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PREVALENCE OF HAEMOPARASITES AND WEST NILE VIRUS SEROPOSITIVITY IN SONG BIRDS (PASSERIFORMES) FROM NORTHWEST MN AND BEHAVIORAL ASPECTS OF MICROFILARIAE

by

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Bachelor of Science, North Dakota State University, 2013

A Thesis

Submitted to the Graduate Faculty

of the

University of North Dakota

in partial fulfillment of the requirements

for the degree of

Master of Science

Grand Forks, North Dakota
August
2015

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

Jefferson Vaughan

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

Wayne Swisher
Dean of the School of Graduate Studies

July 23, 2015
Date

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Department Biology

Degree Master of Science

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Chad A. Stromlund
July 8, 2015

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ABSTRACT

Mosquitoes transmit arboviruses, haemosporidian protozoa (e.g., malaria), and filarioid nematodes. Many of these mosquito-borne pathogens occur together and transmission cycles are occurring concurrently. Despite this, mosquito pathogen transmission cycles are largely studied independently of one another. Considering the large temporal and spatial overlap among these various transmission cycles, the question becomes, what kinds of interactions are occurring between parasite transmission cycles and arboviral transmission cycles? Also, some species of mosquito are incapable of transmitting some species of arbovirus. Therefore, there must be natural barriers to prevent arbovirus infection and transmission. These barriers include the midgut barrier and the salivary gland barrier. Parasites that develop within mosquitoes have the potential to disrupt (overcome, etc.) these barriers. To further understand these interactions, I surveyed the song birds in northwest MN and screened them for haemoparasites and West Nile virus seropositivity. I focused on the behavioral aspects of microfilariae (MF) because they are known to disrupt the midgut barrier of their arthropod hosts. I found high prevalence and polyparasitism of haemoparasites infecting song birds in northwest MN. It was determined that MF of the American robin, *Culex pipiens* mosquitoes and West Nile virus was not an important system for enhanced viral transmission. Melatonin may play a role in the regulation of nocturnal periodicity behavior of MF, but other factors most likely contribute as well.

CHAPTER I

INTRODUCTION

Mosquitoes transmit arboviruses, haemosporidian protozoa (e.g., malaria), and filarioid nematodes. Many of these mosquito-borne pathogens occur together and transmission cycles are occurring concurrently. Despite this, mosquito pathogen transmission cycles are largely studied independently of one another. Considering the large temporal and spatial overlap among these various transmission cycles, the question becomes, what kinds of interactions are occurring between parasite transmission cycles and arboviral transmission cycles?

To understand potential interactions, it is helpful to understand the normal route of arboviral infection in mosquitoes. Briefly, mosquitoes become infected by feeding on an animal that has virus circulating in its blood (i.e., viremic). The virus binds to the surface of the midgut epithelial cells and enters. Within the epithelium, the virus replicates many times and then is released on the other side into the body cavity (hemocoel) of the mosquito. At that stage, the mosquito is considered to have a 'disseminated' viral infection. An analogous process of viral attachment, invasion, replication and release occurs in the salivary glands of the mosquito. Only when this process of midgut and salivary gland infection occurs does the mosquito become 'infectious' and is able to transmit the virus by bite.

Some species of mosquito are incapable of transmitting some species of arbovirus. Therefore, there must be natural barriers to prevent arbovirus infection and transmission. These barriers include the midgut barrier and the salivary gland barrier. Parasites that develop within mosquitoes have the potential to disrupt (overcome, etc.) these barriers. There are two types of mosquito-borne parasites that have been examined experimentally and shown to overcome these barriers, microfilarial nematodes and hemosporidian protozoa (Paulson et al. 1992; Vaughan and Turell 1996; Vaughan et al. 2012).

Microfilarial enhancement occurs during the viral acquisition phase by disruption of the mosquito midgut barrier to viral dissemination. Sporozoite enhancement affects the transmission phase by disruption of the mosquito salivary gland barrier to oral secretion of the virus. Each form of enhancement requires the penetration of a different barrier so there are different requirements and restraints of the two forms. Microfilarial enhancement requires concurrent ingestion of microfilariae (MF) and virus from a dually infected host. This is dependent on the prevalence of MF infected hosts in the community. Sporozoite enhancement does not share these constraints. However, sporozoite enhancement requires a competent mosquito vector that allows the plasmodia to complete sporogonic development inside the mosquito. This constraint does not apply to MF enhancement. Thus, a competent vector is not required and the MF only need to be capable of penetrating the mosquito midgut.

Microfilarial Enhancement

Simultaneous ingestion of MF and arboviruses (viruses transmitted by arthropod vectors) by blood-feeding arthropods has been shown to significantly enhance the transmission of viruses compared to when the arthropod vector ingests an equal dose of the virus alone (Turell et al. 1984; Vaughan and Turell 1996; Vaughan et al. 1999). This mechanism has been termed

microfilarial enhancement of arboviral transmission. This can occur when arthropod vectors consume a bloodmeal from a dually-infected vertebrate (i.e. concurrently microfilaremic and viremic). After ingestion, the MF penetrate the arthropod midgut (Figure 1) which allows direct passage of the virus into the hemocoel. Once in the hemocoel, the virus can disseminate and move into the salivary glands of the mosquito causing it to be infective for the virus (Turell et al. 1984). Thus the virus can pass the midgut barrier without having to invade the cells and replicate.

There are two ways this mechanism can enhance virus transmission; 1) increase vector competence and 2) decrease the extrinsic incubation period (Vaughan et al. 2012). The midgut barrier of the mosquito is the primary constraint against viral infection, but if the barrier is perforated by MF, the virus can then easily cross the barrier (Turell et al. 1984; Vaughan et al. 2012). Direct transfer of virus into the hemocoel can also decrease the amount of time it takes for the vector to become infectious for the virus (i.e. extrinsic incubation period) and allow the vector to transmit the virus to new hosts in a shorter period of time (Vaughan et al. 2012). Vaughan et al (2012) also describes four requirements for MF enhancement of arboviral transmission; 1) the vertebrate host must be dually infected with the virus and MF, 2) the MF must penetrate the midgut of the arthropod after a bloodmeal is taken, 3) a sufficient amount of virus must pass into the hemocoel through the MF perforations to establish infection, and 4) the arthropod must be able to transmit the virus by bite.

Several studies have provided proof of this concept in laboratory settings. One of the earliest studies used gerbils infected with *Brugia malayi* and *Aedes taeniorhyncus* as the vector for Rift Valley Fever to show enhancement (Turell et al. 1984). Normally, *Aedes taeniorhyncus* is refractory to Rift Valley Fever virus because of a strong midgut barrier. But when the MF

were present, *Aedes taeniorrhyncus* became infected and transmitted the virus at significantly higher rates (5% transmission with no MF infection and 31% transmission with MF infection) (Turell et al. 1984). Likewise, Vaughan and Turell (1996) used *Aedes* mosquitoes and *Brugia* MF in gerbils to show enhanced transmission for Eastern Equine Encephalitis. A third study

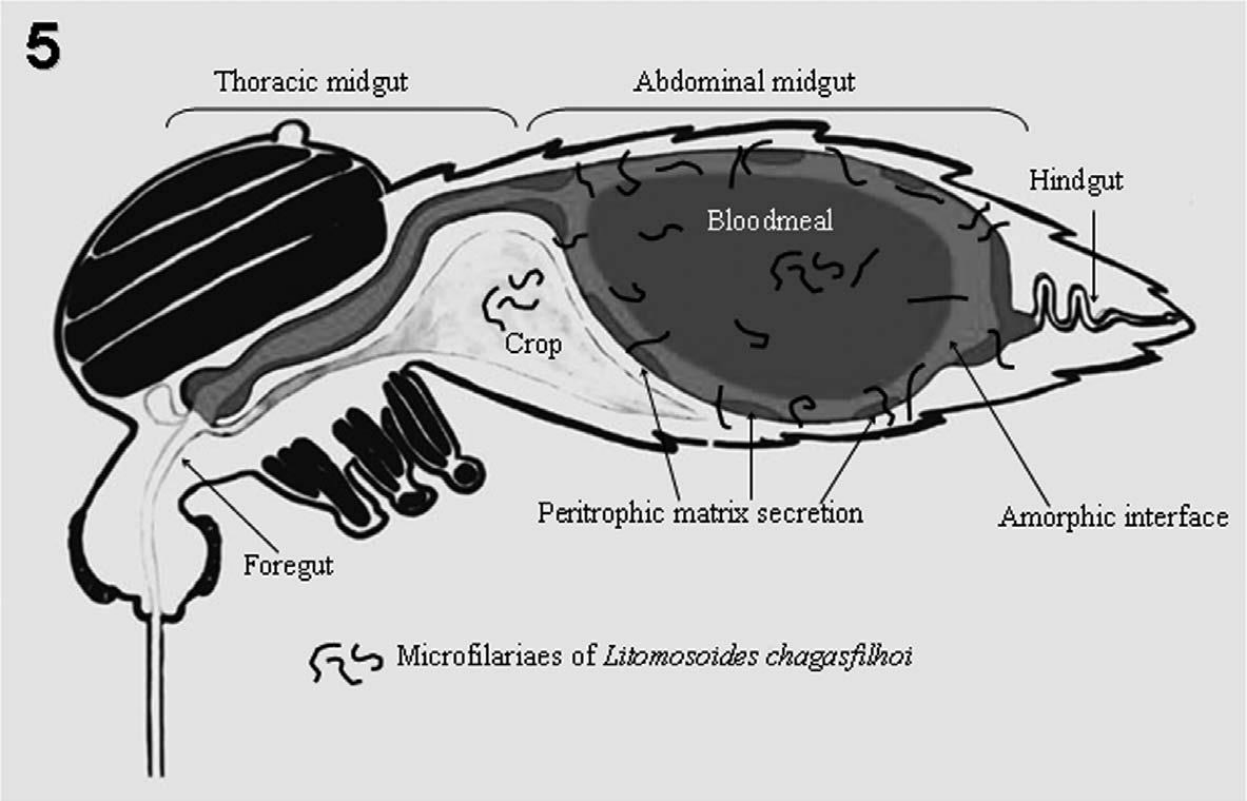

Figure 1. Illustration of the invasive process of microfilaria in the mosquito midgut. (Santos et al 2006).

showed this concept worked by using all the same players except Venezuelan Equine Encephalitis virus was used (Vaughan et al 1999).

Sporozoite Enhancement

Much less is known about the ability of malaria parasites to enhance arboviral transmission. Paulson et al. (1992) reported that when mosquitoes were previously infected with avian malaria (*P. gallinaceum)*, the transmission of LaCrosse virus was significantly enhanced. LaCrosse virus can produce disseminated infections in *Aedes hendersoni* mosquitoes, but

transmission was rare due to the salivary gland barrier (Hardy et al. 1983). Vaughan and Turell (1996) reported that *Anopheles stephensi* mosquitoes are unable to transmit Rift Valley Fever virus due to a salivary gland barrier, but Rift Valley Fever inoculated *A.* stephensi with salivary glands infected with *P. berghei* sporozoites transmitted virus. It appears that enhanced transmission occurred due to sporozoite disruption of the salivary gland barrier increasing the vector competence of the mosquito host. To date, there is much more information in the literature about midgut barriers than salivary gland barriers. Considering the high prevalence and wide geographical distribution of malaria parasites, it warrants consideration that sporozoite enhancement may play a role in arboviral transmission.

