurrence. In general, reported mortality rates have been much higher during the larval stages, resulting in recruitment failure (Brunner et al. 2015; Cunningham et al. 2007; Echaubard et al. 2014; Gray et al. 2009). However, adults are known to be reservoirs and likely sources of transmission among sites, sometimes with little apparent impact on their health (Gray et al. 2009).

Infections were also geographically widespread, likely because *Ranavirus* infects a variety of ectothermic hosts, including all tested amphibian species in our study and at least some fishes and reptiles that can spread infections. Several experimental studies demonstrated *Ranavirus* transmission among various ectothermic classes (Bayley 2013; Mao et al. 1999; Picco et al. 2010; Schock et al. 2008). These studies often result in nearly all amphibians dying, but infected turtles and fish persisting without mortality. If infected fishes or reptiles are nearby, amphibians can become infected by direct contact with infected individuals or surrounding environment (Gray et al. 2009).

I found *Ranavirus* prevalence varied significantly among amphibian species, ecoregion and land use when pooled across all amphibian species. There are many studies confirming amphibian species vary in susceptibility to *Ranavirus* because of phylogeny, life history, and their ecology (Brunner et al. 2015; Hoverman et al. 2011). *Ranavirus* prevalence was also somewhat more common in the Missouri Coteau than other areas, followed by sites in the Red River Valley. Pasture and grassland sites had lower *Ranavirus* prevalence than cropland when pooled across all amphibian species. The cause of differences in infection risk is not known; there are a variety of factors that might differ geographically across the state, and our study was not designed to reveal underlying causation.

From the logistic regression results, the Missouri Coteau ecoregion was more likely to have *Ranaviru*s infected amphibians. The Missouri Coteau contains a large number of prairie pothole wetlands located in close proximity, possibly increasing opportunities for transmission (Fig 1). However, the Northern Glaciated Plain ecoregion is contained within the Prairie Pothole Region and also contains a high density of wetlands. From the spatial autocorrelation analysis, I detected a clear pattern that wetlands in close proximity are more likely to be similar in the

presence or absence of infected frogs. However, this is not simply an effect of local proximity, given that the spatial dependency extended to 40km. The spatial autocorrelation pattern may simply reflect the broader geographic differences in prevalence: it is reasonable to infer that if an infection becomes established at a site in the Missouri Coteau, additional transmission is more likely at nearby sites than more distant sites, many of which will be located in other ecoregions. In other words, some portion of the autocorrelation pattern may be confounded with broad scale geographic variation that also underlies the ecoregion effect. Spatial factor 18 was probably included in the best model for the same reason: it corresponds to some extent with ecoregion.

Land use, wetland length and area, and percent and number of wetlands were not significant predictors for *Ranavirus* occurrence. The latter landscape factors correspond to the finest scale of spatial structure, but were not represented either in the spatial autocorrelation analysis or the construction of spatial factors. This result also supports the inference that spatial effects were largely driven by ecoregion differences and not local wetland connectivity. The underlying cause for an ecoregion effect is not clear. Possibly the density of wetlands, which varies from moderate in the Missouri Coteau to very high in the Northern Glaciated Plains, to low in the Red River Valley, does impact wetland-to-wetland transmission, but in a complex, nonlinear manner.

There are many caveats in this study for any inference I can make about the ecology of amphibian-*Ranavirus* interactions. These include lack of repeated sampling at each site at different times of seasons to increase detectability, uneven sample sizes at different life stages, species, land use categories and ecoregions. Future studies should incorporate other variables hypothesized to affect *Ranavirus* occurrence such as amphibian abundance, fish and reptile presence, and more detailed measurements of wetland and landscape characteristics, such as

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aquatic vegetation, water chemistry, and surrounding land use. These results were also only compiled from two years of field data and likely capture only a snapshot in time of *Ranavirus* occurrence throughout North Dakota, and certainly do not capture temporal dynamics.

Continued disease surveillance is needed, coupled with routine amphibian monitoring because *Ranavirus* is a well-established cause of mass mortality and recruitment failure in amphibian populations (Miller et al. 2009, Price et al. 2014). As far as we know, North Dakota does not have a routine, standardized amphibian monitoring program at this time, but even a modest monitoring program at select sites would be useful and provide a starting point for surveillance. This becomes particularly important in the face of climate change and other environmental perturbations that might alter local habitats in such a way that exposes animals to localized stressors, or alters demographic connectivity among amphibian populations or movement patterns of potential disease carriers. Furthermore, other ectotherms (reptiles and fishes) are also susceptible to *Ranaviruses* (Gray and Chinchar 2015) and monitoring programs for amphibians would also provide useful information for those taxa.

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CHAPTER III

PREVALENCE AND GEOGRAPHIC DISTRIBUTION OF CHYTRID FUNGUS IN NORTH DAKOTA AMPHIBIANS

ABSTRACT

Chytrid fungus has been implicated in amphibian species extinctions and nearly half of all amphibian species declining worldwide. To our knowledge, no amphibian survey has ever been done for chytrid fungus in North Dakota. In this study, I estimated chytrid fungus geographic distribution and prevalence in North Dakota amphibians. I sampled broadly across the state, including all major ecoregions, and land use categories. Six species of amphibians were represented in the total sample including northern leopard frogs (*Lithobates pipiens*), wood frogs (*Lithobates sylvaticus*), boreal chorus frogs (*Pseudacris maculata*), Canadian toads (*Anaxyrus hemiophrys*), Great Plains toad (*Anaxyrus cognatus*), and tiger salamanders (*Ambystoma mavortium*). I used real time PCR to detect chytrid fungus infections and found five infected northern leopard frogs, resulting in 0.007% prevalence pooled across all amphibian species. All infections were found in central North Dakota. I could not estimate ecological predictors for chytrid fungus occurrence because of the low detection of infections among amphibians. Some explanations for low detection are chytrid fungus is rare and localized at a few locations in North Dakota, rapid die-offs of infected amphibians limited our detection ability, and/or our diagnostic and swabbing techniques were inadequate for detection. I recommend continued disease surveillance to confirm low prevalence and monitor the spread of chytrid fungus to uninfected areas.

INTRODUCTION

Batrachochytrium dendrobatidis (Bd) was first discovered in 1997 (Berger et al. 1998) and since then over 500 species of amphibians in 54 countries has been infected (Fisher et al. 2009; Olson et al. 2013). Bd is highly pathogenic and causes the disease chytridiomycosis. This disease is a huge threat to biodiversity (Kilpatrick et al. 2010) and the causative agent for amphibian declines worldwide (Lips et al. 2006; Lips et al. 2008; Muths et al. 2003; Rachowicz et al. 2006). The Global Amphibian Assessment (GAA) proposed 32.5% of all amphibian species are threatened and 92.5% are critically endangered and undergoing declines that are correlated to Bd (Bielby et al. 2008; Stuart et al. 2004). Because chytrid fungus is a threat to amphibian population sizes and biodiversity, I conducted the first statewide survey to estimate the prevalence and geographic distribution of chytrid fungus in North Dakota amphibians.

Bd has a broad geographic distribution, and has been found widely in the Americas, and patchily in Africa, Asia, Australia and Europe (James et al. 2006). Detection biases and gaps in sampling effort still exist across the globe, contributing to the clustering of locations where Bd has been found (James et al. 2006). The geographic distribution of chytrid fungus is likely underestimated because of rapid removal of dead hosts by predation, fast decomposition, sporadic disease outbreaks, and limited number of large-scale and long-term surveillance efforts (Gray et al. 2009; Kilpatrick et al. 2010).

North Dakota is a likely location to suspect disease occurrence because it contains many factors promoting Bd infection, including cool temperatures (Forrest et al. 2011; Holmes et al. 2014; Kilpatrick et al. 2010; Lips et al. 2008; Olson et al. 2013; Raffel et al. 2015; Rohr et al. 2011; Woodhams et al. 2008), pesticide runoff into wetlands and extensive agricultural land use, which combine to reduce habitat availability and increase exposure to environmental stressors

(Gahl et al. 2011; Gaietto et al. 2014; Hanlon and Parris 2013; Paetow et al. 2012). North Dakota also contains many prairie pothole wetlands that are used for multiple recreational activities such as hunting, fishing, and boating that can facilitate the movement of contaminated objects and amphibians to uninfected areas (Addis et al. 2015; Fisher et al. 2012).

OBJECTIVES AND HYPOTHESES

The main goal of this portion of my amphibian disease study was to estimate the geographic distribution and prevalence of Bd in the portion of the northern Great Plains within North Dakota. I tested the following hypotheses regarding Bd occurrence based on previous information known about Bd environmental persistence, transmission and amphibian susceptibility.

Objectives

(1). Estimate the geographic distribution of chytrid fungus in North Dakota.

Hypothesis 1: I predict the geographic distribution of Bd is widespread across North Dakota because Bd has many host reservoirs and long environmental persistence. Bd infects a variety of ectothermic vertebrates (Kilpatrick et al. 2010; Olson et al. 2013; Valencia-Aguillar et al. 2015) increasing the probability of transmission to amphibians. Researchers also found zoospores can adhere, survive, and even proliferate on the toe scales and feathers of waterfowl (Garmyn et al. 2012; Johnson and Speare 2005). North Dakota contains many prairie pothole wetlands that are inhabited by waterfowl potentially spreading infections to uninfected wetlands. Bd can also survive in the environment for long periods of time by the presence of another reservoir species shedding zoospores, persistence in another life stage of the host, and surviving in a

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saprophytic stage by eating dead decaying matter (Kilpatrick et al. 2010) increasing opportunities for amphibian exposure.

(2). Estimate the prevalence of chytrid fungus in North Dakota amphibians.

Hypothesis 2A: I hypothesize Bd prevalence will increase at locations with cool temperatures and increased precipitation (Forrest et al. 2011; Holmes et al. 2014; Kilpatrick et al. 2010; Lips et al. 2008; Olson et al. 2013; Rohr et al. 2011; Raffel et al. 2015; Woodhams et al. 2008). Temperatures between 17-25°C are optimal for Bd growth, but temperatures higher than 29°C or below 0°C are considered lethal (Piotrowski et al. 2004). Several studies have shown Bd prevalence to drop dramatically in warm water and increase in cold water temperatures (Forrest and Schlaepfer 2011; Whitfield et al. 2012).

Hypothesis 2B: The prevalence of Bd will increase at locations with high human disturbance because contaminants in the water can affect Bd infection intensity and prevalence (Gahl et al. 2011; Gaietto et al. 2014; Hanlon and Parris 2013; Paetow et al. 2012). In addition, fungal characteristics such as habitat flexibility, long environmental persistence, and multiple reproductive modes allow humans to spread Bd by transporting contaminated objects such as boots, fishing equipment, research gear and boats to uninfected wetlands (Fisher et al. 2012).

METHODS
Study Design

The main objective was to estimate the geographic extent of infections across a portion of the northern plains in a wide variety of habitats. To achieve this, I sampled amphibians broadly across the state of North Dakota using a stratified sampling design. The four level III ecoregions (Omernik 1987) were the primary sampling strata, with the four general categories of land use

within ecoregion as secondary strata. The routes for collecting trips were selected to cover as much of the state as possible, but were constrained by accessibility of wetlands, and incorporating as much of the active season as possible. Routes were generally driven east to west and collection trips focused on the southern, central, or northern tiers of the state. Wetlands were selected based on availability (sites with water apparent) and accessibility (proximity to roads that were passable under prevailing conditions) and to include a gradient of land uses. I specifically sought out wetlands that were in or adjacent to croplands, pasture, grasslands (including hayland, CRP-type land, or anything that resembled currently untilled, ungrazed land), and woodland. Sample locations (N=171) are indicated in Figure 3. Each symbol on the map represents a location where I collected amphibians.

Amphibian collection efforts spanned two field seasons, 2013 and 2014. The start of each field season depended on the weather and phenology of each amphibian species. In the early field season (April-May), collection focused on adult breeding amphibians. In the mid field season (June-July), I searched for tadpoles and adults and in the late season (August-September), meta-morphs and juveniles were primarily collected along with any additional adults found. The 2013 field season did not begin until late May because of extended snowfall leading to late thawing of wetlands resulting in limited time for collection of early breeders including the boreal chorus frog and the wood frog. I attempted to include all amphibian species and life stages in collection but did not encounter the gray tree frog (*Hyla versicolor)*, American toad (*Anaxyrus americanus*), spadefoot toad (*Spea bombifrons*), and the woodhouse's toad (*Anaxyrus woodhousei*) and collected very few tadpoles.

Field Protocol

I relied on chance encounters to collect amphibians while I walked around the perimeter and waded through wetlands. This was the most efficient method because it limited the time needed at individual wetlands and allowed inclusion of more sites. This method of sampling is subject to substantial bias in terms of which amphibian species were found. Some amphibian species have limited geographic ranges and specific habitat preferences that reduce the likelihood for encounters. For example, the plains spadefoot toad spends most of its time buried underground, limiting our ability to detect them. In addition, the gray tree frog is only found in the eastern margin of the state and is highly arboreal except duing larval stages.

The field protocol from James Cook University was followed to avoid cross contamination in the field and maintain field hygiene when handling amphibians (Speare et al. 2004). I swabbed the skin of each amphibian for Bd detection following Berger and Speare (1998). The swabs were then stored in ethanol-filled vials labeled with the corresponding amphibian ID. Then amphibians were placed in a numbered container corresponding to the amphibian ID to keep track of individuals. In each container, I placed a wet paper towel in with the adults, juveniles and meta-morphs to avoid desiccation. Tadpoles were kept in sufficient water so they remain submerged while being transported. The containers with each individual amphibian were stored in a cooler with ice to avoid over heating. I recorded the snout-vent length of each amphibian and landscape characteristics such as wind speed, humidity, temperature, vegetation structure, GPS coordinates, and land use at each site. I also took a picture of each location for future reference.

Necropsy Protocol

Amphibians collected for necropsy focused on males and abundant species to avoid demographic and population impacts. Five of each species at each location were collected for necropsy and euthanized following an IACUC approved protocol (#1305-2). The blood, liver, spleen and skin swab were also collected from each amphibian for DNA extraction and stored in 95% ethanol at -20°C. The skin swabs were later used for Bd detection. During dissection, the mouth, urinary bladder, lungs, kidneys, liver, intestines, and leg muscles were examined for helminths using a dissecting scope (Ch. 4). I also made blood smears of each necropsied amphibian to later search for blood parasites (Ch.4).

