January 2015

# Prevalence, Statistical Trends And Phylogenetics Of Blood Parasites (haemosporidia: Haemoproteus, Plasmodium And Leucocytozoon) In Songbird Passerines From Grasslands Of Northwest Minnesota

Danielle Kvasager

# PREVALENCE, STATISTICAL TRENDS AND PHYLOGENETICS OF BLOOD PARASITES (HAEMOSPORIDIA: *HAEMOPROTEUS*, *PLASMODIUM* AND *LEUCOCYTOZOON*) IN SONGBIRD PASSERINES FROM GRASSLANDS OF NORTHWEST MINNESOTA

by

Danielle Kay Kvasager
Master of Science, University of North Dakota, 2015

A Thesis

Submitted to the Graduate Faculty

of the

University of North Dakota

in partial fulfillment of the requirements

for the degree of

Master of Science

Grand Forks, North Dakota
December
2015

This thesis, submitted by Danielle Kvasager 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

Vasyl Tkach

Rebecca Simmons

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

Wayne Swisher
Dean of the School of Graduate Studies

November 30, 2015
Date

## PERMISSION

Title Prevalence, Statistical Trends and Phylogenetics of Blood Parasites (Haemosporidia: *Haemoproteus*, *Plasmodium* and *Leucocytozoon*) in Songbird Passerines from Grasslands of northwest Minnesota

Department Biology

Degree Master of Science

In presenting this thesis in partial fulfillment of the requirements for a graduate degree from the University of North Dakota, I agree that the library of this University shall make it freely available for inspection. I further agree that permission for extensive copying for scholarly purposes may be granted by the professor who supervised my thesis work or, in his absence, by the Chairperson of the department or the dean of the School of Graduate Studies. It is understood that any copying or publication or other use of this thesis or part thereof for financial gain shall not be allowed without my written permission. It is also understood that due recognition shall be given to me and to the University of North Dakota in any scholarly use which may be made of any material in my thesis.

Danielle Kvasager
November 10, 2015

## TABLE OF CONTENTS

## LIST OF FIGURES

## LIST OF TABLES

## ABSTRACT

Passerine birds that primarily use grassland habitats are rarely the focus of a parasite study. With many rapidly declining bird populations that breed at even faster decreasing grassland habitat, it is important to know the potential risks to the birds posed by blood parasites. During the breeding seasons of 2009-2011, 150 samples from 148 individual birds (fourteen species) were collected from five grassland sites in northwest Minnesota, USA and surveyed for blood parasites using microscopy and molecular methods. Eighty-five (56.67%) of the 150 samples were infected with at least one of three haemosporidian genera: *Haemoproteus*, *Plasmodium* and *Leucocytozoon*. Seventy (46.67%) of the 150 samples were infected with either *Haemoproteus* or *Plasmodium* (fourteen infections were *Haemoproteus*, forty were *Plasmodium* and sixteen were undetermined due to dual infections or lack of sequences) and 41 samples (27.33%) were infected with *Leucocytozoon*, for a total of 111 infections. *Plasmodium* infections in two juvenile bobolinks provided evidence of active transmission within the study area.

*Haemoproteus*/*Plasmodium* prevalence was significantly higher in May and June than in later collection months (July-Sept.) and dual infections were significantly higher in June compared with other sampling months. Of the three most frequently collected bird species, clay-colored sparrows (*Spizella pallida*) had a significantly greater prevalence of *Haemoproteus* infections than savannah sparrows (*Passerculus sandwichensis*) and bobolinks (*Dolichonyx oryzivorus*). Only bobolinks were classified based on sex and/or age and adult males had

significantly more *Leucocytozoon* and dual infections than adult females or juveniles. Parasite prevalence did not differ significantly between study sites or years.

Phylogenetic reconstructions based on Maximum Likelihood and Bayesian analyses produced three major clades, corresponding to the three haemosporidian genera. Bird host species were well mixed within the trees, indicating that infective vectors fed on bird species opportunistically rather than selectively and that the Haemosporidia were generalists, capable of infecting a wide range of the sampled bird species.

# CHAPTER I

## PREVALENCE OF BLOOD PARASITES (HAEMOSPORIDIA: *HAEMOPROTEUS*, *PLASMODIUM*, AND *LEUCOCYTOZOON*) IN SONGBIRD PASSERINES FROM GRASSLANDS OF NORTHWEST MINNESOTA

### Introduction

*Haemoproteus*, *Plasmodium* and *Leucocytozoon* are closely related genera of single celled, vector-borne organisms. Along with several others, these three genera are found within the Order Haemosporida (Phylum Apicomplexa). Haemosporidia have been found on every continent except Antarctica [1, 2, 3, 4, 5] and in nearly every country where samples have been collected; they are also found in every class of vertebrate animals (mammals, birds, reptiles and amphibians) except fish [6]. Prevalence (proportion of infected individuals) studies help to define naturally occurring compositions of Haemosporidia inhabiting animals. These studies are the first step toward understanding these parasites and the impact they have on their hosts [7].

Though Charles Laveran was the first person to see *Plasmodium* in humans in 1880, it was V. Y. Danilewsky who realized this organism was actually a parasite. Danilewsky laid the foundation for current parasitology in avian malaria by publishing a small paper called 'About blood parasites (Haematozoa)' in 1884 [6]. Ross [8, 9] added a missing piece of the malaria lifecycle puzzle by discovering the role of mosquitoes for *Plasmodium* transmission. In the many years since these discoveries, hundreds of surveys have accumulated into what has been become a vast (yet incomplete) knowledge base about Haemosporidia in nature.

Hundreds of species of *Haemoproteus*, *Plasmodium* and *Leucocytozoon* are now known to parasitize birds. *Haemoproteus* is perhaps the least pathogenic of the three genera in wild birds

[10, 11, 4] with a few exceptions, mainly in captive or non-adapted birds [12, 13, 14, 15, 16, 17]. Most *Haemoproteus* species are transmitted by biting midges (Diptera: Ceratopogonidae), though some are transmitted by hippoboscid flies (Diptera: Hippoboscidae). *Plasmodium* is a well-recognized genus, commonly called avian malaria in birds [18] and is transmitted to by mosquitoes (Dipera: Culicidae). *Leucocytozoon* vertebrate hosts are limited to birds, and this genera is transmitted by black flies (Diptera: Simuliidae) [6]. Though each haemosporidan genus infects only one or two families of insect vectors, a single bird may attract multiple families of insect vectors; hence there is the potential for a bird to be infected by more than one haemosporidian genus. Also, a single species of parasite can infect multiple families of birds.

It is extremely important to know the parasites present in bird populations to allow for the detection of new avian parasites. Discovery of novel parasites is especially important for birds in declining habitats and for populations of birds on islands, where the avifauna are exposed to fewer pathogens than mainland populations. For example, the mosquito, *Culex quinquefasciatus*, was introduced to the Hawaiian Islands in the 1800s and eventually led to the emergence of *P. relictum* in previously unexposed native avifauna. This introduction was implicated as the cause of severe decline, even extinction, for several bird species on the islands [19, 20]. Levin et al. [21] also detailed the recent exposure of the endangered Galapagos penguin (*Spheniscus mendiculus*) to an avian *Plasmodium* lineage that is a close relative to *Plasmodium* lineages that are pathogenic for captive penguins. The suspected cause is the recent introduction of *C. quinquefasciatus* to the Galapagos Islands. *Leucocytozoon* may also cause severe symptoms when they are introduced to new wild avian hosts [22, 23, 6].

**Haemosporidian Life Cycle**

Confirmation of active transmission of these three haemosporidian genera in birds at a geographic location involves discovering parasites in nestlings, fledglings or juveniles that have not yet migrated [24] or in non-migratory birds. Active transmission requires three components to be present: the parasite, a competent dipteran vector and a competent bird.

An infective dipteran transmits the parasites as sporozoites found in their salivary glands into every bird it takes a blood meal from. Each sporozoite travels in the bird's bloodstream until it infects an appropriate tissue cell (e.g. liver, spleen). Once inside, the sporozoite then undergoes asexual reproduction to form a schizont (which contains many merozoites) during a process called schizogony or merogony. The merozoites are released when the tissue cell ruptures. Merozoites will either continue the cycle of infecting tissue cells to produce more schizonts and merozoites or they may infect blood cells and develop into either micro- or macrogametocytes, the male and female sexual forms of the parasite, respectively. The process of gametocyte formation is called gametogony. *Plasmodium* is the only genus that undergoes additional asexual reproduction in the blood. During this process, a portion of the *Plasmodium* merozoites proceed to infect red blood cells to produce more schizonts and merozoites.

Following sporozoite transmission by the vectors, the three haemosporidian genera become detectable in avian blood at different times (i.e. they have different pre-patent periods). *Haemoproteus* becomes detectable in the blood after about 11-21 days, while *Plasmodium* becomes detectable two days to several months after sporozoite infection [6]. *Leucocytozoon* has a pre-patent period of roughly five days [25]. The acute phase when the parasitemia (proportion of infected cells) is highest in the blood happens soon after the infection becomes detectable. High parasitemia can last days or weeks before chronic infection with fewer detectable parasites.

The infection can even become latent or dormant and reside back to the tissue/organ stage [6]. During latency, it is nearly impossible to detect the parasites in the blood. Some triggering events (e.g. migration, weakened immune system, reproductive effort) can cause a relapse that allows detection of the parasite in the blood again.