Of the two types of parasite enhancement, MF enhancement may be the more important because; 1. Midgut barrier to arbovirus infection & dissemination is the more common barrier to arbovirus infection in mosquitoes. 2. In addition to increasing vector competence (i.e., transforming a non-vector species into a vector), MF enhancement can also decreases the extrinsic incubation period (i.e., infected mosquitoes become infectious sooner than normal).

Most studies that have examined parasitic enhancement have used unrealistic model systems – systems in which the virus, mosquito and parasite do not occur together and hence would never interact with one another in nature. One of my objectives was to define naturally-occurring arbovirus, parasite and mosquito species combinations that have direct relevance to the mid-western USA. Selection of the arbovirus was easy. West Nile virus (WNV) is the most common arbovirus in the USA. West Nile virus is an introduced mosquito-transmitted virus that infects mainly songbirds (reservoirs).

West Nile Virus

West Nile virus is a member of the family Flaviviridae along with other viruses that cause dengue, different types of encephalitis, and yellow fever (Nosal and Pellizzari 2003). West Nile virus is capable of causing severe and sometimes fatal neurological illness. Over 150 different species of birds and mammals can be infected by WNV (Barker 2002). Birds from the

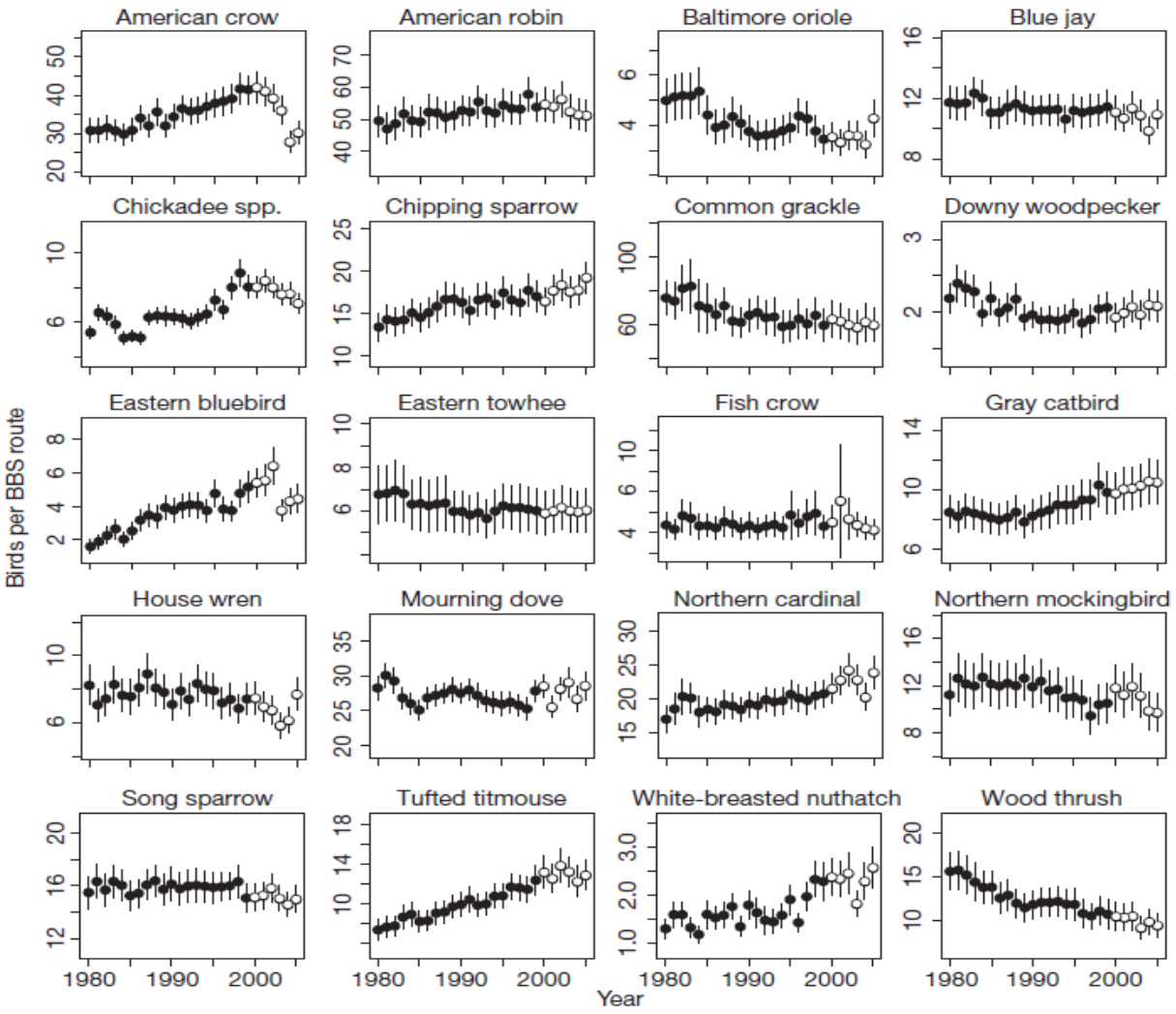

Figure 2**.** Time series of mean abundance per Breeding Bird Survey route adjusted for missing observations and observer variance. Error bars show 2 standard errors. Open circles denote years after WNV was first detected in North America. Population growth rates between 1980 and 1998 that were significantly different from 0 (LaDeau et al. 2007).

family Corvidae are especially susceptible to WNV illness and death, but some species of birds (e.g. American robin: *Turdus migratorius*) are able to develop immunities to WNV (Komar et al. 2003). For this reason, dead crow sightings have been used as markers for WNV activity and testing of the dead crows has been helpful in passive surveillance (Nosal and Pellizzari 2003). West Nile virus has had the biggest impact on certain avian species (e.g., American crow: *Corvus brachyrhynchos*, chickadee: *Poecile spp.*, eastern bluebird: *Sialia sialis*, see Figure 2) since its arrival in North America because WNV's vectors feed predominantly on birds (Molaei et al. 2006).

West Nile virus is designated by the CDC as a biosafety level 3 category pathogen. This means experiments using WNV must be conducted within a BSL-3 laboratory by law. The University of North Dakota does not have a functioning BSL-3 laboratory, so my studies concentrated on the other components of parasitic enhancement. I focused on the primary vector of WNV (i.e. *C. pipiens)*, the reservoir hosts of WNV (i.e. song birds), and their haemohaemoparasites.

Mosquitoes

A few different types of mosquitoes are involved in the transmission of WNV to humans. *Culex pipiens* mosquitoes feed primarily on birds, and are responsible for the transmission of the virus from bird to bird (Molaei et al. 2006). These transmissions create a large reservoir for WNV in the avian community. *Aedes* mosquitoes serve as the bridge for transmissions of WNV between birds and mammals because they are more opportunistic, and feed on both groups (Wood et al. 1979). As part of a surveillance plan, mosquitoes were trapped in the eastern United States to determine which species were responsible for the WNV outbreak in the U.S. In 1999-2000, most WNV positive mosquitoes were *Culex spp.*, which included *C. pipiens, C.*

salinarius, and *C. restuans* (Turell et al. 2005). Turell's designation of *C. pipiens* as a competent vector along with the fact that I have access to a laboratory reared colony of *C. pipiens* makes them an ideal vector model for enhanced WNV transmission. These mosquitoes are the main vectors for WNV, but they also ingest MF from the birds they feed on (Vaughan et al. 2012).

Songbird Hosts of West Nile Virus

The American robin (*Turdus migratorius)* is one of the most abundant and recognizable birds in North America. The species has a wide range in diet depending on the season. In spring and summer robins eat invertebrates, and during the fall and winter they eat primarily fruit (Vanderhoff and Eason 2007). Like most animals, robins are hosts to a number of parasites, but little is known about the filarial nematodes that parasitize the species. Two undescribed species of *Eufilaria* and of Cardiofilaria have been observed in the blood of robins that reside in the Red River Valley near Grand Forks, ND (Vaughan et al. 2012). The identity of these unknown nematodes are important, because some species of MF have trouble penetrating the midgut of *Culex pipiens* whereas other MF species (i.e., *Wuchereria bancrofti)* do not (Michalski et al. 2010). Vaughan et al. (2012) found that 38% of the robins sampled in the Red River Valley were also positive for WNV antibodies, meaning that robins are exposed not only to MF infections, but also to WNV infections. Robins account for the majority of infectious mosquito vectors which also makes them important reservoirs for WNV (Kilpatrick et al. 2006). Thus, the robin is a good model for studying the enhanced transmission of WNV in our region. Also, local robins are infected with MF, they get naturally infected with WNV, they are found in the same geographical regions as the WNV vectors, have a low mortality rate due to WNV infection, and are a favorite host for mosquitoes.

Common grackles (*Quiscalus quiscula)* are another abundant song bird in the United States. Grackles can be observed foraging for seeds and insects in cities and parks across much of the eastern portion of the country. These birds are host to a species of filarial nematode known as *Chandlerella quiscali* (Vaughan et al. 2012). A colony of filaremic grackles are housed in the aviary at the University of North Dakota, and these birds and their MF were used in experiments to determine the physiological characteristics of the MF.

Microfilaria

Microfilariae are an early stage in the developmental cycle of some nematodes in the family Onchocercidae. The adult worms live in the tissue of their definitive host, and instead of laying eggs they release MF directly into the bloodstream (Anderson 2000). From the blood stream, MF are ingested by blood-sucking arthropods, where the MF quickly penetrate the arthropod midgut and pass into the hemocoel. In the hemocoel, the MF develop and go through several molts to the infective L3 stage. When the larvae reach the infective L3 stage, they migrate to the head and exit down the mouthparts and into their new host when the vector feeds.

Some MF are known to show nocturnal periodicity (Vaughan et al. 2012). This is when the MF are only found in the bloodstream at night. It is thought that they enter the general circulation at peak feeding times of their arthropod host. This periodicity should increase the chance the microfilariae are ingested by the correct vector.

All species of filarial nematodes use hematophagous arthropod vectors (Anderson 2000). There are 16 different genera of filarioid nematodes known to infect birds and the typical vectors for avian filarioids include lice (Phthiraptera) and flies (Diptera) (Bartlett 2008). The prevalence of MF infection is known for a few species of birds in the United States. Vaughan et al. (2012)

report that 19% of Common grackles (n=58) were infected with MF in the Red River Valley region of North Dakota and Minnesota. In the east, prevalence of infection is much higher. Prevalence in Indiana was 100% in Common grackles and in Illinois, 98% of grackles were infected (Welker 1962; Granath 1980). Presumably, the vector that transmits this parasite (i.e., biting midges) are more abundant in Indiana and Illinois than in eastern North Dakota.