DNA extraction

I extracted DNA from skin swabs following a modification of Retallick et al. (2006) and Richards-Hrdlicka et al. (2013). The first step was to centrifuge each micro-centrifuge tube containing a swab at 13000 rpm for ten minutes. Next, the ethanol was removed from each micro-centrifuge tube using a clean pipette, while not dislodging or removing the pellet. Then I added 150µl of Prepman Ultra (Applied Biosystem[TM]) and vortexed each tube. After the tubes have been vortexed, I centrifuged the tubes containing Prepman Ultra for two minutes at 13000rpm. The tubes were then boiled at 100°C at 700rpm for ten minutes and later cooled at room temp for two minutes. After the tubes have been cooled, I centrifuged them at 13000 rpm for three minutes and added 20µl of the supernatant to a new tube and stored at -20°C. Before each real-time PCR run, I made a 1:10 dilution of each DNA extract with ultrapure water.

Real-time PCR

I tested for Bd DNA following Boyle et al. (2004) and Richards-Hrdlicka et al. (2013) real time PCR protocols. Each reaction consisted of 5µl of DNA that was diluted 1:10 with

ultrapure water, 10µl SsoAdvanced Universal probes Supermix (works best for CFX384 Touch

Real time PCR Detection System), 1µl of each primer (900nm of each forward and reverse), 2µl

of the probe (250nm), and 1µl of ultrapure water (Boyle et al. 2004; Richards-Hrdlicka et al.

2013). The qPCR protocol was: initial step at 95°C for 2 minutes 30 seconds, denaturing step at

95°C for 10 seconds, and annealing step at 54°C for 30 seconds for 40 total cycles (Boyle et al.

2004). Each qPCR assay included a positive and negative control. The positive control is a

synthetic gene block of Chytrid MGB2 gene 5'-ACG-TTT-TGA-TGC-GAA-ACT-CTC-GTC-

CTT-GAT-ATA-ATA-CAG-TGT-GCC-ATA-TGT-CAC-GAG-TCG-AAC-AAA-ATT-TAT-

TTA-TTT-TTT-CGA-CAA-ATT-AAT-TGG-AAA-TTG-AAT-AAT-TTA-ATT-GAA-AAA-

AAT-TGA-AAA-TAA-ATA-TTA-AAA-ACA-ACT-TTT-GAC-AAC-GGA-TCT-CTT-GGC-

TCT-CGC-AAC-GAT-GAA-GAA-CGC-AG-3' (Boyle et al. 2004). The negative control is ultra

pure water to verify there was no contamination during each assay. The primers and probe used

for real time PCR are shown in Table 11 (Boyle et al. 2004).

Table 11. Primers and probe used for real time PCR

Name	Nucleotide sequence (5'-3')
ITS1-3 Chytr (forward primer)	CCTTGATATAATACAGTGTGCCATATGTC
5.8S Chytr (reverse primer)	AGCCAAGAGATCCGTTGTCAAA
Chytr MGB2 (probe)	6FAM CGAGTCGAACAAAAT 31ABkFQ
External 5.8S (reverse primer)	GTGGTTGTGACGTTGTATTAC

I verified all positive Bd samples by sequencing. I selected a 401bp gene fragment of

MGB2 gene using the forward primer ITS1-3 and reverse primer external 5.8S (Table 11). In

each sequencing reaction, I included 2µl of BigDye sequencing buffer, 1.5µl of each primer, 1-

2µl of PCR template (depending on the strength of the PCR product), and 1µl of BigDye. I ran

all sequences on a Bio rad T100 thermal cycler for 25 cycles: 96°C for 15 seconds, 50°C for 5

seconds, 60°C for 4 minutes. Then all samples were run on an ABI Prism 3100 automated

capillary sequencer.

I collected a total of 705 amphibians from both 2013 and 2014 field seasons combined. The sample size of each species is broken down by life stage during both collecting years in Tables 2 and 3. The northern leopard frog was the most abundant species collected during both years. Tiger salamanders had the smallest overall sample size because I did not use collecting methods suitable for that species.

Objective 1: Estimate the geographic distribution of chytrid fungus in North Dakota

Infections were found near central North Dakota and located at relatively close proximity to each other (Figure 10). In particular, one infected northern leopard frog was found southwest of Carrington, North Dakota (N47.165 and W-99.228), two others were found towards the west side of central North Dakota south of Lake Sakakawea (N47.383 W101.654), the fourth was towards the central east side of North Dakota north of Cleveland, North Dakota (N46.96 W-99.11) and the last was north of Woodworth, North Dakota (N47.175 E-99.27).
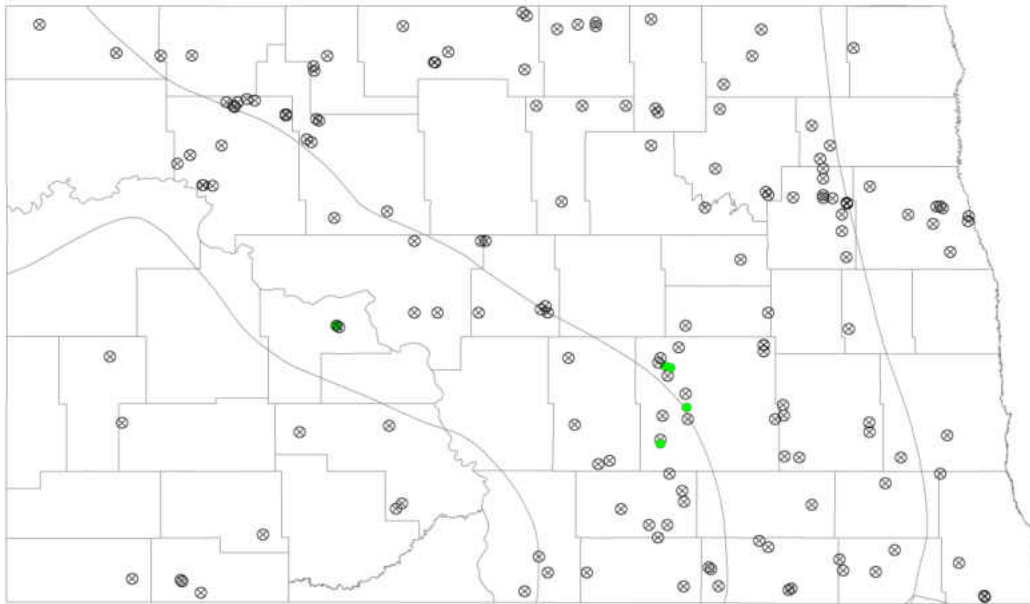
Figure 10. Geographic occurrence of Bd infections across all amphibian species. Green circles represent infected amphibians, open circles "X"s indicate amphibian specimens testing negative. Lines illustrate county boundaries and ecoregions as in Figure 3 (from west to east: NWGP, MC, NGP, RRV).

Objective 2: Estimate the prevalence of chytrid fungus in North Dakota amphibians

I detected Bd in five northern leopard frogs (*Lithobates pipiens*) out of 705 assayed amphibians, resulting in an overall prevalence (percent of infected individuals within a sample) of 0.007%. I could not estimate predictors for Bd occurrence because of the low number of detections.

DISCUSSION

Bd was surprisingly uncommon (0.007%) in North Dakota amphibians and primarily found near central North Dakota. Because of the low detection of infections, it is impossible to make any statistical conclusions that estimate ecological predictors for Bd occurrence. Other nearby states including Minnesota, Iowa, Montana, Nebraska, and Wyoming found variable high to low prevalence of Bd in amphibian populations (Briggs et al. 2010; Moffit et al. 2015; Muths et al. 2008; Olson et al. 2013) Many factors influence Bd occurrence and the complexity of those interactions result in sporadic disease outbreaks that limit our ability to detect infections before mortality events.

Four possible explanations for low Bd prevalence in North Dakota are: 1) Bd is rare in North Dakota, 2) infected amphibians die too rapidly to detect infections, 3) the summer prior to this study (2012) was very dry and reduced Bd survival in the environment, and 4) our diagnostic and swabbing techniques were inadequate for Bd detection. In the following paragraphs I will discuss these explanations for low Bd detection.

Bd could be rare in North Dakota because warmer temperatures and lower precipitation rates are not ideal for zoospore production and persistence outside of the host (Beyer et al. 2014; Puschendorf et al. 2009; Woodhams et al. 2008). North Dakota has a wide variety of temperatures with warm to hot and somewhat humid summers and cold windy winters. Warmer

temperatures reduce zoospore production limiting opportunities for disease transmission because amphibians require direct contact with the zoospores to become infected (Kilpatrick et al. 2010). North Dakota also has lower annual precipitation rates compared to other nearby states, which is unfavorable for Bd growth and persistence outside of the host (Beyer et al. 2014; Puschendorf et al. 2009).

Another possibility is infected animals died before I visited a location and predators or decomposition removed carcasses before I was able to detect infections. I primarily collected post metamorphic individuals and they are most susceptible to infections because they contain greater amounts of keratin. Several studies have demonstrated frequent metamorph and adult mortality while infected with Bd (Berger et al. 1999; Bradley et al. 2002; Kilpatrick et al. 2010; Scheele et al. 2010). Therefore, I could have easily missed a mortality event that occurred earlier in the season and found no evidence at the time of collection.

For Bd to persist in the environment for long periods of time it requires a cool and moist habitat. The summer of 2012 was very dry in North Dakota, with the majority of small ponds drying completely. This could have resulted in failure of Bd to persist and subsequently recolonize those locations. We do not know if the drought during the summer of 2012 was long enough to kill off Bd throughout most of the prairie pothole region. We also do not know how long it would take for Bd to re-establish in those areas given that it infects multiple ectothermic hosts and can be dispersed throughout the area by waterfowl and human movement.

For Bd detection, I used Boyle et al. (2004) and Richards-Hrdlicka et al. (2013) real time PCR protocols to identify individual infections. These protocols are well established and proven successful in various other studies (Briggs et al. 2010; Hrdlicka et al. 2013; Kriger and Hero et al. 2007; Retallick et al. 2006) so false negatives are unlikely. I also found five positive Bd

infections using this protocol so I would expect to detect other infections using the same protocol. However, some studies have also extracted Bd DNA from the mouthparts, sloughed skin (Retallick et al. 2006; Berger et al. 2005), and toe clippings (Boyle et al. 2004) in addition to skin swabs and yielded positive results.

Researchers also discovered different swabbing procedures including swabbing location, number of strokes, pressure applied, and type of swab used could result in variable levels of Bd detection (Simpkins et al. 2014). I followed the swabbing protocol of Berger and Speare (1998) that targets areas highly susceptible to Bd infections including the ventral surface, thighs, feet, as well as the dorsal surface of the amphibian. However, other studies have air-dried the skin swabs for 30 minutes before putting into ethanol vials or did not store in ethanol at all (Adams 2010; Gower et al. 2012). In contrast, I stored the skin swabs immediately into ethanol without drying, which could have accounted for the low detection results. Hyatt et al. 2007 also found fine tipped swabs is best for Bd detection and I did not use these.

I suggest continued disease surveillance for Bd in North Dakota amphibians because it is a well-established cause of mass mortality in amphibian populations worldwide (Lips et al. 2006; Lips et al. 2008; Muths et al. 2003; Rachowicz et al. 2006). Sampling for this study was limited to just two collecting years and future studies should incorporate more years of sampling data. Sampling designs should also include multiple amphibian collection visits per location to increase Bd detection throughout different seasons. Researchers should also extract DNA from mouthparts, sloughed skin and toes to increase probability of Bd detection. In addition, multiple researchers should swab each amphibian with a fine tip swab (Hyatt et al. 2007; Simpkins et al. 2014).

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CHAPTER IV

PREVALENCE AND GEOGRAPHIC DISTRIBUTION OF HELMINTHS IN NORTH DAKOTA AMPHIBIANS

ABSTRACT

Parasites are integral components of all biotic communities and their diversity is an indicator for ecosystem health. Some parasites can also impair the health of amphibians. Knowledge about the diversity, distribution, and host associations of parasites is limited in North Dakota. To understand the ecology of parasites and their role in host populations, I conducted a statewide survey to estimate the prevalence and geographic distribution of parasites in multiple amphibian hosts. I collected amphibians broadly across the state, including all major ecoregions and land use categories. I identified parasites to taxonomic level by both morphology and molecular techniques. Overall, 60.3% of amphibians were infected with digeneans, 17% with nematodes, and 2.8% with cestodes. Common parasite genera included the digeneans *Alaria* (29.2% prevalence), *Echinoparyphium* (23.3%), *Haematoloechus* (13.3%), *Glypthelmins* (11.2%), and the nematodes *Cosmocercoides* (7.7%), and *Rhabdias* (8.2%). Some parasite species were widely distributed, including *Alaria*, *Echinoparyphium*, and *Haematoloechus* and some had more limited distributions including *Glypthelmins* and *Rhabdias*. *Alaria* had a fine scale spatial dependency at distances less than 20km and *Rhabdias* at distances less than 40km, indicating a patchy distribution for both. More complete surveys need to be designed to establish causation for each parasite's occurrence.

INTRODUCTION

Parasites play an important role in ecology because they may shape host population dynamics (Lafferty 2006), alter interspecific competition (Lafferty 1997), influence energy flow (Hatcher et al. 2006; Lefevre et al. 2009; Marcogliese 2002; Prenter et al. 2004) and reflect diversity of biotic communities (Hudson et al. 2006; Lafferty 2003). In general, parasite communities should reflect the hosts that are available to them, therefore a community rich with hosts should also be one rich with parasites (Lafferty 2003). Several studies have shown that parasite species diversity increases as ecosystem health improves (Marcogliese 2002; Thompson et al. 2005).

Even though parasites are useful indictors of ecosystem health, they can also impair the health of their hosts to further their own fitness. In amphibians, digeneans in the family Echinostomatidae (Koprivnikar et al. 2006) and genus *Ribeiroia* (Johnson et al. 2002) are known to have negative health impacts and sometimes reported at high prevalence in aquatic habitats (Beasley et al. 2005; Johnson and Hartson 2009). Several species of the lung nematode *Rhabdias* can also decrease growth rates and survival in toads (Goater et al. 1993; Kelehear et al. 2009; Kelehear et al. 2011). The body size of amphibians is directly correlated with all aspects of performance, so infection with *Rhabdias* appears to impair fitness related traits (Goater et al. 1993). In addition, *Rhabdias* can reach 2/3 the length of the host's lung, occupying a significant portion of the lung cavity, possibly inhibiting the mechanical functioning of the lung, damage the lung lining and cause blockages of blood vessels (Goater et al. 1993). These symptoms have important implications for reproductive success because they often impair amphibians' chorusing ability (Goater and Vandenbos 1997).