Gametocytes, along with other blood components from an infective bird, are ingested by one or more dipterans during subsequent feeding. While in the gut of a dipteran, each macrogametocyte releases itself from the blood cell in which it resides (creating a macrogamete). At the same time, each microgametocyte undergoes rapid asexual reproduction to produce eight flagella-like microgametes that burst out of the blood cell, a process called exflagellation. The microgamete fertilizes a macrogamete to produce a diploid zygote, an elongated structure called an ookinete. The ookinete penetrates the gut and attaches to the outside of the gut wall where it becomes a sphere called an oocyst. The oocyst matures and grows by producing many haploid sporozoites via asexual reproduction. Eventually the oocyst bursts and the sporozoites are released to travel to the salivary glands. This process of sporozoite production is called sporogony. The parasites are now ready to repeat the life cycle when the infective dipteran feeds on another avian host.

Most host-parasite association studies of avian haemosporidians screen for parasites in blood. Taking a peripheral blood sample does not require sacrificing the host, minimizing the impact on avian population dynamics. Most researchers make thin or thick blood smears on microscope slides to examine with microscopy and/or preserve additional whole blood to screen using molecular methods (i.e. PCR based detection methods). Less often, the birds are killed to screen for parasites, but this presents an opportunity to also smear organ samples (e.g. liver and/or spleen) onto slides for microscopy or collect tissue samples for molecular testing.

Sampling organs can be very helpful because sometimes parasites are in the latent phase and can only be detectable in the tissues.

**Detection Methods**

**Microscopy.** Prior to 2000 the main method available to screen for avian malaria in birds was microscopy which yields morphological data. Several morphological and lifecycle differences help to distinguish between the three genera in a blood smear. *Haemoproteus* and *Plasmodium* are most similar because both produce hemozoin granules (malaria pigment) in gametocytes (Figure 1.1a-b). One major difference between these two genera is the presence of a schizont on a blood smear; a schizont indicates a *Plasmodium* infection. *Haemoproteus* infections only form schizonts within tissues and not in blood. If only gametocytes are seen and there are enough of them to clearly define their morphology, the use of a dichotomous key such as the one by Valkiūnas (2005) [6] is required to determine the species of *Haemoproteus*. In addition, morphological comparisons should be made to newly described *Haemoproteus* species in more recent studies. An infection that is detected by the presence of trophozoites is nearly impossible to identify to genus (Figure 1.1d). Infections of more than one species and/or genus in the same bird get very difficult to identify. *Leucocytozoon* is easily identified, because unlike members of the other two genera, it does not produce hemozoin and staining of the parasite's cytoplasm results in fairly uniform, yet slightly mottled color. *Leucocytozoon* also tends to distort the host cell and displace the host cell's nucleus more drastically than the other genera (Figure 1.1c). For members of all three genera, the larger the parasite the more likely the parasite will migrate to the edges of thin blood smears during slide preparation. Because members of *Leucocytozoon* are larger than *Haemoproteus* and *Plasmodium*, they are more likely than the other two genera to be found at the edges of a blood smear slide (personal observation).

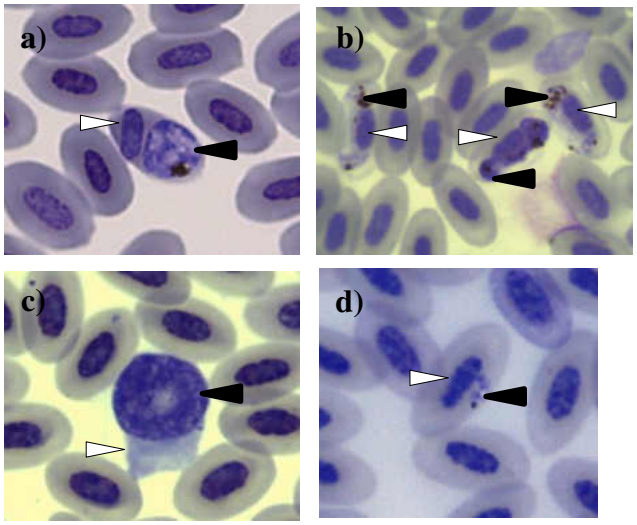



Figure 1.1: 1000-1500x magnification of three haemosporidian parasite genera and a trophozoite from Giemsa-stained, thin blood smears.
(a) *Plasmodium* sp.
(b) *Haemoproteus* sp. (the two light colored parasites are microgametocytes and the dark one is a macrogametocyte)
(c) *Leucocytozoon* sp.
(d) trophozoite (young parasite)
Black arrow= parasite
White arrow= host nucleus

**Molecular methods.** Polymerase chain reaction (PCR) has become an important molecular tool to screen birds for Haemosporidia and is often used in conjunction with microscopy. Using both microscopy and molecular results can help to determine 1) which genus and species each parasite belongs to, 2) if a bird is infected with one or more haemosporidian genera or species (dual infection) and 3) whether there is enough variability in morphology and DNA to determine if the parasite is a separate species from those already discovered.

Sequencing of PCR products yields RNA and DNA sequences. Most RNA sequences are sourced from ribosomal RNA (rRNA). There are three DNA sources: nucleus, mitochondria and apicoplast. Though several primer pairs were published in the 1980s and 1990s, most were intended to amplity RNA from primate *Plasmodium* [26, 27, 28, 29]. Bensch et al. (2000) [30] helped to revolutionize avian malaria screening by designing a primer set to amplify a 478 nucleotide portion of the mitochondrial cytochrome b (mt-cytb) gene from avian *Haemoproteus* and *Plasmodium* Every year since, more primers are designed to amplify different nucleotide fragments from one or both of these two genera. Some primers amplify *Leucocytozoon* fragments

homologous to those of *Haemoproteus* and *Plasmodium*, making it possible to compare the same fragment in all three genera [31].

Some other molecular methods or variations of PCR used to screen bird samples include restriction fragment length polymorphism (RFLP) [32], serological testing [19, 33], quantitative PCR, real time PCR and amplifying DNA via PCR from a single cell that is selected using a laser [34]. There is also the post-PCR manipulation of cloning (e.g. TA cloning) which, in the case of avian Haemosporidia, is often used to separate *Haemoproteus* and *Plasmodium* infections in the same bird.

More recently and not yet as common, researchers have used primers to screen wild caught insect vectors of Haemosporidia to identify prevalence and determine potential vector competency (mosquitoes: [35, 36, 37, 38, 39, 40, 41, 42, 43, 44] [45, 46, 47], black flies: [48, 49, 46], biting midges: [50, 51, 52, 46]). However, the presence of parasite DNA does not confirm the ability of the vector to actually transmit the sporozoites from their salivary glands into vertebrate hosts [4, 53].

Occasionally, a genus of parasites is found via PCR in a vector that is not considered a competent host. Mosquitoes are screened more often than other vectors; thus, there are records of *Haemoproteus* being amplified from mosquitoes [36, 41, 42, 44, 46]. This amplification does not prove competency because, at the very least, the entire thorax is often used for DNA extraction. Salivary glands are found in the thorax, but may not actually house the parasite. Valkiūnas et al. [53] experimentally infected mosquitoes with two species of *Haemoproteus* but the development of both species was halted during oocyst development and no sporozoites were present in the salivary glands. Despite the mosquitoes being incompetent and resistant, DNA from *Haemoproteus* was detectable in the mosquito for several weeks. *Plasmodium* DNA is also

occasionally found in biting midges [50, 51, 44], which are competent vectors for *Haemoproteus*. Valkiūnas [4] cautions against the all too frequent use of only PCR to identify Haemosporidia in vectors and urges that microscopy be implemented in addition to DNA-based methods.

The prevalence of Haemosporidia in vectors is always much lower than the prevalence found in avian populations in the same geographic locations [7, 54, 47]. This difference due to the presence of many more potential vectors compared to individual birds. Further, some vectors do not feed on all birds species, and only the females take blood meals in many species of vectors.

**Rationale for Study**

While investigating literature regarding blood parasites in bobolinks (*Dolichonyx oryzivorus*) during 2009, I became aware of deficiencies in blood parasite research. Birds from Minnesota (MN) are rarely screened for blood parasites and grassland birds, even passerines, are rarely the focus of a blood parasite study.

The number of Passeriformes that have been screened for blood parasites globally is quite extensive, though there are many geographic regions yet to be surveyed. Minnesota is certainly one of these regions; I found only three studies that presented data on the prevalence of Haemosporidia in birds from this state, and only one included a passerine species. Micks [55] screened a widespread passerine species, the house sparrow (*Passer domesticus*), and found that *Plasmodium* prevalence was 27.3% (3/11) in MN samples. The other two studies screened non-passeriformes birds. Stuht et al. [56] found that 100% (12/12) of nestling bald eagles from MN were infected with *L. toddi*, while Castle and Christensen [57] found *H. meleadridis*, *P. kempi* and *L. smithi* in 29% (5/17), 44% (8/18) and 6% (1/18), respectively, in MN wild turkeys.

Three studies from nearby North Dakota (ND) had prevalence data for birds but two studies were the same ones referenced above. Micks [55] found *Plasmodium* in 20% (3/15) of house sparrows while Castle and Christensen [57] found *P. kempi* in 100% (2/2) of ND wild turkeys. Wetmore [58] found *P. pedioecetii* [59] in the blood of a sharp-tailed grouse from ND.