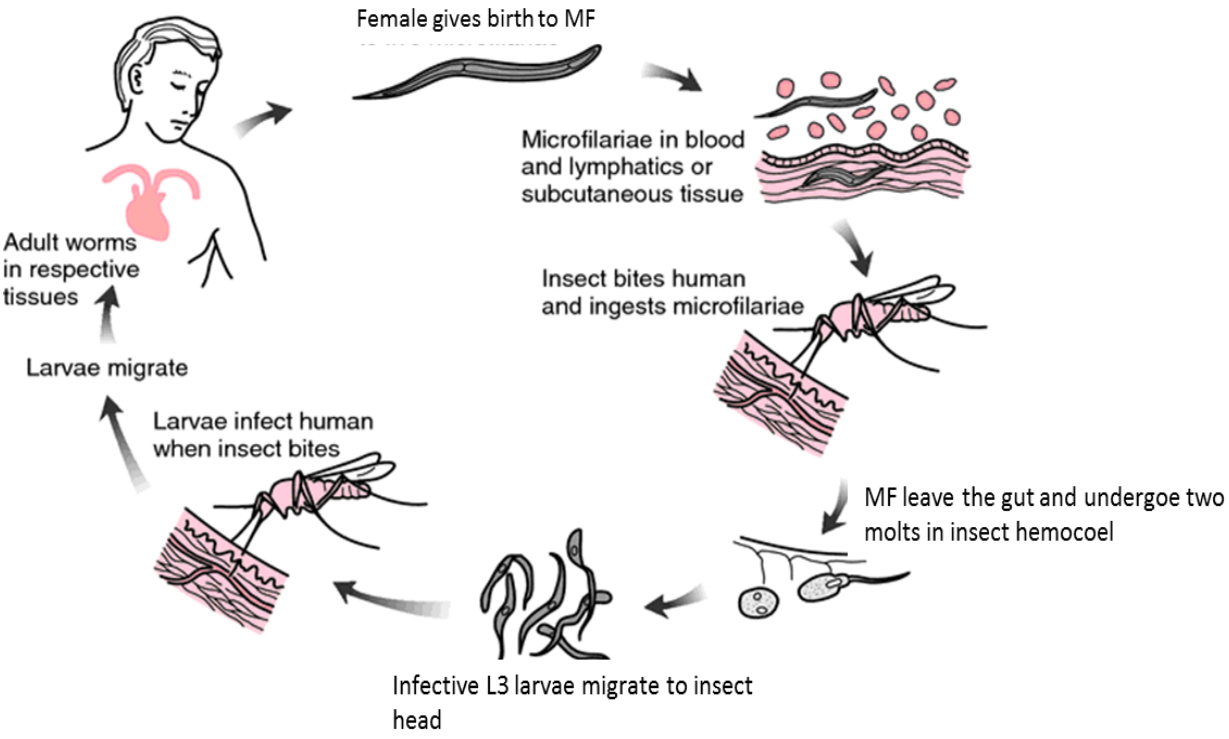

Figure 3**:** Generalized life cycle of a filarial nematode (Modified from CDC 2013).

Plasmodium

The genus *Plasmodium* is made up of a group of intracellular parasites belonging to the phylum Apicomplexa and the order Haemospororida. Avian malaria infections are caused by more than 40 species of *Plasmodium* that differ in host range, geographic distribution, and mosquito vectors. The life cycle begins when infective sporozoites are inoculated by a mosquito vector into a susceptible host (Huff and Coulston 1944). The sporozoites invade macrophages

and undergo an initial generation of asexual reproduction (merogony). Sporozoites mature into merozoites that invade the cells of the lymphoid-macrophage system to begin a second round of merogony. Mature merozoites are released that are capable of invading circulating erythrocytes. These first two rounds of merogony are referred to as the preerythrocytic stages. Merozoites that invade circulating erythrocytes develop into either schizonts or gametocytes that are infective to mosquito vectors. Gametocytes remain in circulation and do not develop until they are ingested by a vector. Once ingested, the gametocytes undergo gametogenesis and then male gametocytes undergo exflagellation to produce up to 8 flagellated microgametes. Once a microgamete fertilizes a macrogamete, a zygote develops which is capable of penetrating the midgut to form an oocyst. Oocysts undergo asexual reproduction (sporogony) and produce thousands of sporozoites. The sporozoites are released into the hemocoel and migrate to the salivary glands where they can gain access to a new host during the vectors next bloodmeal. Species that infect birds have a wide range and are found on every continent except Antarctica, where mosquito vector do not occur. Reports of diminished health in birds with infections are rare, but pathogenic infections do occur where parasites or mosquito vectors have been introduced to naïve bird populations (Warner 1968; Woodworth et al. 2005; Foster et al. 2007).

Haemoproteus

Species of *Haemoproteus* are closely related to *Plasmodium*. They are a group of vector-borne intraerythrocytic parasites and are one of the most widespread blood parasites of wild birds. Some species are transmitted by hippoboscid flies while others are transmitted by ceratopogonid flies in the genus *Culicoides* (Sergent and Sergent 1906; Fallis and Wood 1957). Unlike *Plasmodium*, species of *Haemoproteus* undergo asexual reproduction (merogony) within tissues rather than erythrocytes. The significance of *Haemoproteus* as a disease agent is highly

unknown. Recently, they are receiving more attention as models for effects of disease on host fitness, but the lack of basic knowledge of the genus has hindered these efforts.

Leucocytozoon

Leucocytozoon is a genus of parasitic Haemosporidians that use Simuliid flies as vectors. The life cycle begins when sporozoites are injected into the bird during a bloodfeed. Sporozoites enter hepatocytes and develop into small schizonts. Schizonts produce merozoites in 4-6 days that enter erythrocytes or macrophages. In the erythrocyte, the merozoites develop into round gametocytes and in the macrophages the merozoites develop into megaloschizonts. Megaloschizonts divide into primary cytomeres which multiply into smaller cytomeres and finally multiply by schizogony into merozoites. Merozoites at this stage will penetrate leukocytes or developing erythrocytes to become elongated gametocytes. At this point a non-infected fly will feed on an infected bird and ingest the elongated gametocytes. The elongated gametocytes become a macrogametocyte (female) and a microgametocyte (male). The macrogametocyte and microgametocyte form an ookinete that penetrates an intestinal cell of the black fly and matures into an oocyst. The oocyst produces sporozoites that leave and migrate to the salivary glands of the black fly, thus starting the life cycle over again. *Leucocytozoon* has been known to cause anemia, leukocytosis, hepatomegaly, and splenomegaly in their hosts (Roberts and Janovy 2009).

Borrelia burgdorferi

Borrelia burgdorferi is a spirochete bacteria that is the causative agent of Lyme disease. It is the most prevalent vector-borne disease in the United States (CDC 2015). The bacteria are transmitted by ticks in the genus *Ixodes* (Bugdorfer et al. 1985). Spirochetes are ingested by

larval or nymphal ticks while they feed on an infected host, and then they may infect other vertebrate hosts after they molt and blood-feed at the subsequent larval or adult stages. Ixodid ticks are known to infect a number of vertebrate hosts including lizards, birds, and mammals (Castro and Wright 2007). It is well known that mammals serve as primary reservoirs for *B. burgdorferi*, but little is known about the importance of birds as reservoirs (Brown and Lane 1992). Birds are potentially important reservoirs because they are abundant, diverse, hosts to vector ticks, and migrate great distances, but the ability of *B. burgdorferi* infected birds to infect feeding ticks is not well known (Mather et al. 1989; Ginsburg et al. 2005).

Trypanosomes

The genus *Trypanosoma* is the most abundant and important genus among the kinetoplastids. Mammalian trypanosomes are studied extensively due to the diseases they cause (i.e. Chagas disease and sleeping sickness), but little is known about their avian relatives (Kirchoff 2001; Taylor et al. 2007). Avian trypanosomes remain understudied because they are mostly harmless to their hosts (Macfie and Thomson 1929; Baker 1976). Avian trypanosomes occur in every continent excluding the polar regions. Their life cycle is digenetic and suggested vectors include blood sucking arthropods (simuliids, hipoboscids, mosquitoes, biting midges or mites) (Baker 1976; Molyneux 1977). However, complete life cycles have only been confirmed in a few species and the vector has not always been determined (Baker 1956; Votypka and Svobodova 2004; Votypka et al. 2011).

Specific Aims

1. Determine the prevalence of hemoparasite infection along with WNV seropositivity (presence of WNV antibodies in blood) in common songbirds inhabiting woodland habitats within northwestern Minnesota.
2. A comparison of methods including,
    - Microfilarial densities in venous blood taken from the same bird.
    - Microfilarial densities in blood taken from the general circulation (i.e., venous blood) versus blood taken from dermal capillaries (mosquito bloodmeals).
    - Quantifying the number of microfilariae ingested per mosquito.
    - Prevalence of microfilarial penetration into the mosquito hemocoel.
3. Investigate behavioral aspects of microfilarial parasites that parasitize common songbirds. Specifically,
    - Determine the nocturnal periodicity of microfilariae found in the circulatory system of American Robins, and the ability of microfilariae to penetrate the midgut of *Culex pipiens* mosquitoes fed on microfilaremic robins.
    - Determine the effect of melatonin antagonist (i.e., Luzindole) and melatonin agonist (i.e., Ramelteon) on altering the nocturnal periodicity of microfilariae in the Common Grackle.

CHAPTER II

PREVALENCE OF HAEMOPARASITES AND WEST NILE VIRUS SEROPOSITIVITY IN SONG BIRDS (PASSERIFORMES*)* FROM NORTHWEST MN

Introduction

Avian blood parasites and their hosts can be useful models to help understand important ecological, evolutionary, and behavioral questions (Buchanan et al. 1999). Studies have shown that haemosporidians can have negative effects on their host's survival, reproductive success, and body condition (Davidar and Morton 1993; Merino et al. 2000; Hatchwell et al. 2001). Haemosporidian prevalence also varies between locations which may lead to different selection pressures on bird populations (Allander and Bennett 1994; Valkiunias 1997). However most host parasitemias are low in wild birds and traditional microscopy methods have underestimated the prevalence of infection (Tham et al. 1999).

Microfilariae (MF) and trypanosomes are two other blood parasites that utilize avian hosts. Both parasites cause serious disease in humans, but are mostly harmless to their avian hosts (Macfie and Thomson 1929; Baker 1976). Although simultaneous ingestion of microfilariae (MF) and arboviruses (viruses transmitted by arthropod vectors) by blood-feeding arthropods has been shown to significantly enhance the transmission of viruses compared to when the arthropod vector ingests an equal dose of the virus alone (Turell et al. 1984; Vaughan and Turell 1996; Vaughan et al. 1999). Microscopy has traditionally been used to detect these

parasites in the blood do to their relatively large size. Trypanosomes are still much smaller than MF, so polymerase chain reaction (PCR) techniques are most appropriate for detection.

West Nile Virus (WNV) is a newly emerging arbovirus that is found on four continents, Africa, Asia, Europe, and North America. The introduction of the virus into North America has also led to high levels of mortality in wild and captive birds (Kramer and Bernard 2001). Kramer and Bernard (2001) documented more than 60 species of birds infected with WNV that died in 2000, and passerines accounted for the highest number of deaths. The results were unexpected, because in countries where WNV is enzootic, the virus has caused minimal disease in avian species (Kramer and Bernard 2001).

Borrelia burgdorferi is a spirochete bacteria that is the causative agent of Lyme disease. It is the most prevalent vector-borne disease in the United States (CDC 2015). It is well known that mammals serve as primary reservoirs for *B. burgdorferi*, but little is known about the importance of birds as reservoirs (Brown and Lane 1992). Birds are potentially important reservoirs because they are abundant, diverse, hosts to vector ticks, and migrate great distances, but the ability of *B. burgdorferi* infected birds to infect feeding ticks is not well known (Mather et al. 1989; Ginsburg et al. 2005).