Given the importance of parasites both as indicators of ecosystem health and in relation to host population ecology, I conducted a statewide survey to estimate the prevalence and geographic distribution of parasites found in six of the more commonly encountered North Dakota amphibian species. I also tested for associations between parasite occurrence and ecological features such as ecoregion, land use, wetland length, area, and density. There have been two previous parasite surveys in the Sheyenne National Grasslands (Gustafson et al. 2013) and Nelson County, North Dakota (Pulis et al. 2011). To our knowledge this is the first survey of amphibian parasites of this scope in North Dakota.

North Dakota is a likely location for parasite occurrence because it contains many prairie pothole wetlands that are inhabited by a wide range of hosts necessary for parasite life cycles, including snails, waterfowl and other birds, amphibians, and mammals (Fig 1). In addition, agricultural development can result in pesticide runoff into wetlands, which compromises the immunological response in amphibians (Gendron et al. 2003; Kiesecker 2002; Rohr et al. 2008) and contributes to the overall habitat loss and fragmentation of wetlands (Greer and Collins 2008). North Dakota also contains ephemeral ponds that periodically dry during the summer or fall, resulting in seasonal habitat reductions and clustering of amphibians (Greer and Collins 2008). The incidence of parasites should increase when amphibians are clustered at fewer locations because the probability of transmission is high.

OBJECTIVES AND HYPOTHESES

I tested for associations between helminth occurrence in North Dakota and several ecological factors. I selected the ecological factors based on information already known about helminth occurrence. These hypothesized factors do not operate independently and are often

confounded with each other. Consequently, estimating the independent contributions of each may be challenging or even not feasible in this study.

(1) Estimate the geographic distribution of helminths in North Dakota.

<u>Hypothesis 1</u>: Helminth genera will vary in occurrence across the state because of different ecological conditions and distributions of host species. For instance, helminths might be more common in hosts that inhabit the Missouri Coteau and Northern Glaciated Plains ecoregions because these contain many prairie pothole wetlands (Fig 1). Prairie pothole wetlands are suitable habitats for many parasite hosts (snails, amphibians, birds, etc) necessary for reproduction and development. In addition, hosts vary in geographic distribution across the state and their parasites will exhibit the same pattern.

(2) Estimate the prevalence of helminths in North Dakota amphibians.

<u>Hypothesis 2A</u>: Helminth prevalence will vary among host species because helminths have different host requirements. For example, *Echinoparyphium rubrum* will be found in amphibians that inhabit the same wetlands or surrounding areas as birds, mammals, and turtles. In contrast, some parasites require specific hosts to complete their lifecycle, such as *Rhabdias bakeri* that only infects wood frogs.

<u>Hypothesis 2B</u>: Land use will influence helminth occupancy. The occupancy of parasites should be higher in hosts that inhabit wetlands surrounded by cropland because amphibians are more susceptible to infections when exposed to agricultural chemicals (Carey and Bryant 1995; Carey et al. 2003; Christin et al. 2003, 2004; Gendron et al. 2003; Kiesecker 2002; Rohr et al. 2008). Atrazine runoff and eutrophication also increases algae production leading to the increase in biomass of the first intermediate host, a gastropod (Johnson et al. 2007; Rohr et al. 2008). A larger gastropod can harbor

more parasites potentially increasing parasitic load (Johnson et al. 2007; Szuroczki et al. 2009) and the likelihood of infection in the second intermediate host, amphibians (Rohr et al. 2008; Szuroczki et al. 2009). However, atrazine also negatively impacts the parasites by reducing survival and infectivity (Koprivnikar et al. 2006; Pietrock and Marcogliese 2003; Rohr et al. 2008).

Hypothesis 2C: Wetlands at close proximity to each other are more likely to contain infected hosts than wetlands at further distances because of increased habitat connectivity and movement of intermediate and definitive hosts. If amphibians are moving between wetlands they are more likely to consume infected hosts or have a parasite penetrate its skin because they are exposed to more habitats and hosts suitable for parasites.

Hypothesis 2D: Wetlands with smaller length and area should also have higher helminth prevalence because hosts including snails and amphibians prefer this wetland size. However, other parasite hosts such as waterfowl and mammals will visit larger wetlands increasing transmission opportunities for parasites that require these hosts in their lifecycle.

Hypothesis 2E: Helminth occupancy should be higher in the Missouri Coteau and Northern Glaciated Plains ecoregions because they contain many prairie pothole wetlands located fairly close to one another (Fig 1). A higher percentage of wetlands provide more habitats for parasite hosts and will likely result in increased movement between wetlands. Increased amphibian movement should increase opportunities for parasite transmission throughout the surrounding area by depositing parasite eggs with their feces, contacting snail cercaria, or increasing the chances of being eaten by a definitive host, such as a bird.

In contrast, a lower percentage of wetlands might restrict and concentrate hosts to fewer wetlands increasing the chances of transmission, if an infected host is nearby (Greer and Collins 2008).

METHODS

Study Design

The main objective was to estimate the geographic extent of infections across a portion of the northern plains in a wide variety of habitats. To achieve this, I sampled amphibians broadly across the state of North Dakota using a stratified sampling design. The four level III ecoregions (Omernik 1987) were the primary sampling strata, with the four general categories of land use within ecoregion as secondary strata. The routes for collecting trips were selected to cover as much of the state as possible, but were constrained by accessibility of wetlands, and incorporating as much of the active season as possible. Routes were generally driven east to west and collection trips focused on the southern, central, or northern tiers of the state. Wetlands were selected based on availability (sites with water apparent) and accessibility (proximity to roads that were passable under prevailing conditions) and to include a gradient of land uses. I specifically sought out wetlands that were in or adjacent to croplands, pasture, grasslands (including hayland, CRP-type land, or anything that resembled currently untilled, ungrazed land), and woodland. Sample locations (N=171) are indicated in Figure 3. Each symbol on the map represents a location where I collected amphibians.

Amphibian collection efforts spanned two field seasons, 2013 and 2014. The start of each field season depended on the weather and phenology of each amphibian species. In the early field season (April-May), collection focused on adult breeding amphibians. In the mid field season (June-July), I searched for tadpoles and adults and in the late season (August-September),

meta-morphs and juveniles were primarily collected along with any additional adults found. The 2013 field season did not begin until late May because of extended snowfall leading to late thawing of wetlands resulting in limited time for collection of early breeders including the boreal chorus frog and the wood frog. I attempted to include all amphibian species and life stages in collection but did not encounter the gray tree frog (*Hyla versicolor)*, American toad (*Anaxyrus americanus*), spadefoot toad (*Spea bombifrons*), and the Woodhouse's toad (*Anaxyrus woodhousei*) and collected very few tadpoles.

Field protocol

I relied on chance encounters to collect amphibians while I walked around and waded through wetlands. This was the most efficient method because it limited the time needed at individual wetlands and allowed inclusion of more sites. This method of sampling is subject to substantial bias in terms of which amphibian species were found. Some amphibian species have limited geographic ranges and specific habitat preferences that reduce the likelihood for encounters. For example, the plains spadefoot toad spends most of its time buried underground, limiting our ability to detect them. In addition, the gray tree frog is only found in the eastern margin of the state and is highly arboreal except during larval stages. One species, the tiger salamander *Ambystoma mavortium*, is unlikely to be encountered by this method and is best sampled using traps or seines. Hence, any inference related to this host species will be limited.

The field protocol from James Cook University was followed to avoid cross contamination in the field and maintain field hygiene when handling amphibians (Speare et al. 2004). After amphibians were collected, each individual was assigned a numerical ID for future reference along with a photograph to confirm species identification and placed in a numbered container. In each container, I placed a wet paper towel in with the adults, juveniles and

metamorphs to avoid desiccation. Tadpoles were kept in sufficient water so they remained submerged during transport. The containers with each individual amphibian were then stored in a cooler with ice to avoid over-heating. I recorded the snout-vent length of each amphibian, GPS coordinate of the location, and environmental characteristics such as wind speed, humidity, temperature, vegetation structure, and land use, and also took a picture of the location for future reference.

Necropsy Protocol

Amphibians collected for necropsy focused on males and abundant species to avoid demographic and population impacts. Five of each species at each location are collected for necropsy and euthanized following an IACUC approved protocol (#1305-2). All amphibians were necropsied following standard endoparasite collecting procedures (Bennett 1970) and further optimized for amphibian necropsy. The blood, liver, spleen and skin swab were also collected from each amphibian for DNA extraction and stored in 95% ethanol at -20°C. Blood smears were taken immediately to avoid coagulation and allowed to air dry. After the blood dried they were fixed with 100% methanol, and later stained with 10% buffered Geisma for parasite detection. During dissection, the mouth cavity, leg muscles and body cavity were examined for helminths and the bladder, lungs, kidneys, liver, and complete digestive tract were removed and placed in clean Petri dishes with saline. The complete digestive tract was dissected by cutting lengthwise down the intestine and the inner lumen was scraped out with a clean microscope slide and examined using a stereomicroscope. Each organ was later dissected and examined for helminths using stereomicroscopy. Once the parasites were found, I heat killed them in hot water for all digeneans and hot saline for all nematodes. Then I fixed all parasites in 70% ethanol and labeled each vial with the corresponding frog ID number.

Morphological Identification

To identity parasites to the lowest possible taxonomical level, worms were rehydrated in distilled water and stained with alum carmine. Acid ethanol was then used to remove any excess stain to clearly distinguish taxonomic structures. Digeneans were then dehydrated in a graded ethanol series of increasing concentrations of 70%, 80%, 90%, 95%, and 100% (twice). The length of time each worm spent in each ethanol concentration depended on the size of the worm. Next, the digeneans were cleared in clove oil and mounted permanently in Damar gum. Nematodes were cleared in glycerin–phenol (3:1) solution and temporarily mounted on slides for morphological identification (Kruse and Pritchard 1982). All parasites used for morphological identification were photographed and sometimes measured using a DIC equipped Olympus BX-51 microscope and Rincon HD software (Imaging Planet, Goleta, California). Original descriptions and related literature were used to identify helminths (Baker 1978; Bolek and Janovy 2008; Bolek et al. 2009; Cort 1915; Faust 1918; Ingles 1936; Kuzmin et al. 2003; Ogren 1953; Rankin 1944; Slimane and Dessett 1997; Stafford 1902a; Stafford 1902b; Stafford 1905; Tkach et al. 2006; Walton 1929).

Molecular Identification

Immature digenean and nematode identifications were completed by molecular techniques because taxonomic traits are not always visible. A portion or sometimes the entire parasite was used for species differentiation. I followed Tkach and Pawlowski (1999) protocol for all parasite extractions. I slightly modified this protocol for metacercaria DNA extraction. First, I placed one metacercaria into a microcentrifuge tube and aspirated most of the ethanol with a pipette. The specimens were then dried for 20 minutes at 60°F to remove any remaining ethanol from the tissues. Next, 60µl of pure H_2O was added to the tube to rehydrate the

metacercariae. The metacercariae were then broken apart by sonication using a UP100H compact ultrasonic processor (Hielscher USA, Inc., Ringwood, NJ) at 80-100% for 20 seconds. After sonication, 250µl of Zymo Cell Lysis Buffer was added to the tubes and samples were allowed to lyse for at least one hour. The remaining extraction steps follow Tkach and Pawlowski (1999) protocol. All of the DNA extracts were then stored at -20°C after being eluted with ≥25 µl of pure H2O.

Various nuclear and mitochondrial DNA regions were amplified depending on the group of parasite following Tkach and Snyder (2003). For digenean and nematode identifications, DNA fragments of approximately 1400 base pairs covering the 28S nuclear ribosomal DNA gene were amplified by PCR in Eppendorf Mastercycler thermocycler. Each PCR reaction contained 1µL of each forward and reverse primer at a concentration 10pM/µl, 12.5µl of OneTaq 2xMaster Mix with stand buffer, 1-3µl of template genomic DNA extract, and enough nuclease free water to total the reaction to 25µL (typically 7.5-9.5µl). The 28S thermocycler PCR protocol is: initial step 94°C for 30 seconds, 40 cycles of the denaturing step at 94°C for 30 seconds, annealing step 53°C for 40 seconds, extension step 68°C for 2 minutes, and final extension at 68°C for 5 minutes. Table 12 shows a list of 28S primers used for species differentiation.

I also amplified regions of the ITS gene because it provides greater variability than the 28S gene. The ITS thermocycler protocol was: 30 sec initial denaturation at 94°C; 40 cycles of 30 sec denaturation at 94°C, 1min and 45 sec annealing at 55°C, 2 min extension at 68°C; and 5 min final extension at 68°C. Table 12 also lists the ITS primers used for amplification.

Mitochondrial genes including cox1, 12S, and nad1 were also used for species differentiation because they contained higher interspecific variation than nuclear ribosomal genes, which was needed for some parasites. The mitochondrial thermocycler protocol was 30

sec initial denaturation at 94°C; 40 cycles of 30 sec denaturation at 94°C, 30 sec annealing at 45°C, 1 min extension at 68°C; and 5 min final extension at 68°C. Table 12 also lists the mitochondrial primers used.

For nematodes, regions of the 18S gene are amplified because this region is somewhat represented in public sequence databases. I compared our sequences to submitted sequences in databases and added any new sequences of unrepresented nematodes. The thermocycler PCR protocol for 18S genes: 30 sec initial denaturation at 94°C; 40 cycles of 30 sec denaturation at 94°C, 35 sec annealing at 53°C, 2 extension min at 68°C; and 5 min final extension at 68°C. Table 12 also lists 18S primers.

