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Mosquito Fauna And Prevalence Of Avian Malaria In The Turtle Mountains, North Dakota

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MOSQUITO FAUNA AND PREVALENCE OF AVIAN MALARIA IN THE TURTLE MOUNTAINS, NORTH DAKOTA

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Kelsey Jo Morin
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ABSTRACT

Avian malaria is a disease caused by intracellular parasites transmitted by mosquitoes and is detrimental to individual birds and their populations. Although it is sub-lethal in most instances, avian malaria can result in anemia, lower fertility, and reduced migration ability. The Turtle Mountains is a unique ecoregion in North Dakota and provides habitat for various bird and mosquito species that differ from other parts of the state. Previous research has defined the mosquito fauna and occurrence of avian malaria in the Red River Valley region of eastern North Dakota and Northwest Minnesota; however, no research has been conducted in the Turtle Mountains. Mosquito population dynamics are vital to understanding the transmission dynamics of malaria.

This research had three objectives. First, the species composition, seasonal abundances, and malaria infection status of host-seeking mosquitoes were determined throughout two summer seasons. Abundances and species compositions of mosquitoes differed from 2019 to 2020, but there were no haemosporidian infections identified. Second, blood from local and migrating birds in the Turtle Mountains was collected to establish the occurrence of avian malaria in the region. This study determined the prevalence of avian haemosporidian parasites in both migratory birds and birds that remain permanent residents throughout the seasons. Avian haemosporidians were present in both local and migratory birds captured in the Turtle Mountains. Based on this research in the Turtle Mountains, it appears that the avian haemosporidian transmission occurs for migratory birds prior to their arrival the Turtle Mountain

ecoregion. Third, laboratory studies determined the relative competencies of different mosquito species to support the development of *Plasmodium*. Despite successful infection of *Plasmodium* in some *Culex pipiens*, the parasite was unable to fully develop into sporozoites within the vector. *Plasmodium* was unable to develop into oocysts in *Aedes aegypti* or *Culex tarsalis*.

Future studies should focus on climate change and its impact on the mosquito populations of the Turtle Mountains. The introduction of a new competent vector or the increase of a current competent vector would greatly influence the transmission of avian parasites. Increased transmission of current parasites or the introduction of a new parasite species would negatively impact bird populations of the area.

CHAPTER I

INTRODUCTION

There are three major types of vector-borne blood parasites that typically infect birds – intracellular protozoa (Haemosporidia), extracellular protozoa (Trypanosomatidae), and larval nematodes (microfilariae).

Haemosporida

Haemosporida is an order of parasites in the phylum Apicomplexa. There are four families of Haemosporida: Plasmodiidae, Haemoproteidae, Leucocytozoidae, and Garniidae. Haemosporidians are protozoan parasites transmitted from host to host by hematophagous Diptera (true flies) (Valkiunas 2005). Birds have the highest haemosporidian diversity (Valkiunas 2005, Greiner *et al.* 1975). The majority of haemosporidian parasitizing birds fall into three genera: *Plasmodium, Haemoproteus,* and *Leucocytozoon*. Passeriformes (songbirds) are parasitized by the largest number of haemosporidian species (Valkiunas 2005).

Plasmodium

Plasmodium is an intracellular parasite transmitted by mosquitoes and causes the disease Malaria. *Plasmodium* is the most well studied haemosporidian because of its importance to human health. There is a lot known about the basic biology & transmission of these parasites from human hosts to *Anopheles* mosquito vectors. However, *Plasmodium* is a large genus and contains species that parasitize mammals, reptiles, and birds. In fact, avian malaria is one of most widespread and common blood protozoan infections of birds. Compared to the *Plasmodium*

species that infect humans, there is much less known about the basic biology and transmission of avian *Plasmodium*.

Originally avian *Plasmodium* was used as a surrogate model for human malaria during the early discovery and development of natural and synthetic anti-malarial drugs. After the discovery of rodent malaria in Africa during the 1950s, the studies relating to avian haemosporidians decreased and rodent malaria largely replaced avian malaria as a model system for use in drug and vaccine studies (Valkiunas 2005).

Avian *Plasmodium* is transmitted by several genera of mosquito vectors (Diptera: Culicidae). Avian *Plasmodium* infections are not often lethal but can have negative impacts on the host, such as anemia and enlargement of the liver and spleen (Palinauskas *et al*. 2008, Valkiunas 2005). In cases of novel parasite introduction, *Plasmodium* has been found to cause death (Levin *et al*. 2009, van Riper *et al*. 1986, Warner 1968).

The *Plasmodium* life cycle begins when sporozoites are injected into the host bird from the mosquito vector when blood feeding (Figure 1.1). Inside the vertebrate host, *Plasmodium* parasites develop intracellularly. Development with host erythrocytes is called erythrocytic development, whereas development within other cell types is called exo-erythrocytic development. Within the bird host, the *Plasmodium* lifecycle can be divided into three main categories. The first category is primary exo-erythrocytic merogony (=asexual replication). This category has two generations. The first generation occurs when sporozoites, introduced from the bite of an infectious mosquito, infect reticuloendothelial cells of the bird. Merogony produces daughter cells known as merozoites, that are released into the blood upon completion of the asexual replicative cycle. A second generation of primary exoerythrocytic merogony occurs within macrophages. The progeny merozoites are released from the infected macrophages and

can then infect erythrocytes to produce sexual stages of the parasite (=gametocytes). Gametocytes do not replicate and there is only one gametocyte, either male or female, per infected erythrocyte. Gametocytes are the stage that is infectious to mosquitoes. However, the life cycle of *Plasmodium* within birds can have additional pathways. The merozoites can also go onto the second or third lifecycle category. The second lifecycle category is erythrocytic merogony (asexual replication) producing a second round of merozoites that can infect erythrocytes and produce gametocytes. The third lifecycle category can occur from either the merozoites produced from the primary-exoerythrocytic stages or the erythrocytic merogony from the secondary-exoerythrocytic merogony. These lifecycle stages occur when merozoites infect endothelial cells of capillaries. The phanerozoites produced from the secondary-exoerythrocytic stages can enter the second lifecycle stage category (erythrocytic merogony), re-enter the third lifecycle stage category (secondary-exoerythrocytic merogony), or can infect erythrocytes and become gametocytes.

After gametocytes are formed in the host, the bird becomes infectious to mosquitoes. Once gametocytes are ingested by the mosquito in the bloodmeal, gametocytes will exit the erythrocytes to form gametes. The female gametocyte forms a single macrogamete and the male gametocyte forms several highly active flagellar-like microgametes (*i.e.*, exflagellation). With fertilization of the macrogamete, a zygote is formed. The zygote transforms into a banana-shaped, motile ookinete which moves through midgut and rounds up on the outer gut wall to form a sphere known as an oocyst. As the oocyst grows, progeny are produced within the cyst by asexual reproduction, culminating in hundreds of eyelash-shaped sporozoites. When released into the hemocoel from the oocyst, sporozoites penetrate the salivary glands of the mosquito and

can then be passed into a new host during blood feeding by the infectious mosquito (Valkiunas 2005).

Haemoproteus

Haemoproteus is a genus of parasites that infect reptiles, amphibians, and birds. Avian *Haemoproteus* species are currently divided into two subgenera: *Haemoproteus* and *Parahaemoproteus*. Taxonomic treatment of *Parahaemoproteus* differs among researchers; several works suggest that *Parahaemoproteus* should be elevated to genus status (Martinsen *et al*. 2008, Galen *et al.* 2018). Avian *Haemoproteus* is transmitted by hippoboscid flies (Hippoboscidae) and *Parahaemoproteus* is transmitted by biting midges (Ceratopogonidae) (Valkiunas 2005). Asexual stages of *Haemoproteus* occur within tissues rather than erythrocytes (Valkiunas 2005). *Haemoproteus* does not typically present clinical symptoms in birds (Valkiunas 2005). However, as research continues to develop, it has been found that this parasite can cause death in novel hosts (Donovan *et al*. 2008, Cannell *et al*. 2013).

Leucocytozoon

Avian *Leucocytozoon* is transmitted by black flies (Simuliidae) (Valkiunas 2005). *Leucocytozoon* are unique in the Haemosporida order because they only parasitize avian hosts. The *Leucocytozoon* genus is also unique, because gametocytes can be formed in leucocytes, not just erythrocytes (Valkiunas 2005). Although anemia is associated with all Haemosporida infections, anemia is intensified in a *Leucocytozoon* infection due to anti-erythrocyte factor in the plasma (Valkiunas 2005).

Microfilaria

Microfilariae are the pre-larval form of vector-borne filarial nematodes in the Family Onchocercidae. Filarial nematodes infect all classes of vertebrates, except fish (Anderson 2000).

Microfilariae live in the blood or skin of the host, where they are available to hematophagous vectors. The developmental site for the adult worms is species specific and can include the body cavity, the circulatory/lymphatic system, or air sacs. Arthropod vectors known to transmit microfilariae include biting midges, blackflies, fleas, horse and deer flies, mosquitoes, lice, louse flies, mites, and ticks (Anderson 2000). Some microfilarial species that inhabit the blood also show changes in bloodstream circulation within the host. These microfilariae show nocturnal periodicity and enter peripheral circulation at peak arthropod feeding time (Anderson 2000, Vaughan *et al*. 2012).

Trypanosoma

Trypanosomes (Trypanosomatidae) are unicellular flagellate parasites that are transmitted by mosquitoes, biting midges, bugs, and mites (Baker 1976). The lifecycles of trypanosomes vary but involve a hematophagous vector and a lifestage within a vertebrate host. Avian trypanosomes are transmitted by ingestion of an infected vector by the host (Votýpka, J., & Svobodová 2004, Fialová *et al*. 2021). Trypanosomes have very negative impacts on mammalian hosts but are mostly harmless to their avian hosts (Baker 1976). These parasites are abundant and very diverse in avian species throughout the world but are poorly studied (Zídková *et al*. 2012).

Turtle Mountains

The Turtle Mountains are located in the north central part of North Dakota. The area is oval shaped (Fig. 1.2) and has a distinct change in altitude and habitat compared to the surrounding area. The Turtle Mountains are 400-800 feet above the surrounding plain (Hendricks & Laird 1943, Deal 1970). The Turtle Mountains are high enough in relation to the surrounding area, resulting in more precipitation than other regions (Deal 1970). The elevated area is due to an underlain of rocks by the Cretaceous Fox Hills and Hell Creek Formations. The movement of

glacial ice over the obstruction caused glacier shearing and debris covered stagnant ice formations. The ice above the Turtle Mountains melted slower and more unevenly than the surrounding area. Accumulations of this glacial debris and precipitation formed the hilly topography and scattered lakes known today (Deal 1970, Leonard 1919).

Today the Turtle Mountains are home to the Turtle Mountain Band of Chippewa Native American tribe. Most of the land is in tribal trust status and individual trust status (Sanstead 1997). The rest of the land is held by individual ownership. The Turtle Mountains are filled with deciduous trees (birch, oak, elm, willow) and woodland lakes (Sanstead 1997) (Figure 1.3). The Turtle Mountains are unique due to their habitat and due to the policies surrounding the area. The area is politically protected through the tribal government. The Turtle Mountains of North Dakota were deemed unsuitable for economic development due to the scattered resources and the difficulty associated with cultivation (Deal 1970, King & Brauch 1981). In addition to the lack of suitability for agricultural development in the area, the tribe occupying the land is dedicated to preserving the environment. The tribe clearly demonstrates this through their political actions. The Turtle Mountain Chippewa Tribal Constitution states "The Turtle Mountain Band of Chippewa are interconnected to the environment and to the integrity of the ecosystem, including but not limited to the fish, wildlife, plants, trees and the environment." The combination of the notable habitat and the political protections of the area create a rare opportunity for research.

Study Objectives

The purpose of this research was to establish a baseline of avian blood parasites in different bird hosts and mosquito vectors that are present in the Turtle Mountains, North Dakota. First, the species composition, seasonal abundances, and malaria infection status of host-seeking mosquitoes were determined throughout two summer seasons. Second, blood from local and

migrating birds in the Turtle Mountains was collected to establish a baseline for avian blood parasites in the region. Blood samples were used for microscopic and molecular detection of avian Haemosporidian parasites, microfilariae, and *Trypanosoma*. Third, laboratory studies were used to determine the relative abilities of different mosquito species to support the development of the *Plasmodium* parasite. This dissertation will serve as a baseline for any future research in the Turtle Mountains and for comparative studies on avian malaria throughout the different ecoregions of the northern Great Plains.

Compliance Documentation:

This research was conducted in compliance with tribal, state, federal, and university regulations concerning the collection of mosquitoes and birds.