The number of species in Passeriformes screened for blood parasites is extensive; however, grassland passerine species are extremely undersampled, to the point that there are no records of many grassland passerine species having been screened. In the United States, grassland passerine populations are declining due to the shrinking of preferable habitat. Many authors have voiced concern regarding apparent broadscale declines in abundance of most grassland bird species (e.g.: [60, 61]). Knopf [60] summarized breeding bird survey (BBS) data from 1966-1991 and found that savannah sparrows, clay-colored sparrows and grasshopper sparrows declined from 1966-1991, significantly for the latter two species. Peterjohn and Sauer [61] also summarized BBS data from 1966-1996 and found that savannah sparrows, bobolinks and grasshopper sparrows were significantly decreasing while Le Conte's sparrows and sedge wrens both showed nonsignificant increases (however, their article made it clear that they were skeptical about the validity of the increases). Both studies noted that of all the BBS bird groups, grassland birds contained the lowest proportion of species (17-23%) that showed a positive trend in population growth. This low population growth is even more troubling considering that the average proportion of all bird species that are increasing in population was approximately 51%.

The habitat of northwest MN where birds were sampled for this study is called tallgrass aspen parkland and is considered a natural transition zone between the prairie grassland and coniferous forest habitats [62]. The steady conversion of the grasslands to cropland is a major contributing factor to grassland habitat loss in MN. Because of the habitat loss, grassland

passerines must either increase population density at the remaining optimum grasslands (causing overcrowding) or choose inferior breeding sites (which could lead to poorer health) during the breeding season. These factors could cause the introduction of new blood parasites to grassland passerines to have much more dire consequences. The increase in population density could allow the parasites to spread quickly because vectors would have many more hosts to feed on and poorer health of birds at inferior grasslands could make individual birds more susceptible to parasitic infections. This is the first study that monitors blood parasite prevalences to identify any emerging blood parasites in these birds that could affect their population biology.

## Materials and Methods

### Study Sites

Though many sites in northwest Minnesota (MN) were considered for this study, five were chosen based on several characteristics in the summer of 2009. Each site had to be at least 320 acres of continuous grassland to maximize the avian species present and include standing water necessary for vector breeding. The sites had to be sufficiently distant from one another in order to increase the diversity of bird species and parasite lineages between sites.

Figure 1.2 shows the five chosen study sites located throughout three counties in northwest MN: Pennington County (site 1 [47°59’09” N, 96°26’35” W]), Red Lake County (sites 2 [47°57’03” N, 96°07’37” W] and 3[47°54’14” N, 95°47’37” W]) and Polk County (sites 4 [47°38’39” N, 96°05’50” W] and 5[47°41’39” N, 96°20’00” W]). Study sites 1, 2 and 3 were part of the Conservation Reserve Program (CRP) and the two largest sites, 4 and 5, are native tall grass prairies located on Rydell and Glacial Ridge National Wildlife Refuges, respectively. Sites 1 and 3 are the furthest away from each other (about 31 miles in a straight line).

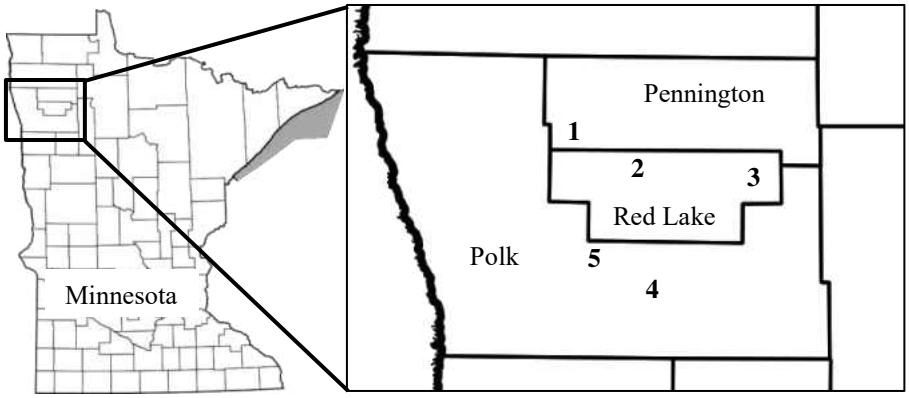



Figure 1.2: Locations of the five grassland study sites in Red Lake, Pennington and Polk Counties of northwest Minnesota, USA where blood samples were obtained from birds.

**Permissions**

Permit number MB072162-2 was issued by the Federal Fish and Wildlife Service and permit numbers 15700 (2009), 16317 (2010) and 17425 (2011) were issued by the State of Minnesota Fish and Wildlife Service, granting permissions to collect migratory bird samples for scientific research. Permissions were granted by landowners to conduct research on the CRPs and Special Use permits 0907 (2009) and 2010-005 (2010 and 2011) were obtained to collect samples on National Wildlife Refuge property. Federal Bird Banding Permit #10760 – B was issued by the Bird Banding Laboratory. The UND IACUC protocol 0904-1c was followed.

**Field Methods**

Blood samples were collected in Aug. 2009, May-Sept. 2010 and May-Sept. 2011 for a total of 150 samples from 148 birds of 14 species. On days when wind was very light, birds were captured using six mist nets (each measured 30’ x 8’) that were placed linearly from just before dawn until noon. The mist nets were left at a study site until birds were sampled for two days. If this criterion was not met after attempting to capture birds for four days, the nets were set up at

the next study site. This pattern continued until sample collection at all five study sites was attempted two to three times throughout the field season.

The site, month and year of capture and species was recorded for each captured bird. Bobolink samples were classified as juvenile (hatched within the collection year and able to fly well), adult male or adult female based on color characteristics. Since recaptures can provide valuable prevalence data [63, 64, 65, 21, 66, 67, 68], birds captured in 2009 and 2010 were banded to identify recaptures during and between the collection years. To limit unnecessary sampling and stress to the birds, they were not resampled if recaptured on the same day or, if applicable, during the following collection days before the nets were moved to the next site. Birds were never recaptured at a time when they met the criteria to be resampled within the same year. Thus, birds in 2011 received a temporary nontoxic mark on their right leg to identify recaptures until the mist nets were moved to the next site. Two savannah sparrows that were sampled in 2010 were resampled in 2011and were negative for parasites both years.

To collect blood, the brachial vein was pricked using a 27 or 30 gauge needle (depending on vein size) and blood was collected into one 80 µl heparinized microcapillary tube (10-70 µl of blood was collect depending on bird size). Pressure was applied to the vein with sterile gauze until bleeding subsided. While the blood was being processed (see below), movement of each bird was restricted for one or two more minutes in a mesh bag to minimize further bleeding or hematoma formation. Birds were then released on site. Total handling time never exceeded ten minutes per bird.

For each bird, a small portion of the blood was used to make one or two thin blood smears that were then air dried. The remaining portion of each blood sample was either stored in

a 1.5 ml microcentrifuge tube with Longmire's lysis buffer [69] and kept at room temperature or absorbed on Whatman filter paper, air dried, stored in separate plastic bags and kept frozen.

**Lab Methods**

**Microscopy.** The thin blood smears were fixed for five minutes in methanol within a week of being made. The smears were stained for 40 mins in a solution of 5% Giemsa stain within a month of being made. Microscopy was used to examine each blood smear for 40 fields of view at 400x magnification to search for *Leucocytozoon* parasites and for 100 fields of view at 1000x magnification to search for *Haemoproteus* and *Plasmodium* parasites. A bird was considered to be positive if at least one cell was obviously infected with a parasite from any of the three genera. *Haemoproteus* and *Plasmodium* were not differentiated using microscopy.

**Molecular Methods.** The initial steps of the DNA extraction process differed depending on the sample storage method. For each blood sample stored in Longmire's lysis buffer, a small portion was transferred to a new 1.5 ml microcentrifuge tube and placed in a heat block at 70°F with the cap open until all liquid was evaporated. Depending on how much residue was left in the tube after drying, 100-300 µl of guanidine thiocyanate extraction buffer [70] was added to the tube. The tube was placed in a heat block at 70°F for one hour until the contents were dissolved; occasional mixing with a vortex and grinding the sample with a sterile pestle facilitated sample dissolution.

For blood samples stored on filter paper, a small disk was removed and transferred to a new 1.5 ml microcentrifuge tube. To elute the blood from the disk, 300 µl of extraction buffer was added to the tube. The tube was placed in a heat block at 70°F for one hour with occasional vortexing, and then the contents were ground using a sterile pestle. The tube was centrifuged at

high speed for two minutes to separate the pulp from the liquid. The supernatant was transferred to a new tube and the pulp discarded.

Once samples were dissolved in extraction buffer, the DNA extraction procedure was standardized. The process was completed using steps described by Tkach and Pawlowski [70] although the volumes of liquids used for each step were doubled because the samples were larger.

Spectrophotometry was used to determine the purity and concentration of each DNA extraction product (Nanodrop 1000). A ratio absorbance at 260nm and 280nm (A260/280) of 1.8-1.95 was considered pure enough to use for PCR. If a sample was not pure enough or if there was no DNA detected, DNA extraction was repeated until a satisfactory result was achieved. If the yield of produced DNA was at a high concentration, a small amount of the sample was transferred to a sterile 1.5 ml tube and diluted with distilled water to approximately 20 ng/µl (Nanodrop 1000).