Table 12. PCR and sequencing primers for 18S, ITS, 28S, Cox1, Nad1, 12S genes C = cestode, D = digenean, and N = nematode

Primer Name	Primer Type	Direction	Helminth Group(s)	Primer Sequence (5' – 3')
18S Primers				
SSUR23	PCR	forward	N	TCTCGCTCGTTATCGGAAT
GI8S4	Internal	forward	N	GCTTGTCTCAAAGATTAAGCC
RITF	PCR	forward	N	GCGGCTTAATTTGACTCAACA
C1800F	PCR	forward	N	CCTAGTAAGTGTGAGTCATCA
N900R	PCR	reverse	N	GGTTCGATTAGTCTTTCGCC
ITS Primers				
ITSF	PCR	forward	C, D	CGCCCGTCGCTACTACCGATTG
ITSF1	Internal	forward	C, D, N	GTCCCTGCCCTTTGTACACACCG
ITS5	PCR	forward	C, D	GGAAGTAAAAGTCGTAACAAGG
M18F1	Internal	forward	C, D	CGTAACAAGGTTTCCGTAG
28S Primers				
LSU5	PCR	forward	C, D, N	TAGGTCGACCCGTGAAYTTAAGCA
1500R	PCR	reverse	C, D	CGAAGTTTCCCTCAGGATAGC
300R	Internal	reverse	C, D	CAACTTTCCCTCACGGTACTTG
ECD2	Internal	reverse	C, D	CCCGTCTTGAAACACGGACCAAG
DIGL2	PCR	forward	D	AAGCATATCACTAAGCGG
1500R1	PCR	reverse	D	GCTACTAGATGGTTCGATTAG
Cox1 Primers				
JB5	PCR	forward	C, D, N	AGCACCTAAACTTAAAACATAATGA AAATG
JB3	PCR	reverse	C, D, N	TTTTTTGGGCATCCTGAGGTTTAT
TRNNR	PCR	reverse	C	TTCYTGAAGTTAACAGCATCA
12S Primers				
60FOR	PCR	forward	C	TTAAGATATATGTGGTACAGGATTAG ATACCC
375R	PCR	reverse	C	AACCGAGGGTGACGGGCGGTGTGTA CC

After amplification of selected genes, I conducted gel electrophoresis to determine the length of each DNA fragment. First, I placed a 1.2% agarose gel into a gel electrophoresis apparatus and filled it with 0.5% Tris/Borate/EDTA (TBE) buffer solution. Then I pipetted 5µl of each PCR product into the gel wells along with 4 µl of the standard DNA ladder to determine the DNA fragment length. I did not add loading dye to each PCR product because it was already included in the OneTaq 2xMaster Mix used for PCR amplification. After the PCR product and DNA ladder are added to the gel, I ran each electrophoresis at 96V for 45minutes or until the PCR product has traveled the majority of the way down the gel. The gel is then stained with ethidium bromide for 10 minutes, rinsed in water for 12 minutes, visualized on a UV transilluminator, and photographed using a digital gel documentation system.

After successful amplification of selected genes, all PCR products were purified using ExoSap PCR clean up enzymatic kit from Affimetrix (Santa Clara, CA, USA) or DNA Clean & Concentrator™ kit from Zymo Research (Irvine, CA, USA) followed according to manufacturer's instructions. After PCR products were purified, I prepared each reaction for sequencing. Each sequencing reaction contained 2µl of BigDye Terminator 5X sequencing buffer, 1.5µl of the appropriate primer, 1 µl of BigDye (Life Technologies), 1-1.5µl of purified PCR product (depending on the strength of PCR reaction), and enough nuclease free water to make the total reaction be 10µl. The sequencing protocol was: 96°C for 15 sec, 50°C for 5 seconds, and 60°C for 4 minutes repeated for a total of 25 cycles. Sequencing reactions were alcohol precipitated and sequenced directly on an automated capillary sequencer ABI Prism 3100. Contiguous sequences were assembled using Sequencher™ ver. 4.2 (GeneCodes Corp., Ann Arbor, Michigan) and aligned using BioEdit alignment software, version 7.0.1 (Hall 1999). Poor quality sequences with high background interference were not used for parasite

identification or species differentiation. Sequences were compared to entries in a public database called NCBI (National Center for Biotechnology Information) Blast.

Data analysis

To summarize the occurrence of parasites in amphibians across the state, I calculated the prevalence (percent of infected individuals within a sample) and occupancy (percent of locations containing infected individuals) for each ecoregion and land use. I also tested for statistical differences in parasite prevalence among amphibian species, ecoregions, and land use categories using exact χ^2 tests (StatXact version 9.0, Cytel, Inc. 2010).

I used ArcGIS version 10.2 (ESRI 2014) to visualize the geographic distribution of parasites and to estimate the wetland neighborhood around sample locations. I created buffers of 250 m and 500 m around each sample location to estimate wetland density, the percent cover of wetlands identified in the National Wetlands Inventory database within buffers. Because wetland densities at these scales proved to be highly correlated, I retained only one for further analysis. I also estimated wetland density as the number of wetlands within each buffer.

To determine if locations with parasite-infected amphibians were spatially dependent I estimated spatial autocorrelation in relation to distance separating sample locations. Distances were estimated from GPS coordinates using the software Passage 2.1 (Rosenburg et al. 2011). Significance of autocorrelations was determined using a permutation test with 1000 permutations.

I used Moran's eigenvector-based maps (Legendre and Legendre 2012) to generate a series of factors reflecting different components of the underlying spatial structure to incorporate into regression models. Spatial factors in this approach are derived based purely on the geographic arrangement of sampling locations. This method allows for more complex aspects of

spatial structure to be represented, rather than simply distance along linear axes (UTM coordinates or latitude and longitude) for each location. For example, some components might appear as simple gradients, whereas more complex aspects of spatial structure would appear as different patterns of patchiness, perhaps differing in scale or spatial arrangement. These spatial factors can then be used as predictor variables in regression models to test for similarities between response variables (e.g., parasite occurrence) and spatial patterns. During the initial spatial factor selection process, I looked at each factor individually and selected those that appeared to have at least some correlation with the parasite's occurrence. I used program SAM (Spatial Analysis in Macroecology version 4, Rangel et al. 2010) to estimate and conduct initial screening of spatial factors using a minimum separation threshold of 10 km between sites.

To test for associations between parasite occurrence and the combined influence of all ecological and spatial predictor variables (Table 1), I constructed generalized linear models with various combinations of predictors. Because presence-absence is a binary response variable, I fit logistic regression models in R version 3.1.2 (R Core 2013). Model selection was based on a best subsets approach (Hosmer and Lemeshow 2000), with AICc as the criterion for comparing models (Burnham and Anderson 2002). The model selection process allowed us to identify factors that have the strongest association with parasite occurrence. Because multiple models had a AICc < 2, I used multi-model inference to generate a model-averaged estimate for each predictor variable compiled from all the component models. I specified ΔAIC of less than two (Burnham and Anderson, 2002) for model averaging, and estimated the contribution of all variables included in these models to the prediction of parasite occurrence.

RESULTS

I collected a total of 705 amphibians during the 2013 and 2014 field seasons combined (Tables 2 and 3). The northern leopard frog was the most abundant species collected during both years. Tiger salamanders had the smallest overall sample size because I did not use collecting methods suitable for that species.

Objective 1: Estimate the geographic distribution of helminths in North Dakota

Some parasites had broad geographic distributions across the state including *Alaria*, *Echinoparyphium,* and *Haematoloechu*s (Fig. 11-12 and 14 respectively). Other parasites had a more limited geographic range including *Glypthelmins* and *Rhabdias* (Fig. 13 and 15 respectively). For example, *Rhabdias* was found in the eastern and northern margins of the state.
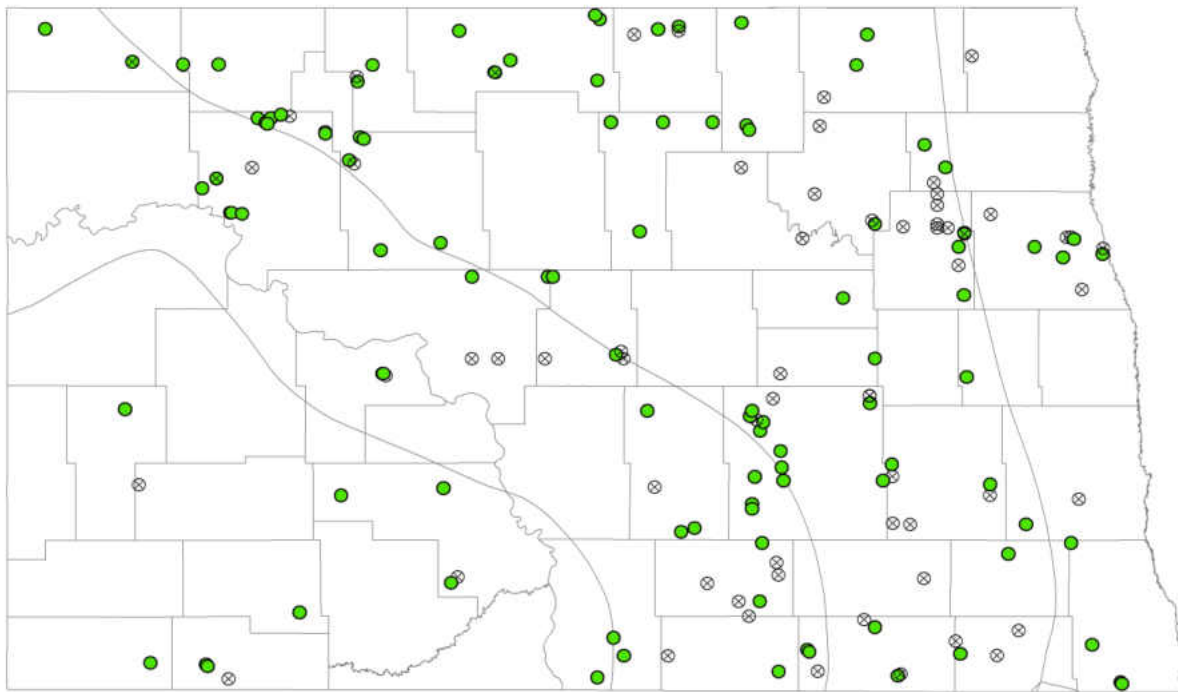
Figure 11. The geographic distribution of *Alaria* infections pooled across all amphibian species. For all of the following maps the green circles represent infected amphibians, open circled "X"s indicate amphibian specimens testing negative. Lines illustrate county boundaries and ecoregions as in Figure 3 (from west to east: NWGP, MC, NGP, RRV).

Figure 12. The geographic distribution of *Echinoparyphium* infections pooled across all amphibian species.

Figure 13. The geographic distribution of *Glypthelmins* infections pooled across all amphibian species.