Turtle Mountain Research Review Board RRB Protocol #90

North Dakota Game & Fish Scientific Permit GNF0493696

U.S. Fish & Wildlife Service Scientific Collection Permit MB072162-0

UND Institutional Animal Care and Use Committee Protocol 1804-2

UND Blood-borne Pathogen Training Program

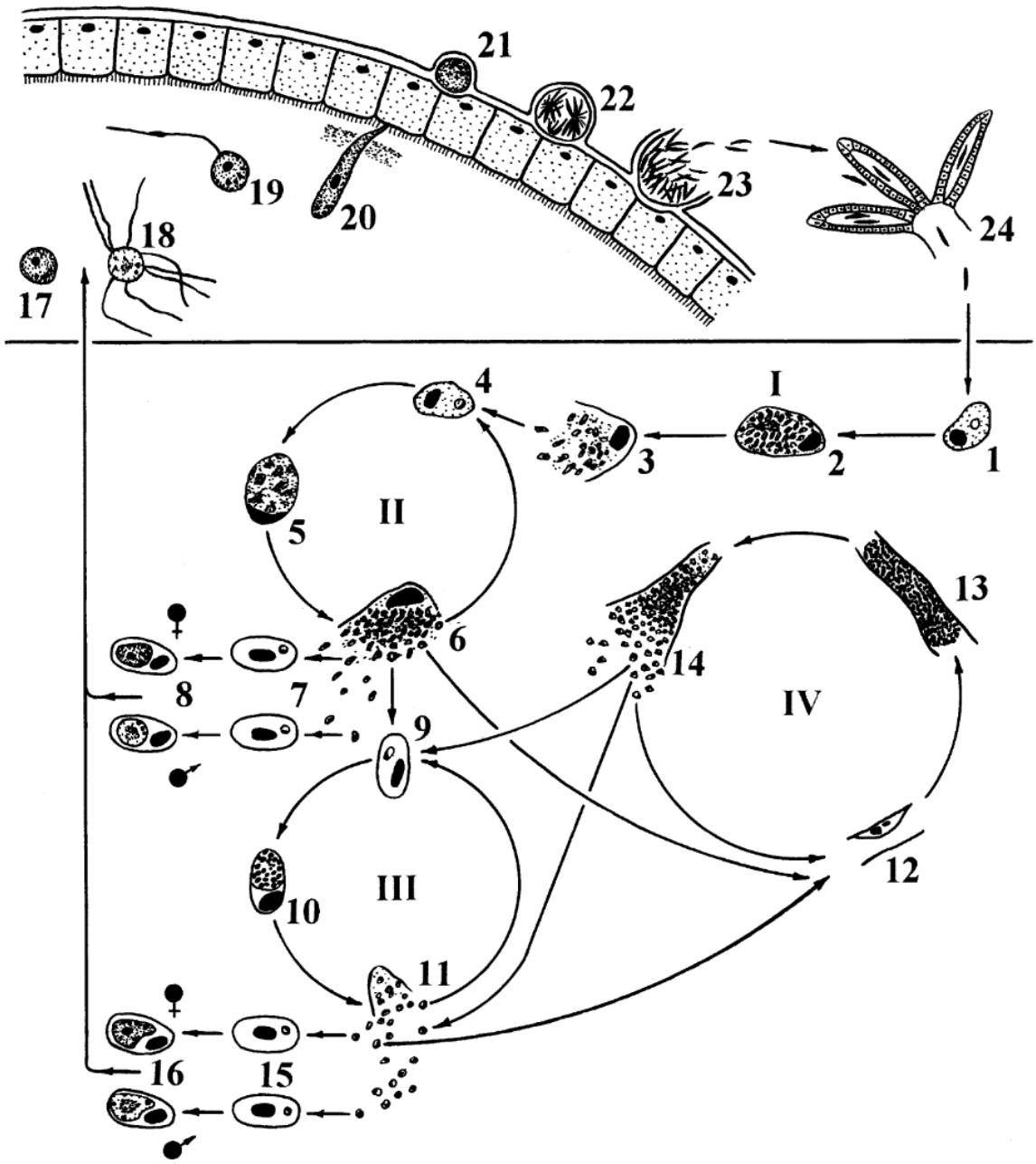

Figure 1.1. Diagrammatic representation of the life cycle of bird malaria parasites (*Plasmodium relictum* as an example): Upper part, in vector; lower part, in bird: I, II – primary exoerythrocytic merogony; III – erythro- cytic merogony; IV – secondary exoerythrocytic merogony; 1 – sporozoite in reticuloendothelial cell; 2, 3 – cryptozoites; 4 – merozoite in macrophage; 5, 6 – metacryptozoites; 7 – merozoites in erythrocytes; 8 – gametocytes; 9 – merozoite in erythrocyte; 10, 11 – erythrocytic meronts; 12 – merozoite in endothelial cell of capillaries; 13, 14 – phanerozoites; 15 – merozoites in erythrocytes; 16 – gametocytes; 17 – macrogamete; 18 – exflagellation of microgametes; 19 – fertilization of macrogamete; 20 – ookinete penetrating the peritrophic membrane; 21 – young oocyst; 22, 23 – sporogony; 24 – sporozoites in the salivary glands of vector (Valkiunas 2005).

Figure 1.2. Location of Turtle Mountains, North Dakota USA. Image was obtained through Google Earth March 2022 (Google, Camera View, ND USA).

Figure 1.3. Turtle Mountains, North Dakota USA Fall 2021. Photo credited to Kelsey Morin.

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CHAPTER II

MOSQUITO FAUNA AND AVIAN HAEMOSPORIDIAN INFECTION OF HOST-SEEKING MOSQUITOES IN THE TURTLE MOUNTAINS, NORTH DAKOTA

INTRODUCTION

Mosquitoes play a major role in disease transmission and are the vectors for many bird parasites. Early data in North Dakota reported the presence of 40 mosquito species (Darsie & Anderson 1985, Darsie & Ward 1989). More recent research has shown low counts of additional species (Mehus 2013, Hanson et al. 2012). Mosquito fauna research previously conducted in the state of North Dakota reports *Aedes vexans* as the dominant species (Anderson *et al*. 2015, Bell *et al*. 2005, Deckert 1995, Mehus 2013, Munro & Saugstad 1939, Post & Munro 1949). However, some environments may support different mosquito species due to differences in their natural histories (*e.g.,* larval physiology, preferred breeding habitat). Therefore, some environments will provide more opportunity for disease transmission by mosquitoes.

The Turtle Mountain region of North Dakota is a unique ecoregion. Despite the interesting landscape, there has been little research on the mosquito fauna of the area. The Turtle Mountain region is in the north-central part of North Dakota. Unlike much of North Dakota, the Turtle Mountains consist of miles of rolling hills, and highly wooded areas. The habitats consist of mostly thick, deciduous forested areas with few, small clearings for pastureland (North Dakota 2012). The ecoregion is surrounded by agricultural crop land and grasslands, which is similar to other ecoregions of North Dakota. These forested areas provide wildlife habitat and

potential areas for mosquito breeding habitats. Pooled water is essential for the development and survival of aquatic mosquito stages. Lakes, sloughs, and varying wetlands are abundant in the Turtle Mountains (Bluemle 2005, King & Brauch 1981). The higher elevation of the region results in higher precipitation than the surrounding grasslands (Bluemle 2005). Tree-holes and covered, woodland pools provide a larval habitat unlike other North Dakota areas. Due to the wetlands and woodlands, the Turtle Mountains provide habitats for a diverse mosquito fauna.

Malaria is a disease caused by an intracellular *Plasmodium* parasite that is transmitted by mosquitoes. The disease affects several groups of vertebrates, including humans and other primates, certain rodents, white-tailed deer, and lizards. But the greatest species diversity of *Plasmodium* is found in birds. Exposure to novel avian parasites has been shown to be detrimental to entire bird populations (Atkinson *et al*. 2000, Levin *et al.* 2009). Understanding the mosquito population of an area is crucial to understand the transmission of avian malaria and to provide a baseline for future research.

The presence of a mosquito species is not equivalent to disease transmission. Understanding the mosquito population will give insights into possible avian *Plasmodium* transmission, but this information does not directly implicate the vector responsible. Mosquito species vary in host preference and parasite susceptibility (Kimura 2010). Despite the importance of the mosquitoes as vectors, little research has been done to implicate natural avian *Plasmodium* vectors (Hughes *et al*. 2010, Inci *et al*. 2012). What little research that has been done did not cover entire summer seasons and did not occur in the Turtle Mountains.

The physical landscape and available water sources play an important role in mosquito species composition. The mosquito abundance and species composition has never been thoroughly recorded in the Turtle Mountains despite its vast differences from the rest of North

Dakota. This study analyzes the population dynamics of the mosquito fauna in the Turtle Mountains over two mosquito seasons. The species composition, population abundances, and timeline of emergence for each species give insight into potential parasite transmission. Despite mosquito collections occurring over entire summer seasons, no avian *Plasmodium* was found. This chapter provides a detailed description of the mosquito populations of the Turtle Mountains and their potential relationship to avian malaria transmission.

METHODS

Permission

Tribal permission was needed to collect specimens and conduct research on Reservation land due to the Turtle Mountain Band of Chippewa Indians Research Protection Act (2014). Permission to work on Turtle Mountain Band of Chippewa tribal land was granted by the Turtle Mountain Research Review Board. Permission was granted for mosquito collections for 2019 and 2020 summers (RRB Protocol #90).

Mosquito Collection

Host seeking mosquitoes were captured using Mosquito Magnet traps (Liberty MM3000 model) (Woodstream, Lititz PA). These traps attract mosquitoes by emitting heat and carbon dioxide. The heat is fueled by a small propane burner. Excess propane is diverted through a catalytic converter to create carbon dioxide. A small electric fan moves the heat and carbon dioxide out into an attractive plume, meant to simulate the body heat and breath of a warm-blooded animal. Another small electric fan turning in the opposite direction creates a slight vacuum above where the carbon dioxide is being emitted. Host seeking mosquitoes detect the carbon dioxide and move up the carbon dioxide gradient toward its source. As mosquitoes swarm around the carbon dioxide source, they are gently vacuumed into a collection bag housed

within the trap. The traps use a 12-volt power supply and are attached to a 5-gallon propane tank. The Mosquito Magnet is expected to cover up to one acre of land, according to the manufacturer.

Mosquito Magnets were placed in seven locations throughout the Turtle Mountain region of North Dakota in Rolette County (Figure 2.1). Figure 2.1A depicts the locations used in the year 2019, and Figure 2.1B depicts the locations used in the year 2020. Trap locations were reduced in Summer 2020 due to unreliable power sources and difficulties that arose during the Summer 2019 season. Locations were backyards throughout the community and were chosen based on landowner permission and power source availability. Traps were placed at least fifteen yards from the home or power source. All traps were placed on land free from insecticides.

Mosquito trapping was conducted in 2019 from May 5 to October 7 and in 2020 from May 4 to September 23 to fully encompass two mosquito seasons. The Mosquito Magnets ran from 7pm to 8am to target night feeding mosquitoes. Each week of trapping had a minimum of three trap nights. Trap-nights were chosen based on suitable weather conditions. The mosquitoes were taken out of the Mosquito Magnets each morning. Mosquitoes were immobilized by putting them into the freezer and then taken out of the mesh mosquito magnet reservoir. Mosquitoes were stored in plastic baggies in a -20°C freezer. Mosquitoes were collected for a total of 202 trap-nights in 2019 and 190 trap-nights in 2020. Mosquitoes were individually identified using dissecting microscopes. We used a dichotomous key to identify each mosquito to species, based on morphological features (Darsie Jr. and Ward 2005).

To compare the abundance of the mosquito fauna collected at Turtle Mountains with what is known at other sites in eastern North Dakota, I calculated the mosquito density collected per trap-night (measure of relative abundance) and Shannon index (measure of diversity) for all mosquitoes identified to species during 2019 and 2020. I compared my estimates of relative

abundance and diversity of mosquitoes with mosquito collections from two ecologically different Steele Co., ND sites and sites within the city of Grand Forks, ND (Mehus 2013, Deckert 1995). One of the Steel Co., ND sites (=FARM) consisted of open agricultural habitat characteristic for most of the Red River Valley. The other site (=FOREST) consisted of a *ca.* 40-acre area of hardwood forest with semi-closed canopy adjacent to the Goose River. The Grand Forks Co. Study was conducted within city limits. For each of the three sites, I calculated Shannon indices (H) (Spellerberg & Fedor 2003) using the following formula:

$$H = - \sum \left[(n_i/N) \times \log_2(n_i/N) \right]$$

where:

n_i = number of mosquitoes of each species collected at each site

N = the total number of mosquitoes collected at each site

DNA Extraction

Mosquitoes were pooled based on date of capture and species and stored in the freezer at -20°C. A maximum of 25 mosquitoes were used per extraction. Approximately 500-700 µl of phosphate buffered saline and 15 to 20 Zirconium Oxide 1.00mm beads (Next Advance, Inc., Troy, NY) were added to each tube. Tubes were shaken using the TissueLyser II (Qiagen, Germantown, MD) vortex machine to macerate the mosquito tissue. DNA was extracted using a Qiagen DNeasy Blood & Tissue Kit (Qiagen, Germantown, MD) following the manufacturers protocols.

Haemosporidian Detection

To screen for general avian Haemosporidia, a real-time polymerase chain reaction protocol was used amplifying a 182 base pair portion of rDNA (Table 2.1) (Bell et al. 2015). The

following cycling conditions were used: 95 °C for 30 s, 35 cycles of 95 °C for 30 s, 53 °C for 35 s, plate read and melt curve. Several positive and negative controls were used in all runs. The positive control was a previously sequenced avian *Plasmodium* positive blood sample. Samples were considered real-time PCR positive if the melt peak curve from amplification was around 78.5°C and a suitable amplification curve was visible, resembling the positive control.

Plasmodium/Haemoproteus **Detection**

If the real-time PCR indicated a positive sample, the extracted DNA was tested for *Plasmodium/Haemoproteus* infection using traditional nested PCR reactions (Table 2.1) (Bell et al. 2015). The following cycling conditions were used for initial PCR: 95°C for 3 mins, 20 cycles of 95°C for 30 s, 50°C for 45 s, 68°C for 60 s, and a final extension at 68°C for 60 s. The following cycling conditions were used for the second PCR: 95°C for 3 mins, 35 cycles of 95°C for 30 s, 50°C for 45 s, 68°C for 60 s, and a final extension at 68°C for 60 s. The same previously tested positive avian *Plasmodium* sample was used as a positive control for *Plasmodium/Haemoproteus* nested PCR. All PCR products were then run onto a 1% ethidium bromide agarose gel to look for amplification.

A subsample of negative samples and any sample with slight amplification were also amplified using a nested PCR protocol (Bell *et al*. 2015). A previously tested, avian *Plasmodium* sample was used as a positive control. The PCR products were then run onto a 1% ethidium bromide agarose gel to look for amplification.

RESULTS

Mosquito Trapping

A total of 15,293 mosquitoes over 392 trap-nights were collected in 2019 and 2020, with 28 mosquito species identified. For Summer 2019, the average number of mosquitoes per trap-

night (n = 6,581, trap-nights = 202) was 32.6. For Summer 2020, the average number of mosquitoes per trap-night (n = 8,712, trap-nights = 190) was 45.9. The population dynamics and species composition varied between years (Figure 2.2, Figure 2.3).

Mosquito Populations in 2019

In the 2019 field season (202 trap-nights), I captured a total of 6,581 mosquitoes and identified 28 mosquito species (Table 2.2). Eight of these species were outside of the known distribution maps, including *Ae. aurifer, Ae. hendersoni, Ae. triseriatus, Ae. trivittatus, An. quadrimaculatus, Cu. incidens, Cu. minnesotae*, and *Cx. salinarius* (Darsie and Ward 2005). The weekly trap-night mosquito mean was 22.7 ± 27.6 mosquitoes. The most abundant species caught throughout the season were *Aedes vexans* (n = 2,204, 33% of 2019 mosquitoes captured) and *Coquillettidia perturbans* (n = 1,972, 30% of 2019 mosquitoes captured).

Seasonality 2019

The first mosquitoes caught in the 2019 season were caught May 21, 2019 (Figure 2.2). Mosquito populations remained low until mid-June and the height of the season was reached in early July. Following the height of the season, the community decreased dramatically in mid-July. The season had three more short increases in numbers. On September 26, 2019, the season ended.

Coquillettidia perturbans did not appear until June 12, 2019 (Figure 2.4). The population grew quickly and reached their maximum number the week of July 22, 2019. *Coquillettidia perturbans* did not appear in the mosquito traps after September 5, 2019. *Aedes vexans* also first appeared in the community during the week of June 12, 2019 (Figure 2.5). *Ae. vexans* reached the height of their season during the week of July 7, 2019. The *Ae. vexans* population decreased and had two more population spikes near the end of the mosquito season. Increases in the *Ae.*

vexans population occurred during the first week of September and the middle of September 2019. The last *Ae. vexans* caught in the 2019 season were on September 26, 2019.

Mosquito Population in 2020

In the 2020 field season (190 trap-nights), a total of 8,712 mosquitoes were captured, and 20 mosquito species were identified (Table 2.2). The weekly trap-night mosquito mean was 31.5 ± 45.9 mosquitoes. *Coquillettidia perturbans* were the most abundant species captured in summer 2020 (n = 6,485, 74% of 2020 mosquitoes captured).

Seasonality 2020

The first mosquitoes of the 2020 season were caught in the first week of June (Figure 2.3). The mosquito community quickly grew and reached the highest capture rate during the last week of June. Following the height of the season, the population rapidly decreased. The community had two more seasonal peaks occurring in mid and late July. The mosquito season ended in the last week of August 2020.

Coquillettidia perturbans appeared the second week of June 2020 (Figure 2.6). Their seasonal peaks matched the overall population trends, including the highest peak during the last week of June, and two smaller peaks in mid and late July. *Coquillettidia perturbans* were caught until the end of the season in the last week of August. The *Aedes vexans* population had no discernable seasonal peaks and abundance remained low throughout Summer 2020 (Figure 2.7).