An initial PCR and two nested PCRs were run for each DNA sample using published methods described by Hellgren et al. in 2004 [31]. A 478 base pair portion of the mt-cytb gene was amplified. Each reaction was run in a total volume of 25 µl; approximately 100 ng of DNA was used as the template. The initial PCR was run with primers HaemNFI (5'-CATATATTAAGAGAAITATGGAG-3') and HaemNR3 (5'-ATAGAAAGATAAGAAATACCATTC-3') [31] that amplified *Haemoproteus*, *Plasmodium* and *Leucocytozoon*. The following thermocycler settings were used for the reaction: 20 cycles of 30 seconds at 94°C, 30 seconds at 50°C and 45 seconds at 72°C and the samples were incubated for 3 minutes at 94°C before the cyclic reaction and for 10 minutes at 72°C after the cyclic reaction. The second PCR was run with nested primers. One primer set, HaemF (5'-

ATGGTGCTTTCGATATATGCATG-3') and HaemR2 (5'-GCATTATCTGGATGTGATAATGGT-3') [30] amplified *Haemoproteus* and *Plasmodium* in one reaction while another primer set, HaemFL (5'-ATGGTGTTTTAGATACTTACATT-3') and HaemR2L (5'-CATTATCTGGATGAGATAATGGIGC-3') [31] amplified *Leucocytozoon* infections in a separate reaction. Two µl of template DNA (product from the initial PCR) was used for each nested PCR. The thermocycler settings for the nested PCRs were identical to the initial PCR except it ran for 35 cycles. Two positives controls (one from a bird known to be infected with *Plasmodium* and another from a bird known to be infected with *Leucocytozoon*) and one negative control (from a bird negative for Haemosporidia) were run for every seven samples.

All products from the nested PCRs were run on ethidium bromide-containing, 1.5% agarose gels and viewed under UV light to identify bands, indicating DNA was amplified. All positive nested PCR products were cleaned up using ExoSAP-IT and 3 µl were directly sequenced using terminator cyclic sequencing with 2 µl of BigDye Master Mix (Applied Biosystems, USA) in total reactions of 10 µl. The reverse primers were used for the sequencing reactions; HaemR2 was used for samples positive for *Haemoproteus*/*Plasmodium* and HaemR2L was used for samples positive for *Leucocytozoon*. The following thermocycler settings were used for the sequencing reactions: 25 cycles of 15 seconds at 96°C, 10 seconds at 50°C and 4 minutes at 60°C and the samples were incubated at 4°C following the cyclic reaction.

Resulting sequence information was utilized for identification by comparing these with published sequences archived in GenBank and MalAvi [71]. If sequences were not able to match published sequences, then unknown were sampled again. If sequence data could not be obtained after three attempts, then that sample was determined to be a false positive. A bird was

considered to have an infection using molecular methods if a sequence was determined to match one of the three genera in this study. The closest matching parasite species (and the percentage of matching nucleotides) was also recorded.

## Results

### Birds Sampled

A total of 150 samples from 148 individual birds were collected. Of the fourteen bird species sampled, the following six species were sampled the most and seem to prefer grassland habitats: savannah sparrow (SAVS, *Passerculus sandwichensis*, n=73), bobolink (BOBO, *Dolichonyx oryzivorus*, n=32), clay-colored sparrow (CCSP, *Spizella pallida*, n=17), Le Conte's sparrow (LCSP, *Ammodramus leconteii*, n=6), grasshopper sparrow (GRSP, *A. savannarum*, n=4) and sedge wren (SEWR, *Cistothorus platensis*, n=4). The remaining eight species had low sample sizes and are considered to be inhabitants of riparian zones; these were included in the data since the individuals are assumed to have spent a considerable amount of time in grasslands. These species are the yellow warbler (YEWA, *Setophaga petechia*, n=4), song sparrow (SOSP, *Melospiza melodia*, n=2), American goldfinch (AMGO, *Spinus tristis*, n=2), common yellowthroat (COYE, *Geothlypis trichas*, n=2), gray catbird (GRCA, *Dumetella carolinensis*, n=1), brown-headed cowbird (BHCO, *Molothrus ater*, n=1), eastern kingbird (EAKI, *Tyrannus tyrannus*, n=1) and willow flycatcher (WIFL, *Empidonax traillii*, n=1).

### Prevalence of Haemosporidia

Besides *Haemoproteus*, *Plasmodium* and *Leucocytozoon*, no other blood parasites that are known to infect passerines in northwest Minnesota (MN) such as trypanosomes or microfilariae were found using microscopy. Table 1.1 shows the infections found in each bird species using a combination of microscopic and molecular methods. All six of the grassland species had at least

one infected individual while three of the eight other species had at least one infected individual. Of the collected samples, 56.67% (85/150) were infected with at least one haemosporidian genus; the total number of infections was 111. *Haemoproteus* or *Plasmodium* was found in

Table 1.1: Overall prevalences of Haemosporidia based on a combination of microscopy and molecular methods. The numbers (percentages below) represent how many individual birds were infected with *Haemoproteus* (*H.*), *Plasmodium* (*P.*) and *Leucocytozoon* (*L.*) infections, dual infected individuals and total infected individuals broken down by the fourteen species of birds sampled (n=150).

| Bird Species | Total Sampled | *H./P.** | *H.* | *P.* | *L.* | Dual Infected | Total Infected |
|---|---|---|---|---|---|---|---|
| Savannah sparrow<br>*Passerculus sandwichensis* | 73 | 37<br>50.7% | 4<br>5.5 | 25<br>34.2 | 23<br>31.5 | 16<br>22 | 44<br>60.3 |
| Bobolink<br>*Dolichonyx oryzivorus* | 32 | 11<br>34.4 | | 7<br>21.9 | 10<br>31.3 | 5<br>15.6 | 16<br>50.0 |
| Clay-colored sparrow<br>*Spizella pallida* | 17 | 14<br>82.4 | 8<br>47.1 | 4<br>23.5 | 5<br>29.4 | 4<br>23.5 | 15<br>88.2 |
| Le Conte's sparrow<br>*Ammodramus leconteii* | 6 | 3<br>50 | 1<br>16.7 | 1<br>16.7 | 1<br>16.7 | 1<br>16.7 | 3<br>50 |
| Grasshopper sparrow<br>*A. savannarum* | 4 | 1<br>25 | | 1<br>25 | 1<br>25 | | 2<br>50 |
| Sedge wren<br>*Cistothorus platensis* | 4 | 1<br>25 | | 1<br>25 | | | 1<br>25 |
| Yellow warbler<br>*Setophaga petechia* | 4 | 1<br>25 | | 1<br>25 | 1<br>25 | | 2<br>50 |
| Song sparrow<br>*Melospiza melodia* | 2 | | | | | | |
| American goldfinch<br>*Spinus tristis* | 2 | 1<br>50 | | | | | 1<br>50 |
| Common yellowthroat<br>*Geothlypis trichas* | 2 | | | | | | |
| Gray catbird<br>*Dumetella carolinensis* | 1 | 1<br>100 | 1<br>100 | | | | 1<br>100 |
| Brown-headed cowbird<br>*Molothrus ater* | 1 | | | | | | |
| Eastern kingbird<br>*Tyrannus tyrannus* | 1 | | | | | | |
| Willow flycatcher<br>*Empidonax traillii* | 1 | | | | | | |
| **Totals** | **150** | **70**<br>**46.7** | **14**<br>**9.3** | **40**<br>**26.7** | **41**<br>**27.3** | **26**<br>**17.3** | **85**<br>**56.7** |

* *H*/*P* column contains sixteen infections for which the exact genus cannot be identified

46.67% (70/150) of samples; of these, 54 of the 70 could be identified to genus. Sixteen infections could not be identified to a genus due to lack of successfully collected sequence (n=11) or multiple base callings (MBCs) in the chromatogram (n=5) indicating that more than one haemosporidian species (and possibly genus) was present. Thus, the overall prevalence of *Haemoproteus* was 9.3% (14/150), *Plasmodium* was 26.7% (40/150) and *Leucocytozoon* was 27.33% (41/150). A total of 95 infections were successfully identified to genus. Dual infections (infected with two genera) occurred in 17.33% (26/150) of samples. Since 56.67% (85/150) of samples were infected with at least one of the three genera, and 17.33% (26/150) of samples were infected with two genera (= dual infection), we can estimate that if a bird was infected with one genus, there is a 30.6% chance that the same bird would be infected with at least one of the other two genera.

**Tentative Identification of Haemosporidia to Species**

The only infections that were tentatively identified to species were those that were the result of single infections and had successful sequence reactions. Ninety-nine infections were sequenced but seven were eliminated for having MBCs (five *Haemoproteus*/*Plasmodium* and two *Leucocytozoon*). Three other reactions were eliminated for sequences that terminated early. Eighty-nine sequences were used for identifications. Table 1.2 shows the number of infections by parasite species and bird species. The 89 sequences included fifteen species of Haemosporidia. The dominant species for each parasite genus was *H. coatneyi* (n=8), *P. cathemerium* (n=14) and *P. circumflexium* (n=12) and *L. majoris* (n=28).