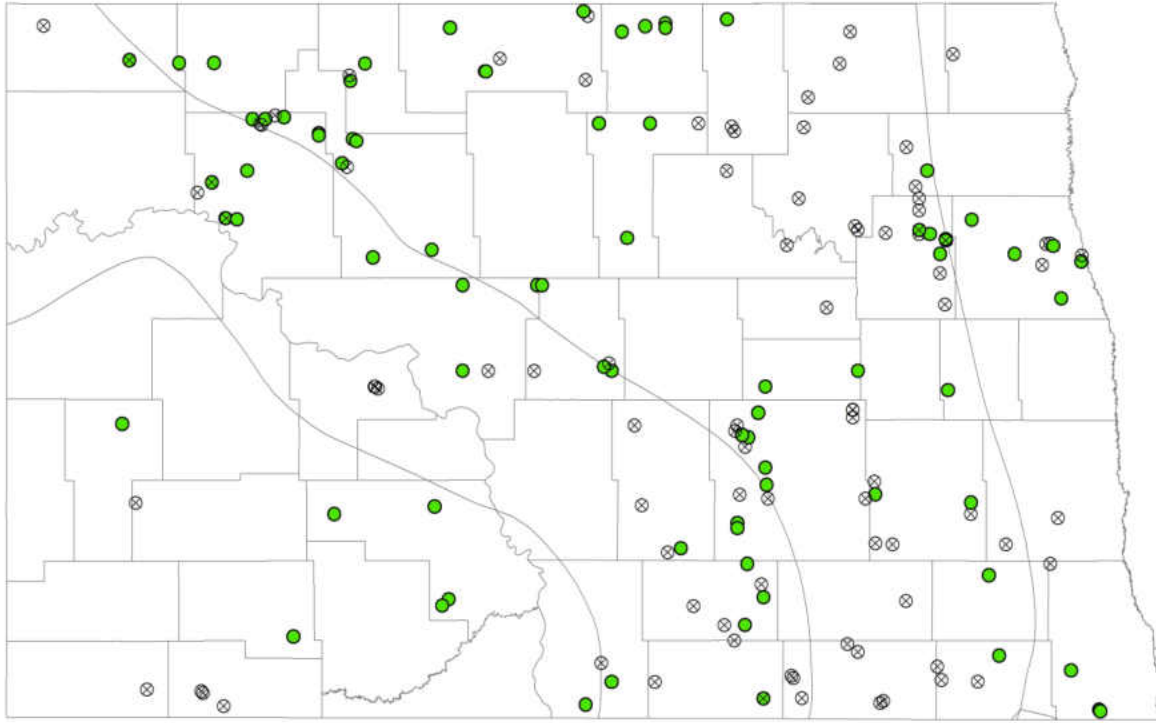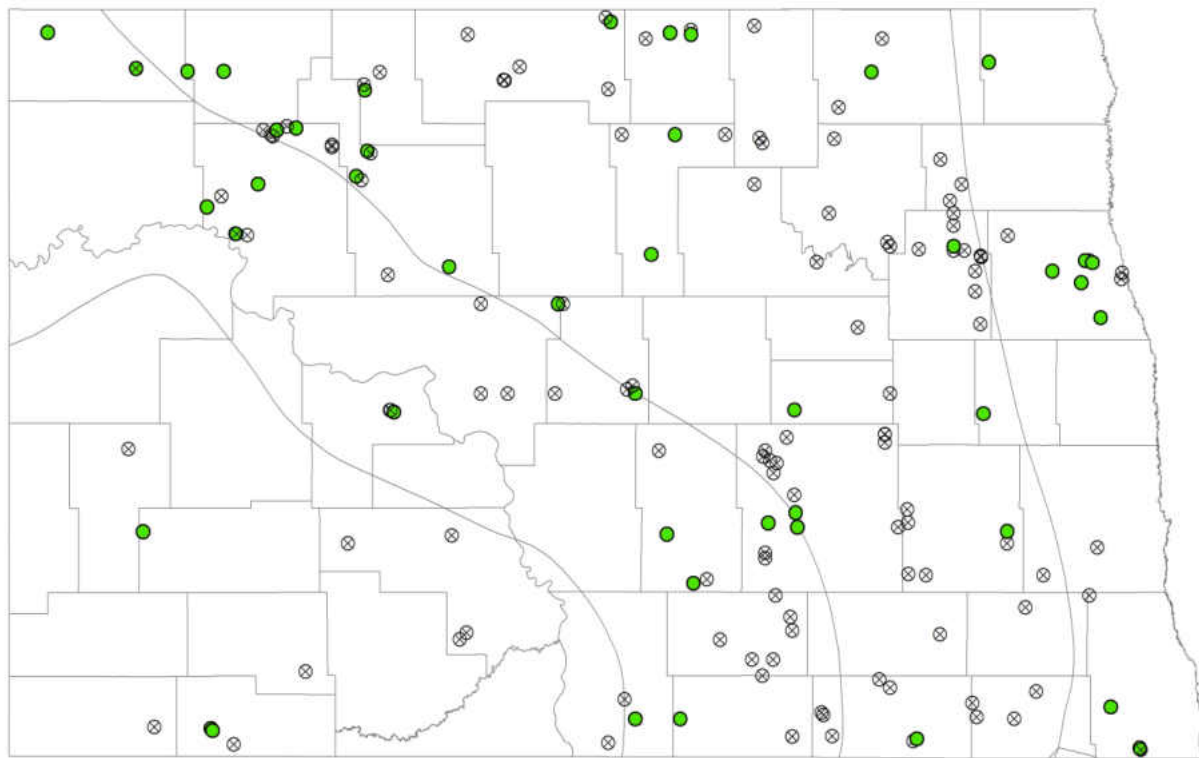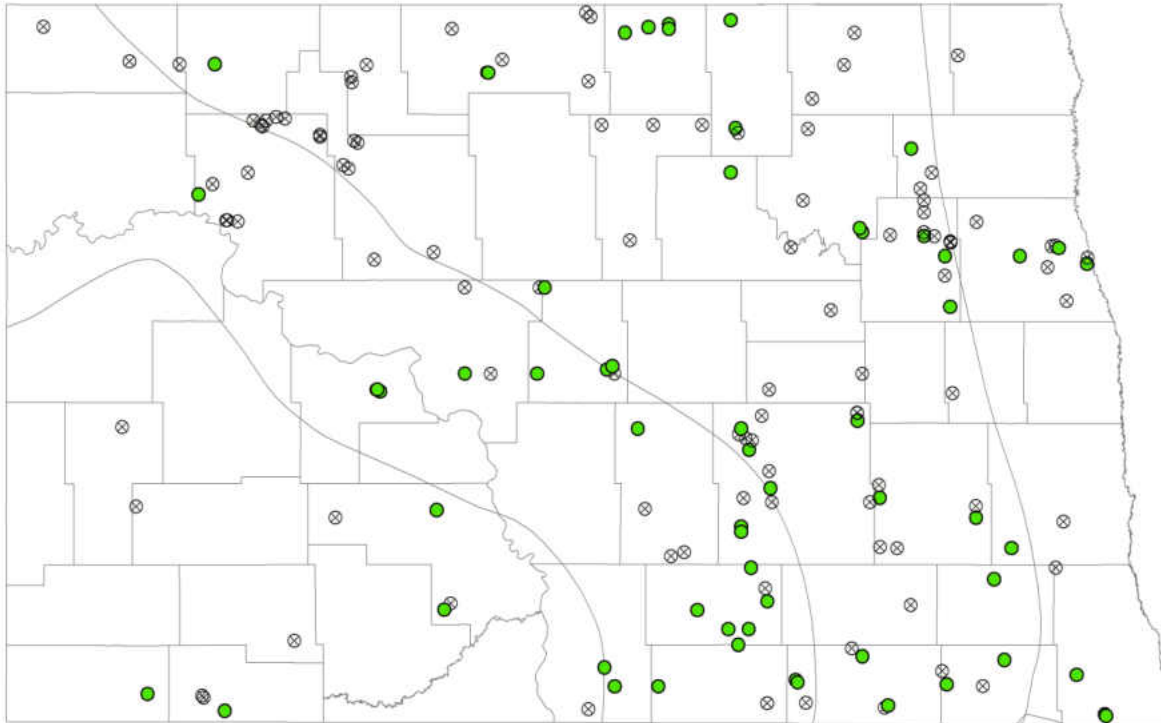**Figure 14**. The geographic distribution of *Haematoloechus* infections pooled across all amphibian species.
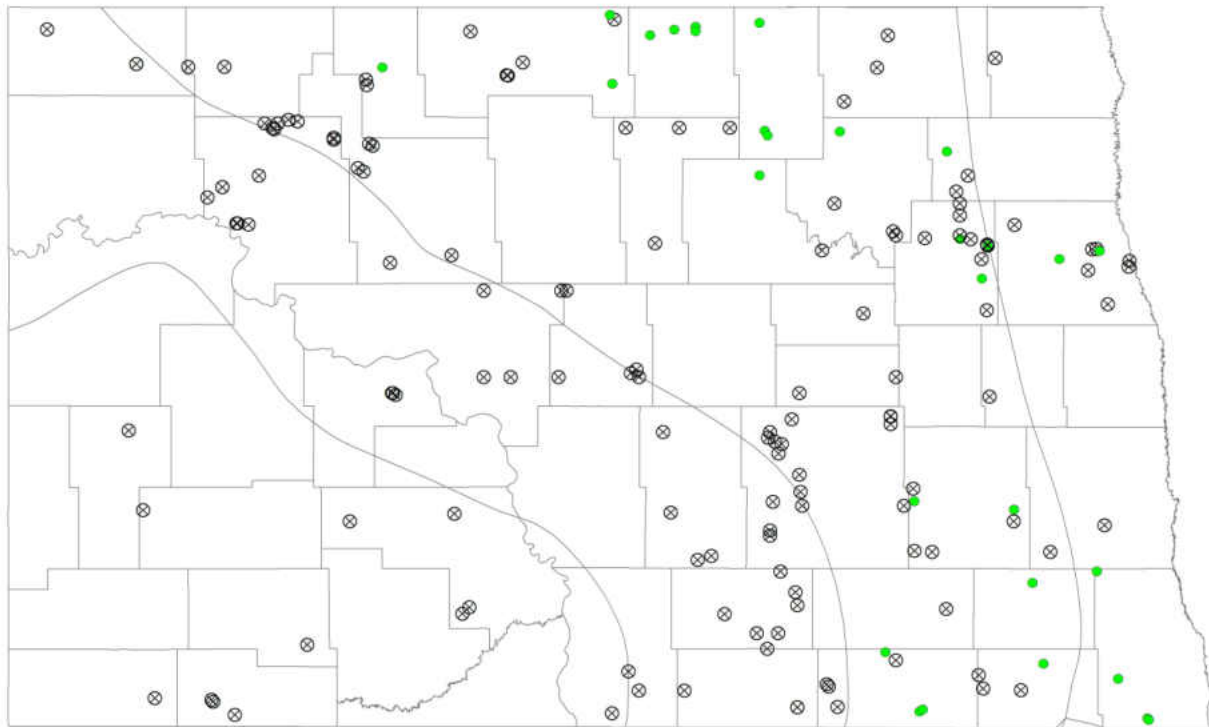
Figure 15. The geographic distribution of *Rhabdias* infections pooled across all amphibian species.

Among all sampled locations, *Alaria* was found at 60% of the sites, *Echinoparyphium* 46.5%, *Rhabdias* 17.1%, *Haematoloechus* 34.7% and *Glypthelmins* 27.1% (Tables 13 and 14). I also found *Alaria*, *Echinoparyphium*, *Glypthelmins*, and *Haematoloechus* occupancy did not vary significantly among ecoregions (Table 13). However, *Rhabdias* had significantly greater prevalence in the RRV compared to elsewhere.

Helminth occupancy appeared to depend somewhat on land use at least for some parasites including *Echinoparyphium*, *Haematoloechus,* and *Rhabdias* (Table 14). However, *Alaria* and *Glypthelmins* did not significantly vary by land use. It is not surprising the pattern of helminth occurrence in each land use varied among parasite species considering each helminth relies on different snail and vertebrate hosts to complete its life cycle.

Table 13. Number and percentage of sites where common helminths were found in at least one amphibian specimen of any species per ecoregion. Ecoregion abbreviations are noted in Figure 3. Some helminth genera are abbreviated: Echino = *Echinoparyphium*, Haem = *Haematoloechus*, Glyph = *Glypthelmins*.
a. N sites where indicated parasite was found in each ecoregion see Tables 32-34 for full names of helminth taxa.

Ecoregion (N sites)	N amphibian specimens	*Alaria*	Echino	*Rhabdias*	Haem	Glypth
NWGP (19)	87	15	10	0	7	5
NGP (79)	317	47	33	14	27	19
MC (43)	140	24	19	4	12	12
RRV (29)	160	16	17	11	13	10
Total (170)	704	102	79	29	59	46
χ^2 (3 df)		3.45	2.80	14.69	2.24	1.19
p (exact, 2 sided)		0.34	0.42	**0.0022**	0.53	0.76
b. Percent of sites (occupancy) where parasite was detected in each ecoregion						
NWGP		78.9	52.6	0.0	36.8	26.3
NGP		59.5	41.8	17.7	34.2	24.1
MC		55.8	44.2	9.3	27.9	27.9
RRV		62.1	62.1	62.1	62.1	62.1
total		60.0	46.5	17.1	34.7	27.1

Table 14. Number and percentage of sites where common helminths were found in at least one amphibian specimen of any species per land use. Helminth abbreviations are noted in Table 13. a. N sites where indicated parasite was found in each land use see Tables 32-34 for full names of helminth taxa.

Land use (N sites)	N amphib specimens	*Alaria*	Echino	*Rhabdias*	Haem	Glypth
Cropland (104)	428	63	51	17	30	27
Pasture (12)	162	10	5	1	9	12
Grassland (43)	66	21	15	8	14	3
Woodland (11)	51	8	8	3	6	4
Total (170)	707	102	79	29	59	46
Exact test (3 df)		4.81	8.275	12.29	10.29	0.99
p (exact, 2-sided)		0.19	**0.04**	**0.008**	**0.014**	0.81
b. percent of sites (occupancy) where parasite was detected in each land use						
Cropland		60.6	49.0	16.3	28.8	26.0
Pasture		83.3	41.7	8.3	75.0	100.0
Grassland		48.8	34.9	18.6	32.6	7.0
Woodland		72.7	72.7	72.7	54.5	36.4
Total		60.0	46.5	17.1	34.7	27.1

Because northern leopard frogs comprised the majority of collected amphibians, I also plotted helminth occupancy by ecoregion (Fig. 16) and land use (Fig. 17) for just the northern leopard frogs. I only included the helminths that occurred at high enough frequency to warrant statistical analysis. I found *Alaria, Echinoparyphium,* and *Haematoloechus* occupancy did not vary significantly by ecoregion and land use for northern leopard frogs.
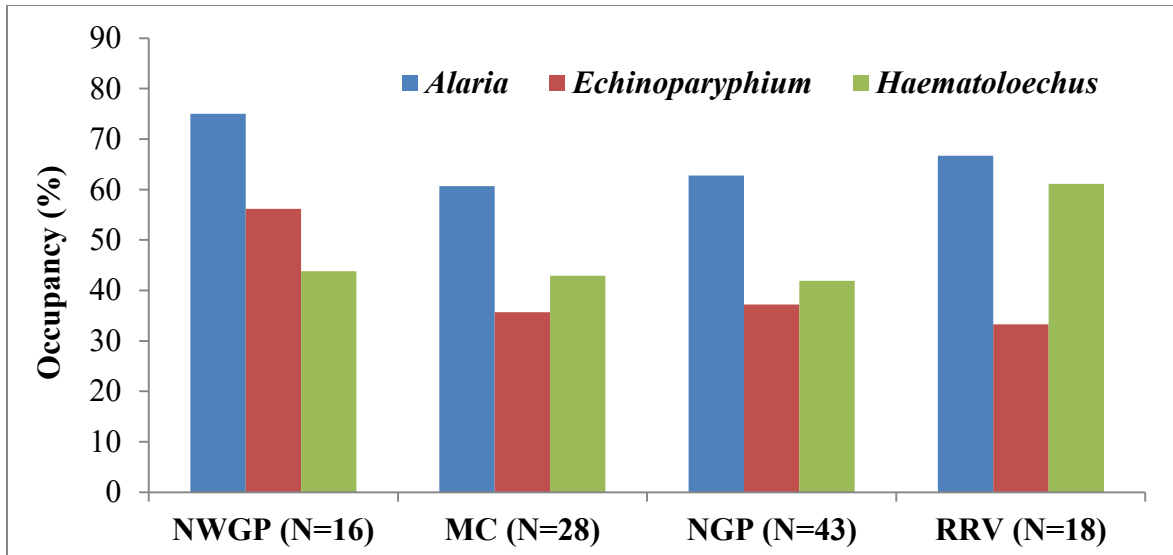
Figure 16. The occupancy of infected northern leopard frogs by ecoregion. The total sample size of locations within each ecoregion is represented by N.
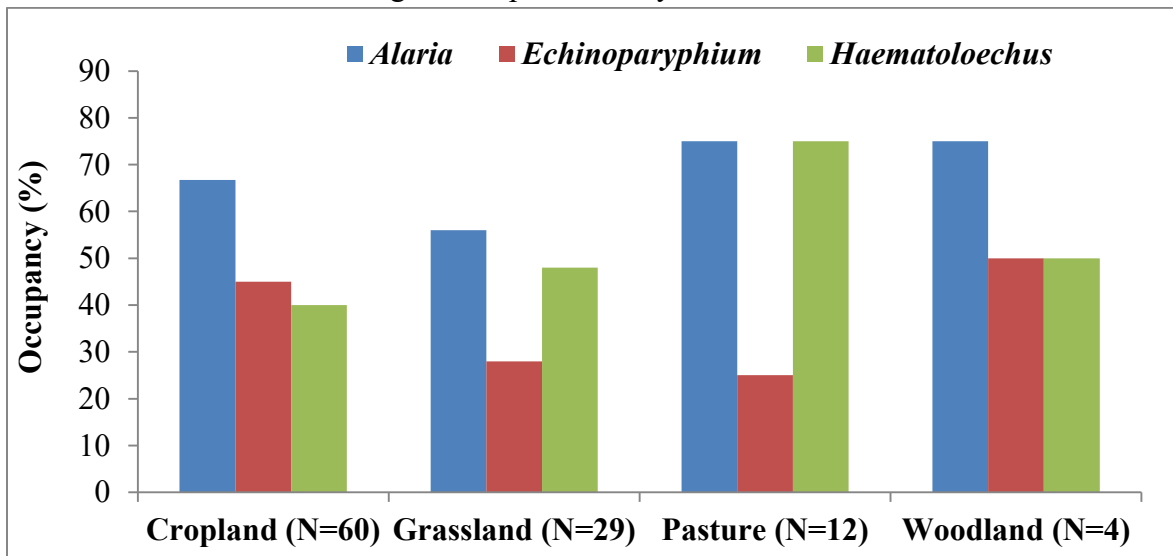
Figure 17. The occupancy of infected northern leopard frogs by land use. The total sample size of locations within each ecoregion is represented by N.