Comparison of Mosquito Abundance and Diversity with Previous Studies

To investigate how my species composition within the Turtle Mountains might compare with that in other areas of North Dakota, I compared my results with results from two previous studies conducted in the Red River Valley by UND Biology graduate students. Each of these studies sampled mosquitoes over two concessive seasons. One study was conducted within the

city limits of Grand Forks during the summers of 1992 and 1993, when the city spray program was minimal (*i.e.,* before the introduction of West Nile virus into the region) (Deckert 1995). The other study was conducted at two sites within Steele County, ND (Mehus 2013). The relative abundance of host-seeking mosquitoes collected in the Turtle Mountain during the Summer 2019 and Summer 2020 (*i.e*., 39 mosquitoes per trap-night) was nearly an order of magnitude less than the abundance of host-seeking mosquitoes collected using similar trapping methods (*i.e.,* CO_2 traps) within the Red River Valley (*i.e*., 302 to 557 mosquitoes per trap-night) (Table 2.4). Averages were log transformed to approximate normality. An unpaired t test was used to compare Turtle Mountains averages to other capture averages. There was significantly less numbers of mosquitoes captured per-trap night in the Turtle Mountains (3.7 mosquitoes ± 0.3) than captured in other North Dakota studies (6.0 mosquitoes ± 0.6); $t(5) = 0.5$, $p = 0.0007$. Of the 15,293 mosquitoes captured in the Turtle Mountains, a total of 14,836 mosquitoes were identified to 28 mosquito species. Based on the Shannon Indices calculated, the Turtle Mountains contained a higher species diversity of mosquitoes (index=2.25) than observed for Grand Forks city (index=1.48) and the rural Steele County farm site (index=1.24). Interestingly, the Steele County Forest site, which is more ecologically similar to the Turtle Mountains than the other two Red River Valley sites, had somewhat lower mosquito abundance than the other Red River Valley sites and a species diversity index (1.95) that most closely approximated that in the Turtle Mountains (Table 2.4). *Aedes vexans*, the inland floodwater mosquito, was the dominant species in Grand Forks city (75% of total mosquitoes) and the Farm site in Steele County (57% of total) whereas *Ae. flavescens* was the dominant species in the Forest site (55% of total). In the Turtle Mountains, the cattail mosquito, *Coq. perturbans*, was the dominant species (57% of total).

Mosquito *Plasmodium* Testing

After extraction, all mosquitoes were tested for general haemosporidian infections using real-time polymerase chain reaction (Table 2.3). A total of 6,581 mosquitoes were tested from 2019 and 8,712 samples from 2020. A total of 430 mosquito pools from 2019 and 446 mosquito pools from 2020 were tested using real-time polymerase chain reaction. Very few mosquito pools showed even slight amplification using real-time polymerase chain reaction. No true positives were validated using the nested polymerase chain reaction.

DISCUSSION

Mosquito Populations

Each mosquito field season had a similar number of trap-nights and showed similar mosquito population abundance trends. The overall population trends for 2019 and 2020 reflect the seasonal weather patterns (Figure 2.2, Figure 2.3). The mosquito season started in early June and reached a seasonal height during the warmest part of the summer, in late June. Smaller population peaks followed. Both the 2019 and 2020 mosquito seasons ended with the appearance of frost (Weather Underground 2022).

Summer season 2019 had a higher species diversity, while summer season 2020 had a higher abundance of mosquitoes captured. However, both population indices differed from other North Dakota mosquito capture data (Bell *et al.* 2005, Deckert 1995, Mehus 2013). First, mosquito abundance in the Turtle Mountains was significantly less than reported elsewhere within the Red River valley region of North Dakota. Second, Shannon indices comparing the diversity of the Turtle Mountains to the Steele County farm and forest sites, indicate that species diversity of mosquitoes in the Turtle Mountains was higher than in the Red River valley region.

Thus, the Turtle Mountains contain a lower abundance but higher species diversity of mosquitoes than in the surrounding area. The higher mosquito species diversity in the Turtle Mountains is likely due to the greater variety of breeding habitats available in the wooded areas (*e.g.,* treeholes) (Ferraguti *et al*. 2016, Young *et al*. 2017). The overall lower abundance of mosquitoes could be due to the wetland type or the slight variation in air temperatures due to the wooded habitat (Gleiser *et al.* 2002, Medlock & Vaux 2015).

Aedes

The Turtle Mountain mosquito population in 2019 was mainly comprised of *Aedes* mosquitoes. This is likely due to the high amount of precipitation throughout 2019 and the life-history of *Aedes* mosquitoes. Many *Aedes* species are considered floodwater mosquitoes that lay eggs on soil, just above the water line. With precipitation, the waterline rises, and the eggs develop (Burkett-Cadena 2013). A paired t-test was conducted to compare average rainfall per month (May to September) in 2019 summer and 2020 summer (Weather Underground 2022). There was not a significant difference between average rainfall per month in 2019 summer (2.0 inches ± 1.8) versus 2020 summer (1.6 inches ± 1.8); $t(4) = 0.28$, $p = 0.80$. However, in 2019 North Dakota had a total of 24.4 inches of precipitation – an unusually high amount, according to the North Dakota Annual Climate Summary. In 2020, North Dakota had substantially less total rainfall – *i.e*., 12.9 inches of precipitation. The high amount of precipitation in 2019 likely contributed to high *Aedes* mosquito populations. Rising water levels at the start of the season, followed by frequent refilling of low-lying areas throughout the summer, probably resulted in continual rounds of egg hatching for this floodwater species.

Aedes vexans was the most abundant mosquito species captured in 2019 and had multiple generations during the season (Table 2.2, Figure 2.5). The population gradually increased

through June and then reached a season high that aligned with the warmest part of the season (North Dakota Annual Climate Summary 2020). A final adult emergence occurred starting in early September. The appearance of multiple *Ae. vexans* generations is consistent with previous data indicating that they are a multivoltine species (Mehus 2013, Read & Moon 1996).

Due to the lower amount of rain that occurred the following summer, *Aedes* populations remained low throughout the 2020 season (Table 2.2). Indeed, the catch rate for *Aedes vexans* in 2020 was so low that discreet generations (*i.e.,* spikes in adult emergence) could not be distinguished throughout the season (Figure 2.7).

Coquillettidia

Coquillettidia perturbans is often called the "cattail mosquito" because it prefers flooded vegetation for egg laying. Their larval forms use specialized siphons to breathe through cattails and other vegetation (Bosak & Crans 2002). Sloughs and other permanent water sources are present throughout the Turtle Mountains and provide the preferred cattail habitat (Bluemle 2005). For both years, *Coq. perturbans* exhibited a single, somewhat extended, peak in adult emergence (Figure 2.6, Figure 2.7). This univoltine pattern resembles the pattern observed elsewhere in northern latitudes – *e.g.,* Red River Valley (Mehus 2013) and New Brunswick, Canada (Lewis & Bennett 1980). The ample amount of preferred and environmentally stable larval breeding habitat explains the consistency and success of *Coq. perturbans* in the Turtle Mountains in 2019 and 2020 despite marked differences in rainfall between the two seasons.

The emergence of the *Coq. perturbans* population was synchronous, likely due to the fact that this species overwinters as larvae (Lounibos & Escher 1983). For both 2019 and 2020 the adult population grew quickly and had a high prevalence throughout the season (Figure 2.4,

Figure 2.6). This timeline is consistent with the lifecycle of *Coq. perturbans* in eastern Canada (Carpenter & LaCasse 1955, Lewis & Bennett 1980).

Other Notable Species

Although *Aedes aurifer, Ae. hendersoni, Ae. triseriatus, Ae. trivittatus, An. quadrimaculatus, Cu. incidens, Cu. minnesotae*, and *Cx. salinarius* were collected in 2019 and 2020, these species have not been generally well documented in North Dakota. Nevertheless, the life histories of these mosquito species correspond well to the various types of aquatic habitats present in the Turtle Mountains (Darsie & Ward 2005). For example, *Aedes aurifer* mosquitoes are often found near cattail marshes (Rayburn *et al*. 2004, Burkett-Cadena 2013). *Aedes hendersoni, Ae. triseriatus* and *Ae. trivittatus* all benefit from water-filled tree-holes for larval habitat and overwintering (Burkett-Candena 2013). *Anopheles quadrimaculatus, Cu. incidens, Cu. minnesotae*, and *Cx. salinarius* use more permanent water bodies for egg laying (Burkett-Cadena 2013). Although these species were not particularly abundant, it can be hypothesized that they were breeding in the area (as opposed to being blown in from surrounding areas) due to the congruent match between their life-histories and the varied habitats of the Turtle Mountains.

Avian *Plasmodium* Vector

The Turtle Mountains has the potential to be an area of avian *Plasmodium* transmission due to the presence of mosquitoes. Several mosquito species found in the Turtle Mountains reported to be capable of harboring avian *Plasmodium* include *Ae. campestris*, *Ae. canadensis*, *Ae. stimulans*, *Ae. triseriatus*, *An. quadrimaculatus*, *Cq. perturbans*, *Cx. morsitans*, *Cx. pipiens*, *Cx. restuans*, and *Cx. tarsalis* (Santiago-Alarcon *et al*. 2012). Of these, mosquitoes in the genera *Culex* and *Culiseta* are more likely to be vectors because of their greater susceptibility to

infection and predilection for feeding on birds. Despite the potential for avian transmission in the Turtle Mountains, I did not find any *Plasmodium*-positive mosquitoes.

To transmit *Plasmodium*, a mosquito must first bite an infected host and ingest the parasite. Different mosquito species have different feeding habits and host preferences. Only a small number of potential vectors for avian malaria prefer to feed on birds (Molaei *et al*. 2008, Mehus 2013, Abella-Medrano *et al*. 2018, Kimura *et al*. 2010). Most studies find that most mosquito species feed on mammals. The mosquitoes that normally feed on birds (*i.e*., ornithophilic) include *Culex* and *Culiseta*. Therefore, the lack of *Plasmodium* positive mosquitoes may be due to the relatively low density of ornithophilic mosquito species. For 2019, only 3% of the mosquito population were comprised of species that would be considered ornithophilic (*i.e., Culex* and *Culiseta*). In 2020 there were even fewer *Culex* and *Culiseta*, comprising only 1% of that year's total mosquito catch.

Vector Incrimination

Although several of the mosquito species found in the Turtle Mountains have been proven to be capable of supporting avian *Plasmodium* development in a lab setting, that does not necessarily mean these mosquitoes are natural vectors (Santiago-Arlacan *et al.* 2012). Each population of mosquitoes, even those of the same species, may differ in their vector competence. I was unable to detect avian *Plasmodium* in the sampled mosquito community of the Turtle Mountains, which may be due to the low number of competent, bird-feeding, mosquitoes.

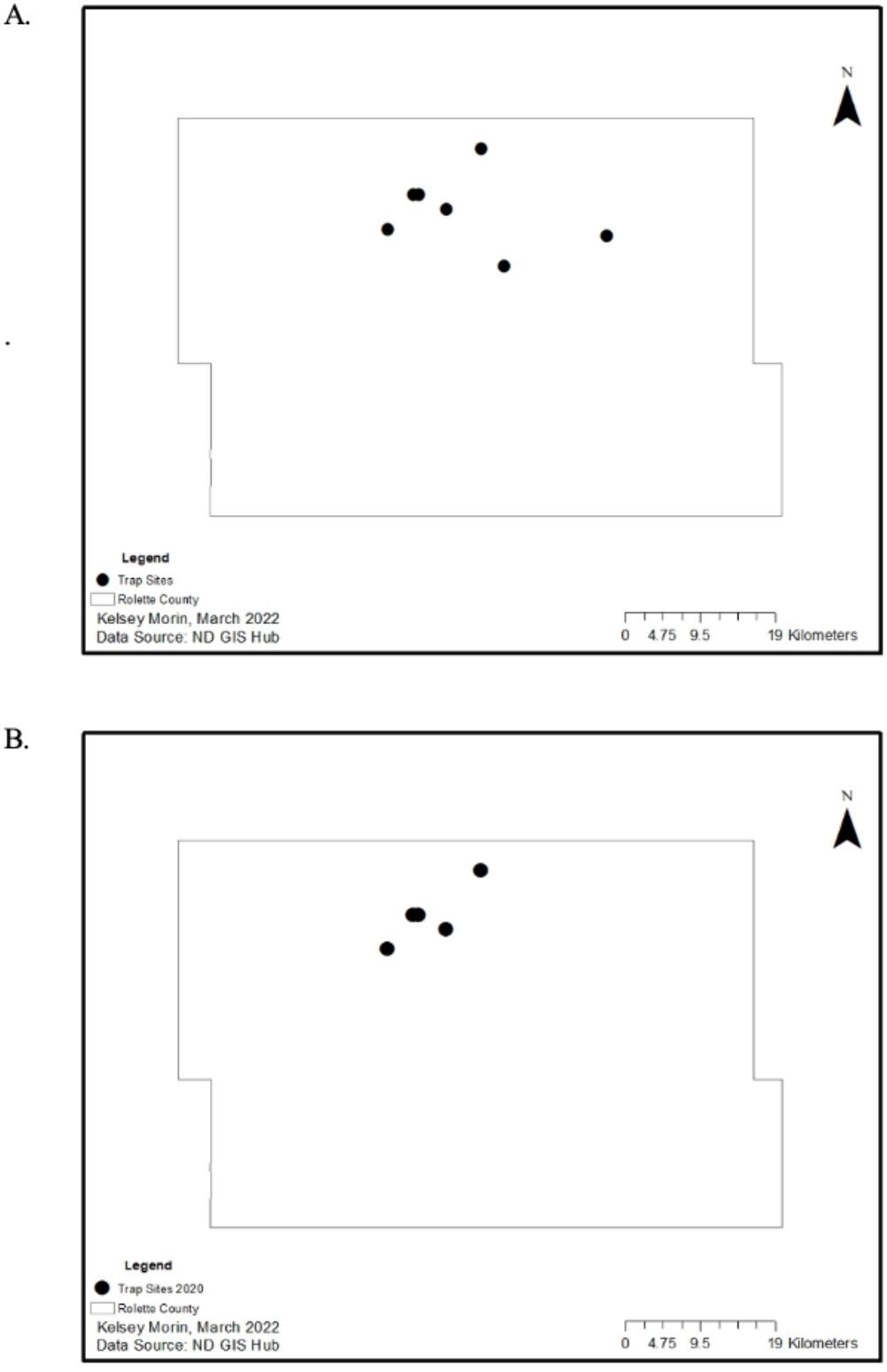

Figure 2.1. Mosquito Magnet trap locations in Rolette County, North Dakota USA. A. Seven trap locations used in Summer 2019, B. Five trap locations used in Summer 2020.