**False Results**

Fifty-one false negatives were recorded using microscopy. Twelve false negatives were recorded using molecular methods. Molecular testing has a greater chance of producing false

Table 1.2: Numbers of infections identified to each parasite species using molecular methods. Aside from one exception (see table below), a blastn with each sequence from this study resulted in a 97%-100% match to a sequence from a parasite identified to species. The other six bird species (WIFL, EAKI, BHCO, COYE, AMGO and SOSP) not listed in the table had no infections identified to species.

| | *Haemoproteus coatneyi* | *H. fringillae* | *H. homobelopolskyi* | *H. tartakovskyi* | *H. pallidus* | *Plasmodium cathemerium* | *P. circumflexum* | *P. polare* | *P. relictum* | *P. homopolare* | *P. lutzi* | *P. nucleophilum* | *P. unalis* | *Leucocytozoon majoris* | *L. fringillinarum* | Unidentified Infections* | **Total Infections** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Savannah sparrow *Passerculus sandwichensis* | 3 | | | | | 10 | 7 | 5 | 2 | | | | 1 | 15 | 6[b] | 11 | **60** |
| Bobolink *Dolichonyx oryzivorus* | | | | | | 3[a] | | | 2 | 1[a] | | 1 | | 10 | | 4 | **21** |
| Clay-colored sparrow *Spizella pallida* | 4 | 1 | 1 | 1 | | 1 | 3 | | | | | | | 2 | 2 | 4 | **19** |
| Le Conte's sparrow *Ammodramus leconteii* | 1 | | | | | | 1 | | | | | | | | 1 | 1 | **4** |
| Grasshopper sparrow *A. savannarum* | | | | | | | | | 1 | | | | | | | 1 | **2** |
| Sedge wren *Cistothorus platensis* | | | | | | | 1 | | | | | | | | | | **1** |
| Yellow warbler *Setophaga petechia* | | | | | | | | | | | 1 | | | 1 | | | **2** |
| American goldfinch *Spinus tristis* | | | | | | | | | | | | | | | | 1 | **1** |
| Gray catbird *Dumetella carolinensis* | | | | | 1 | | | | | | | | | | | | **1** |
| **Total Infections** | **8** | **1** | **1** | **1** | **1** | **14** | **12** | **5** | **5** | **1** | **1** | **1** | **1** | **28** | **9** | **22** | **111** |

[a]= 1 infection was from a juvenile bobolink
[b]= 1 infection was only a 95% match
*= Includes the infections not able to be identified due to being microscopy positive only

positives than microscopy [72, 73]; indeed there were fourteen false positives. These false positives occurred when a band was visible on the gel but sequencing was unsuccessful. Of these fourteen, nine were caused by HaemF/HaemR2 nested primers amplifying *Leucocytozoon* (for more about the primers in this study amplifying unintended genera see [74, 75, 72, 76]) and five were caused by sequences matching non-parasite DNA.

### Comparison of Microscopy and PCR

Figure 1.3 shows that molecular methods were significantly more likely than microscopy to detect infections in samples collected in 2009 and 2010. There was no significant difference for samples collected in 2011. Combining data from all three years, molecular methods were always significantly more sensitive than microscopy for parasite detection. Additionally, considering microscopy only, 34% (51/150) of samples were infected with at least one genus and using molecular methods only, 53% (79/150) of samples were infected.

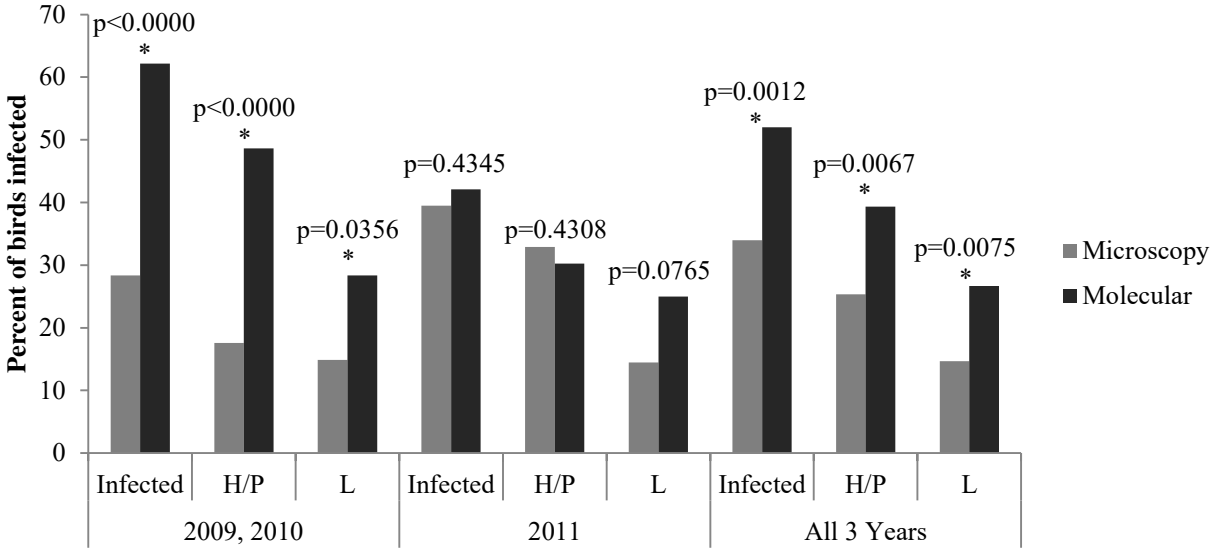



Figure 1.3: Comparison between microscopy and molecular methods' ability to detect infections. The bar heights show the percentages of birds infected with at least one genus (Infected), *Haemoproteus* or *Plasmodium* (H/P) and *Leucocytozoon* (L) for 2009/2010 and 2011 separately and all three years combined. A Fisher's Exact test was run with the data for each pair of bars and p-values are labeled above each pair. An * indicates a significant p-value <0.05.

## Discussion

### Prevalence of Haemosporidia

One of the largest compilations of blood parasite works is by Valkiūnas (2005) [6], in which he summarized Haemosporidia prevalence by genus for each zoogeographical region. He determined that in the Holarctic region (the majority of the northern hemisphere) the prevalence

of *Haemoproteus* was 17.9%, *Plasmodium* was 2.9% and *Leucocytozoon* was 16.2%. By comparison, the current study of 150 samples from 148 birds found that the prevalence of *Haemoproteus* was 9.3%, *Plasmodium* was 26.7% and *Leucocytozoon* was 27.3%. In another frequently cited work, Greiner et al. (1975) [77] compiled studies that screened samples from North American birds and found that 19.5%, 3.8% and 17.7% of birds were infected with *Haemoproteus*, *Plasmodium* and *Leucocytozoon*, respectively, which is fairly similar to what Valkiūnas (2005) [6] found. Greiner et al. (1975) [77] also divided the continent into seven regions. Region 2, the Great Plains, includes the area for the current study. The prevalence of *Haemoproteus*, *Plasmodium* and *Leucocytozoon* was approximately 22%, 4% and 3%, respectively. In all of the preceeding cases, compared to this study, *Haemoproteus* prevalence was found to be higher overall, while *Plasmodium* and *Leucocytozoon* were lower. The Minnesota (MN) grassland passerines must have more contact with infective mosquitoes and black flies than with biting midges compared to other birds in general and/or are more susceptible to *Plasmodium* and *Leucocytozoon* than to *Haemoproteus*.

Greiner et al. (1975) [77] also reported that 36.9% (21,048/57,026) of the North American birds screened were positive for *Haemoproteus*, *Plasmodium*, *Leucocytozoon*, microfilariae, *Trypanosoma*, and/or *Haemogregarina*/*Lankestrella*. The overall prevalence within Region 2 birds was approximately 27% (*Haemoproteus*, *Plasmodium* and *Leucocytozoon* only). Both of these are lower than the 56.67% (85/150) found in this study. Perhaps the use of PCR is responsible for a much higher prevalence found here. Alternatively, MN grassland passerines could be in contact with infective vectors more or are more susceptible to haemosporidian infections than other birds.

## Host-Genus Associations

Several of the host-genus associations found in this study are new records. Table 1.3 shows all of the previously published and current associations for the bird species sampled in this study. The first six bird species listed on the table prefer grasslands; very few host-genus associations had been found previously with these six species compared to those found for the

Table 1.3: List of the currently known host-genus associations for the fourteen bird species sampled in this study. A black square (■) means the association was found in the current study. A black circle (●) means the association was retrieved from the MalAvi database, indicating that one or more mt-cytb sequences exist for the parasite genus. A black triangle (▲) means the association was found in one or more sources other than the current study or MalAvi database. The gray highlight indicates the host-genus association is new. The letter(s) in parentheses following each resource is/are the genus/genera found.

| Bird Species | *Haemoproteus* (*H*) | *Plasmodium* (*P*) | *Leucocytozoon* (*L*) | Resource |
|---|---|---|---|---|
| Savannah sparrow *Passerculus sandwichensis* | ■▲ | ■▲ | ■▲ | [78, 77] |
| Bobolink *Dolichonyx oryzivorus* | ●▲ | ■● | ■ | [77] |
| Clay-colored sparrow *Spizella pallida* | ■▲ | ■ | ■▲ | [77] |
| Le Conte's sparrow *Ammodramus leconteii* | ■ | ■ | ■ | |
| Grasshopper sparrow *A. savannarum* | ▲ | ■▲ | ■ | [79, 77] |
| Sedge wren *Cistothorus platensis* | | ■ | | |
| Yellow warbler *Setophaga petechia* | ●▲ | ■●▲ | ■▲ | [80, 77, 81] |
| Song sparrow *Melospiza melodia* | ●▲ | ●▲ | ▲ | [79, 7, 80, 78, 77, 82, 83] |
| American goldfinch *Spinus tristis* | ▲ | ▲ | ▲ | [78, 77, 80] |
| Common yellowthroat *Geothlypis trichas* | ●▲ | ●▲ | ●▲ | [78, 77, 80, 82] |
| Gray catbird *Dumetella carolinensis* | ■●▲ | ●▲ | ▲ | [84, 78, 77, 80, 82, 85, 65] |
| Brown-headed cowbird *Molothrus ater* | ▲ | ●▲ | ▲ | [79, 7, 77, 80] |
| Eastern kingbird *Tyrannus tyrannus* | ▲ | ▲ | ▲ | [79, 78, 77] |
| Willow flycatcher *Empidonax traillii* | ▲ | | ▲ | [77] |

eight other bird species that are considered inhabitants of riparian zones. Of the seventeen total host associations from this study, fourteen of those are with grassland species because these species were most extensively sampled. Seven associations found in this study are new, all of which are with grassland birds. Most notably, I found no evidence that blood parasites had ever been found in Le Conte's sparrows or sedge wrens before my current study.