Analysis of spatial patterns

In analysis based on combined detections for *Alaria* across all amphibian host species, I found evidence for spatial autocorrelation (non-independence in disease occurrence) among wetlands closer than 20 km, but not at greater distances, indicating a moderately patchy distribution (Fig. 18). The genera *Echinoparyphium*, *Haematoloechus*, and *Glypthelmins* did not

show a significant relationship in their occurrence with distance between sampled wetlands (Figs. 19-21). However, *Rhabdias* showed a significant finer scale spatial dependency at wetlands less than 40 km, but not at greater distances, also indicating a patchy distribution (Fig. 22).
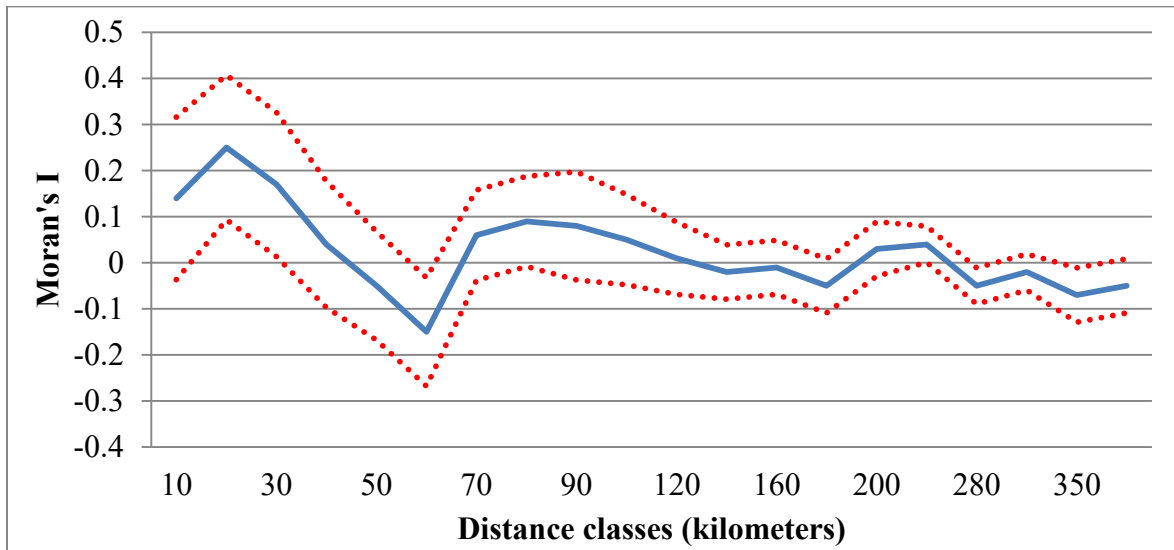
Figure 18. Spatial correlogram (solid line) for locations with *Alaria* infections pooled across all amphibian species. Lower and upper 95% confidence intervals are represented by the dotted lines. The number of pairs of points ranged from 113-290 from 10-60km and up to over 1800 beyond that.
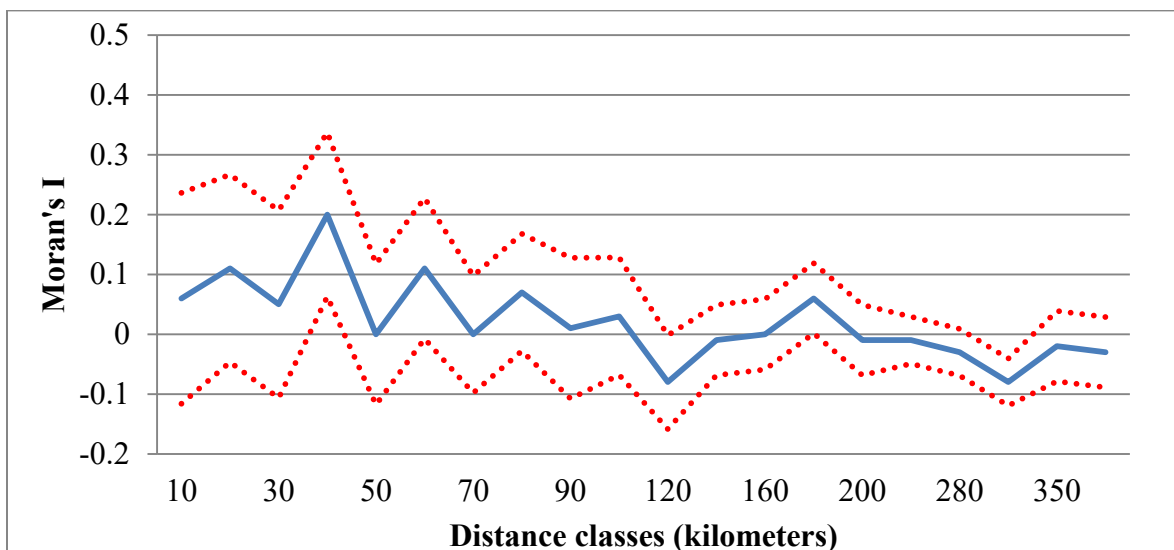
Figure 19. Spatial correlogram (solid line) for locations with *Echinoparyphium rubrum* infections pooled across all amphibian species. Lower and upper 95% confidence intervals are represented by the dotted lines. The number and pairs of points ranged from 113-290 from 10-60km and up to over 1800 beyond that.
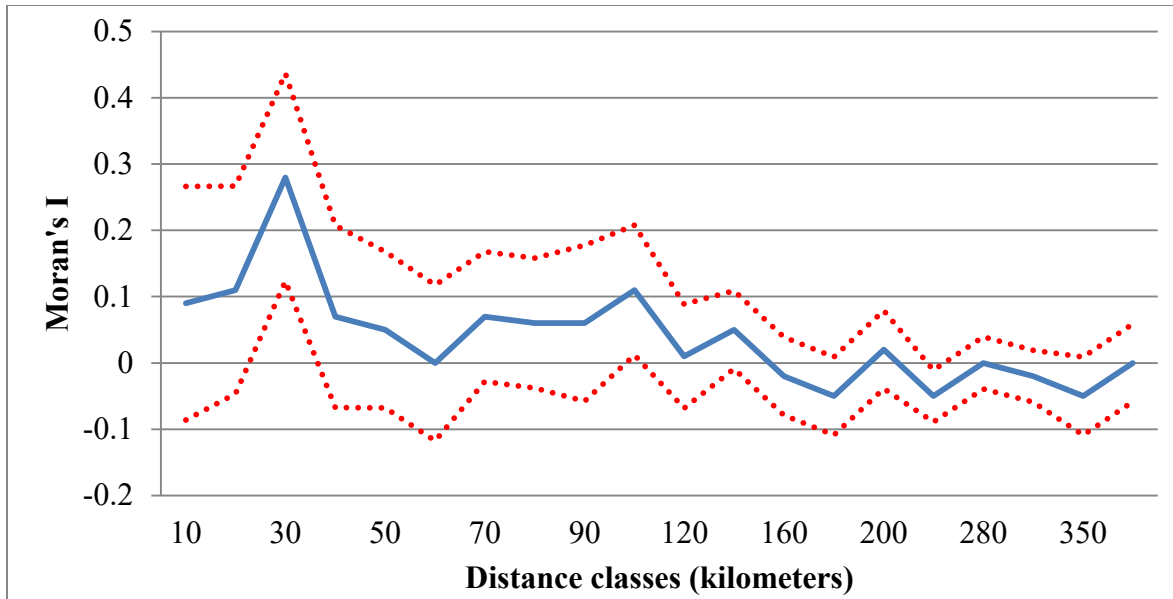
Figure 20. Spatial correlogram (solid line) for locations with *Haematoloechus* infections pooled across all amphibian species. Lower and upper 95% confidence intervals are represented by the dotted lines. The number of pairs of points ranged from 113-290 from 10-60km and up to over 1800 beyond that.
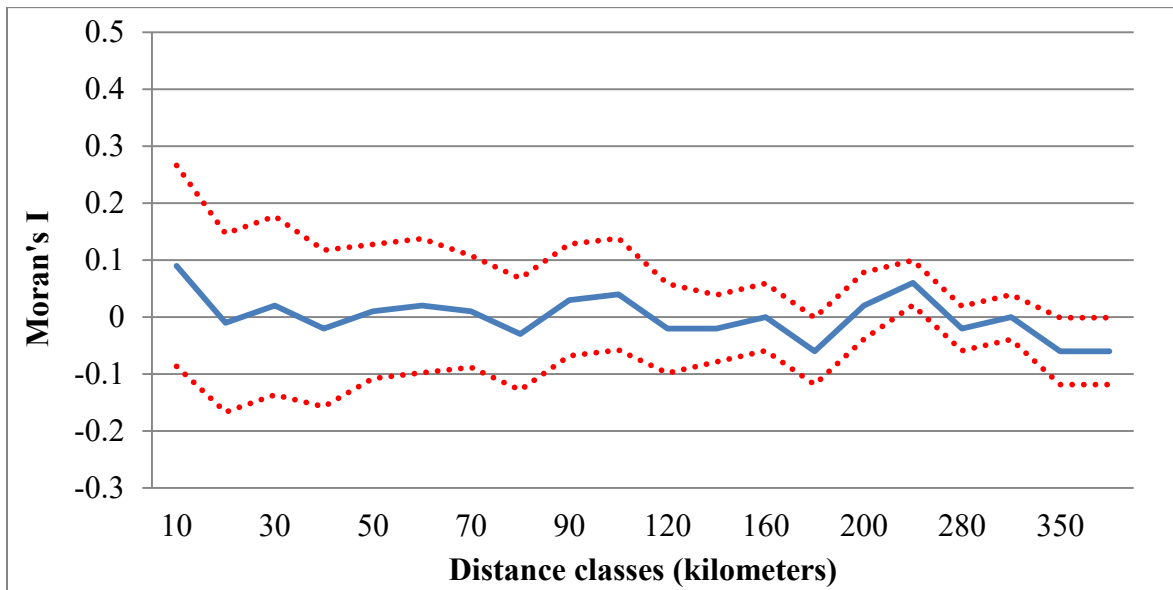
Figure 21. Spatial correlogram (solid line) for locations with *Glypthelmins* infections pooled across all amphibian species. Lower and upper 95% confidence intervals are represented by the dotted lines. The number of pairs of points ranged from 113-290 from 10-60km and up to over 1800 beyond that.
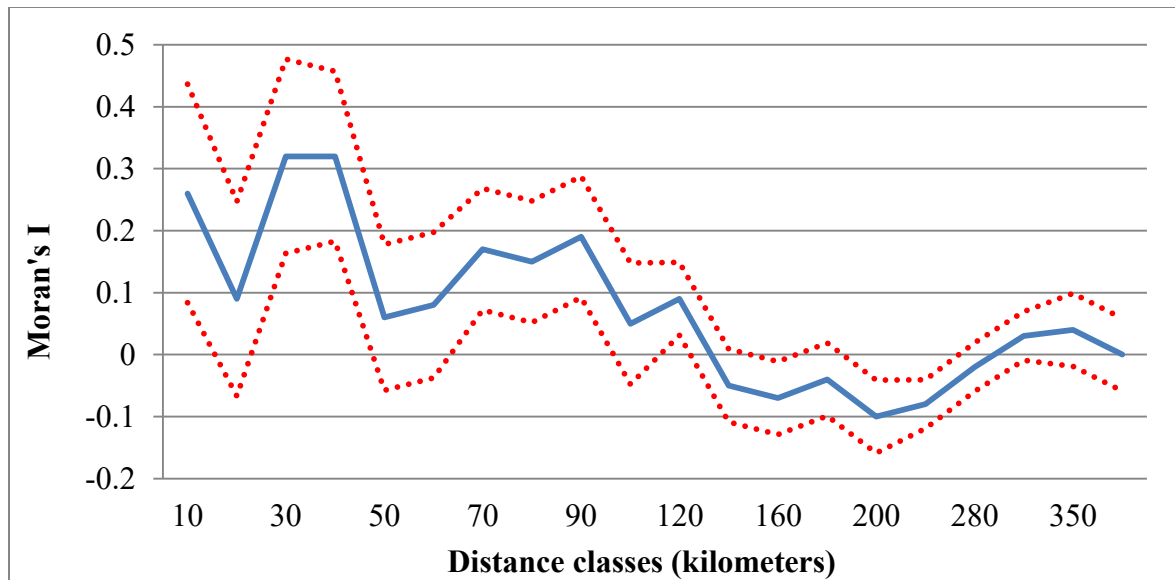
Figure 22. Spatial correlogram (solid line) for locations with *Rhabdias* infections pooled across all amphibian species. Lower and upper 95% confidence intervals are represented by the dotted lines. The number of pairs of points ranged from 113-290 from 10-60km and up to over 1800 beyond that.

Objective 2: Estimate the prevalence of helminths in North Dakota amphibians

Across all amphibians collected, 565 were infected with helminth endoparasites, resulting in a prevalence of 80.1%. The majority of amphibians were infected with digeneans (60.3%) followed by nematodes (17.4%) and cestodes (2.8%). I did not detect blood parasites in any of the 705 collected amphibians.

The major helminth groups found in each amphibian species are summarized in Table 15. The few tiger salamanders in our sample lacked flukes but had a high prevalence of intestinal nematodes, as well as cestodes. The two toad species and wood frogs, all largely terrestrial except during breeding, also tended to carry some nematodes, although these were mainly lungworms (*Rhabdias* sp.). All of the anuran species carried some species of digenean.

Table 15. Prevalence of helminth parasites at the broadest taxonomic level in amphibian host species.

Amphibian species (sample size, N)	Digenea prevalence (%)	Nematoda prevalence (%)	Cestoda prevalence (%)
Tiger salamanders (N = 14)	0	71.4	14.3
Great Plains toads (N = 29)	58.6	34.5	20.7
Canadian toads (N = 45)	42.2	22.2	22.2
Northern leopard frogs (N = 402)	63.2	13.2	0.5
Wood frogs (N = 109)	67.9	35.8	0
Boreal chorus frogs (N = 106)	61.3	0.01	0

A more taxonomically refined compilation of the helminth community across all amphibian species for common helminth genera includes these estimates of prevalence for helminth taxa: 29.2% for *Alaria*, 23.3% for *Echinoparyphium*, 13.3% for *Haematoloechus*, 11.2% for *Glypthelmins*, 7.7% for *Cosmocercoides* and 8.2% for *Rhabdias*. Uncommon helminths with less than 2.5% prevalence included *Cephalogonimus americanus*, *Megalodiscus temperatus*, *Apharyngostrigea pipientis*, *Lechriorchis tygarti*, Diplostomatidae sp, *Telorchis bonnerensis*, *Mesocestoides* sp., *Oswaldocruzia* spp, *Eustrongylides* sp., Spirurida sp., and *Spiroxys* sp. These parasite taxa are even further broken down by infection prevalence per amphibian host in Tables 32-34.