Objective

The objective of this study was to determine the prevalence of hemoparasite infection along with WNV seropositivity (presence of WNV antibodies in blood) in common songbirds inhabiting woodland habitats within northwestern Minnesota.

Methods

Site Description

The study was conducted at two sites in northwest Minnesota. Site one was located in Huss township in Roseau county and site two was located in Silverton township in Pennington county. The region has a temperate climate and the dominant habitat is aspen parkland with interspersed bur oak (*Quercus macrocarpa),* black ash (*Fraxinus nigra),* tamarack (*Larix laricina)* and white spruce (*Picea glauca).* Site one was predominately aspen parkland and site two was a patchy mixture of aspen parkland and farmland.

Bird Sampling

Birds were captured by mist netting during the breeding season from April 18^{th,} 2014 to June 25th, 2014. Three Avinet (Avinet Inc., Dryden, New York) mist nets (8-12m, 38mm mesh size) were placed at one of the sites on sampling days. Nets were placed on the edge of forest openings and were observed from dawn until dusk. Captured birds, except robins, were immediately processed using the screening method described below. Robins were held in cages and screened at midnight to increase the probability of detecting MF. Negative birds were released the next morning in the same location as capture.

Bird Screening

Birds were identified to species and blood was drawn from the brachial vein into heparinized capillary tubes. The tubes were spun in a hematocrit centrifuge to separate the cells from sera. Hematocrit readings were recorded and the spun tubes were screened using microscopy to detect motile MF and trypanosomes at the cell pack interface (Collins 1971). Trypanosomes were also screened using molecular techniques to compare the sensitivity of the two methods. American robins that were infected with MF were moved to an aviary at the

University of North Dakota for further study. Blood and sera taken from captured birds was extruded into separate .5 mL tubes and stored at -20^0 C for later molecular analysis.

Molecular Diagnosis

Genomic DNA was extracted from the blood samples using a DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA). The extracts were tested using a nested polymerase chain reaction (PCR) targeting the cytochrome b gene for haemosporidians, 18S rRNA gene for trypanosomes and 5S-23S rRNA spacer region for *B. burgdorferi.* Five microliters of DNA extract, 5.5 µL of purified water, 1 µL of each primer (Table 1) and 12.5 µL of oneTaq Quick-Load 2X MM with standard buffer (New England Biolabs, Ipswich, MA) were used in the first round of PCR reactions for all three groups. Three microliters of DNA product from the first reactions were used in the nested round and 2 µL of water were added to make a 25 µL reaction. The cycling conditions for both rounds of the haemosporidian protocol involved an initial 3 minute denaturation at 95°C, followed by 35 amplification cycles, each consisting of a 30 second denaturation at 95°C, a 45 second annealing at 53°C, and a 1 minute extension at 68°C. These cycles were followed by a 5 minute extension at 68°C. Cycling conditions for the first round of trypanosome DNA amplification involved an initial 5 minute denaturation at 95°C, followed by 40 amplification cycles, each consisting of a 1 minute denaturation at 95°C, a 30 second annealing at 50°C, and a 1 minute extension at 72°C. These cycles were followed by a 10 minute extension at 65°C. The nested round for trypanosomes involved an initial 3 minute denaturation at 96°C, followed by 25 amplification cycles, each consisting of a 30 second denaturation at 96°C, a 1 minute annealing at 51°C, and a 30 second extension at 72°C. These cycles were followed by a 7 minute extension at 72°C. Cycling conditions for the initial round of *B. burgdorferi* DNA amplification involved an initial 4 minute denaturation at 88°C, followed by

40 amplification cycles, each consisting of a 1 minute denaturation at 88°C, a 2 minute

Table 1. Primers used to amplify hemoparasite DNA in bird blood, Roseau Co. MN, 2014.

Target Organism	Target Gene	Primer Name	5' – 3' Sequence	Amplicon Size
Haemosporidia	Cytochrome b	H332F (Initial)	GAGAATTATGGAGYGGATGGTG	380 bp
Haemosporidia	Cytochrome b	HAEMNR3 (Initial)	ATAGAAAGATAAGAAATACCATTC	380 bp
Plasmodium/ Haemoproteus	Cytochrome b	H350F (Nested)	GGTGTTTTAGATATATGCATGC	380 bp
Plasmodium/ Haemoproteus	Cytochrome b	HAEMR2 (Nested)	GCATTATCTGGATGTGATAATGGT	380 bp
Plasmodium/ Haemoproteus	Cytochrome b	FIFI (Sequencing)	GGGTCAATTGAGTTTCTGG	380 bp
Plasmodium/ Haemoproteus	Cytochrome b	F2R (Sequencing)	CTTTTTAAGGTTGGGTCACTT	380 bp
Leucocytozoon	Cytochrome b	L350F (Nested)	GGTGTTTTAGATACTTA	380 bp
Leucocytozoon	Cytochrome b	L890R (Nested)	TACAATATGTTGAGGTGTTTG	380 bp
Leucocytozoon	Cytochrome b	L545R (Sequencing)	ACAAATGAGTTTCTGGGGA	380 bp
Leucocytozoon	Cytochrome b	L825R (Sequencing)	GCAATTCCAAATAAACTTTGAA	380 bp
Trypanosoma	18s rRNA	Tryp763 (Initial)	CATATGCTTGTTTCAAGGAC	700 bp
Trypanosoma	18s rRNA	T870R (Initial)	TCCTTTGTTATCCCATGCTTTC	700 bp
Trypanosoma	18s rRNA	T100F (Nested)	ACGTAATCTGCCGCAAA	700 bp
Trypanosoma	18s rRNA	T800R (Nested)	GGCGCCCCCTGGCATGC	700 bp
Trypanosoma	18s rRNA	T300F (Sequencing)	GACTCAATTCATTCCGTG	700 bp
Trypanosoma	18s rRNA	T435R (Sequencing)	CTGCTGCCCTCCGTAGAA	700 bp
Borrelia burgdorferi	5s-23s rRNA	rrf-rrl1 (Initial)	CTGCGAGTTCGCGGGAGA	250 bp
Borrelia burgdorferi	5s-23s rRNA	rrf-rrl2 (Initial)	TCCTAGGCATTCACCATA	250 bp
Borrelia burgdorferi	5s-23s rRNA	Nested 1 (Nested)	GAGTAGGTTATTGCCAGGGTTTTATT	250 bp
Borrelia burgdorferi	5s-23s rRNA	Nested 2 (Nested)	TATTTTTATCTTCCATCTCTATTTTGCC	250 bp

annealing at 55°C, and a 1 minute extension at 72°C. These cycles were followed by a 10 minute extension at 72°C. The nested round for *B. burgdorferi* involved an initial 4 minute denaturation at 94°C, followed by 40 amplification cycles, each consisting of a 1 minute denaturation at 94°C,

a 1 minute annealing at 55°C, and a 1 minute extension at 72°C. These cycles were followed by a 10 minute extension at 72°C (Lane et al.; 2004Newman et al. 2015). Nested products were run on 1.25% agarose gel using electrophoresis. Positive samples were purified using ExoSap PCR clean-up enzymatic kit from Affimetrix (Santa Clara, CA). Purified samples were sequenced using ABI BigDye technology, ethanol precipitation and an ABI Prism 3100 automated capillary sequencer. Quality sequences were identified using Basic Local Alignment Search Tool (BLAST).

The sera were assayed for the presence of WNV antibodies by ELISA using the methods of Blitvich et al. (2003). Blitvich et al. (2003) describe the most effective assays for the detection of WNV serum antibodies were those performed with MAbs 3.1112G and 2B2. If a sample shows > 30% inhibition, then this is evidence of seropositivity against West Nile virus.

Phylogenetic Analysis

Phylogenetic analysis was performed on sequences obtained from haemosporidians and trypanosomes to determine if there were any specific host associations with the different groups of parasites. Sequences were trimmed and edited using Sequencher ver. 4.2 (GeneCodes Corp., Ann Arbor, MI) and aligned using BioEdit, version 7.0.1.The alignments were refined in MacClade, version 4. Bayesian inference (BI) was used to perform the phylogenetic analysis and was carried out in the MrBayes, version 2.01. Posterior probabilities were approximated over 5,000,000 generations for both data-sets, log-likelihood scores plotted and only the final 75% of trees were used to produce the consensus trees by setting the ‘‘burnin’’ parameters at 1,250,000 generations. The TIM2+G model for haemosporidians was used for the analysis based off of the results obtained from jModelTest, version 0.1.1 (ref), and the JC+G model was used for trypanosomes.

Results

A total of 110 birds were screened for haemoparasites and WNV seropositivity. A complete list of all the bird species screened and the prevalence of each infection is given in Table 2. A total of 77% of all birds screened had at least 1 infection (Table 3). Trypanosomes had the highest prevalence, infecting 58% of the birds screened. Though, the original prevalence was determined to be 40% when screened by microscopy. Molecular methods were determined to be more sensitive as they detected 20 infections that were missed using microscopy (X^2=7.28, p=0.007). The prevalence of the other infections are as follows: WNV 29%, *Plasmodium* 24%, *Leucocytozoon* 18%, *Haemoproteus* 19%, MF 9%, *B. burgdorferi* 5%.

Polyparasitism (multiple concurrent infections including WNV seropositivity) was discovered in 55% of the individuals screened (Table 3). Every gray catbird and white-throated sparrow that were screened were found to have multiple infections. Polyparasitism didn't seem to have an effect on the birds' health based on hematocrit measurements (p=0.7371). Mean hematocrit for infected birds was 51.2% (n=72, sd=0.0450) and for non-infected birds was 51.5% (n=20, sd=3.3930).

Phylogenetic analysis showed specific host relationships for *Plasmodium* and *Haemoproteus* groups, but *Leucocytozoon* and Trypanosomes didn't show any specificity (Figs. 4 and 5). One *Plasmodium* group was found to primarily infect robins and the only other bird that this group infected was a Swainson's thrush which belongs to the same family, Turdidae (Fig. 4). Another *Plasmodium* group primarily infected sparrows from the genus *Zonotrichia (*Fig. 4). Different groups of *Haemoproteus* infected only catbirds, only orioles, and primarily sparrows (Fig. 1).

Table 2. Prevalence of vector-borne hemoparasites and seropositivity to West Nile virus in birds collected in Roseau County, MN April – June, 2014.