**Haemosporidia Species**

Species identification in the current study should be considered tentative since they have not been confirmed morphologically and are based solely on closest matching DNA sequences with those published in GenBank and MalAvi. There are many haemosporidian species that have no mt-cytb sequences available for comparison. Nevertheless, I obtained 89 sequences in this study that closely matched published sequences representing fifteen different haemosporidian species. There were fifteen parasite species that were tentatively matched to 89 of the sequences in this study. All fifteen species had been previously reported from Passeriformes but from what I could find in the literature, there were no microscopy or molecular records for five of these in the USA prior to this work (*H. homobelopolskyi*, *H. pallidus*, *H. tartakovskyi*, *P. lutzi* and *P. unalis*). These species were described in 2011, 1991, 1986, 1939 and 2013, respectively. It was no surprise that the two most recently described species have not been found in the USA but it was unexpected to not find other records of *P. lutzi* since it was described in 1939 and was identified in this study. *Plasmodium lutzi* is very similar to *P. relictum* [6], so it is possible it has been found but was misidentified. *Haemoproteus pallidus* and *H. tartakovskyi* have been found mainly in Russia and Europe. According to MalAvi [71], the ten other species have been found in the USA previously: *H. coatneyi*, *H. fringillae*, *P. cathemerium*, *P. circumflexum*, *P. homopolare*, *P. nucleophilum*, *P. polare*, *P. relictum*, *L. fringillinarum* and *L. majoris*.

**Evidence of Active Transmission**

Two juvenile bobolinks tested positive for *Plasmodium* using DNA sequencing. This indicates that active transmission of *Plasmodium* is occurring within the study area. One sequence was a 100% match to *P. cathemerium* and the other matched 98% to *P. homopolare*. *Plasmodium cathemerium* is very widespread geographically and infects a wide range of bird species spanning several different Orders, mainly Passeriformes. Natural vectors are unknown but sporogony has been completed in at least seventeen experimentally infected mosquito species [6]. *Plasmodium homopolare* was described in 2014 and has been found in passerines from Colombia and California, USA, but there is no information on its vector.

It is unknown whether the bobolinks were infected as nestlings or once they left the nest. One thing that could affect whether birds get infected as nestlings is how the nest is constructed. Ribeiro et al. [86] suspects that birds raised in open nests are more exposed to vectors than those in closed nests though the parasite prevalence was not significantly different in the study. Of the six grassland bird species in this study, grasshopper sparrows and sedge wrens construct closed nests while the other four species, including boblinks, construct open nests. The length of time that a bird spends as a nestling is also a factor; a longer stay can result in more exposure to vectors [6]. Unfortunately, information on how many days each grassland bird species spends as a nestling was only found for savannah sparrows, clay-colored sparrows, and bobolinks. These three species have similar durations as nestlings [87], so the nestling time may not have been a significant reason for the infections in the juvenile bobolinks.

Other authors have found similar evidence of active transmission in other bird species within the contiguous USA; a portion of these studies are listed here (*Haemoproteus*: [80] (Vermont), [82] (New Jersey), [65] (Ohio), [88] (Florida), [89] (Florida), [90] (South Carolina),

[91] (New York and Georgia), [83] (California); *Plasmodium*: [7] (California), [92] (Kansas), [80] (Vermont), [82] (New Jersey), [93] (Michigan), [91] (New York and Georgia), [83] (California), [94] (north central USA); *Leucocytozoon*: [80] (Vermont), [82] (New Jersey), [95] (Wisconsin), [89] (Florida), [83] (California)). The only local study was by Stuht et al. in 1999 [56] which reported that 100% (n=12) of nestling bald eagles from northern MN were infected with *L. toddi*, showing active transmission via black flies. Some researchers even made specific attempts to find active transmission such as Cosgrove et al. [75] who screened 195 blue tits in the U.K. specifically for *Haemoproteus* or *Plasmodium* but turned up with zero infections. Though neither genus was found, the study did find one *Leucocytozoon* infection.

**Critical Review of Methods Used**

**Mist nets.** A note of caution about the use of mist nets in grasslands regards the cardinal direction positioning of the nets. Initially, the nets were set up in straight line from north to south. Unfortunately, there is nearly nothing to block wind in grasslands and it almost always comes from the west. This left me unable to capture any birds on days when the wind was greater than five mph because the wind would make the nets billow to the fullest extent. This caused the pocket that is supposed to exist at the bottom of each of the five tiers of all nets to be absent. All birds, even the heavier bobolinks, merely bounced off of the billowed tiers as there was no pocket for them to fall into and get trapped in. Eventually, I oriented the nets in a straight line from west to east to minimize the billowing from the wind and allow more birds to be captured, even on slightly windy mornings.

The use of mist nets caused the respective sample size of each bird species to not reflect the actual population composition of birds at the study sites. For example, of the most sampled bird species, I observed first-hand that the mist nets seemed to capture savannah sparrows best

and many clay-colored sparrow and most sedge wren individuals were too small to be captured by the mist nets. Also, Le Conte's sparrows were frequently encountered but were very difficult to persuade to fly into the nets.

In birds, Haemosporidia blood infections can either be at a chronic or acute stage. Birds in the acute stage tend to be sick due to higher parasitemia so these birds may be lying low trying to survive and are not easily persuaded to fly into nets [96, 6]; therefore they are not sampled. If a disproportionate number of individual birds per species are in the acute stage or if a certain species of birds tends to get sicker than the others, the proportion of individuals per bird species that were sampled may be different than what was actually at each site. Possibly, a combination of methods for obtaining birds (mist nets, shooting, ground traps, playback recordings etc.) would have been best.

The above conditions also skew the actual representation of parasites. If there is a specific genus or species of parasite causing severe acute infections, this genus or species does not get accurately represented in the data because the birds are not sampled. On the other side of this, a genus or species may cause chronic infections with fewer parasites than others, making it easier to catch the birds but more difficult to come across parasites on thin smears or for PCR to successfully amplify DNA.

**PCR.** A downside to this study is that *Haemoproteus* and *Plasmodium* were never found in the same host because the nested primer pair (HaemF and HaemR2) amplified both genera and they were not differentiated using microscopy. It does not affect the overall prevalence of 57% or the prevalence of *Leucocytozoon* (27%) for example, but it means there could have been more *Haemoproteus*, *Plasmodium* and dual infections than the 14, 40 and 26 that were found, respectively. If both genera infected the same bird and the difference in parasitemia was large

enough, evidence of the dual infection in an electrophoretogram (more than one peak at one or more nucleotide positions or multiple base callings [MBCs]) may not have been evident. However, it is known that three of the five sequences that had MBCs in this study were amplified by the *Haemoproteus*/*Plasmodium* primers, so it is possible that in these three cases the MBCs were caused by the host being infected with both *Haemoproteus* and *Plasmodium*. Only one of those three birds was not infected with *Leucocytozoon*, so had it been two genera causing the MBCs, the bird would have been considered dual infected.

Three methodological changes could have given the best estimate of prevalence and better assisted with parasite identification: 1) use a combination of primer pairs to screen for parasites because some primers may pick up an infection that others miss but this is rarely done for other studies [97, 98, 99, 100, 101], 2) ensure that at least one primer pair amplifies only *Haemoproteus* and another for only *Plasmodium* and 3) have someone very skilled in discerning between *Haemoproteus* and *Plasmodium* using morphology assist with microscopy.

**Sample preservation.** Though collecting blood on filter paper is a method that has been used for the past 50 years [102], I found that it is certainly inferior compared to storing the blood in Longmire's lysis buffer. The difficulty with using filter paper is the limited amount of blood that can be eluted off the paper for DNA extraction [103]. The Longmire's lysis buffer was much easier to work with as the blood was already in a liquid solution, so the difficulty of having to elute the blood was eliminated. Also, more infections were amplified from the blood stored in Longmire's lysis buffer (see below) but it is unclear if this is directly related to the blood storage method.

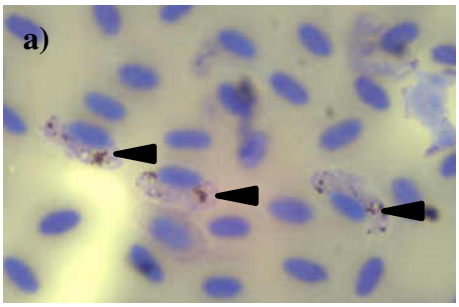
**Comparison of microscopy and PCR.** As was shown in Figure 1.3, for all three collection years together, PCR was always significantly more sensitive at detecting infections

than microscopy. Samples from 2009 and 2010 showed the same trend but samples from 2011 indicated that, although PCR was always more sensitive than microscopy, the difference was not significant. I believe that a combination of the following two explanations are responsible for the non-significance between methods in 2011: 1) the ability of the observer to detect infections using microscopy was significantly improved in 2011 compared to 2009 and 2010 and 2) blood samples in 2011 were stored on filter paper, which is suspected to be an inferior storage method compared to the Longmire's lysis buffer that was used in 2009 and 2010 and this possibly caused the molecular method to miss some infections that microscopy also missed.