After molecular screening of parasite taxa, I discovered some helminth genera had several different species. For *Alaria*, I sequenced 31 DNA extracts from multiple amphibian hosts including northern leopard frogs, wood frogs, and boreal chorus frogs and found at least 3 different species. These three species of *Alaria* do not show any host specificity. For *Haematoloechus,* I detected 3 different species including *Haematoloechus varioplexus*, *Haematoloechus medioplexus*, and *Haematoloechus longiplexus. H. varioplexus* and *H. medioplexus* were found in several different amphibian species. *H. longiplexus* was only detected in one northern leopard frog therefore it is difficult to compare host specificities. The nematode

genus *Oswaldocruzia* also contained at least 3 species shown by the difference in nucleotide

bases ranging from 0.2-19.8% in the Cox1 gene (Table 16). For the genus *Spiroxys*, I detected at

least 3 different species with the difference in nucleotide bases ranging from 5.4-23.9% (Table

17). This is the first time the Cox1 gene has been sequenced from *Oswaldocruzia and Spiroxys*.

These results demonstrate the importance of sequencing data in determining the taxonomic status

of parasites.

Table 16. The percentage (%) of variable sites based on pairwise comparison of *Oswaldocruzia* taxa at 420 base pairs of partial Cox1 gene.

Individual ID and amphibian species	5176 Great Plains Toad	5177 Wood frog	5178 northern leopard frog	5179 Canadian toad	5180 tiger salamander
5176 Great Plains Toad	Identical	1.91	19.8	19.5	19.8
5177 Wood frog	1.91	Identical	20	19.8	20
5178 northern leopard frog	19.8	20	Identical	0.2	0.7
5179 Canadian toad	19.5	19.8	0.2	Identical	0.5
5180 tiger salamander	19.8	20	0.7	0.5	Identical

Table 17. The percentage (%) of variable sites based on pairwise comparison of *Spiroxys* taxa at 351 base pairs of partial Cox1 gene.

Individual ID and amphibian species	5181 boreal chorus frog	5182 tiger salamander	5185 tiger salamander
5181 boreal chorus frog	Identical	19.7	5.4
5182 tiger Salamander	19.7	Identical	23.9
5185 tiger salamander	5.4	23.9	Identical

In contrast, we only found one species of some Digenea and Nematoda genera even with

repeated sequencing in multiple host species. These digenean genera include *Apharyngostrigea,*

Cephalogonimus, Echinoparyphium, Glypthelmins, Lechriorchis, Megalodiscus and *Telorchis.*

The nematode genera with only one species found include *Cosmocercoides* and *Eustrongylides.*

This is also the first time Cox1 and 18S gene has been sequenced from *Cosmocercoides*.

Among the nematode taxa, the genus *Cosmocercoides* varied significantly across host

species, and primarily infected tiger salamanders (35.7%) (Table 32). *Rhabdias* also varied significantly across host species, with the highest prevalence (25.7%) in wood frogs and Great Plains toads (20.7%). *Spiroxys* was primarily found in tiger salamanders (35.7%), but one infected boreal chorus frog was found.

For the digeneans, *Alaria* prevalence varied significantly among host species, with the highest value in northern leopard frogs (37.8%), followed by wood frogs (23%), and Great Plains toads (20.7%) (Table 33). *Echinoparyphium* prevalence also varied significantly among host species, occurring at the highest in wood frogs (44%). *Glypthelmins* primarily infected boreal chorus frogs (40.5%) and all other hosts had less than 11% prevalence. *Haematoloechus* prevalence also varied significantly among host species, with the highest prevalence in Great Plains toads (17.2%) and northern leopard frogs (18.4%). I also found one *Telorchis bonnerensis* in a Great Plains toad, although this helminth usually infects tiger salamanders.

Cestodes were uncommon in most amphibian species. I found the genus *Mesocestoides* in Great Plains toads and northern leopard frogs at prevalence of less than 4% (Table 34). The cestode family Proteocephalidea varied significantly among host species, but primarily infected the Great Plains toads, Canadian toads, and tiger salamanders.

Helminth species richness within individual hosts ranged from 0 to 5 taxa and varied little among host species (Fig. 23). It was most common to find 0-2 different species of parasites within an individual host than 3-5 species. In fact, only one northern leopard frog had 5 different species of helminths.
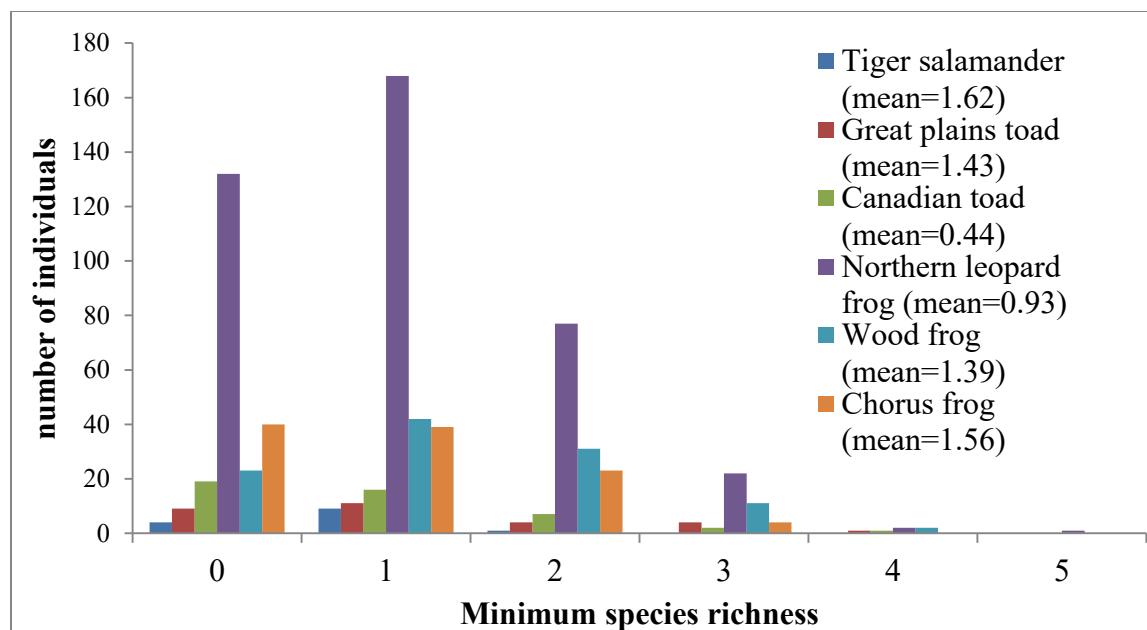
Figure 23. Minimum number of helminth taxa found in each host individual, reported separately for each amphibian species. E.g., the purple bars illustrate the number of leopard frogs with 0-5 helminth taxa per frog. More than half of leopard frogs were infected with at least one helminth species (note that this does not quantify infection intensity, or the number of individual parasites found within the frog). The mean number of helminth taxa per host individual is given in the legend.

Combined effects of geographic and ecological factors on helminth occurrence

I included all predictor variables listed in Table 1 in logistic regression models except the spatial factors because most parasites tended to show no spatial pattern in their occurrence. For *Alaria* and *Echinoparyphium*, the top models with AICc < 2 included land use, Nwetlands500 and Pcwet500 (Tables 18 and 20, respectively). Both of these sets of models explained very little of the variation in *Alaria* and *Echinoparyphium* occurrence (based on the similarity of residual and null deviance). To synthesize the information content of the top component models, I used multi-model inference to obtain a model-averaged estimate generated from the top component models for each predictor variable. For both *Alaria* (Table 19) and *Echinoparyphium* (Table 21) occurrence, the 95% confidence intervals of all predictor variables included zero. The top models for *Glypthelmins* occurrence included Pcwet500 and Nwetlands500 (Table 22) but these models

also explained very little of the variation, and again, none of the coefficients of predictor variables were significantly different from zero in multi-model inference (Table 23). For *Haematoloechus* occurrence, the top model included land use and the interaction between land use and Pcwet500 and Nwetlands500 (Table 24). These top models contribute very little information about the variation in *Haematoloechus* occurrence based on the similar values between the residual and null deviance. After using multi-model inferences to synthesize the information from the top models, I found pasture, relative to cropland, was the only variable with an estimate effect that departed from zero (Table 25).

Table 18. The top models (AICc < 2) for *Alaria* occurrence pooled across all amphibians. The null deviance is 230.65

Model	Variable	Residual Deviance	df	AICc	delta	weight
1	Null	----	1	232.67	0	0.45
2	Land use	226.01	4	234.25	1.58	0.20
3	Nwetlands500	230.42	2	234.49	1.82	0.18
4	Pcwet500	230.50	2	234.57	1.90	0.17

Table 19. Model averaged coefficients (ΔAIC < 2) for *Alaria* occurrence pooled across all amphibians

Variable	Estimate	Standard Error	P
Intercept	**0.429519**	**0.209328**	**0.0416**
Land use [Grassland]	-0.476083	0.365153	0.1955
Land use [Pasture]	0.774410	0.688183	0.2639
Land use [Woodland]	0.551267	0.706113	0.4383
Nwetlands500	-0.006696	0.014083	0.6369
Pcwet500	-0.572903	1.506338	0.7057

Table 20. The top models (AICc < 2) for *Echinoparyphium* occurrence pooled across all amphibians. The null deviance is 236.07.

Model	Variable	Residual Deviance	df	AICc	delta	weight
1	Null	----	1	238.09	0	0.27
2	Land use	229.97	4	238.21	0.12	0.25
3	Nwetlands500	235.80	2	239.52	1.43	0.13
4	Land use_Pcwet500	229.27	5	239.63	1.54	0.12
5	Land use_Nwetlands500	229.49	5	239.85	1.76	0.11
6	Nwetlands500	235.80	2	239.87	1.78	0.11

Table 21. Modeled averaged coefficients (ΔAIC < 2) for *Echinoparyphium* occurrence pooled across all amphibians.

Variable	Estimate	Standard Error	P
Intercept	-0.028745	0.243542	0.907
Land use[Grassland]	-0.580861	0.375731	0.125
Land use [Pasture]	-0.437773	0.603835	0.472
Land use[Woodland]	1.040128	0.707464	0.144
Pcwet500	-1.232427	1.535677	0.426
Nwetlands500	-0.008612	0.014383	0.552

Table 22. The top models (AICc < 2) for *Glypthelmins* occurrence pooled across all amphibians. The null deviance is 199.14.

Model	Variable	Residual Deviance	df	AICc	delta	weight
1	Null	----	1	201.16	0	0.42
2	Pcwet500	197.19	2	201.26	0.10	0.40
3	Nwetlands500	198.84	2	202.91	1.75	0.18

Table 23. Model averaged coefficients (ΔAIC < 2) for *Glypthelmins* occurrence pooled across all amphibians

Variable	Estimate	Standard Error	P
Intercept	**-0.85489**	**0.27769**	**0.0022**
Pcwet500	-2.47690	1.83782	0.1809
Nwetlands500	-0.00874	0.01616	0.5914

Table 24. The top models (AICc < 2) for *Haematoloechus* occurrence pooled across all amphibians. The null deviance is 220.26.

Model	Variable	Residual Deviance	df	AICc	delta	weight
1	Land use	210.43	4	218.67	0	0.49
2	Land use_Pcwet500	209.22	5	219.58	0.91	0.31
3	Land use_Nwetlands500	210.11	5	220.48	1.80	0.20

Table 25. Model averaged coefficients (ΔAIC < 2) for *Haematoloechus* occurrence pooled across all amphibians.

Variable	Estimate	Standard Error	P
Intercept	**-0.998889**	**0.284633**	**0.000488**
Land use[Grassland]	0.167340	0.391672	0.671462
Land use [Pasture]	**1.729073**	**0.640633**	**0.007375**
Land use[Woodland]	1.068319	0.645108	0.100176
Pcwet500	1.761266	1.595700	0.273205
Nwetlands500	0.008503	0.069	0.572846

I also estimated parasite occurrence in northern leopard frogs with the same ecological variables because they comprised the largest sample size of collected amphibians. The top models with AICc < 2 for *Alaria* occurrence included the null and Nwetlands500 (Table 26). After using multi-model inferences to synthesize the information from the top models, I found none of the predictor variables significantly explained the variation in *Alaria* occurrence (Table 27). The top models for *Echinoparyphium* included the null, Pcwet500, and Nwetlands500. All of the top models contributed similar information based on similar weights and residual deviances (Table 28). After using multi-model inference to obtain a model-averaged estimate for each predictor variable, I found no significant predictors for *Echinoparyphium* occurrence (Table 29). The top models For *Haematoleochus* occurrence included the intercept and land use (Table 30). These models explained very little variation in *Haematoloechus* occurrence based on the similar null and residual deviance values. However, pasture was a significant predictor for *Haematoloechus* occurrence (Table 31).

Table 26. The top models (AICc < 2) for *Alaria* occurrence in northern leopard frogs. The null deviance is 136.27

Model	Variable	Residual Deviance	df	AICc	delta	weight
1	Null	----	1	138.31	0	0.73
2	Nwetlands500	136.14	2	140.26	1.95	0.27

Table 27. Model averaged coefficients (ΔAIC < 2) for *Alaria* occurrence in northern leopard frogs

Variable	Estimate	Standard Error	P
Intercept	**0.579565**	**0.259119**	**0.027**
Nwetlands500	0.007885	0.021826	0.721

Table 28. The top models (AICc < 2) for *Echinoparyphium* occurrence in northern leopard frogs. The null deviance is 140.48.

Model	Variable	Residual Deviance	df	AICc	delta	weight
1	Null	----	1	142.52	0	0.43
2	Pcwet500	138.67	2	142.79	0.27	0.37
3	Nwetlands500	139.96	2	144.08	1.56	0.20

Table 29. Model averaged coefficients (ΔAIC < 2) for *Echinoparyphium* occurrence in northern leopard frogs

Variable	Estimate	Standard Error	P
Intercept	-0.28184	0.31694	0.378
Pcwet500	-2.51747	1.93528	0.199
Nwetlands500	-0.01542	0.02157	0.480

Table 30. The top models (AICc < 2) for *Haematoloechus* occurrence in northern leopard frogs. The null deviance is 144.79.