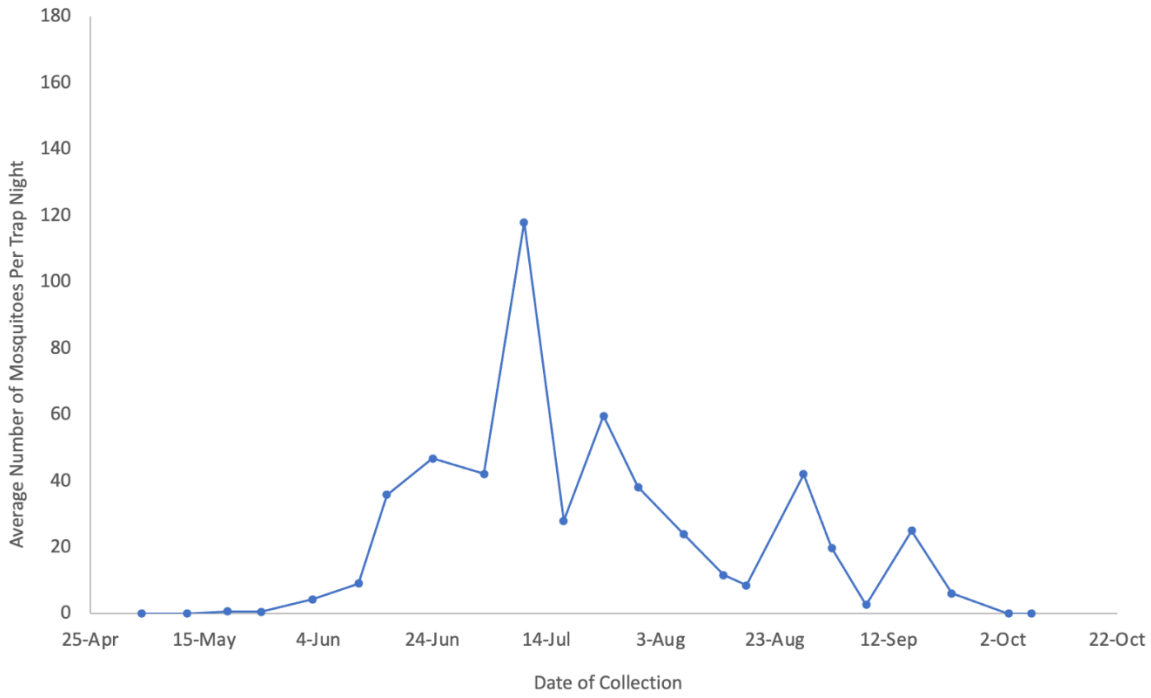

Figure 2.2. Weekly trends in total mosquito population during the Summer 2019 Rolette County Turtle Mountains, North Dakota.

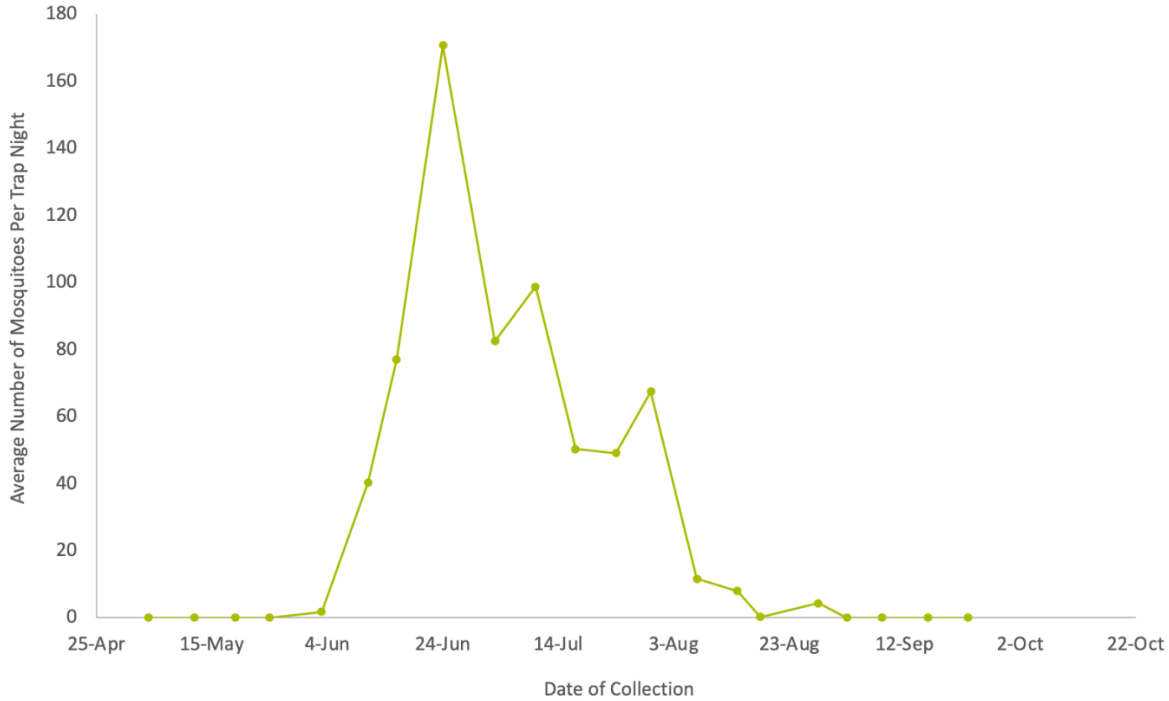

Figure 2.3. Weekly trends in total mosquito population during the Summer 2020 Rolette County Turtle Mountains, North Dakota.

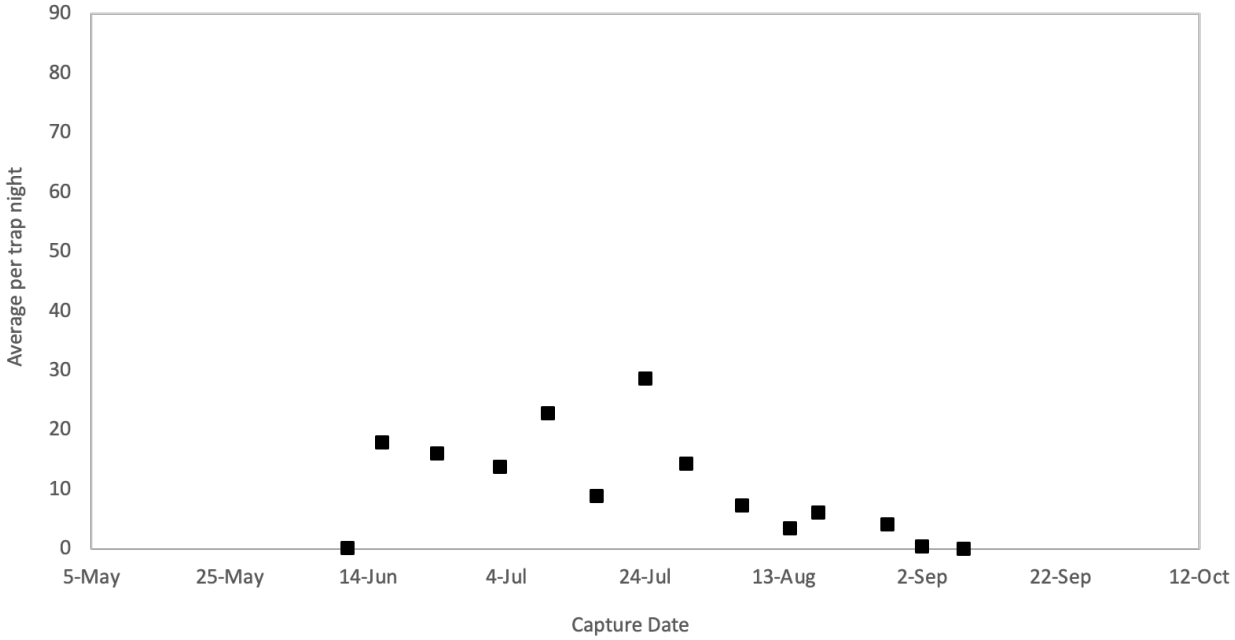

Figure 2.4. Weekly trends of *Coquillettidia perturbans* population captured in 2019 Rolette County Turtle Mountains, North Dakota.

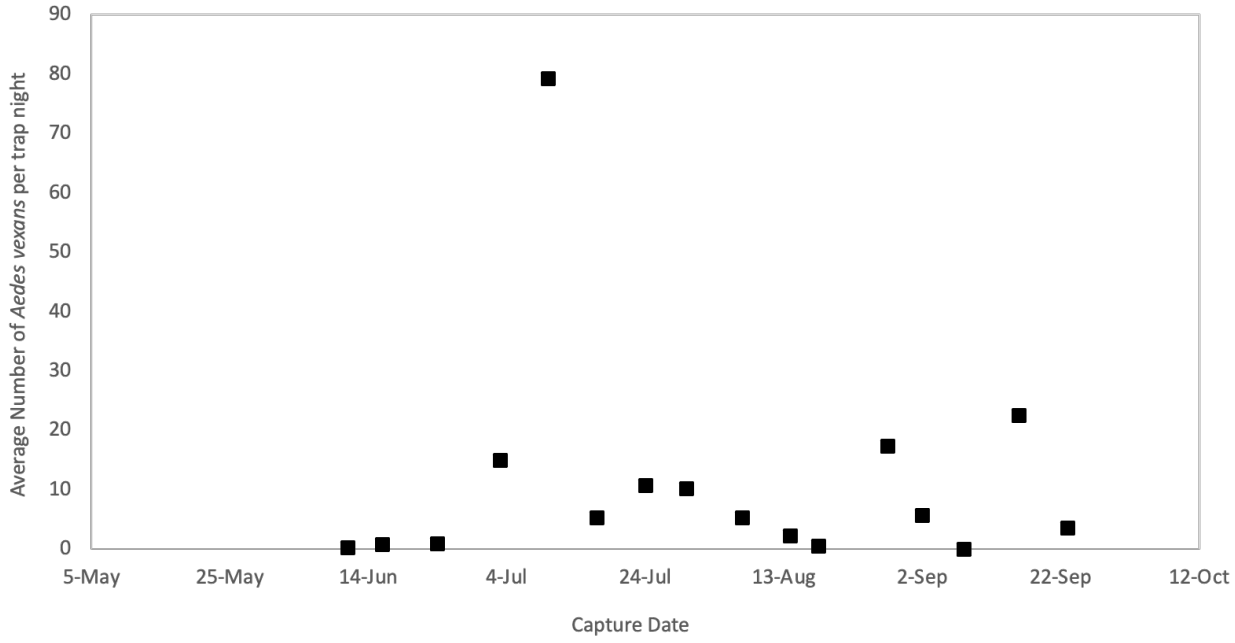

Figure 2.5. Weekly trends of *Aedes vexans* population captured in 2019 Rolette County Turtle Mountains, North Dakota.

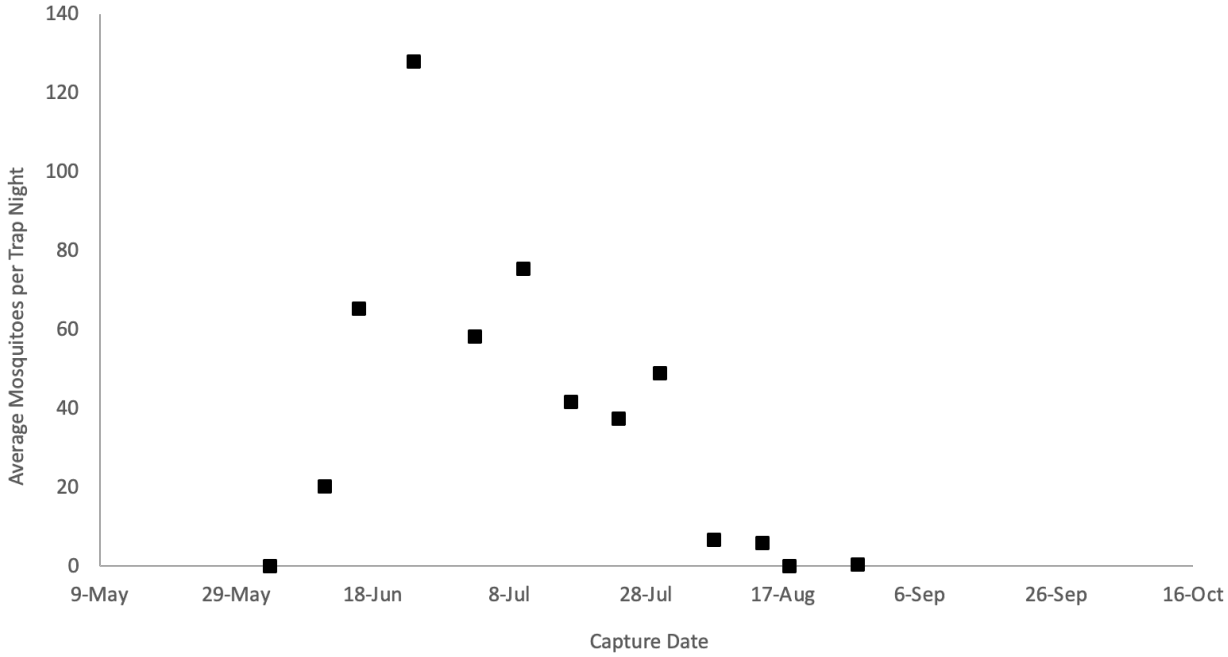

Figure 2.6. Weekly trends of *Coquillettidia perturbans* population captured in 2020 Rolette County Turtle Mountains, North Dakota.

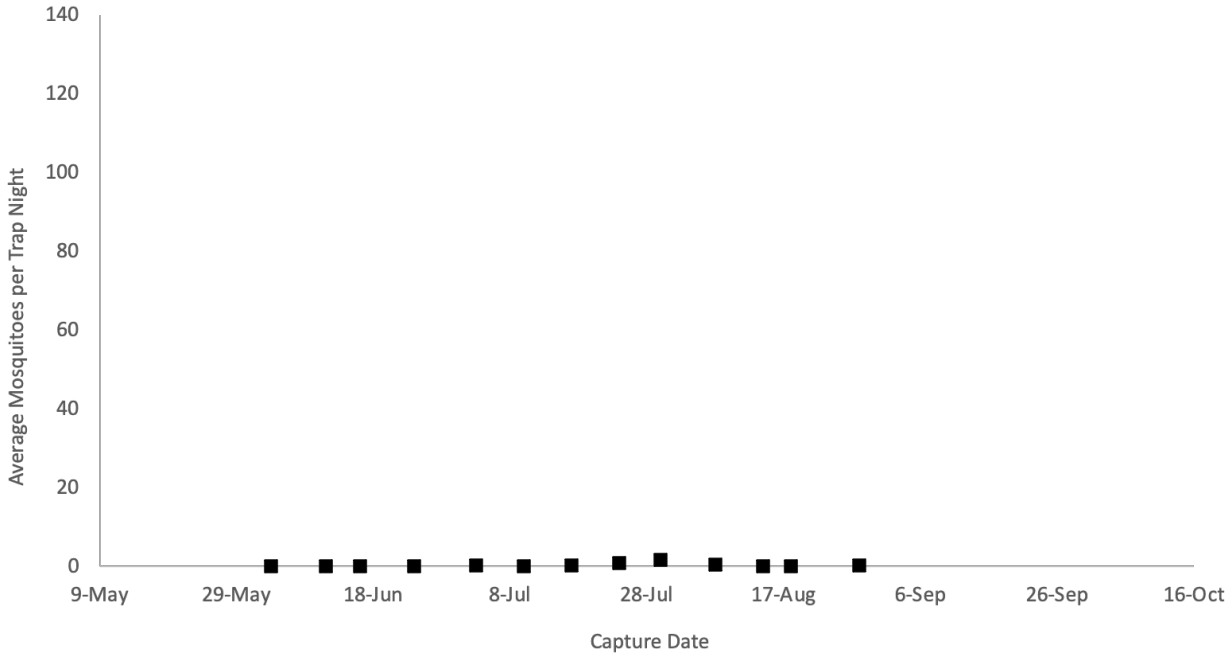

Figure 2.7. Weekly trends of *Aedes vexans* population captured in 2020 Rolette County Turtle Mountains, North Dakota.