Most other studies that compare microscopy and PCR also find that PCR is more sensitive, sometimes significantly [97, 104, 98, 105, 86, 106, 107, 99, 108, 109] or, less often, both methods are equally as good [30, 110]. Zehtindjiev et al. [110] found microscopy to be more sensitive than PCR in identifying dual infections which is impossible for many primers (as discussed above). Garamszegi [111] found that the overall prevalences as determined by PCR and microscopy were not significantly different; however, when the genera were considered separately, PCR was significantly more sensitive in detecting *Plasmodium*. Valkiūnas et al. [73] did not find a significant difference between the two methods used to assess prevalence for *Haemoproteus*, *Plasmodium* and *Leucocytozoon*. They, along with Jarvi et al. [97], even suspect that both methods can underestimate prevalence for all three genera due to dual infections and/or low parasitemia. Jarvi et al. [97] knew exactly what the infection prevalence was in the birds they tested as they were experimentally infected, giving them a way to calculate just how accurate serological, PCR and microscopy methods were in detecting infections. Serology was best, then PCR, then microscopy, being able to detect 97%, 61-84% (depending on the primers used) and 27% of infections, respectively.

It is beneficial and recommended to use both methods, however, because morphology from microscopy and DNA sequences from PCR help to identify genus and/or species of parasites. Though morphology should definitely be used as well to identify genus, at this point, there are enough sequences for many DNA fragments in GenBank and MalAvi databases that a quick comparison will identify the genus as long as there is no question that the sequences are accurate. Unfortunately, not all species have DNA sequences available and there are many haemosporidian species in GenBank that are clearly misidentified [112, 113]. Identification of species based on DNA alone is not optimal unless an exact match is found to another sequence that has the species defined using proper methods. As long as morphology is very clear, it may be best to identify species using available dichotomous keys [6] with comparison of morphology with newly identified species. If there is a corresponding DNA sequence, then it can be added to databases with a complete identification. Using both methods can also find if a parasite in question is a novel morphospecies or parasite lineage [114, 115, 116, 117].

It is also highly beneficial and recommended to use a combination of molecular and microscopy methods to screen for parasites [97, 118, 73, 119, 111, 116, 120, 108] because they give the best estimate of prevalence. The results of this study support this recommendation, because had only microscopy been used, 51 infections (32 *Haemoproteus* /*Plasmodium*, 19 *Leucocytozoon*) would have been missed and the prevalence would have been 34%. If only PCR had been used, twelve infections (eleven *Haemoproteus* /*Plasmodium* and one *Leucocytozoon*, all in 2011*)* would have been missed and prevalence would have been 53%. The prevalence was 57% because both methods were used (Table 1.1).



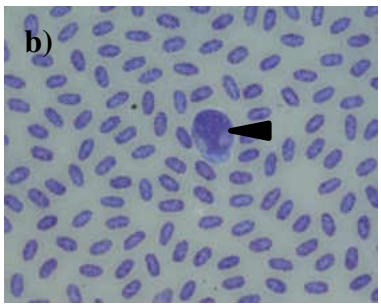




Figure 1.4: Two examples of poorly fixed slides.
(a) *Haemoproteus*
(b) *Leucocytozoon*
Black arrow= parasite

DNA was possibly too overwhelming for the PCR to amplify enough parasitic DNA, 2) there may have been mismatched bases between the template and primer sequences and/or 3) the preserved parasite DNA on the filter paper was too degraded. The six most likely reasons for the 51 false negatives via microscopy are 1) fixing and staining of slides produced less than optimal results (Figure 1.4a-b) making parasites difficult to discern, 2) insufficient fields of view were studied, 3) observer inexperience, 4) low parasite intensity 5) higher sensitivity of PCR methods and/or 6) there was no active blood infections (the sporozoites may have been injected and began the tissue phase of infection but then died in the resistant bird, leaving the DNA in the blood which is then detected up by PCR). Valkiūnas et al. [73] suspects that reasons 1, 2 and 3 for microscopy false negatives may best explain the low microscopy estimates in most studies. A benefit of microscopy is the absence of false positives.

# CHAPTER II

## STATISTICAL TRENDS OF BLOOD PARASITES (HAEMOSPORIDIA: *HAEMOPROTEUS*, *PLASMODIUM* AND *LEUCOCYTOZOON*) IN SONGBIRD PASSERINES FROM GRASSLANDS OF NORTHWEST MINNESOTA

### Introduction

Most Haemosporidia studies that focus on blood parasite prevalence in wild bird populations report either the presence or absence of a variety of trends in the data. Trends that are commonly tested for are driven by parasite genus and time (temporal), space (spatial), bird species, sex, age and health in relation to the prevalence of blood parasites. Generally, discovering trends is not the main purpose of a study, but sometimes a study is actually designed to test for a specific trend.

Occasionally, while reviewing other studies, it was difficult to decide whether I should report that there was a lack of a trend or not when significance was not tested for. For example, the author(s) may report that parasite prevalence in male and female birds was 57% and 51%, respectively but not provide statistical test results. In this case I would conclude that there was a trend of male biased parasitism. If another study found male and female parasite prevalence was 57% and 49%, respectively and reported that statistical testing was not significant, I would have concluded that there was no difference in prevalence between sexes. For the vast majority of the studies I list as lacking a trend were place there because statistical testing concluded there was no significance, but had testing been done on some of the trends that I concluded were present may not be significant either. This situation occurred more frequently with less recent studies,

because it was less likely that the authors would have done statistical tests on the data. In general, trends from studies prior to 1980 are scarce.

I found that when overall prevalence was used to test a trend, it would not be significant. The insignificance with overall prevalence was often caused by a trend of one genus cancelling out an opposing trend of another genus. For example, a theoretical avian population was sampled during spring and summer (Mar.-Aug.) and is infected primarily with *Plasmodium* and *Leucocytozoon*. *Plasmodium* prevalence becomes very high in the spring due to relapse/stress and *Leucocytozoon* prevalence becomes very high in the summer due to being infected at or near the study site during the sampling period. Considering both genera together, prevalence would be high each month, with no month being significantly higher than others. Testing the two genera separately, however, would show that *Plasmodium* is significantly more prevalent in the spring and prevalence of *Leucocytozoon* is significantly higher in the summer.

**Parasite Genera Trend**

Most studies report the prevalence of blood parasites by genus but they rarely statistically test for a trend to find out if the prevalence of one or more genus/genera is significantly different from the other(s) (exceptions: [83, 121, 122]). However, a main comment to make is that *Haemoproteus* is the most prevalent haemosporidian genus, both worldwide [123, 6] and in North America [77], a contrasting finding compared to the results of this study.

**Temporal Trends**

**Months.** Temporal trends are reported on quite often for studies that span several months. Studies usually find one of three temporal trends related to high Haemosporidia prevalence: highest early in the breeding season, Mar.-Jun. [124, 125, 82, 64, 126, 65, 127] or highest later in the breeding season, Jul.-Sept. [128, 129, 93, 130, 131, 91, 132], or they find both

peaks in the same study [78, 133, 63, 88, 134, 91]. The early peak is associated with a relapse of existing infections in after hatch year birds [125] caused by the stress of migration and/or reproduction efforts. The later peak is caused by adults and juveniles being infected at the collection sites during the study. Alternatively, Schrader et al. [134] had a different hypothesis for the cause of a later peak in woodpeckers; they suspect it was due to another relapse brought on by the stress of subsequent reproductive effort. Results always show that prevalence is lowest during the winter months.

Freeman-Gallant et al.'s [135] study is most notable because they studied savannah sparrows in New York but found no significant difference in *Plasmodium* prevalence between collection months (May-Jul.). Very few other studies find that prevalence was not significantly higher during certain months or seasons [136, 137, 138].

Interestingly, several studies that identify haemosporidian infections in vectors by month found that the prevalence climbs higher later in the bird breeding season [37, 39, 42, 44, 45]. Atkinson's [139] study is one of the few where there was no temporal trend because prevalence in southern Florida biting midges remained around 2% year round.

**Years.** It is not uncommon for sample collection to span more than one year in which case there is the potential to have a significant prevalence difference between years, as has been found on occasion [133, 140, 67] but most authors found no significant difference [141, 136, 63, 142, 129, 143, 88, 144, 145, 146] [94, 147, 21, 148].

Few haemosporidian studies occur across several years, allowing determination of long term trends of regular (stable) or irregular (unstable) patterns to the oscillation of parasite prevalence. Bensch et al. [149] found that parasite prevalence was stable over a seventeen year period of monitoring parasites in a population of great reed warblers. Fallon et al.'s [138] study

found that parasite prevalence was generally stable over the course of a year and over a ten year period but after the ten years, only one lineage was gained at a study site and one was lost at another site.

**Spatial Trends**

Many researchers have more than one location from which samples are collected, allowing for statistical testing on parasite prevalence by site. Authors of studies with sites that are very similar and only vary by geographic location are usually interested in parasite prevalence of one or more bird species preferring that particular habitat; the presence or absence of a spatial trend (if it is tested for at all) generally is not a main purpose of the study. Geographically distant sites are more likely to display spatial trend.

Only two studies that were not compilations were found that included Minnesota (MN) in spatial trend testing. Castle and Christensen [57] screened turkeys and found that *Plasmodium* and *Haemoproteus* prevalence was significantly higher in Wisconsin and MN compared to Michigan and Missouri. Pagenkopp et al. [94] sampled common yellowthroats across the USA and parts of Canada and grouped sample sites into five regions. Prevalence differed significantly between five regions: the north central region which includes MN had highest prevalence at 78.47% (*Plasmodium* prevalence was very high but *Haemoproteus* and *Leucocytozoon* was very low).