Model	Variable	Residual Deviance	df	AICc	delta	weight
1	Null	----	1	146.83	0	0.43
2	Land use	139.69	4	148.09	1.27	0.23
3	Nwetlands500	144.48	2	148.60	1.77	0.18
4	Pcwet500	144.70	2	148.82	1.99	0.16

Table 31. Model averaged coefficients (ΔAIC < 2) for *Haematoloechus* occurrence in northern leopard frogs

Variable	Estimate	Standard Error	P
Intercept	-0.20921	0.29085	0.4762
Land use[Grassland]	0.19783	0.45702	0.6689
Land use [Pasture]	**1.50408**	**0.71686**	**0.0382**
Land use[Woodland]	0.40547	1.03414	0.6985
Nwetlands500	-0.01149	0.02088	0.5866
Pcwet500	0.52588	1.74420	0.7657

DISCUSSION

Parasites were frequently found in amphibians throughout North Dakota, with considerable variation in prevalence of different classes and species of helminths, along with differences in infection rates among host species. For example, *Cosmocercoides* was more prevalent in tiger salamanders than any other host species. *Rhabdias* was also more common in the toads and wood frogs. These results are consistent with past studies because amphibians that prefer a terrestrial habitat usually have a helminth fauna dominated by nematodes (Fransden 1974; Kuc and Sulgostowska 1988). The route of transmission in nematodes by either consumption (*Cosmocercoides*) or penetration (*Rhabdias*) does not require an aquatic intermediate snail host.

In contrast, all digeneans require a molluscan (usually snail) intermediate host to complete their lifecycle. Amphibians are exposed to snails during aquatic stages of development (larvae and tadpoles) or during the breeding season for adults, which likely contributed to all anurans infected with some species of digenean. Amphibian host species also differed in cestode infection rates. I primarily found infections in toads and tiger salamanders and very rarely the northern leopard frogs. Amphibians become infected with cestodes by consuming infected arthropods or crustaceans. Toads and salamanders might inhabit locations more suitable for infected arthropods or crustaceans resulting in higher prevalence of cestodes.

I also discovered some helminth genera including *Alaria*, *Haematoloechus*, *Oswaldocruzia*, and *Spiroxys* had several different species. Within the genus *Oswaldocruzia* I likely found 3 species because of the differences between nucleotide bases (Table 16). The *Oswaldocruzia* sequence from Thompson is very different from the *Oswaldocruzia* detected in Pickard (19.8% difference in bases), Eastman (19.5%), and Nelson County (19.8%). The 3

different species were also found far from one another including the cities Thompson, Homen, Pickard, Eastman and Nelson County. Given the morphology data alone, two of the three species resemble *Oswaldocruzia pipiens* and *Oswaldocruzia andersoni*. However, taxonomic status is questionable because no researchers have submitted molecular sequences of these species. In fact, this is the first time Cox1 or 18S gene has even been sequenced for *Oswaldocruzia* and the results are indicating greater species diversity.

The broad distribution of parasites across the state closely resembles the host distributions required of each parasite. For example, *Alaria* was found broadly across the state (Fig. 11), most likely because their definitive hosts (Canidae) also have broad geographic distributions. Similarly, *Echinoparyphium* is widespread (Fig. 12) and their definitive hosts are waterfowl and many mammals including muskrats, raccoons, foxes, and coyotes that also have broad geographic distributions across the state. In contrast, *Glypthelmins* has somewhat of a more limited distribution (Fig. 13), perhaps because the definitive and intermediate hosts are amphibians and snails, which differ in geographic distribution across the state depending on the species of amphibian and snail. *Haematoloechus* has a broad distribution across the state and so does the Northern leopard frog, one of its hosts. *Rhabdias* was primarily found at the northern and eastern sides of the state (Fig. 15) in a pattern resembling the wood frog distribution because *Rhabdias bakeri* (the most prevalent *Rhabdias* species) is specific to wood frogs who are the only hosts in this lifecycle. *Rhabdias americanus* was another prevalent species and is specific to toads that are also found in these areas.

Parasite distributions also appeared to reflect finer scale aspects of host space use. For example, *Alaria* and *Echinoparyphium* hosts (birds and medium-sized mammals) travel long distances and spread parasite eggs in their feces throughout North Dakota, resulting in boad

dispersal of parasite eggs throughout the host home range. In contrast, *Glypthelmins* hosts are amphibians and snails that do not travel long distances or must be aided in dispersal by waterfowl, connected streams, or by mammals in the case of snails (Leeuwen et al. 2012). Therefore, it is more likely these hosts infect nearby wetlands. *Haematoloechus* has a geographically widespread distribution (Fig. 14), likely because the intermediate hosts (odonates) have high dispersal rates between ponds (Conrad et al. 2006), which increases the opportunities for consumption by amphibians. *Rhabdias* also had a fairly limited fine scale distribution across the state because amphibians are the only hosts in its lifecycle and they have smaller home ranges.

Helminth occupancy varied significantly by land use for some species, including *Echinoparyphium*, *Haematoloechus*, and *Rhabdias*. *Echinoparyphium* was found at more sites in cropland than other land use categories, ignoring woodland because of the low sample size. *Haematoloechus* was found at more sites surrounded by pasture and *Rhabdias* at more sites surrounded by grassland. The cause of differences in parasite occupancy is not known; there are a variety of factors that might differ geographically across the state, and our study was not designed to reveal underlying causation. In addition, *Rhabdias* occupancy was significantly higher in the Red River Valley compared to all other ecoregions likely because we primarily collected *Rhadias bakeri*, which is specific to wood frogs that are also found in the Red River Valley.

Other variables used in logistic regression models including ecoregion, wetland length, wetland area, Nwetlands500 and Pcwet500 were not significant predictors for any of the parasites' occurrence. Perhaps these variables did not vary enough across the region with helminth occupancy to generate an effect. Other factors, including host presence, density, and

habitat use might be better predictors for parasite occurrence because these factors play a role in parasite transmission.

Alaria and *Rhabdias* were the only parasites to show significant spatial dependency in their infections. These results suggest the connectivity between wetlands is an important factor involved in the transmission of *Alaria* and *Rhabdias* parasites. Wetlands closer together are likely to be similar in the presence or absence of these two helminths, because the movement of amphibians to nearby wetlands increasing the probability of transmission. For example, if amphibians are visiting multiple wetlands they have a greater probability of contacting cercaria shed from snails or being eaten by a predator from the family Canidae in the case of *Alaria*. *Rhabdias* likely has a spatial dependency because amphibians are the only host in its lifecycle and they have smaller home ranges that would restrict infections to nearby wetlands. Other parasites (*Echinoparyphium*, *Haematoloechus*, and *Glypthelmins*) were not spatially autocorrelated probably because other factors such as surrounding topography and amount of human disturbance limited movement or accessibility of wetlands for definitive or intermediate hosts to a particular wetland.

These results were only compiled from two years of data and captured only a limited view of parasite occurrence. This study has many caveats including uneven sample sizes between the different life stages, species, land use categories, and ecoregions. It would be interesting to look at other variables affecting parasite occurrence such as non-amphibian host occurrence and density. Future studies should include more years for data collection to estimate seasonal and yearly changes in parasite occurrence. Continued sampling of parasite communities is important because it can provide important insights into biotic communities because parasite diversity is an indicator of ecological integrity and general environmental health. Higher parasite species

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richness results in richer, more diverse biotic communities (Hatcher et al. 2012, Hudson et al. 2006). Therefore, parasite surveys can provide a relatively inexpensive method to index ecosystem integrity.

It is also important to continue conducting parasite surveys in North Dakota because some parasite species found are known to impair amphibian health including *Echinoparyphium* infecting the host's kidneys, and the two taxa infecting the host's lungs, *Haematoloechus* and *Rhabdias* (Kelehear et al. 2011, Koprivnikar et al. 2006, Szuroczki and Richardson 2009). Additionally, infection risk may be amplified by exposure to a variety of environmental stressors, some of which are likely or certain to be encountered on agricultural landscapes (Gendron et al, 2003, King et al. 2007, Koprivnikar et al. 2006). Consequently, parasite surveys can provide useful information about factors that might influence amphibian demography and distribution.

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APPENDICES

Table 32. Nematode prevalence (%) per amphibian species. The sample size of each amphibian species is represented by the letter N. The genus *Rhabdias* is host specific. *Rhabdias americanus* is found in the toads, *Rhabdias bakeri* is found in wood frogs and *Rhabdias ranae* is found in northern leopard frogs.

Nematoda Taxa	Tiger salamander (N=14)	Great Plains toad (N=29)	Canadian toad (N=45)	Northern leopard frog (N=402)	Wood frogs (N=109)	Boreal chorus frog (N=106)	χ^2	P
Cosmocercoides variabilis	35.7	6.9	4.4	7.4	11.9	0	28.69	0.000293
Eustrongylides sp.	0	0	0	0.25	0	0	na	
Oswaldocruzia spp.	0	6.9	6.7	2.2	5.5	0	10.97	0.0567
Rhabdias spp.	0	20.7	15.6	4.2	25.7	0	72.45	< 0.0001
Spirurida *sp.*	0	3.4	0	0	0	0	na	na
Spiroxys sp.	35.7	0	0	0	0	0.9	na	na

Table 33. Digenean prevalence (%) per amphibian species. The sample size of each amphibian species is represented by the letter N.

Digenea taxa	Tiger Salamander (N=14)	Great Plains Toad (N=29)	Canadian toad (N=45)	Northern leopard frog (N=402)	Wood frog (N=109)	Boreal chorus frog (N=106)	χ^2	P
Alaria spp.	0	20.7	13.3	37.8	23	16	37.62	< 0.00001
Apharyngostrigea pipientis	0	0	0	1.5	3.7	0	6.758	0.2086
Cephalogonimusamericanus	0	0	0	0.1	0	0.9	na	na
Diplostomatidae *sp.*	0	0	0	2.2	0	0	na	na
Echinoparyphium rubrum	0	13.8	20	17	44	33	47.97	< 0.00001
Glypthelmins pennsylvaniensis	0	10.3	4.4	5.7	7.3	40.5	109.5	<0.00001
Haematoloechus spp.	0	17.2	6.7	18.4	11	0	30.04	0.000067
Lechriorchis tygarti	0	0	0	1.2	1.8	0	na	na
Megalodiscus temperatus	0	0	0	1.2	0	0	na	na
Telorchis bonnerensis	0	3.4	0	0	0	0	na	na

Table 34. Cestode prevalence (%) per amphibian species. The sample size of each amphibian species is represented by the letter N.

Cestoda taxa	Tiger Salamander (N=14)	Great Plains toad (N=29)	Canadian toad (N=45)	Northern leopard frog (N=402)	Wood frog (N=109)	Boreal chorus frog (N=106)	χ^2	P
Mesocestoides sp.	0	3.4	0	0.25	0	0	na	na
Proteocephalidea *sp.*	14.3	17.2	22.2	0.25	0	0	117.1	< 0.00001

Table 35. Life cycle characteristics for all identified helminths. Helminths that do not use a first or second intermediate host are marked as not applicable N/A.

Parasite taxa	Life cycle	Transmission to amphibians	Site of infection in amphibians	First intermediate	Second intermediate	Definitive	Life cycle reference
Nematoda							
Cosmocercoides variabilis (Harwood 1930)	Direct	Trophic or penetration	Intestines Bladder	N/A	N/A	Amphibian Mollusk	(Ogren 1953)
Eustrongylides sp. (Jagerskiold 1909)	Complex	Trophic	Bladder	Arthropod	Fish and amphibian	Bird Mammal	(Olsen 1974)
Oswaldocruzia spp. (Slimane & Durette-Desset, 1997)	Direct	Penetration	Intestines	N/A	N/A	Amphibian	(Baker 1987)
Rhabdias spp. (Walton 1929)	Heterogonic	Penetration	Lungs	N/A	N/A	Amphibian	(Langford and Janovy 2009)
Spirurida *sp* (Anderson 1976)	Complex	Trophic	Intestines	Arthropod	N/A	Vertebrate	(Olsen 1974)
Spiroxys sp. (Rudolphi 1819)	Complex	Trophic	Intestines	Arthropod	N/A	Vertebrate	(Olsen 1974)
Digenea							
Alaria spp. (Bosma 1931)	Complex	Penetration	Leg muscle Body Cavity	Snail	Amphibian Snake	Mammal (Canidae)	(Olsen 1974)
Apharyngostrigea pipientis (Faust 1918)	Complex	Penetration	Body Cavity	Snail	Fish Frog Snail	Fish Frog Bird Mammal	(Olsen 1974)
Cephalogonimus americanus (Stafford 1902)	Complex	Penetration	Intestines	Snail	Amphibian Fish Reptile	Amphibian Fish Reptile	(Lang 1968)
Diplostomatidae (Dubois 1968)	Complex	Penetration	Intestines Liver	Snail	Fish Amphibian	Fish Bird Mammal	(Olsen 1974)

Continued

Species							
Echinoparyphium rubrum (Cort 1915)	Complex	Penetration	Kidneys	Snail	Turtle Amphibian	Bird Mammal	(Olsen 1974)
Glypthelmins pennsylvaniensis (Cheng 1961)	Complex	Trophic	Intestines	Snail	Amphibian Skin	Amphibian	(Olsen 1974)
Haematoloechus spp. (Stafford 1902)	Complex	Trophic	Lungs	Snail	Odonate	Amphibian	(Olsen 1974)
Lechriorchis tygarti (Talbot 1933)	Complex	Penetration	Liver Bladder	Snail	Amphibian	Snake	(Cort et al. 1952)
Megalodiscus temperatus (Stafford 1905)	Complex	Trophic	Intestines	Snail	Amphibian skin	Amphibian	(Olsen 1974)
Telorchis bonnerensis (Waitz 1960)	Complex	Penetration	Intestines	Snail	Snail Amphibian	Turtle Snake Salamander	(Schell 1962)
Cestoda							
Mesocestoides sp. (Goeze 1782)	Complex	Trophic	Body Cavity	Arthropod	Amphibian Bird Reptile Mammal	Mammal Bird Reptile Amphibian	(Foronda et al. 2007)
Proteocephalidea *sp.* (Mola 1928)	Complex	Trophic	Intestines	Crustacean	N/A	Fish, Amphibian Reptile	(Scholz 1999)