Table 2.1. Primers used to detect Haemosporidia in mosquitoes.

Organism and Target Gene	Primer
Real-time PCR Primers	
Haemosporidia rDNA (313-501) 182 bp	Forward: R330F 5′ - CGTTCTTAACCCAGCTCACG - 3′ Reverse: R480RL 5′ - GCCTGGAGGTWAYGTCC - 3′
Nested PCR/ Sequence Primers	
Plasmodium/Haemoproteus Cytochrome b (3703-4273) 526 bp	Forward: H332F 5′ - GAGAATTATGGAGYGGATGGTG - 3′ Reverse: HaemNR2 5′ - AGAGGTGTAGCATATCTATCTAC - 3′
Plasmodium/Haemoproteus Cytochrome b (3719-4243) 477 bp	Forward: H350F 5′ - GGTGTTTTAGATATATGCATGC - 3′ Reverse: HaemR2 5′ - GCATTATCTGGATGTGATAATGGT - 3′

Table 2.2. Mosquito species captured in Rolette County Turtle Mountains, North Dakota Summer 2019, and Summer 2020.

Mosquito Species	Year 2019	Year 2020	Totals	% of 2019 Total	% of 2020 Total	% of Total Captured
Ae. aurifer	8	1	9	0.1%	0.0%	0.1%
Ae. campestris	1	1	2	0.0%	0.0%	0.0%
Ae. canadensis	16	26	42	0.2%	0.3%	0.3%
Ae. cinereus	253	366	619	3.8%	4.2%	4.0%
Ae. dorsalis	118	47	165	1.8%	0.5%	1.1%
Ae. flavescens	2	0	2	0.0%	0.0%	0.0%
Ae. hendersoni	155	10	165	2.4%	0.1%	1.1%
Ae. intrudens	214	210	424	3.3%	2.4%	2.8%
Ae. nigromaculis	1	0	1	0.0%	0.0%	0.0%
Ae. riparius	438	744	1182	6.7%	8.5%	7.7%
Ae. sticticus	0	1	1	0.0%	0.0%	0.0%
Ae. stimulans complex	334	378	712	5.1%	4.3%	4.7%
Ae. triseriatus	210	104	314	3.2%	1.2%	2.1%
Ae. trivittatus	15	0	15	0.2%	0.0%	0.1%
Ae. vexans	2204	44	2248	33.5%	0.5%	14.7%
An. earlei	185	87	272	2.8%	1.0%	1.8%
An. punctipennis	1	0	1	0.0%	0.0%	0.0%
An. quadrimaculatus	1	0	1	0.0%	0.0%	0.0%
Co. perturbans	1972	6485	8457	30.0%	74.4%	55.3%
Cx. pipiens	23	44	67	0.3%	0.5%	0.4%
Cx. restuans	23	2	25	0.3%	0.0%	0.2%
Cx. salinarius	1	2	3	0.0%	0.0%	0.0%
Cx. tarsalis	23	10	33	0.3%	0.1%	0.2%
Cs. impatiens	1	0	1	0.0%	0.0%	0.0%
Cs. incidens	2	0	2	0.0%	0.0%	0.0%
Cs. inornata	37	3	40	0.6%	0.0%	0.3%
Cs. minnesotae	1	0	1	0.0%	0.0%	0.0%
Cs. morsitans	32	0	32	0.5%	0.0%	0.2%

Table 2.2
Continued

Mosquito Species	Year 2019	Year 2020	Totals	% of 2019 Total	% of 2020 Total	% of Total Captured
An. unidentified	6	2	8	0.1%	0.0%	0.1%
Ae. unidentified	195	85	280	3.0%	1.0%	1.8%
Cx. unidentified	56	27	83	0.9%	0.3%	0.5%
Cs. unidentified	4	0	4	0.1%	0.0%	0.0%
Unidentified	49	33	82	0.7%	0.4%	0.5%
Yearly Total	6581	8712	15293			
Species Total	27	20				

Table 2.3. Mosquitoes captured in 2019 and 2020 from Rolette County Turtle Mountains, North Dakota. Mosquitoes were pooled based on species and trap week. Pools were tested using real-time PCR and Nested PCR to detect avian haemosporidian.

Mosquito Genus	n =	No. Pools Tested with Real-time PCR	No. Pools Tested with Nested PCR	*Plasmodium/Haemoproteus* Positive Samples
Aedes				
2019	4164	256	57	0
2020	2017	142	7	0
Anopheles				
2019	193	23	23	0
2020	89	12	1	0
Coquillettidia				
2019	1972	87	21	0
2020	6485	264	18	0
Culex				
2019	126	27	16	0
2020	85	19	4	0
Culiseta				
2019	77	27	12	0
2020	3	3	3	0
Unidentified				
2019	49	10	6	0
2020	33	6	3	0
2019 Total	6581	430	135	0
2020 Total	8712	446	36	0

Table 2.4. Relative abundance (number per trap-night) and species diversity (Shannon Index) of host-seeking mosquitoes collected by carbon dioxide baited traps in different habitats within northeast North Dakota.

Location	Trapping Periods	Total Number Mosquitoes	Number Mosq. per Trap Night	Number Mosquito Species	Shannon Index	Ref.
Rolette Co., Turtle Mountains	May 5 – Oct 7 2019 May 4 – Sept 23 2020	14,836	39	28	2.25	Present Study
Steele Co. Forest	15 May- 15 Aug. 2009 15 May- 15 Aug. 2011	41,755	302	18	1.95	Mehus 2013
Steele Co. Farm	15 May- 15 Aug. 2010 15 May- 15 Aug. 2011	82,940	557	15	1.24	Mehus 2013
Grand Forks City	May 20 – July 30 1992 July 2 – Sept 22 1993	87,084	435	26	1.48	Deckert 1995

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CHAPTER III

PREVALENCE OF AVIAN BLOOD PARASITES IN LOCAL AND MIGRATORY PASSERINE BIRDS OF THE TURTLE MOUNTAINS, NORTH DAKOTA

INTRODUCTION

Avian parasites can be detrimental to individual bird health and can reduce entire populations. The majority of chronic Haemosporidia infections in adult birds do not have pronounced clinical symptoms (Valkiunas 2005). However, the presence of avian Haemosporidia can reduce reproductive success, shorten lifespans, and increase mortality (Asghar *et al*. 2015, Groff *et al*. 2019). In few instances, the presence of avian Haemosporidia blood parasites has also been found to cause overall bird population declines (Dadam *et al*. 2019, Niebuhr *et al*. 2016). Dipteran vectors transmit haemosporidian parasites. *Plasmodium* is transmitted by mosquitoes, *Haemoproteus* by louse flies, *Parahaemoproteus* by biting midges, and *Leucocytozoon* by black flies. Recent research indicates that *Haemoproteus* and *Parahaemoproteus* may be two unique genera, but for the purpose of this study we will refer to *Haemoproteus* as inclusive of both subgenera (Galen *et al*. 2018).

Other avian blood parasites transmitted by blood sucking arthropods are also be found in bird populations. *Trypanosoma* is considered a mostly benign protozoan parasite that has several possible vectors, including mosquitoes, biting midges, and mites (Baker 1976). Microfilariae are

larval stages of filarial nematodes that are transmitted by biting midges, blackflies, mosquitoes, or another hematophagous arthropod (Anderson 2000).

Research on the presence of these avian blood parasites has been done in North America. In a landmark study based on the microscopic examination of blood smears from 57,026 North American birds of 388 species (55 families), the overall prevalence of Haemosporidia for wild birds was reported to be; *Plasmodium* at a 3.8% prevalence, *Haemoproteus* at a 19.5% prevalence, and *Leucocytozoon* at a 17.7% prevalence (Greiner *et al*. 1975). Microfilaria prevalence was reported at 3.1%, and *Trypanosoma* was reported at 3.9% prevalence (Greiner *et al*. 1975). A more recent publication combining data from the Northern hemisphere reported similar prevalence for Haemosporidia; *Plasmodium* 2.9%, *Haemoproteus* 17.9%, and *Leucocytozoon* 16.2% (Valkiunas 2005).

The prevalence of avian blood parasites in the passerine birds of Minnesota do not reflect the overall average prevalence for blood parasites in North America or the Northern hemisphere. A study looking at grassland songbirds in Minnesota reported the following prevalence of Haemosporidia: *Plasmodium* 29%, *Haemoproteus* 10%, and *Leucocytozoon* 29% (Kvasager 2015). Research conducted in aspen parkland and farmland habitats in Minnesota reported avian blood parasite prevalence: *Plasmodium* 24%, *Haemoproteus* 19%, *Leucocytozoon* 18%, microfilaria 9.0%, and *Trypanosoma* 58.0% (Stromlund 2015). Despite the geographic proximity of these studies, the prevalence of avian blood parasites differed. The bird fauna and parasite prevalence were likely influenced by the habitat of these areas.

Although previous research has been done in Minnesota, no research of avian blood parasites has been conducted in the Turtle Mountains of North Dakota. The Turtle Mountains is in a completely unique ecoregion of North Dakota and has distinctive forested habitats (Bryce *et*

al. 1996). The Turtle Mountains are comprised of abundant wetlands, and experience more rain than the surrounding areas, resulting in a mixture of wooded areas that provide forest cover (Bryce *et al*. 1996, Seabloom 2011). The uniqueness of this habitat results in a distinctive array of avian and vector species compared with other areas in North Dakota.

The objective of this study was to determine the prevalence of avian haemosporidian and related blood parasites in resident and migrating birds within the Turtle Mountains of North Dakota. This study used real-time polymerase chain reactions, traditional nested PCR and sequencing to determine the prevalence of *Plasmodium*, *Haemoproteus* and *Leucocytozoon* in 98 passerine birds caught in the Turtle Mountains. Microfilariae and trypanosome prevalence was determined using microscopic capillary tube detection in 57 of the 98 birds. *Plasmodium*, *Haemoproteus*, and *Leucocytozoon* were found in both local and migratory bird species. The most prevalent avian blood parasite was microfilaria, which was found in both local and migratory bird species. The least prevalent blood parasite was *Trypanosoma*, which was found in one migratory bird species.

METHODS

Permission

Collection of avian blood samples from the Turtle Mountain reservation in North Dakota was in accordance with the Turtle Mountain Band of Chippewa Indians Research Protection Act (2014). Permission to capture and test birds for blood parasites was granted by the Turtle Mountain Research Review Board for 2020 and 2021 (RRB Protocol #90).

Blood Samples

Birds were captured in Rolette County, North Dakota (Turtle Mountains) (Figure 3.1). Birds were caught using baited 4-cell Potter traps and 30’ x 8’ mist nets. Ground traps were used

throughout non-raining days. Traps were baited with mixed bird seed and bread. One or two mist nets were used on days with little to no wind. For morning trapping, the nets were put up before sunrise and taken down at approximately noon. For evening trapping, the nets were put up before sunset and taken down after sunset.

Blood was drawn from the brachial vein of each bird using a 27-gauge needle and collected in an 80 µl heparinized capillary tube. Birds were banded using a color corresponding to the month of capture and band information was recorded.

When laboratory equipment was near capture location, capillary tubes were closed on one end using Critoseal Sealent (Mccormick Scientific LLC, Los Angeles, CA). Then tubes were centrifuged for approximately 4 mins to separate red blood cells from sera. After being centrifuged, capillary tubes were viewed under a compound microscope at 100X. The buffy coat interface between the cells and sera was screened to detect the presence of microfilaria and trypanosomes (Collins 1971). Centrifuged capillary tubes were also used to determine hematocrit. All blood samples from capillary tubes were put into 1.5ml tubes with 500µl of 100% ethanol. Collected blood was stored at -20ºC.

Haemosporidia Testing

A small amount of blood (ca. 5 µl) sample was dried in a separate 1.5 ml test tube on a heating block at 21 °C. After drying the sample, DNA was extracted using a Qiagen DNeasy Blood and Tissue kit (Qiagen, Germantown, MD) following manufacturer's instructions. To test for general avian Haemosporidia, a real-time polymerase chain reaction protocol was used amplifying a 182 base pair portion of rDNA (Table 3.1) (Bell *et al.* 2015). The following cycling conditions were used: 95 °C for 30 s, 35 cycles of 95 °C for 30 s, 53 °C for 35 s, plate read and melt curve. Several positive and negative controls were used in all runs. The positive control was

a previously sequenced avian *Plasmodium* positive blood sample. Samples were considered real-time PCR positive if the melt peak curve from amplification was between 75°C and 80°C.

If the real-time PCR indicated a positive sample, the extracted DNA was tested for *Plasmodium/Haemoproteus* and *Leucocytozoon* infection using traditional nested PCR reactions targeting the cytochrome *b* gene (Table 3.1) (Bell et al 2015). The following cycling conditions were used for initial PCR: 95°C for 3 mins, 20 cycles of 95°C for 30 s, 50°C for 45 s, 68°C for 60 s, and a final extension at 68°C for 60 s. The following cycling conditions were used for the second PCR: 95°C for 3 mins, 35 cycles of 95°C for 30 s, 50°C for 45 s, 68°C for 60 s, and a final extension at 68°C for 60 s. All PCR products were then run onto a 1% agarose ethidium bromide gel to look for amplification.

For *Plasmodium/Haemoproteus* sequencing, nested PCR samples were cleaned with ExoSAP-IT (Affymetrix Inc., Santa Clara, CA) according to the manufacturer's instructions. Samples were sequenced using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems Inc. - ABI, Foster, CA) 10 µl reactions and a 3100 Genetic Analyzer (Applied Biosystems Inc. - ABI, Foster, CA). The following cycling conditions were used: 25 cycles of 96°C for 15 s, 50°C for 5 s, 60°C for 4 mins.

Phylogenetic Analysis

Plasmodium/Haemoproteus sequences from 13 birds caught in the Turtle Mountains, North Dakota and 3 birds caught in Grand Forks County, North Dakota were used to recreate a phylogeny that was used to identify these parasites and to observe the genetic variation within these samples. *Plasmodium/Haemoproteus* forward and reverse sequences were visualized and assembled using Sequencher v5.0 (Gene Codes Corporation, Ann Arbor, MI). Phylogenetic analyses were completed using CIPRES Science Gateway V 3.3. Sequences were aligned using

ClustalW v2.1 with standard parameters (Thompson *et al*. 1994). FastTreeMP v2.1.10 was used to reconstruct the phylogeny (Prince *et al.* 2009). FigTree v1.4.4 was used to visualize and edit the appearance of the phylogeny (Rambout 2007). Sequences identities were verified with GenBank and MalAvi databases (Bensch *et al.* 2009, Sayers *et al*. 2019).