Species	(n)	*Plasmodium*	*Haemoproteus*	*Leucocytozoon*	*Trypanosoma*	Microfilaria	*Borellia burgdorferi*	West Nile Virus
American Robin	28	57%	0%	4%	54%	18%	0%	43%
Gray Catbird	10	0%	50%	50%	100%	20%	0%	40%
Cedar Waxwing	6	0%	0%	17%	83%	17%	0%	50%
Eastern Pheobe	6	0%	17%	0%	17%	0%	0%	0%
Savannah Sparrow	6	0%	17%	0%	0%	0%	0%	17%
Yellow-bellied Sapsucker	6	0%	0%	0%	0%	0%	0%	33%
Baltimore Oriole	5	0%	100%	20%	100%	0%	20%	40%
Palm Warbler	5	20%	0%	0%	20%	0%	40%	0%
White-throated Sparrow	5	60%	40%	20%	100%	20%	0%	0%
Song Sparrow	4	50%	0%	25%	50%	0%	0%	50%
Dark-eyed Junco	3	0%	0%	0%	0%	0%	0%	0%
Swainson's Thrush	3	33%	67%	67%	67%	0%	33%	33%
American Goldfinch	2	0%	0%	0%	100%	0%	0%	0%
Blue Jay	2	0%	50%	100%	100%	0%	0%	0%
Brown-headed Cowbird	2	50%	0%	50%	100%	0%	0%	50%

Table 2 cont'd.

Species	(n)	*Plasmodium*	*Haemoproteus*	*Leucocytozoon*	*Trypanosoma*	Microfilaria	*Borellia burgdorferi*	West Nile Virus
Least Flycatcher	2	0%	0%	0%	100%	0%	0%	0%
Veery	2	0%	50%	50%	50%	0%	0%	0%
Warbling Vireo	2	0%	100%	50%	50%	0%	0%	0%
Alder Flycatcher	1	0%	0%	0%	100%	100%	0%	0%
Barn Swallow	1	0%	0%	0%	100%	0%	0%	100%
Black-capped Chickadee	1	100%	0%	100%	100%	0%	0%	0%
Brewer's Blackbird	1	0%	0%	0%	0%	0%	0%	100%
Brown Thrasher	1	0%	0%	100%	100%	0%	0%	0%
Clay-colored Sparrow	1	0%	100%	100%	0%	0%	0%	0%
Common Yellowthroat	1	0%	0%	0%	100%	0%	0%	0%
Gray-cheeked Thrush	1	0%	0%	0%	100%	0%	100%	0%
Red-eyed Vireo	1	0%	0%	0%	100%	0%	0%	100%
White-crowned Sparrow	1	100%	0%	0%	100%	0%	0%	0%

Table 3. Polyparasitism in species with greatest sample sizes collected in Roseau County, MN April – June, 2014.

Species	% with 1 or more infections	% with 2 or more concurrent infections	% with 3 or more concurrent infections	% with 4 or more concurrent infections
American Robin (28)	86%	57%	29%	4%
Gray Catbird (10)	100%	100%	50%	10%
Cedar Waxwing (6)	100%	33%	33%	0%
Savannah Sparrow (6)	33%	0%	0%	0%
YB Sapsucker (6)	33%	0%	0%	0%
Baltimore Oriole (5)	100%	100%	60%	20%
Palm Warbler (5)	40%	20%	0%	0%
Song Sparrow (5)	75%	75%	25%	0%
WT Sparrow (5)	100%	100%	20%	20%
All Birds (110)	77%	55%	25%	5%

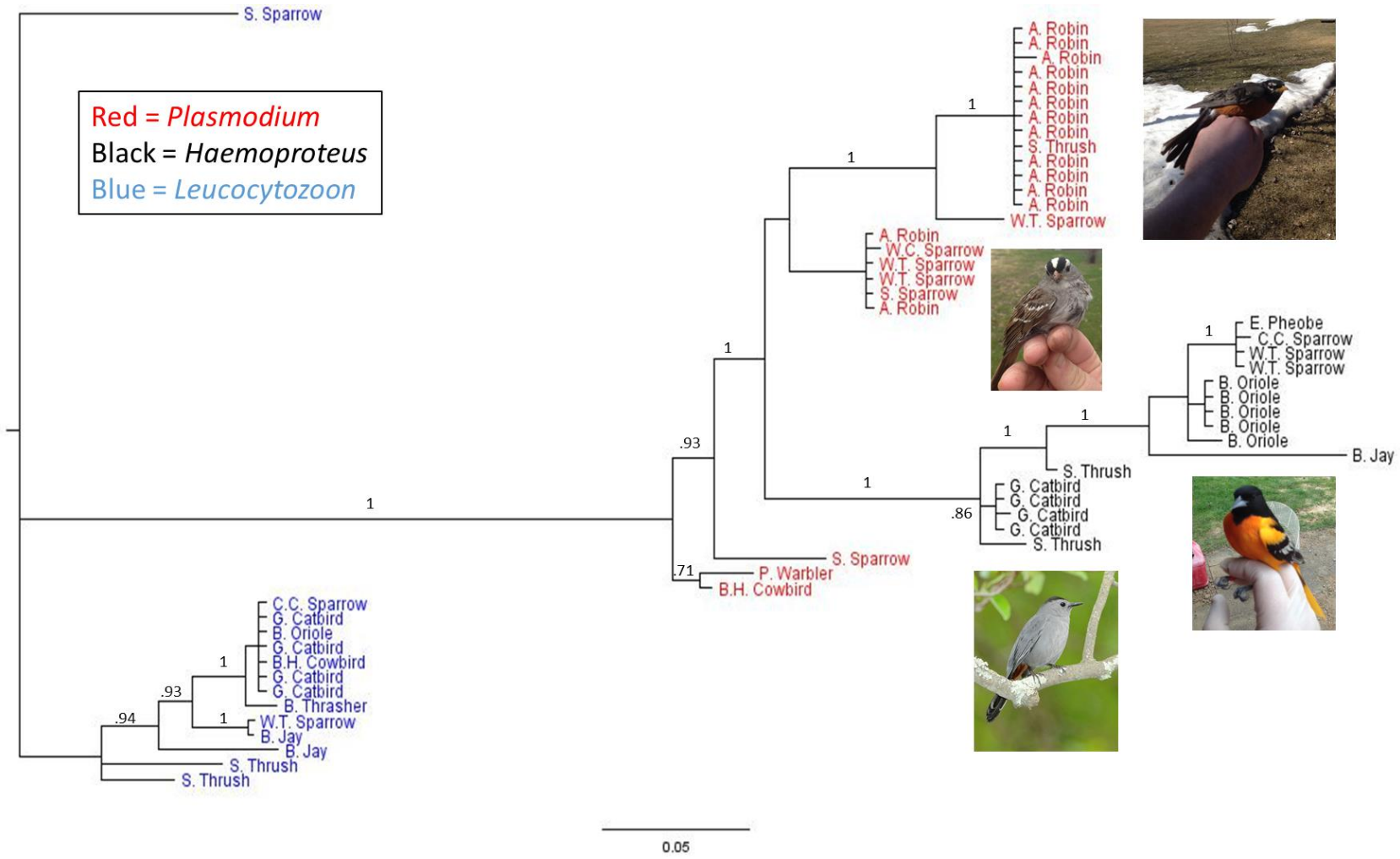

Figure 4. Phylogenetic tree of haemosporidians and the host they infect. The tree depicts a good host/parasite association for *Plasmodium* and *Haemoproteus*, but very little host association for *Leucocytozoon*.

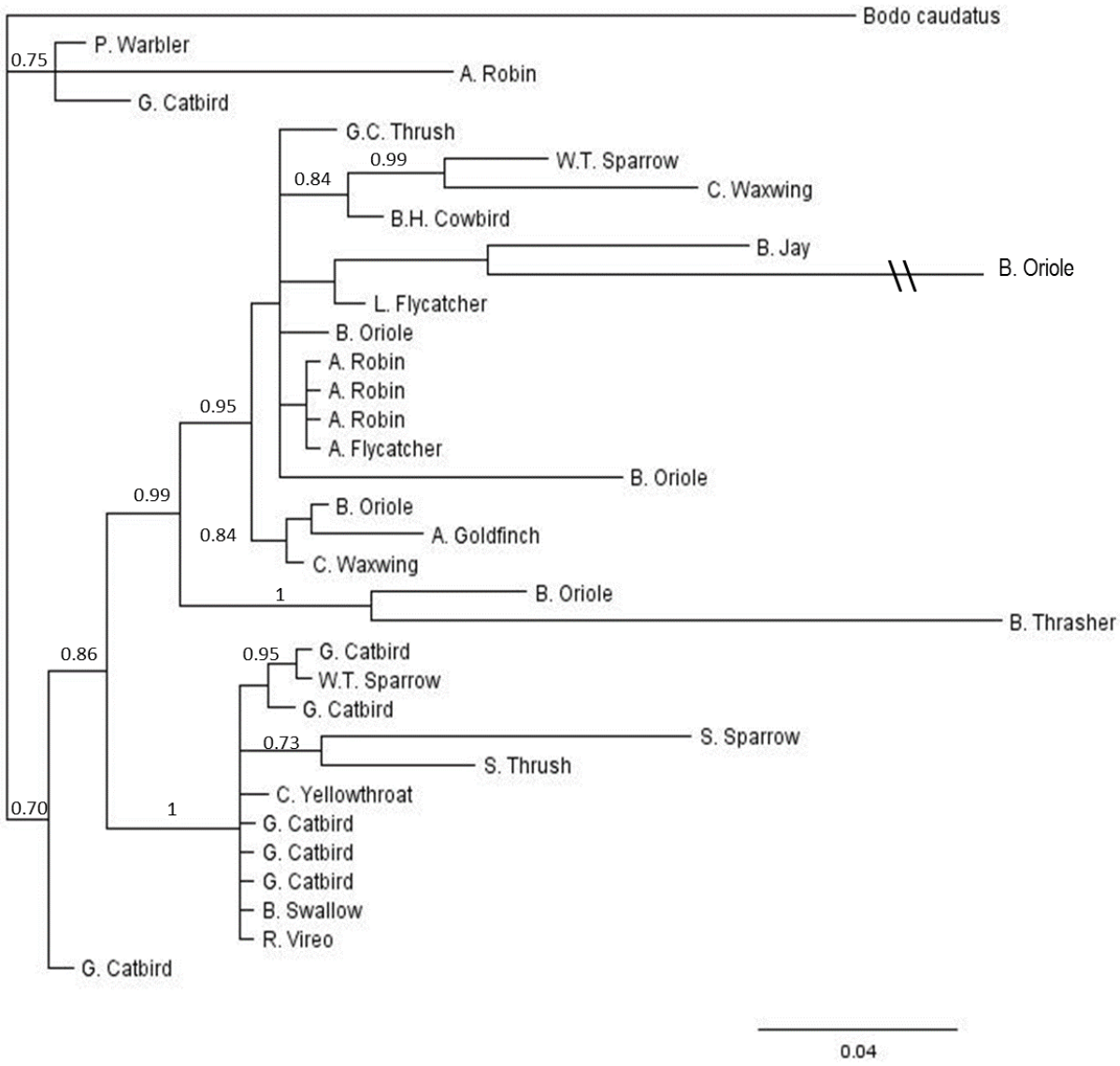

Figure 5. Phylogenetic tree of trypanosomes and the hosts they infect. The tree depicts little parasite/host association.

Discussion

My findings indicate a high prevalence of hemoparasite infection. The high prevalence may be attributed to the environment and the availability of competent vectors. Although no formal survey was undertaken to describe the local vector fauna, there are certainly abundant mosquito populations present that could serve as vectors for *Plasmodium* and potentially for *Trypanosoma* parasites. Black flies (vectors for *Leucocytozoon*) are not abundant nor a nuisance to humans, but they are known to be present (pers. obs.). The *Culicoides* (vectors for *Haemoproteus*) fauna are unknown. To further understand the ecology of these infections, a survey of the vector community would give further insight to possibility of local transmission. Three resident non-migratory birds (2 blue jays and 1 chickadee) were infected with *Leucocytozoon* suggesting the occurrence of local transmission. There is some evidence of local transmission of *Plasmodium* in juvenile bobolinks, but a vector survey would provide further information on the local transmission of other haemoparasites (Kvasager 2010).