RESULTS

Birds Captured

A total of 98 passerine birds were captured in the Turtle Mountains. A total of 73 birds were captured between May 2020 and September 2020. A total of 25 birds were captured between July 2021 and October 2021. There was a total of 21 species captured and identified to species based on morphological features (Table 3.2) (Peterson 2008). House Sparrows (*Passer domesticus*) were the most abundant bird species captured for both seasons. Twenty of the House Sparrows were female and five were male. Four of the 21 species captured are considered local to the Turtle Mountains and non-migratory: Black-capped Chickadees (*Poecile atricapillus*), White-breasted Nuthatches (*Sitta carolinensis*), American Robins (*Turdus migratorius*), and House Sparrows (*Passer domesticus*) (Peterson 2008). Although American Robins year-round range includes the entire United States, it should be noted that their movement in the area is not well tracked. For the purposes of this study, American Robins will be considered a local species to the Turtle Mountain region (Peterson 2008).

Blood Parasites

Avian *Haemosporidia* prevalence for the 21 species captured in 2020 and 2021 are in Table 3.2. Avian *Plasmodium*/*Haemoproteus* were found in 21.4% (n = 98) of the birds, and *Leucocytozoon* was found in 4% (n = 98) of the birds. *Plasmodium* was successfully detected in nine individual birds, and *Haemoproteus* was detected in seven birds. One

Plasmodium/Haemoproteus infection detected in a Brown Creeper (*Certhia americana*) could not be identified to genus. Of the commonly captured bird species, American Robins had the highest prevalence of haemosporidian infection, comprised exclusively of a single species; *Plasmodium unalis* (50%, n = 8).

A total of 64 passerine birds captured in the 2020 field season were tested for *Trypanosoma* and microfilariae (Table 3.3). The total prevalence of *Trypanosoma* was 2%, which was the lowest prevalence of blood parasites observed. The total prevalence of microfilariae was 16%. One of the 64 birds had both *Trypanosoma* and microfilariae present in their blood. Not all birds were tested for *Trypanosoma* and microfilariae due to the time sensitive nature of microscopic detection and sampling.

Polyparasitism was found in three individual birds: 98 birds tested for Haemosporidia, and 57 birds tested for microfilaria and *Trypanosoma* (Table 3.3). Two of the three Chipping Sparrows (*Spizella passerina*) tested had concurrent infections of *Haemoproteus* and microfilariae. One of the three Barn Swallows (*Hirundo rustica*) tested had a concurrent infection of microfilariae and *Trypanosoma* parasites.

A two-tailed t-test was conducted to compare differences of hematocrit of infected (M = 47.58% $\pm$ 5.37, n = 19) vs. uninfected birds (M = 48.78% $\pm$ 5.86, n = 37). Hematocrit was unaffected by blood parasite infection ($p = 0.4575$, $t = 0.75$, $df = 54$).

Phylogenetic Analysis

The resulting phylogeny revealed two main lineages of parasites (Figure 3.2). Species of *Plasmodium* parasites clustered independently from species of *Haemoproteus*. The phylogeny also indicates that some species of *Plasmodium* are exclusive to one avian host or locality. *Plasmodium unalis* was found to only infect American Robins (Figure 3.2). *Plasmodium*

cathemerium was found in birds caught in the Turtle Mountains and birds caught in Grand Forks County, North Dakota (Figure 3.2).

Birds Not Included in Counts

Immature birds were not included in the parasite counts, but their blood was tested for all avian parasites. An immature Chipping Sparrow (*Spizella passerina*) and an immature American Robin were captured and tested. Neither bird had signs of avian Haemosporidia, microfilariae, or *Trypanosoma*. Only one bird was caught twice in the same season. The bird was a male House Sparrow that was originally caught and tested negative in May 2020. The same male House Sparrow was caught again in July 2020. The House Sparrow did not have any parasites at the second catch date.

DISCUSSION

Avian blood parasites are present in local and migratory birds of the Turtle Mountains. House sparrows, Black-capped Chickadees, American Robins, and White-breasted Nuthatches are the four birds that can be considered local to the Turtle Mountains, North Dakota (Peterson 2008). At least one form of avian blood parasite was found in every local species. The overall prevalence of avian Haemosporidia of local bird species in the Turtle Mountains was 19.1% (n = 47) (12.8% *Plasmodium*, 2.1% *Haemoproteus*, and 4.3% *Leucocytozoon*). The prevalence of avian Haemosporidia of migratory birds in the Turtle Mountains is 21.6% (n = 51) (5.9% *Plasmodium*, 11.8% *Haemoproteus*, and 3.9% *Leucocytozoon*). These prevalence rates are lower than the prevalence rates reported in North America and in Minnesota, but they suggest the presence of a local vector (Greiner *et al.* 1975, Kvasager 2015, Stromlund 2015, Valkiunas 2005).

Chi-Square tests were performed to assess difference between the prevalence of each haemosporidian genus (*Plasmodium*, *Haemoproteus*, *Leucocytozoon*) versus locations of bird capture (Turtle Mountains, Minnesota Grassland, and Minnesota Aspen Parkland) (Table 3.5) (Kvasager 2015, Stromlund 2015). All comparisons were statistically significant and indicate a lower prevalence of all three haemosporidian genera in birds in the Turtle Mountains than either Grassland songbirds or Aspen Parkland songbirds ($p<0.05$). Although research in these areas did not include collections of possible vectors, it can be hypothesized that these areas have a higher abundance of competent vectors (Kvasager 2015, Stromlund 2015). The Turtle Mountains have a lower abundances of competent mosquito vectors (*e.g., Culex* spp.) and therefore, the low prevalence of haemosporidian genera is a logical consequence (Chapter II).

The phylogenetic analysis (pairwise sequence comparison) clearly separated the genera *Plasmodium* and *Haemoproteus* (Figure 3.2). Several unidentified *Plasmodium* sequences were similar to *Plasmodium cathemerium*. This cluster included *Plasmodium* infections of bird hosts caught in Grand Forks County and the Turtle Mountains. The addition of the Grand Forks County *Plasmodium* sequences indicates that the parasites detected in the Turtle Mountains birds are common and not unique to the area.

Of the seven *Plasmodium* positive birds caught in the Turtle Mountains, *Plasmodium unalis* was exclusively found in the four American Robins collected (Figure 3.2). However, *Plasmodium unalis* has been reported in several other passerine hosts in North and South America (Mantilla *et al*. 2013, Kvasager 2015, DeBrock *et al*. 2021). American Robins had a higher prevalence of *Plasmodium* than other commonly captured birds (50%, n = 8). The higher prevalence of *Plasmodium* may be due to the host preference of *Culex* mosquitoes. *Culex* mosquitoes are the vector for avian *Plasmodium*, and previous research has shown they prefer to

feed on American Robins over other avian species (Kilpatrick *et al.* 2006, Levine *et al.* 2016). If *Culex* mosquitoes are more attracted to American Robins, it is reasonable that American Robins would have a higher *Plasmodium* prevalence than other bird species less attractive to *Culex* vectors.

Both *Plasmodium* and *Haemoproteus* infections were found in local avian hosts before the opportunity for transmission in that summer season. Mosquitoes in the Turtle Mountains did not appear until the first week of June 2020 (Chapter II, Figure 2.3). A female House sparrow tested positive for *Plasmodium* on May 29, 2020, and a Chickadee tested positive for *Haemoproteus* June 2, 2020. The presence of these parasites before the appearance of the mosquito population suggests members of Haemosporidia may overwinter in local birds in the Turtle Mountains.

Based on Chapter II, the abundance of mosquitoes able to transmit *Plasmodium* in the Turtle Mountains is low (Table 2.2, Table 2.3). Despite this, avian Haemosporidia has been found to be endemic to the area. The presence of avian *Plasmodium* in the Turtle Mountains is likely due to the movement of the bird hosts. Birds can travel beyond the Turtle Mountains habitat and can be exposed to competent vectors carrying *Plasmodium*. This is especially exhibited with the American Robin. Although American Robins are considered local bird species, they have been shown to have large travel distances to search for food (Peterson 2008, Jahn *et al*. 2019).

Microfilariae and *Trypanosoma* were not found in the local birds of the Turtle Mountains. Of the 25 local birds tested for microfilaria and *Trypanosoma*, none of them had either blood parasite. This may be due to the lack of competent vectors in the area. It may also be due to detection methods. Microfilaria have been found to show nocturnal periodicity, and due to the

morning and evening blood draw times, the detection rates may have been reduced (Anderson 2000, Vaughan *et al.* 2012).

The two immature birds captured during the 2020 season do not support the idea of local parasite transmission, but do not rule out the possibility. The presence of parasites in these birds would have verified the presence and competence of a local vector. However, young birds have had less time for exposure to vectors and less time to develop a parasite. The sampling of only two immature birds is not indicative of a population. The lack of parasite infection in the immature birds does not necessarily indicate a lack of disease transmission in the Turtle Mountains, it may indicate a low transmission rate.

Hematocrit of birds was not altered by blood parasites. Low hematocrit is a common indicator of poor bird health, particularly in conjunction with the presence of haemosporidian parasites (Valkiunas 2005). If the parasites are not greatly impairing the host's health and hematocrit is normal, it is a likely the host has successfully passed the initial phase of infection. After one or more initial waves of high parasitemia, haemosporidian-infected hosts often develop immunotolerance, unable to completely clear the infection but able to keep the parasitemia in check and preventing it from reaching dangerously high levels. The successful transmission of the parasites without killing the host can be an indicator that there is an evolutionary relationship between them, and that these parasites are not novel to the area.

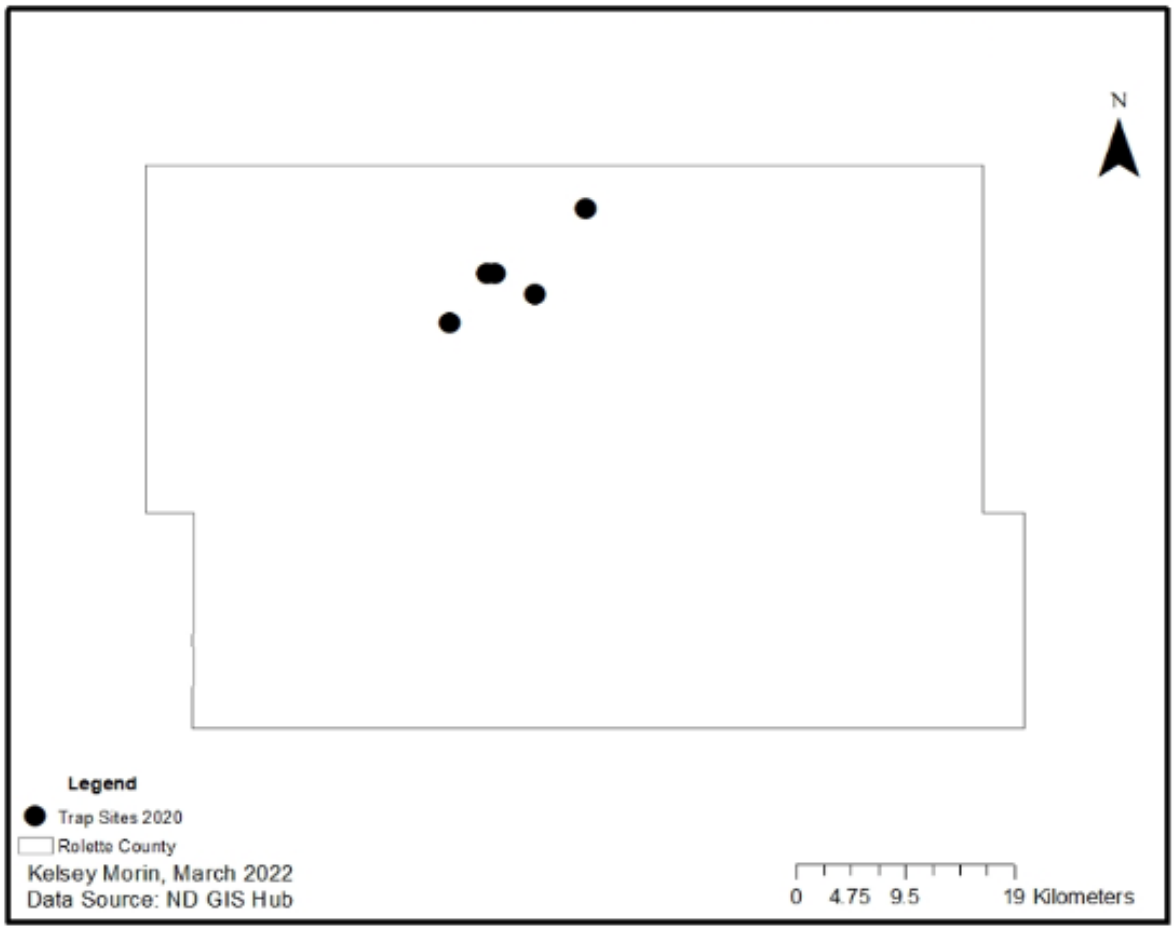

Figure 3.1. Locations for bird trapping in Turtle Mountains Rolette County, North Dakota (May – Sept 2010 and July – October 2021).

Figure 3.2. Maximum likelihood phylogenetic tree of *Plasmodium* and *Haemoproteus* parasites and the hosts they infect. Bird hosts captured in Turtle Mountains Rolette County, North Dakota (n = 13) and Grand Forks County (GFC), North Dakota (n = 3). Scale bar refers to the phylogenetic distance of 0.05 nucleotide substitutions per site. Numbers on the branches indicate bootstrap values.