Leucocytozoon infections were much more prevalent than observed in the neotropics (White et al. 1978). Overall *Leucocytozoon* prevalence for temperate species was 18.2% (n=110), compared to an overall prevalence of neotropical species of 1.8% (n=11,384). When comparing the prevalence of infection between migratory and non-migratory species, I found that prevalence is higher in non-migratory temperate birds (100%, n=3) and migratory neotropic birds (3.8%, n=477) (White et al. 1978). Prevalence for migratory temperate species was 16% (n=107) and for non-migratory neotropic species was 1.7% (n=10,907) (White et al. 1978).

Prevalence of infection varied among parasite and host species. Most species sampled had a high prevalence of one parasite or another, but yellow-bellied sapsuckers (n=6) and savannah sparrows (n=6) had very few infections. The ecology of each species may have

contributed to the low prevalence of infection. Sapsuckers are cavity nesters and may be exposed to fewer feeding vectors than a species that nests in the open. Savannah sparrows are a grassland species and were sampled in a forested habitat. This habit may not be suitable for the vectors that transmit haemoparasites to the species. It is not certain these are the reasons these species had so few infections due to the low sample size. More samples are needed to make a robust conclusion.

Trypanosome infections were much more prevalent when the same bird species were compared to a review done by Reiner et al. (1975). Prevalence for American robins was 54% (n=28) in my study compared to 12% (n=1323) in the Greiner et al. (1975) review. Cedar waxwing infection prevalence was 83% (n=6) compared to 25% (N=80) (Greiner et al. 1975). Baltimore oriole infection prevalence was 100% (n=6) compared to 47% (N=19) (Greiner et al. 1975). Eastern phoebe infection prevalence was 17% (n=6) compared to 0% (N=7) (Greiner et al. 1975). When comparing the four species in common, Greiner et al. (1975) found an overall infection prevalence of 13% (n=1429) and I found the prevalence to be 59% (n=46). There was a significant difference in the overall infection prevalence between the Greiner review and my study ($Chi^2=74.2$, $p<0.0001$). This raises the question of why is there such a difference between the infection prevalence of the same bird species between Greiner's review in 1975 and my study in 2014? I suspect it is due the sampling methods used in each study. Greiner data relied mostly on blood smears to detect trypanosomes in the blood and I used microcapillary tubes and PCR. Birds were first screened using the microcapillary method and I detected a 41% (n=46) prevalence. A second screening using PCR techniques detected 20 extra infections raising the prevalence to 59% (n=46). This data suggests that the microcapillary tube method detects a

significantly higher amount on trypanosome infections than blood smears ($Chi^2=29.8$, $p<0.0001$). When using PCR techniques, the difference becomes even greater ($Chi^2=75.3$, $p<0.0001$).

Parasite infection did not have a significant effect on the health of the hosts (hematocrit). Reports of diminished health in birds with infections are rare, but pathogenic infections do occur where parasites or mosquito vectors have been introduced to naïve bird populations (Warner 1968; Woodworth et al. 2005; Foster et al. 2007). This could reflect a long evolutionary relationship between the parasite and host. It may be difficult to actually detect the health effects caused by parasite infection due to the temporal variability of the parasitemia. There is a brief acute stage of infection when parasites appear in the blood at a high density and the hosts may suffer mortality from the high parasitemia (Atkinson and Van Riper 1991; Valkiunias 2005). All sampled birds may have survived this acute stage and developed adequate immune responses to the parasites.

The haemosporidians all show a different degree of host associations, while trypanosomes show no association at all (Fig. 4 and 5). Of the haemosporidians, *Haemoproteus* showed the strongest host association followed by *Plasmodium* and *Leucocytozoon.* One possibility for this difference is that *Leucocytozoon* may have a much broader host range than the other two haemosporidians. Haemosporidians may also show differences in host associations depending on the community of hosts that is sampled. Trypanosomes may be more promiscuous when it comes to their host's as shown by the lack of association in the data. It is possible that the gene (18S rRNA) used for my analysis is not phylogenetically informative. Utilizing the sequences from other genes may provide a much more informative analysis in which there may be some host associations.

Prevalence of *B. burgdorferi* infection was low, but song birds could still play a role in the spread of Lyme disease. Birds can freely travel great distances and I sample a few birds with juvenile *Ixodes scapularis* ticks feeding around their eyes even though I wasn't screening for exoparasites. Further studies on the ability of *B. burgdorferi* infected song birds to transmit the spirochetes is needed to better understand their role as a reservoir.

Prevalence and polyparasitism is high but without obvious health impacts to the hosts.

CHAPTER III

COMPARISON OF METHODS FOR QUANTIFYING MICROFILARIAE AND PREVALENCE OF MIDGUT PENETRATION

Introduction

Microfilariae (MF) are an early stage in the developmental cycle of some nematodes in the family Onchocercidae. Some MF show nocturnal periodicity (Vaughan et al. 2012). This is when the MF are only found in the bloodstream at night. It is thought that they enter the general circulation at peak feeding times of their arthropod host. This periodicity should increase the chance the microfilariae are ingested by the correct vector. There are 16 different genera of filarioid nematodes known to infect birds and the typical vectors for avian filarioids include lice (Phthiraptera) and flies (Diptera) (Bartlett 2008).

Simultaneous ingestion of microfilariae (MF) and arboviruses (viruses transmitted by arthropod vectors) by blood-feeding arthropods has been shown to significantly enhance the transmission of viruses compared to when the arthropod vector ingests an equal dose of the virus alone (Turell et al. 1984; Vaughan and Turell 1996; Vaughan et al. 1999). This mechanism has been termed microfilarial enhancement of arboviral transmission. This can occur when arthropod vectors consume a bloodmeal from a dually-infected vertebrate (i.e. concurrently microfilaremic and viremic). After ingestion, the MF penetrate the arthropod midgut which allows direct passage of the virus into the hemocoel. Once in the hemocoel, the virus can

disseminate and move into the salivary glands of the mosquito causing it to be infective for the virus (Turell et al. 1984). Thus the virus can pass the midgut barrier without having to invade the cells and replicate. Screening and quantifying microfilaremia of infected vertebrate hosts can be accomplished using several different methods.

Traditional methods of quantifying MF densities in host blood require counting the individual worms under a microscope and converting the count to a standard volume of blood (e.g. 20 µL). One method requires drawing blood into a heparinized capillary tube, spinning the tube in a hematocrit centrifuge, and counting the motile MF at the cell pack interface (Collins 1971). Mosquitoes can also be utilized to determine MF densities by allowing them to feed on a microfilaremic host, dissecting out the midgut, and examining the engorged midgut under a microscope to count the MF (Vaughan et al. 2012). To determine if MF have penetrated the midgut of engorged mosquitoes, the carcasses of the dissected mosquitoes were put on a slide and covered with a glass slip and examined for MF. If MF are observed in the carcass, it is assumed that midgut penetration has occurred (Vaughan et al. 2012).

This study investigated different methods to determine the best way to quantify host microfilaremia and the prevalence of midgut penetration in mosquitoes.

Objective

Conduct a comparison of methods including,

- Microfilarial densities in venous blood.
- Accurate counts of MF densities.
- Microfilarial densities in blood taken from the general circulation (i.e., venous blood) versus blood taken from dermal capillaries (mosquito bloodmeals).

- Prevalence of microfilarial penetration into the mosquito hemocoel.

Methods

Microfilaremia of Venous Blood

One way to quantify microfilaremia in venous blood is to collect host blood in a capillary tube, centrifuge the tubes for two minutes in a hematocrit centrifuge and then simply count the MF that are present at the interface of the red blood cells and serum (buffy coat). Each centrifuge microcapillary tube is examined using a compound microscope at 100X. By carefully rotating the tube while it is held in place on the microscope stage, it is possible to count all of the living MF concentrated at the buffy coat, as well as along the entire length of the tube minus the opaque cell pack. The question becomes; is the count representative of all the MF that are present in the blood sample? To answer this, I first counted the MF with the method described above, then emptied the entire contents of the tube into ca. 100 ul of lysis buffer (5% acetic acid) and prepared wet mounts of the sample. Wet mounts were examined at 100x magnification and MF were systematically counted. Thus, each sample had paired MF counts – one taken directly from the centrifuged blood still in the tube, and another taken from the blood after being expelled on to a microscope slide. A paired t-test was conducted to see if there was a significant difference between MF counts resulting from the two methods.

Obtaining Accurate Counts of MF in Blood

One way to quantify MF in blood is to spot blood on a microscope slide, place a cover slip on the blood, and count the living MF. Seeing the MF often depends on detecting their writhing movements, especially in thick preparations. A second way to quantify MF in blood is

to first lyse the blood cells with a 5% acetic acid (ca. apple cider vinegar) prior to making the wet mount. It lyses the erythrocytes and clarifies the preparation but also kills the MF. Another advantage to the acetic acid is that the samples are pickled and can be stored at room temperature for up to a week. The question becomes; which of these two methods is more accurate? To answer this, I fed *C. pipiens* mosquitoes on a microfilaremic grackle using the same methods stated earlier. I chose to use mosquito blood meals as the source of blood because I could feed many mosquitoes on a single bird and, since each mosquito was a replicate, I could get more replicates per bird than I could by bleeding birds. The engorged mosquitoes were split into two equal groups. The midguts were dissected out of the mosquitoes, midguts from the first group (n=20) were put in 20 µL of MEM media and midguts from the second group (n=30) were put in 25 µL of 5% acetic acid solution. The midgut solutions were then placed under cover slips on slides and observed at 100X magnification to count MF. Means of the counts were calculated and the means of the two groups were compared using a two sample t-test.

Comparing MF Densities, Venous Blood vs Capillary Blood

Microfilaremic common grackles (*Quiscalus quiscula)* were used for comparing methods of MF density quantification. The birds were housed in an aviary at the University of North Dakota and kept on a 12 hour light/dark cycle. At night, blood was collected from the brachial vein in heparinized capillary tubes immediately before birds were anesthetized with a mixture of ketamine (20 mg/kg intramuscular [IM]) and xylazine (4mg/kg IM) injected with a 27 gauge needle into the pectoral muscles. Then *C. pipiens* mosquitoes were allowed to feed on them for 45 minutes in enclosed 1 m square mesh cages. Densities for the general circulation were determined by spinning the capillary tubes in a hematocrit centrifuge to separate the cells and serum. The tubes were then placed under a microscope to count the motile MF at the plasma cell

pack interface (Collins 1971). Densities for dermal capillaries were determined by counting MF ingested by the mosquitoes. Engorged mosquitoes were collected with an aspirator and put in holding cages to be incubated at 12^0 C until they could be dissected. Mosquitoes were dissected the following day and the midguts were placed into a 25 µL 5% solution of acetic acid to lyse the red blood cells. The acetic acid solutions containing the lysed contents of the midguts were placed on slides with a cover slip and viewed under a microscope at 100X magnification. The geometric mean was calculated from the counts of MF ingested by mosquitoes. The tube counts and geometric means for each bird were normalized to number of MF/20 µL and a t-test was performed to determine if there was any statistical difference between the mean number of MF counted for each method.