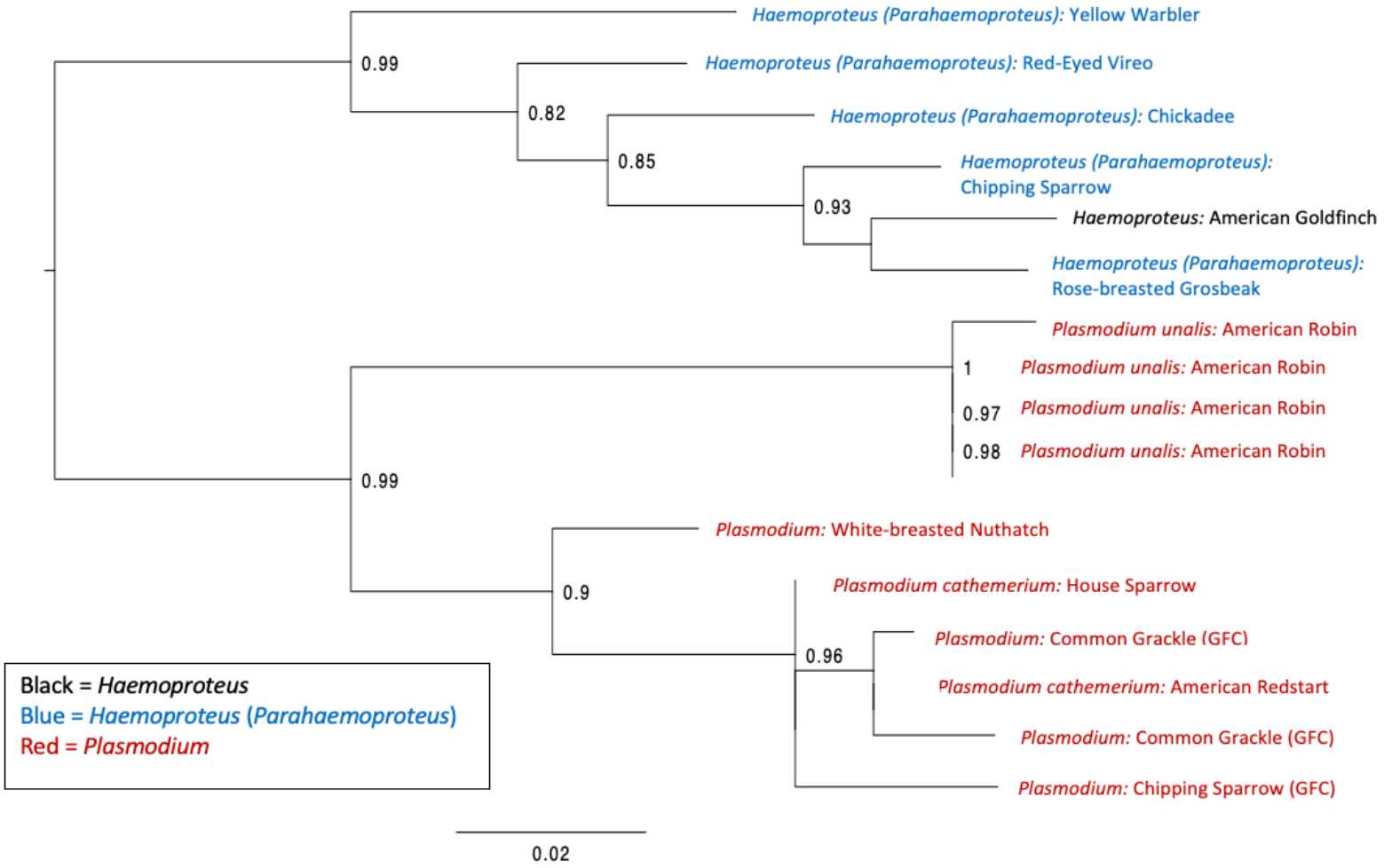

Table 3.1. Primers used for avian Haemosporidia detection in avian blood.

Organism and Target Gene	Primer
Real-time PCR Primers	
Haemosporidia rDNA (313-501) 182 bp	Forward: R330F 5′ - CGTTCTTAACCCAGCTCACG - 3′ Reverse: R480RL 5′ - GCCTGGAGGTWAYGTCC - 3′
Nested PCR/ Sequence Primers	
Plasmodium/Haemoproteus Cytochrome b (3703-4273) 526 bp	Forward: H332F 5′ - GAGAATTATGGAGYGGATGGTG - 3′ Reverse: HaemNR2 5′ - AGAGGTGTAGCATATCTATCTAC - 3′
Plasmodium/Haemoproteus Cytochrome b (3719-4243) 477 bp	Forward: H350F 5′ - GGTGTTTTAGATATATGCATGC - 3′ Reverse: HaemR2 5′ - GCATTATCTGGATGTGATAATGGT - 3′
Leucocytozoon Cytochrome b (3693-4310) 526 bp	Forward: HaemNFI 5′ - CATATATTAAGAGAAITATGGAG - 3′ Reverse: HaemNR3 5′ - ATAGAAAGATAAGAAATACCATTC - 3′
Leucocytozoon Cytochrome b (3721-4283) 478 bp	Forward: L350F 5′ - GGTGTTTTAGATACTTA - 3′ Reverse: L890R 5′ - TACAATATGTTGAGGTGTTTG - 3′

Table 3.2. Prevalence of avian Haemosporidia parasites detected in 98 songbirds (21 species) captured in Turtle Mountains, North Dakota (May – September 2020, n = 73) (July – October 2021 n = 25).

Bird Species	No. Examined	*P./H.*	*Plasmodium*	*Haemoproteus*	*Leucocytozoon*
House Sparrow	25	1	1	0	1
Chipping Sparrow	15	2	0	2	0
Black-Capped Chickadee	12	1	0	1	1
American Robin	8	4	4	0	0
Gray Catbird	6	1	0	1	0
American Goldfinch	5	1	0	1	0
Barn Swallow	4	0	0	0	0
American Redstart	3	1	1	0	0
Brown Creeper	2	1*	0	0	1
Cedar Waxwing	2	0	0	0	0
Northern Waterthrush	2	0	0	0	0
Purple Finch	2	0	0	0	0
Red-Eyed Vireo	2	1	0	1	0
White-breasted Nuthatch	2	1	1	0	0
Yellow Warbler	2	1	1	0	1
Brown-Headed Cowbird	1	0	0	0	0
Dark-Eyed Junco	1	0	0	0	0
Hermit Thrush	1	1	1	0	0
Least Flycatcher	1	0	0	0	0
Rose-Breasted Grosbeak	1	1	0	1	0
White-Throated Sparrow	1	0	0	0	0
Total	98	17	9	7	4
Prevalence		17.3%	9.2%	7.1%	4.1%

* *P/H* column contains one infection for which the genus could not be distinguished between *Plasmodium* or *Haemoproteus*.

Table 3.3. Polyparasitism of Haemosporidia, microfilaria, and trypanosomes in the blood of 21 species of songbirds collected in Turtle Mountains Rolette County, North Dakota Summer 2020 and Summer 2021.

Bird Species	No. Birds	Haemosporidia	MF	*Trypanosoma*	# Birds with one or more infections	# Birds with two concurrent infections
House Sparrow	11	2	0	0	2	0
Chipping Sparrow	7	2	2	0	2	2
Black-Capped Chickadee	7	1	0	0	1	0
American Robin	7	4	0	0	4	0
Gray Catbird	2	0	0	0	1	0
American Goldfinch	4	0	1	0	1	0
Barn Swallow	3	0	2	1	2	1
American Redstart	1	0	0	0	0	0
Brown Creeper	2	2	0	0	2	0
Cedar Waxwing	2	0	2	0	2	0
Northern Waterthrush	2	0	0	0	0	0
Purple Finch	2	0	1	0	1	0
Red-Eyed Vireo	2	1	0	0	1	0
White-breasted Nuthatch	2	1	0	0	1	0
Brown-Headed Cowbird	1	0	1	0	1	0
Hermit Thrush	1	1	0	0	1	0
Rose-Breasted Grosbeak	1	1	0	0	1	0
Totals	57	15	9	1	23	3

Table 3.4. Comparison of haemosporidian prevalence in songbirds collected from other habitats in grassland and aspen parkland habitats of western Minnesota, 2010-2014.

UND Student	USGS Eco-Region	Habitat	No. Birds Examined	No. Species	*Plasmodium*	*Haemoproteus*	*Leucocytozoon*
Danielle Kvasager (2015)	Lake Agassiz Plain	Grassland	136	6	29%*	10%*	29%*
Chad Stomlund (2015)	Lake Agassiz Plain / Peatlands	Aspen Parkland	110	28	29%*	18%*	17%*
Kelsey Morin (2022)	Northern Glaciated Plains	Turtle Mountains	98	21	9%*	7%*	4%*

* = Significantly different for Chi-square tests at $p < 0.05$.

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APPENDIX A

Haemosporidian sequences collected from birds captured in Turtle Mountains Rolette County, ND and Grand Forks County, ND. Haemosporidian lineages in this table were used to create the phylogenetic tree (Figure 3.2).

Bird Species	Capture Date	County	Haemosporidia GenBank Identification	GenBank Reference Sequence	Haemosporidia MalAvi Identification	Lineage Identification	Accession number
American Goldfinch	2021	Rolette	*Haemoproteus sp.*	KF314768.1	*Haemoproteus sp.*	LOXLEU01	KF314768
American Redstart	2020	Rolette	*Plasmodium cathemerium*	MW876849.1			
American Robin	2020	Rolette	*Plasmodium unalis*	KY653814.1	*Plasmodium unalis*	TUMIG03	EF011189
American Robin	2020	Rolette	*Plasmodium unalis*	*KY653814.1*	*Plasmodium sp.*	TUMIG23	KM598221
American Robin	2020	Rolette	*Plasmodium unalis*	KY653814.1	*Plasmodium sp.*	TURASS03	JN819329
American Robin	2020	Rolette	*Plasmodium unalis*	KY653814.1	*Plasmodium unalis*	TUMIG03	EF011189
Black-capped Chickadee	2020	Rolette	*Parahaemoproteus majoris or vireonis*	JN4728.1	*Haemoprtoeus majoris or vireonis*	PHYBOR04	MG726191
Chipping Sparrow	2020	Rolette	*Parahaemoproteus sp.*	FJ168561.1 KF314761.1	*Haemoproteus sp.*	SPIPAS02	MF077649
Chipping sparrow	2018	Grand Forks	*Plasmodium cathemerium*	MN114079.1 MK077679.1	*Plasmodium cathemerium*	SEIAUR01	DQ838988

Bird Species	Capture Date	County	Haemosporidia GenBank Identification	GenBank Reference Sequence	Haemosporidia MalAvi Identification	Lineage Identification	Accession number
Common Grackle	2018	Grand Forks	*Plasmodium sp.*	KT193633.1	*Plasmodium cathemerium*	SEIAUR01	DQ838988
Common Grackle	2018	Grand Forks	*Plasmodium sp.*	KT193633.1	*Plasmodium cathemerium*	SEIAUR02	DQ838989
House Sparrow (F)	2020	Rolette	*Plasmodium cathemerium*	KX867107.1	*Plasmodium cathemerium*	SEIAUR01	DQ838988
Red Eyed Vireo	2020	Rolette	*Parahaemoproteus sp.*	GQ395676.1 GU256262.1	*Haemoproteus sp.*	CHIPAR01	KJ466081
Rose-Breasted Grosbeak	2020	Rolette	*Parahaemoproteus sp.*	MN459654.1	*Haemoproteus sp.*	PIRLUDO8	MK78314B
White-Breasted Nuthatch	2020	Rolette	*Plasmodium sp.*	MK783167.1	*Plasmodium sp.*	TROAED24	KJ620788
Yellow Warbler	2020	Rolette	*Parahaemoproteus sp.*	MN459605.1	*Haemoproteus sp.*	TURFUL01	MT724520

CHAPTER IV

VECTORAL COMPETENCE OF *AEDES AEGYPTI*, *CULEX PIPIENS*, AND *CULEX TARSALIS* TO TRANSMIT AVIAN *PLASMODIUM* FROM NATURALLY INFECTED HOSTS

INTRODUCTION

Understanding vector competence is important to fully understand parasite transmission. Vector competence involves several factors for successful development of avian *Plasmodium* within a mosquito for transmission to avian hosts. Within the mosquito, gametocytes must escape from the bird's red blood cells, undergo sexual reproduction, then migrate toward mosquito midgut cells forming oocysts. Oocysts will burst, releasing developed sporozoites into the mosquito haemocoel. Sporozoites then invade the mosquito salivary glands and are transmitted to the next avian host when the mosquito feeds. Presence of oocysts are indicative of successful parasite development in the vector, while presence of sporozoites indicate that this vector is capable of transmitting the parasite (Valkiunas 2005).

When avian *Plasmodium* gametocytes are picked up by mosquitoes there are several factors that influence the success of the parasite. The number of gametocytes in the blood meal will influence the efficiency of sexually reproduction in the mosquito midgut (Bradley *et al.* 2018, Churcher *et al*. 2013). During its development in the vector, the parasite must evade the mosquito immune system (Cirimotich 2010). *Plasmodium* must also be able to asexually

reproduce, and relies on the mosquito to provide adequate nutrition (Habtewold *et al*. 2021). After successfully completing its development to the sporozoite stage, the parasite is transmitted to a new vertebrate host with the mosquito saliva.

Very little work has been done in the Turtle Mountains with mosquitoes and no previous research has looked at the transmission of *Plasmodium* in the area. This study used wild-caught birds to simulate avian *Plasmodium* transmission to various mosquito vectors. Several avian and mosquito species were used for feeding trials to investigate vector competence and development of *Plasmodium* within a mosquito vector. Despite hundreds of mosquito dissections, full development of an avian *Plasmodium* parasite was not detected.

METHODS

Birds

Birds were captured in Grand Forks County and the Rolette County (Turtle Mountains) using baited ground traps and mist nets. The ground traps were baited with mixed bird seed and bread. The mist nets were put up each day right before dawn. Blood was drawn from the brachial vein of each bird and collected in a heparinized capillary tube. Collected blood was stored at -20°C in 1.5ml tubes with 500µl of 100% ethanol. Birds caught were put into individual cages and transported to the University of North Dakota and held in an aviary until Haemosporidia testing.

Plasmodium Detection

A small amount of (ca. 5 µl) blood was taken from the sample and dried in a test tube on a heating block at 21°C. After the sample was dried, DNA was extracted using a Qiagen DNeasy Blood and Tissue kit (Qiagen, Germantown, MD). To quickly test for avian Haemosporidia, the real-time polymerase chain reaction protocol from Chapter II was used (Table 4.1) (Bell *et al*.

2015). The positive control was a previously tested *Plasmodium* positive sample. Samples were considered real-time PCR positive if the melt peak curve from amplification was between 75°C and 80°C. If the blood did not show a Haemosporidia infection, the bird was released. If the bird was positive, the extracted DNA was tested for *Plasmodium/Haemoproteus* infection using the nested PCR protocol mentioned in Chapter II (Table 4.1) (Bell *et al*. 2015). The same previously tested positive avian *Plasmodium* sample was used as a positive control for the nested PCR. The PCR products were then run onto a 1% ethidium bromide agarose gel to look for amplification. Positive PCR samples were cleaned with ExoSAP-IT (Affymetrix Inc., Santa Clara, CA) according to the manufacturer's instructions. Samples were sequenced using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems Inc. - ABI, Foster, CA) 10 µl reactions and a 3100 Genetic Analyzer (Applied Biosystems Inc. - ABI, Foster, CA). Sequences were trimmed and analyzed using Molecular Evolution Genetic Analysis (MEGA) for macOS. Sequences were compared to known sequences on GenBank and MalAvi. Viable sequences were also compared to birds captured in the Turtle Mountains during the Summers of 2020 and 2021, to establish likely species (Chapter II). Birds found positive for *Plasmodium* were used for mosquito feeding trials.

Mosquitoes

The *Aedes aegypti* (COSTA RICA strain), *Culex pipiens*, and *Culex tarsalis* (YOLO strain) mosquitoes were reared in the University of North Dakota insectary. *Aedes aegypti* and *Cu. tarsalis* eggs were obtained from BEI Resources (Manassas, VA USA). *Culex pipiens* eggs were obtained in 2015 from a colony established at the Colorado State University (Fort Collins, CO USA). The insectary was on a 12-hour light/12-hour dark photoperiod. The insectary was maintained at 26°C. Mosquito eggs were kept in 8.5in by 11in plastic containers until pupation.

Larvae were fed finely ground Tetra fish food daily. Before emerging to the adult stage, the pupae were put into cubic foot mesh cages, with sugar and water available.