Prevalence of Midgut Penetration

The carcasses of the mosquitoes from the MF ingestion comparison experiment were used to compare the two methods. Only carcasses that had clean midgut dissections were used to ensure they were not contaminated by rupturing the midgut. Twenty of the carcasses were placed on a slide, submersed in MEM media and covered with a cover slip. The carcass was examined at 100X magnification to observe MF in the carcass. If a MF was observed in the carcass, it was assumed that it had penetrated the midgut. Thirty of the carcasses were ground up and DNA was extracted using the method explained by Tkach and Pawlowski (1999). Extracted DNA was tested using PCR techniques. Three microliters of DNA extract, 8.5 µL of purified water, .5 µL of each primer and 12.5 µL of oneTaq Quick-Load 2X MM with standard buffer (New England Biolabs, Ipswich, MA) were used in the PCR reactions. The cycling conditions for protocol involved an initial 2 minute denaturation at 95°C, followed by 40 amplification cycles, each consisting of a 45 second denaturation at 95°C, a 30 second annealing at 55°C, and

a 45 second extension at 72°C. These cycles were followed by a 7 minute extension at 72°C. Products were run on 1.25% agarose gel using electrophoresis. The proportion of midgut penetration detected by each method was compared using a Chi squared test.

Results

Counting MF directly from the centrifuged blood (total elapsed time ca. 15 minutes per sample) was more efficient than emptying the blood into lysis buffer and counting MF on a wet mount (total elapsed time ca. 2 to 3 hours per sample). There was no significant difference in MF counts obtained from centrifuged blood still in the tube versus blood expelled on to a microscope slide (paired t-test, T=-0.64, df=13, p=0.5334).

Acetic acid lysis is a superior method, when compared to MEM media wet mounts, to quantify the number of MF ingested by a mosquito during a blood feed. Mosquitoes were fed on the same bird at the same time and blood meals were processed using either acetic acid lysis or no lysis. The acetic acid lysis yielded significantly higher MF counts, when compared to MEM media wet mounts (two-sample t-test; t=3.53, df=48, p=0.0009). The means and standard deviations of MF counts for the lysis method and the no lysis method were 112$\pm$64.3 (n=30) and 57.6$\pm$30 (n=20) respectively. In terms of processing time, both methods were relatively equal but the acetic acid method had the advantage in that samples were preserved and did not have to be processed and counted the same day.

The mosquito feeding method provided significantly higher MF counts than the capillary tube method for quantifying MF densities in their host (two-sample t-test; t=-3.76, df=28.5, p=0.008). Means and standard deviations of MF per unit volume (i.e. 20 µL) for capillary tube

samples versus mosquito midgut contents were 10.5±66.8 (n=29) and 97.5±714 (n=29) respectively.

There was no significant difference between microscopy and PCR methods in determining the percentage of mosquito midguts penetrated by ingested grackle MF (Chi^2=0.18, p=0.67). Microscopy detected a 10% rate of penetration (n=20); and PCR detected a 6.7% rate of penetration (n=30).

Discussion

There is no difference in MF densities whether you spin the hematocrit tube and count MF at the buffy coat or if you empty the contents into acetic acid and prepare wet mounts. The major difference is the amount of time it takes to count a sample. Emptying the contents of the tube and preparing a wet mount to count MF can take 2-3 hour per sample. Quickly spinning the tube for a few minutes and counting the Mf at the buffy coat takes 15-20 minutes. It is much more efficient to spin the tube and observe the MF at the buffy coat. The centrifuge method is great for screening a large number of individuals.

Microfilariae were detected at significantly higher densities in mosquito midguts using the acetic acid method. The MEM method didn't provide a clear enough view of the MF in the blood to make an accurate count. Utilizing acetic acid to lyse the erythrocytes cleared the field of view so that a significantly higher amount of MF could be observed. Both methods took relatively the same amount of time to screen a sample, but the main advantage of the acetic acid method was you didn't have to rely on movement of the MF to observe them. The acetic acid killed and preserved the MF so the samples could be kept at room temperature for weeks before counting.

During peak microfilaremia, MF are more densely aggregated in the dermal capillaries (where mosquitoes feed) than in the general circulation (brachial vein). It is possible the MF congregate in capillary beds at peak times where their arthropod vector prefers to feed. This would increase the likelihood that they would be ingested and transmitted to a new vertebrate host. Counting MF in capillary tubes is still an efficient method to screen for MF or to make quick comparisons of MF densities.

Both methods were equally successful in determining the prevalence of midgut penetration in mosquitoes. This could be due to the low frequency of penetration by the MF. Even though both methods were equally as sensitive, using microscopy to observe MF in the carcasses of mosquitoes is a tedious and time consuming task. The PCR method, even though there are costs for reagents, allows you to screen many samples at once. Other tasks may be completed when waiting for the thermocycler to finish amplifying the DNA.

CHAPTER IV

BEHAVIORAL ASPECTS OF MICROFILARIAE

Introduction

Microfilariae (MF) are an early stage in the developmental cycle of some nematodes in the family Onchocercidae. The adult worms live in the tissue of their definitive host, and instead of laying eggs they release MF directly into the bloodstream (Anderson 2000). From the blood stream, MF are ingested by blood-sucking arthropods, where the MF quickly penetrate the arthropod midgut and pass into the hemocoel. In the hemocoel, the MF develop and go through several molts to the infective L3 stage. When the larvae reach the infective L3 stage, they migrate to the head and exit down the mouthparts and into their new host when the vector feeds.

Some MF are known to show nocturnal periodicity (Vaughan et al. 2012). This is when the MF are only found in the bloodstream at night. It is thought that they enter the general circulation at peak feeding times of their arthropod host. This periodicity should increase the chance the microfilariae are ingested by the correct vector.

The mechanism that drives nocturnal periodicity is not completely understood. There is a correlation between the concentration of melatonin in a host's blood and peak microfilaremia for that species, but measurements were never taken from the same individual. This study examined

the behavioral aspects of robin MF and the effects of a melatonin antagonist and a melatonin agonist on the nocturnal periodicity of MF from Common Grackles (*Quiscalus quiscula).*

Objective

- Determine the nocturnal periodicity of microfilariae found in the circulatory system of American Robins (*Turdus migratorius*, hereafter robins), and the ability of microfilariae to penetrate the midgut of *Culex pipiens* mosquitoes fed on microfilaremic robins.
- Determine the effect of melatonin agonist (i.e., Ramelteon) and melatonin antagonist (i.e., Luzindole) on altering the nocturnal periodicity of microfilariae in the Common Grackle.

Methods

Nocturnal Periodicity of Robin MF

Microfilaremic robins were captured during the infection prevalence survey (Chapter 2) and kept in the outdoor aviary at the University of North Dakota. Nocturnal periodicity was determined by periodically bleeding 4 robins using the same brachial vein method described earlier. Blood was extruded into 100 µL of 5% acetic acid solution to lyse the erythrocytes and then was placed on a glass slide and covered with a glass slip to count MF under 100X magnification. Birds were bled once from each wing daily over a three day period to allow for recovery between bleeds. Bleed times were 8 PM and 10 PM for day 1, 12 AM and 2 AM for day 2, and 4 AM and 6AM for day 3. A bar graph was created to show the MF densities at each time frame.

Ability of Microfilariae to Penetrate the Midgut of *Culex pipiens* Mosquitoes

To determine if robin MF could penetrate the midgut of *C. pipiens* mosquitoes, the PCR method was used to determine the prevalence of mosquito midguts penetrated by robin MF (Chapter 3). The robins used in this study were the same subjects used in the nocturnal periodicity study. Mosquitoes were allowed to feed on 3 microfilaremic robins. The birds were anesthetized with a mixture of ketamine (20 mg/kg intramuscular [IM]) and xylazine (4mg/kg IM) injected with a 27 gauge needle into the pectoral muscles. Then *C. pipiens* mosquitoes were allowed to feed on them for 45 minutes in enclosed 1 m square mesh cages. Mosquitoes were dissected and ingested MF were counted using the acetic acid method. Mosquito carcasses were then screened for MF using the same protocols stated earlier for molecular detection of MF (Chapter 3). Only carcasses that had clean midgut dissections were used to ensure they were not contaminated by rupturing the midgut. Any carcasses that were PCR positive for MF were assumed to have exhibited midgut penetration.

Luzindole Study

This experiment used Luzindole to try disrupt the nocturnal periodicity of *C. quiscali* MF infecting 4 grackles. Densities are expected to decrease during nighttime hours after treatment with Luzindole. Prior to treatment, a baseline measurement was taken at midnight to determine the MF densities of each bird. Birds were anesthetized with a mixture of ketamine (20 mg/kg intramuscular [IM]) and xylazine (4mg/kg IM) injected with a 27 gauge needle into the pectoral muscles. At 2:00 AM, *C. pipiens* mosquitoes were allowed to feed on them for 45 minutes in enclosed 1 m square mesh cages. Engorged mosquitoes were collected with an aspirator and put in holding cages to be incubated at 12^0 C until they could be dissected. Mosquitoes were dissected the following day and the midguts were placed into a 25 µL 5% solution of acetic acid to lyse the red blood cells. The acetic acid solutions containing the lysed contents of the midguts

were placed on slides with a cover slip and viewed under a microscope at 100X magnification. The geometric mean was calculated from the counts of MF ingested by mosquitoes. The counts and means for each bird were normalized to number of MF/20 µL. The mosquito feed was repeated following the treatment of two grackles with Luzindole and two grackles with just DMSO (control). Five mg of Luzindole was added to 400 µL of DMSO and the birds received 25 mg/ kg body weight of the solution (Vachharajani et al. 2003). Intramuscular injections were administered in the breast muscle at dusk and midnight. Means from pre- and post-treatment groups were compared using a two-sample t-test. Wing bleed counts were also taken before and after treatment. These counts were compared using a paired t-test.

Ramelteon Study

This study used Ramelteon to try disrupt the nocturnal periodicity of *C. quiscali* MF infecting grackles and was repeated with a different vehicle for Ramelteon. Daytime densities are expected to increase following treatment with Ramelteon. Prior to treatment, a baseline measurement was taken at 1:15 AM and 3:30 PM to determine the MF densities of each bird and to ensure the MF were still on cycle. The same methods as the luzindole study were used to feed mosquitoes on the grackles and quantify the number of MF ingested. The only difference is the feeds took place between 3:30 and 4:00 PM. The mosquito feed was repeated following the treatment of four grackles with Ramelteon and three grackles with just DMSO (control). Two mg of Ramelteon was added to 1000 µL of DMSO and the birds received 0.4 mg/ kg body weight of the solution (Karim et al. 2006). It was later found that DMSO may have had a physiological effect on the birds and MF, so the study was repeated with water as the vehicle for Ramelteon. Means were compared using two-sample t-tests. Wing bleed counts were also taken before and after treatment. These counts were compared using a paired t-test.

Results

Robin MF exhibited peak densities in the blood at 2:00 AM and were hardly detectable at 8:00 PM and 6:00 AM (Fig. 6). This is clear evidence that this species of MF exhibits the pattern of nocturnal periodicity even though each bird was characterized with different peak densities. The lack of a peak density for bird one was due to the low microfilaremia of that individual. Bird one was never observed to have more than 2 MF/20 µL at any time during the study. The PCR method was utilized to determine the ability of robin MF to penetrate the midgut

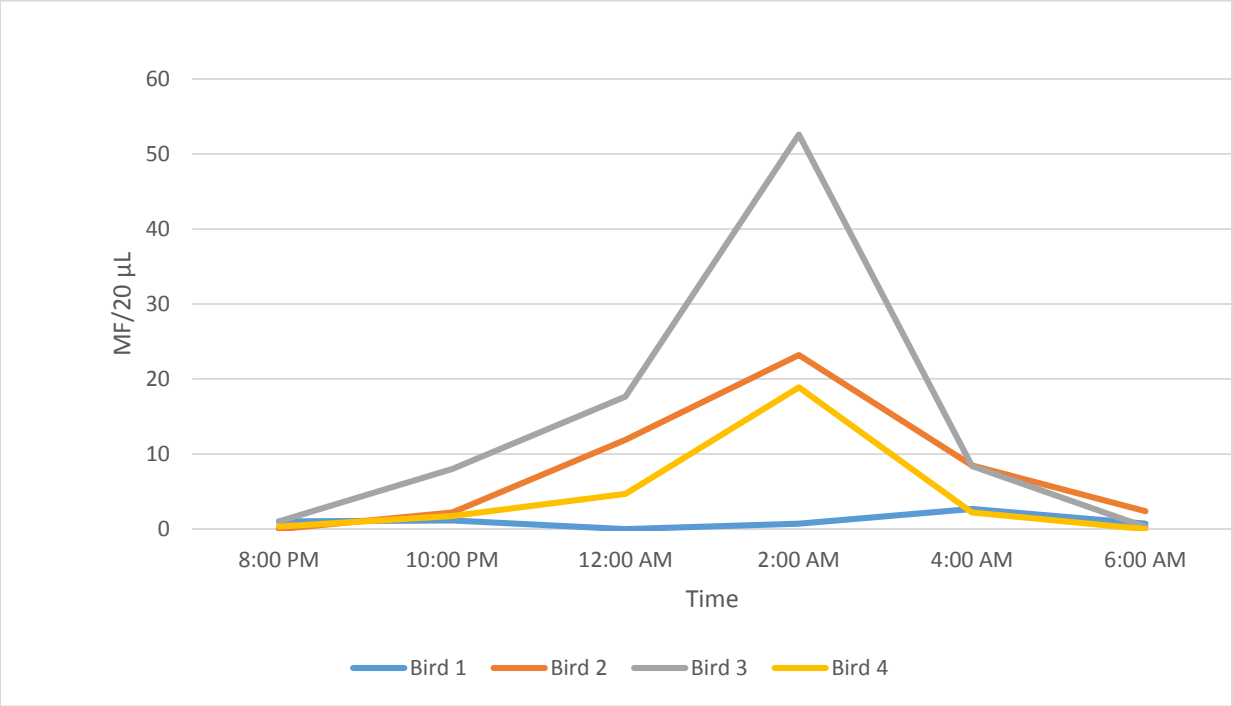

Figure 6. Line chart showing nocturnal periodicity of MF found in the circulatory system of American Robins. At each time frame, four robins were bled and the number of MF/20 µL were estimated.

of *C. pipiens* mosquitoes. Gel electrophoresis did not provide any positive bands for MF DNA in the carcasses of the 102 mosquitoes tested from three different microfilaremic robins. It appears that robin MF are unable to penetrate the midgut of *C. pipiens* mosquitoes. There were no significant differences between the pre-treatment densities of MF ingested by mosquitoes and the post-Luzindole treatment densities (bird17-t=-0.24, df=40, p=0.815; bird68-t=0.22,df=10.1,

p=0.831). A significant difference was detected between pre- and post-treatment densities for control bird 37 (t=-2.79, df=31.5, p=0.009). Control bird 53 also experienced a post-treatment increase in MF densities but, the increase was insignificant (t=-1.25, df=31, p=0.220) (Table 4).

Table 4. Results from Luzindole study using mosquitoes to quantify MF densities. Data presented is the mean number of MF ingested with the standard deviation in parentheses.

Treatment	Bird No.	Pre-treatment (NIGHT)	Post-treatment (NIGHT)
Luzindole	17	92.6 (63.7)	103.0 (60.7)
Luzindole	68	163.4 (307.5)	190.0 (162.1)
Control (DMSO)	53	300.4 (270.3)	411.1 (227.0)
Control (DMSO)	37	81.1 (61.5)	150.5 (101.6)

Table 5. Results from Luzindole study using wing bleeds to quantify MF densities.

		Ratio of Night [MF]/Dusk [MF]		
Treatment	Bird No.	Pre-treatment (DUSK)	Pre-treatment (NIGHT)	Post-treatment (NIGHT)
Luzindole	17	10.4	46.9	5.2
Luzindole	68	3.9	43.9	1.9
Control (DMSO)	53	7.1	20.5	24.0
Control (DMSO)	37	24.0	38.4	21.0

A significant difference between the night-to-dusk ratios were observed one day prior to treatment when compared to those observed two hours after the second treatment (Post-treatment NIGHT) in the Luzindole-treated birds (bird no. 17 and 68) (Table 5, paired t-test, t=-279.0, df=1,

Table 6. Results from Ramelteon 1 study using mosquitoes to quantify MF densities. Data presented is the mean number of MF ingested with the standard deviation in parentheses.

Treatment	Bird No.	Pre-treatment (DAY)	Post-treatment (DAY)
Ramelteon	2	1.8(1.5)	52.8(31.4)
Ramelteon	11	0.1 (0.3)	33.1 (23.1)
Ramelteon	15	0.4 (1.8)	39.0 (41.2)
Ramelteon	37	3.0 (5.3)	3.7 (4.4)
Control (DMSO)	17	0.3 (0.5)	49.5 (29.7)
Control (DMSO)	53	5.3 (4.7)	51.4 (14.9)
Control (DMSO)	68	7.7 (4.6)	63.8 (36.4)

Table 7. Results from Ramelteon 1 study using wing bleeds to quantify MF densities.

		Ratio of Day [MF]/Night [MF]		
Treatment	Bird No.	Pre-treatment (NIGHT)	Pre-treatment (DAY)	Post-treatment (DAY)
Ramelteon	2	2.7	0.33	0.15
Ramelteon	11	63.0	0.01	0
Ramelteon	15	8.0	0.15	0.11
Ramelteon	37	2.6	0.54	0.27
Control (DMSO)	17	29.7	0.10	0.04
Control (DMSO)	26	60.0	0	0
Control (DMSO)	53	44.0	0.28	0.17
Control (DMSO)	68	22.5	0.24	0.31

Table 8. Results from Ramelteon 2 study using mosquitoes to quantify MF densities. Data presented is the mean number of MF ingested with the standard deviation in parentheses.

Treatment	Bird No.	Pre-treatment (DAY)	Post-treatment (DAY)
Ramelteon	11	0.1 (0.3)	230.4 (119.2)
Ramelteon	15	0.4 (1.8)	30.7 (16.8)
Ramelteon	53	5.3 (4.7)	377.1 (193.7)
Control (water)	17	0.3 (0.5)	51.0 (23.8)
Control (water)	37	3.0 (5.3)	44.7 (44.6)
Control (water)	68	7.7 (4.6)	122.5 (114.2)

Table 9. Results from Ramelteon 2 study using wing bleeds to quantify MF densities.

		Ratio of Day [MF]/Night [MF]		
Treatment	Bird No.	Pre-treatment (NIGHT)	Pre-treatment (DAY)	Post-treatment (DAY)
Ramelteon	11	63.0	0.01	0.02
Ramelteon	15	8.0	0	0.26
Ramelteon	23	119.7	0	7.3
Ramelteon	30	116.5	0.01	0.01
Ramelteon	53	44.0	0.08	0.03
Control (water)	17	29.7	0.06	0
Control (water)	26	60.0	0	0
Control (water)	37	2.6	0.15	0.13
Control (water)	68	22.5	0.43	0.22

p=0.002). No such difference was observed in the control treated birds (bird no. 53 and 37) (Table 5, paired t-test, t=-0.67, df=1, p=0.67). The mosquito feed trials for the Ramelteon studies did not provide any credible data because the control groups were also stimulated to

appear in the peripheral circulation during the day (tables 6 and 8). No significant difference was observed in the Ramelteon treated birds in experiment 1 (bird no. 2, 11, 15, and 37) (Table 7, paired t-test, t=-2.05, df=3, p=0.13). Likewise, no significant difference was observed in the Ramelteon treated birds in experiment 2 (bird no. 11, 15, 23, 30, and 53) (Table 9, paired t-test, t=1.04, df=4, p=0.36).

Discussion

The MF of the American robin exhibited nocturnal periodicity which is common for species of avian MF. The arthropod vector for this species of MF must prefer to feed between dusk and dawn with peak feeding time occurring between 12 AM and 2 AM.

Robin MF failed to penetrate the midgut barrier of *Culex pipiens* mosquitoes according to results from PCR and gel electrophoresis. This would indicate that this species of MF does not play a role in the enhanced transmission of arboviruses.

Nocturnal periodicity is a typical trait of avian MF, but it is uncertain what the physiological basis for this behavior is. I have evidence that a melatonin agonist (Ramelteon) can induce MF to reverse their periodicity, but slight changes in periodicity were observed in the control group as well. Both experiments that used DMSO as a vehicle for the anesthesia exhibited and increase in MF densities for the control groups. It is possible that the difference was caused by a reaction to the DMSO vehicle. In the second Ramelteon experiment, water was used as a vehicle to prevent a reaction from the control group. Increases in MF densities were again observed for the control group as well as significant increases in the treatment group. This indicated something else besides the DMSO was causing the MF to be off cycle. A later experiment showed that the ketamine:xylazine anesthesia stimulated the MF to appear in the peripheral circulation during the day. Five microfilaremic grackles housed in the outdoor aviary

were bled at 2:30 PM and had microfilaremias ranging from 0-3 MF per 20 µL of blood. Forty-five minutes after being injected with ketamine:xylazine anesthesia, a second blood draw was taken at 3:15 PM the same day and revealed microfilaremias ranging from 108-352 MF per 20 µL of blood. For this reason, wing bleeds were used to determine the effects of the drugs on the behavior of the MF, because anesthesia complicated matters and was not necessary to use it for wing bleeds. Reliance on wing bleed counts led to much smaller sample sizes for the data, but it was necessary because the mosquito data were spurious due to drug interactions causing abnormal behavior in the MF.

Leading up to the experiments, extra microfilaremic grackles were captured to increase the sample size. This caused overcrowding in the aviary where the grackles were housed. It is possible the situation stressed the birds and altered their circadian rhythm which in turn altered the cycle of the MF. Further studies are necessary to determine if melatonin is the main regulating factor for nocturnal periodicity in avian MF.

To determine how much of a role melatonin plays in regulating nocturnal periodicity, further studies must be done. One such study would be to cut out the pineal gland of the birds and observe what effect that may have on the periodicity of the MF.

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