Mosquito Feeding Trials

Two Common Grackles (*Quiscalus quiscula*), two Pine Siskins (*Spinus pinus*), one American Robins (*Turdus migratorius),* and one Chipping Sparrow (*Spizella passerina*) were weighed and anesthetized using a mixture of ketamine and xylazine before exposure to mosquitoes. Common Grackles, Pine Siskins, and the Chipping Sparrow received intramuscular injections of 2mg ketamine: 0.4 mg xylazine per 100 g body weight. American Robins received 5 mg ketamine: 0.2 mg xylazine per 100 g body weight. They were injected using a 27-gauge needle into one of their pectoral muscles. Anesthesia volume varied based on bird weight and species. After the birds were completely anesthetized, they were moved into a cubic foot mesh mosquito cage and mosquitoes were allowed to feed on them for approximately 45 minutes. All fully blood engorged mosquitoes were gently aspirated into smaller gallon size cages and provided a source of sucrose and water. Mosquitoes were maintained in the 26°C insectary. Mosquitoes for Robin feeding trial #2 were maintained post feed at 24°C or 26°C.

Mosquitoes' midguts were dissected 9-12 days after initial feed to look for oocysts. Mosquitoes were aspirated into ethanol to immobilize them, then immediately moved to Minimum Essential Medium cell culture media (Gibco). Mosquitoes were dissected on a microscope slide using a dissecting scope and a pair of fine-tipped jeweler's forceps. After midgut was removed, a wet mount was created using a droplet of cell media and coverslip. Midgut was viewed using a compound microscope to visualize oocysts.

Mosquitoes were dissected 13-21 days after feed to look for sporozoites. Mosquitoes were aspirated into ethanol to immobilize them, then immediately moved to cell media.

Mosquitoes were dissected on a microscope slide using a dissecting scope and a pair of 27-gauge needles. The pair of removed salivary glands were prepared as a wet mount using a droplet of cell medium and coverslip. The salivary glands were viewed using a compound microscope. Salivary glands were lightly pressed under the coverslip to burst the glands open to release any potential *Plasmodium* sporozoites. After salivary gland removal, the same mosquito carcasses were dissected for midguts to look for oocysts.

Bloodmeal Analysis

To verify *Plasmodium* being ingested by mosquitoes, mosquito midguts were removed from engorged mosquitoes in American Robin Trials # 1-3. The blood from the mosquito midguts was dried in 1.5 ml tubes, DNA was extracted, and a real-time PCR was conducted using the same protocols as previously stated for blood drawn directly from a bird.

RESULTS

Birds from Grand Forks County, ND

All five birds captured in Grand Forks County, North Dakota tested positive by nested PCR for *Plasmodium*. Based on the phylogenetic comparison in Chapter III (Figure 3.2), the Common Grackles and Chipping Sparrow were likely infected with *Plasmodium cathemerium*. The species of *Plasmodium* infecting the Pine Siskins could not be determined.

A total of 292 *Cx. pipiens* mosquitoes successfully fed on the five *Plasmodium* positive birds and were later dissected for oocysts 9 to 12 days later (Table 4.2). The positive mosquito infection came from a mosquito fed on the Chipping Sparrow and produced two small oocysts. The positivity rate for mosquitoes that fed on the Chipping Sparrow was 4%. The mosquitoes that fed on the two Pine Siskins and two Common Grackles failed to infect *Cx. pipiens* mosquitoes.

American Robin from Rolette County, ND

Three mosquito feeding trials were conducted at different times using a single *Plasmodium unalis*-infected American Robin. Trial 1 tested *Cx. pipiens* only; Trial 2 compared *Ae. aegypti* and *Cx. pipiens* fed concurrently and incubated at different temperatures; and Trial 3 compared *Cx*. *pipiens*, *Cx. tarsalis*, and *Ae. aegypti* mosquitoes. Analysis of mosquito blood meals performed at all trials on blood-engorged mosquitoes directly after feeding indicated that all four species of mosquitoes ingested *Plasmodium unalis* at the time of feeding (Table 4.3).

Trial #1 resulted in a 14% prevalence of oocysts in the 42 *Culex pipiens* dissected at 8-12 days post blood meal (Table 4.3). However, none of the mosquito salivary glands dissected from 7 mosquitoes at days 13-21 contained sporozoites even though two of the mosquitoes had oocysts on their midguts, indicating that they were infected but not infectious. The densities of oocysts on infected midguts were low ($X = 3 \pm 2$; range 1-7).

During Trial #2, The American Robin failed to infect any mosquitoes at either of the two developmental temperatures when examined for oocysts at days 8-12 (Table 4.3). Likewise, none of the 24 surviving mosquitoes dissected 13-21 days post blood meal had sporozoites in their salivary glands or oocysts on their midguts.

During Trial #3, oocysts failed to form in *Aedes aegypti* or *Culex tarsalis* but did occur in *Cx. pipiens* when fed concurrently on the same bird (Table 4.3). *Plasmodium* developed in *Cx. pipiens* with an oocyst prevalence of 13%. All positive mosquitoes ($n = 3$) had fewer than 5 oocysts per midgut ($X = 2 \pm 2$). No sporozoites or oocysts were found at 13-21 days post bloodmeal. *Culex tarsalis* did not survive to salivary gland dissection date.

DISCUSSION

Culex spp. are the primary vector for avian *Plasmodium* (Valkiunas 2005). In this study *Culex pipiens* were the only species to support *Plasmodium* development (Table 4.2 and Table 4.4). All feeds done on the American Robin and the passerine birds of 2018 produced lower infection rates and oocyst loads than expected. The only bird to produce positive *Plasmodium* infection from the 2018 feeds was a Chipping Sparrow. Although the Chipping Sparrow produced one positive infection, the parasite load was low. Trials with the American Robin also yielded low infection results. Normally, dozens of oocysts can be found on a single mosquito midgut (Sekar *et al.* 2021). Lack of oocyst development may be due to the mosquito's immune response and the natural population bottleneck that occurs in several *Plasmodium* species (Alavi *et al*. 2003).

Oocyst prevalence across all feeds were low, but this does not explain the lack of sporozoite development. As mentioned previously, oocyst density is lower than the density of the parasite during other life stages. Mosquitoes with oocyst development did not become infectious vectors (Table 4.4). The lack of sporozoites does not match with the indicated vectoral capacity of *Ae. aegpti, Cx. pipiens*, and *Cx. tarsalis* (Santiago-Alarcon *et al.* 2012). However, it should be noted that mosquito vector competence trials with *P. unalis*, have never been reported. For the mosquito to become infectious to a new host, the *Plasmodium* sporozoites must invade the mosquito salivary glands.

A possible reason for lack of transmission may be the use of colony mosquitoes. Colony mosquitoes may not accurately represent wild mosquito populations. Lab conditions can lead to inbreeding and genetic differences from uncolonized mosquitoes (Hoffmann & Ross 2018). These genetic differences can lead to a reduced susceptibility to initial parasite infection and

overall development (Mohanty *et al.* 2018). Laboratory feeds of avian hosts have shown successful transmission of *Plasmodium* to *Culex pipiens.* A study using wild caught *Culex pipiens* produced a 100% prevalence of avian *Plasmodium* infection (Kazlauskiene *et al*. 2013).

Although this study used colony established mosquitoes, mosquito survival is a concern. *Plasmodium* oocysts develop 8–12 days post bloodmeal and sporozoites develop 13 days after bloodmeal. Low sampling and mosquito survival may have influenced the prevalence of the parasite observed.

Another possible reason for low rates of parasite development in the mosquitoes is low parasitemia in the peripheral blood of the bird. A prolonged avian *Plasmodium* infection may lead to lowered parasitemia (Valkiunas 2005). The blood of the 2018 birds was tested immediately before mosquito feeds and showed positive avian *Plasmodium* infection in the blood. The American Robin feeds were all accompanied by mosquito blood meal analyses indicating the presence of *Plasmodium*. The tests done concurrently with the mosquito feeds verify the presence of *Plasmodium* in the blood. The birds were not in a latent infection period. However, the molecular detection of *Plasmodium* does not indicate which parasitic life stage was present. Asexual stages ("meronts") can be found in the peripheral blood of avian hosts, but this life stage is not infectious to mosquitoes – only gametocytes infect mosquitoes. Subsequently, gametocytes may not have been picked up with each mosquito blood meal. Without gametocytes, the parasite would not have been able to develop into oocysts.

Temperature manipulation in the American Robin Trial #2 did not improve the susceptibility of the mosquitoes (Table 4.3). Although the effect of temperature was examined during one of the American Robin feeds, and no oocysts or sporozoites were formed, temperature may still be a component determining the efficiency of parasite development.

Plasmodium development is dependent on temperature (Valkiunas 2005). Lack of parasite development may have been a result of previously mentioned factors.

Notably, most studies of successful avian *Plasmodium* infection of mosquitoes have taken the blood of a wild-caught bird diagnosed with *Plasmodium* and inoculated that blood intravenously into an uninfected bird – typically a canary – which is then used as the host to infect mosquitoes (Carlson *et al.* 2018, Kazlauskiene *et al.* 2021, Palinauskas *et al*. 2016, Sekar *et al*. 2021). Few studies have fed mosquitoes directly on wild-caught birds. The lack of successful transmission in this study may not be reflective of previous laboratory studies, because this work utilized naturally infected avian hosts to infect the mosquitoes. Avian *Plasmodium* transmission may be different based on mode of avian host infection. Although this study did not confirm full development of avian *Plasmodium* transmission in a mosquito vector, it demonstrated the complexity of vector competence. Further research into this laboratory transmission will benefit avian *Plasmodium* epidemiology.

Table 4.1. Primers used for Haemosporidia testing and *Plasmodium/Haemoproteus* detection.

Organism and Target Gene	Primer
Real-time PCR Primers	
Haemosporidia rDNA (313-501) 182 bp	Forward: R330F 5′ - CGTTCTTAACCCAGCTCACG - 3′ Reverse: R480RL 5′ - GCCTGGAGGTWAYGTCC - 3′
Nested PCR/ Sequence Primers	
Plasmodium/Haemoproteus Cytochrome b (3703-4273) 526 bp	Forward: H332F 5′ - GAGAATTATGGAGYGGATGGTG - 3′ Reverse: HaemNR2 5′ - AGAGGTGTAGCATATCTATCTAC - 3′
Plasmodium/Haemoproteus Cytochrome b (3719-4243) 477 bp	Forward: H350F 5′ - GGTGTTTTAGATATATGCATGC - 3′ Reverse: HaemR2 5′ - GCATTATCTGGATGTGATAATGGT - 3′

Table 4.2. *Plasmodium* oocyst prevalence in *Culex pipiens* fed on three species of songbirds captured in 2018 (Grand Forks Co., ND).

Bird Species	Bird ID	% Infected (n)
Pine Siskin	1	0% (29)
Pine Siskin	2	0% (17)
Chipping Sparrow	3	4% (24)
Common Grackle	4	0% (98)
Common Grackle	5	0% (124)

Table 4.3. Limited sporogonic development of *Plasmodium unalis* in four species of mosquitoes fed concurrently on an infected American Robin caught in Rolette Co. ND and maintained in captivity at Starcher Hall Animal Facilities.

Trial No. and Date of Feed	Mosquito Species	Day 0 Gametocyte / Merozoite	Holding Temp	Days 8-12 Oocyst	Days 13-21 Oocyst	Days 13-21 Sporozoite
No. 1 October 2020	*Cx. pipiens*	100% (5)	26 C	14% (42)	40% (7)	0% (7)
No. 2 November 2020	*Ae. aegypti*	100% (3)	26 C	0% (10)	0% (4)	0% (4)
			24 C	0% (12)	0% (12)	0% (12)
	Cx. pipiens	100% (3)	26 C	0% (18)	0% (2)	0% (2)
			24 C	0% (9)	0% (6)	0% (6)
No. 3 June 2021	*Ae. aegypti*	100% (3)	26 C	0% (35)	0% (7)	0% (7)
	Cx. pipiens	100% (4)		13% (27)	0% (4)	0% (4)
	Cx. tarsalis	100% (2)		0% (19)	NT	NT

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CHAPTER V

CONCLUSIONS AND FUTURE DIRECTIONS

The Turtle Mountains of North Dakota is a unique ecoregion. There has been no prior research in this area regarding avian *Plasmodium* or related blood parasites. Research has been conducted in the Red River Valley and Northwestern Minnesota, but due to the differences in habitat, the Turtle Mountains may provide distinctive habitats to facilitate parasite transmission. My research involved three related studies that explored if the Turtle Mountains are unique in the Great Plains region.

The first goal of this dissertation was to identify the species composition and avian *Plasmodium* prevalence of the host-seeking mosquitoes of the Turtle Mountains, North Dakota. I identified over 15,000 mosquitoes belonging to 27 different species. The two main species of mosquitoes occurring in the Turtle Mountains were *Aedes vexans* and *Coquillettidia perturbans*. Overall, the Turtle Mountains had a higher diversity of mosquito species, but a generally lower abundance of mosquitoes in comparison to studies conducted in the other parts of North Dakota. Despite the capability of several mosquito species collected to be a vector for avian *Plasmodium*, *Plasmodium* and other haemosporidians were not detected in any of the mosquitoes.

The second goal of this dissertation was to determine the haemosporidian prevalence of local and migrating birds of the Turtle Mountains. Avian blood parasites were present in local and migratory birds of the Turtle Mountains. The presence of parasite infections in local bird species before migratory birds arrive indicate there is transmission occurring within endemic

birds. However, Chapter 1 showed no evidence for transmission, indicating that there may be no competent vectors in the area. The local birds of the Turtle Mountains may display low parasite prevalence because they only become infected when they leave the Turtle Mountains habitat into the surrounding agricultural plains.

The third goal of this dissertation was to compare the relative abilities of different mosquito species to support the development of the malaria parasite. Five avian *Plasmodium* positive birds caught in Grand Forks County were used to infect *Culex pipiens* that were reared at the University of North Dakota. Of the 292 mosquito midguts examined, only one mosquito was found to have a viable infection that resulted in oocysts on the midgut surface. An American Robin caught in Rolette County from the Turtle Mountains, that was positive for *Plasmodium unalis,* was used to infect laboratory mosquitoes. The American Robin was able to successfully infect *Culex pipiens,* but the *Plasmodium* parasite was unable to infect *Ae. aegypti* and *Culex tarsalis*. Despite successful infection of *Plasmodium* in some *Culex pipiens* to the oocyst state, the parasite was unable to fully develop to the sporozoite stage within the vector. Sporozoites would indicate that the vector has become infectious and is able to continue to transmit the parasite. The lack of transmission was likely due to the low parasitemia of the hosts. Studies focusing on vector competence and transmission of bird malaria typically rely on laboratory-infected hosts with high parasitemia. To generate birds with high parasitemia for experimental mosquito feedings, investigators take blood from a wild bird with an known infection and inoculate a small amount of the blood into an uninfected, naïve bird (= "sub-inoculation" technique). I used naturally infected wild birds, with lower parasitemia, in my experimental mosquito feedings. This approach may have resulted in less successful transmission to the vectors.

My research provides a foundation for understanding the mosquito fauna and avian blood parasite transmission in the Turtle Mountains. As the regional climate continues to change and extreme weather increasingly impacts bird and mosquito populations, this research can be used as a baseline to make comparisons about mosquito species diversity, species abundance, and population dynamics. These factors are particularly important in the unique ecosystem of the Turtle Mountains, where tribal land policies protect the area from agricultural development. Furthermore, the region is unique due to its potential lack of competent mosquito vectors which reduces likelihood of host-vector interactions. As such, the Turtle Mountains will likely remain an area of shelter for birds despite increasing national anthropogenic development that brings potential vectors of avian malaria.