Other global studies found significant difference in prevalence between similar sampling sites (USA: [150, 91, 145]; Canada: [151]; North America: [135, 152]; Africa: [120, 153]; Spain: [154]; Lesser Antilles: [155, 156]; Phillippines: [121]; Brazil: [136]). Not unexpectedly, many studies that include sites of similar habitat do not find a significant spatial trend (Michigan: [93];

Canada: [64]; Jamaica: [63]; Russia: [157]; Uganda: [158]; Australia: [159]; Australo-Papuan region: [160])

Spatial trends can get more complex because many studies are designed to show the presence or absence of a spatial trend by choosing sites with opposing characteristics (sometimes the sites still have a very similar habitat). Researchers have chosen sites at opposite sides of a country [161], urban and rural sites [162], natural and expanded ranges of a bird species [145, 163], breeding and wintering grounds [164], low and high elevations [165, 166, 167, 168, 169, 170, 171, 172, 148], deforested and undisturbed locations [173, 174], large and small habitat fragments [86, 175], tropical and temperate zones [176, 107], humid and dry forests [171], island and continental land [177, 178], and freshwater/inland and marine/coastal environments [179, 180] for the main purpose of testing if there is any significant difference in parasite prevalence between the two types of collection sites. Not surprisingly, the vast majority of these studies did find a significant spatial trend.

**Bird Species Trends**

It is certainly possible that differences in prevalence between species will exist in a study that includes a large number of samples. Unfortunately, of the studies found that did test for this trend, less than half tested for significant differences between two or more species or subspecies [158, 145, 181, 81, 127]. Many other studies group species by higher taxonomic group (genus: [122]; family: [83, 182]) and test for differences in prevalence between these categories instead of by species. Others grouped their bird species based on diet [86, 175], feeding preference [82], habitat preference [171], ant following [183] or other characters, then test for differences between the groups. Catergorization creates difficulties for comparing results between studies

that sampled the same species as I did, because it is not possible to separate a category into individual species.

**Bird Sex Trends**

Due to differences between male and female birds, there is the possibility that one may be more susceptible to haemosporidian infection. Whether authors were looking at overall prevalence or each genus separately, some studies have found a trend of higher prevalence in females [125, 184, 185] and males [186, 127]. Even though it was not statistically significant, the most notable study found male biased parasitism in savannah sparrows infected with *Plasmodium* in Canada and New York [135]. Most studies find no significant difference in parasite prevalence or intensity between males and females [142, 93, 82, 137, 187, 188, 126, 154, 88, 134] [189, 190, 86, 158, 159, 157, 191, 192, 91, 181] [120, 66, 193, 194].

It is generally well known that in vertebrates, females tend to have higher immunocompetence and be less parasitized than males [195, 196, 197]. The immunocompetence handicap hypothesis suggests that, among its other functions, high testostertone in males can actually cause immunosupporession [198, 199, 200, 201, 202] so males are less able to fight off parasitic infections. In birds, occurrence of female bias may be due to longer exposure of females to vectors while sitting on the nest [185]. Of the 30 studies referenced in the preceeding paragraph, eighteen provided enough information to determine whether one sex had a higher prevalence than the other (whether significant or not). Seven found male bias while nine found female bias and two found that the sexes had the same prevalence. Perhaps it was due to chance that the split was nearly even but maybe for birds in general, the added prevalence in females due to exposure on the nest equals the addition in males due to immunosuppression.

**Bird Age Trends**

The age of a bird may also affect whether or not it is likely to have a parasitic infection. Fourteen studies found that for at least one parasite genus, older birds tend to have higher prevalence [78, 133, 184, 82, 64, 203, 126, 154, 204, 88] [179, 157, 205, 186], while only two found that juvenile birds have higher prevalence [80, 191]. Thirteen other studies found no significant difference in prevalence between age classes [141, 137, 187, 188, 189, 131, 65, 190, 152, 86] [158, 159, 91]. It was rather surprising to find how many studies reported no significant difference. The studies that found infections to be very low or absent in juveniles but also very low in adults are not the surprising ones. The remarkable ones are those that reported high prevalence is adults and high prevalence in juveniles. This would indicate a high level of active transmission, most notably in juveniles.

Higher prevalences in older birds could be caused by relapse of existing infections and/or the result of cumulative years of exposure [125, 206, 6] while higher prevalences in younger birds may be caused by them being more susceptible to acquiring parasites due to more exposure to vectors as nestlings and/or lack of immunity [207, 208, 25, 209]. For juveniles to have higher infection prevalence, however, greatly depends on whether they are in an area with infective vectors.

**Bird Health Trends**

Many studies have found a significant negative or positive correlation between haemosporidian infections and a variety of different measures of health. Some of the most common measurements of health are body condition, body mass, reproductive success (# of eggs laid, egg volume, laying date, fledgling success etc), male plumage color, bird survival and immune functions. Though health trends are an important part of research into the effects of

blood parasites on birds, health measurements were not taken for the current study so unfortunately it will not be discussed further

## Materials and Methods

At this point, all field data had been collected for each sample such as the study site, month and year of collection, species, sex and/or age of bird, if determined. Lab data had also been collected. Of the 95 infections that were identified to genus, 14 were *Haemoproteus*, 40 were *Plasmodium* and 41 were *Leucocytozoon*.

A two-column dataset was created using Microsoft Excel in CSV format in order to test for a parasite genera trend using the 95 infections identified to genus. The first column was the dependent variable (categories= infected and uninfected), which indicates the status of the 95 infections for each category (*Haemoproteus*, *Plasmodium* and *Leucocytozoon*) of the independent variable (parasite genus) in column two for a total of 285 rows of data. The dataset was loaded into the R program [210] and a Pearson's chi-squared ($\chi^2$) test was performed on a contingency table of the data (Table 2.1). The resulting p-value was significant ($< 0.05$), so the data was subdivided and retested [211] (pp. 466-467). This was done by studying Table 2.1 to identify the independent variable category that most differed from the values in the other categories (*Haemoproteus* was the obvious choice). The *Haemoproteus* category and its associated dependent variable information were removed from the dataset and a $\chi^2$ test was run on the remaining dataset. The R program automatically determined that Yates Correction for Continuity was needed since there were only two categories left in the independent variable. As expected, the resulting p-value was not significant ($> 0.05$), so all the data about *Haemoproteus* was put back into the dataset exactly as it was before and all other independent variable categories (*Plasmodium* and *Leucocytozoon*) were combined into one category by changing the

names to 'Other'. The dataset was once again loaded into the R program and a $\chi^2$ test was run (Yates Correction for Continuity was once again automatically applied). The resulting p-value was significant, so the category that caused the significance in the first $\chi^2$ test was identified as *Haemoproteus*.

Testing for the other trends was done just slightly differently. For these tests, there are five dependent variables (categories= yes and no): Infected with *Haemoproteus* /*Plasmodium,* Infected with *Haemoproteus*, Infected with *Plasmodium*, Infected with *Leucocytozoon* and Dual Infected (infected with more than one genus). The dependent variable called Infected with *Haemoproteus*/*Plasmodium* exists because it contains sixteen additional infections that were caused by either *Haemoproteus* or *Plasmodium* but the exact genus could not be determined due to multiple base callings in the sequence (n= 5) or complete lack of sequence (n= 11) caused by molecular false negatives. The five independent variables are Month (categories= May, Jun., Jul., Aug. and Sept.), Year (categories= 2009, 2010 and 2011), Study Site (categories= the 5 study sites), Species (categories= SAVS, CCSP and BOBO) and Sex/Age (categories= adult male, adult female and juvenile).

Again, datasets were created using Microsoft Excel in CSV format. Month, Year and Study Site refer to when (temporal) and where (spatial) birds were sampled and a dataset of these variables was created using all samples (n=150). Another dataset was created for the Species variable and consists of savannah sparrow (SAVS), bobolink (BOBO) and clay-colored sparrow (CCSP) samples (n=73, 32 and 17, respectively) since these species were sampled most. The third dataset was made for the variable Sex/Age which consists of only bobolink samples (n=32) since only this species was categorized based on sex and/or age.

Each sample could only belong to one category of each independent variable but may belong to more than one dependent variable (if infected with more than one genus). For example, bird # 23 that was captured in June 2010 at study site three was an adult male bobolink and was determined to be infected with *Plasmodium* and *Leucocytozoon*. Bobolink samples are included in all three of the datasets and based off of the prevalence data it is determined that this sample is to be assigned to four of the five dependent variables: Infected with *Haemoproteus*/*Plasmodium*, Infected with *Plasmodium*, Infected with *Leucocytozoon* and Dual Infected. Based on the information about this bird above, it was assigned to one category for each independent variable as follows: Sex/Age= Adult Male, Study Site= 3, Year= 2010, Month= Jun., Species= BOBO. Each infected sample was evaluated in this fashion until all were assigned to appropriate variables and categories. Table 2.2 was created as a visual aid that sums up the dependent and independent variable information in the datasets. Each of the three datasets was loaded in the R program and the five independent variables were tested against the five dependent variables (25 initial tests total) using contingency tables. The tests were done using the Pearson's chi-squared ($\chi^2$) test or Fisher's exact test when appropriate.

The Pearson's chi-square test is applicable for circumstances where the sample size ($n$) is $\geq 10$, the number of categories ($k$) in the independent variable is $\geq 3$ and $n^2/k \geq 10$ [211]. The Fisher's Exact Test is appropriate when the dataset does not conform to the aforementioned conditions (i.e. there are too few samples or categories). The Pearson's chi-square test would have been applicable for all 25 tests but the Fisher's exact test was used for the five Month and five Year tests because the sample size was so low for September (n=5) and 2009 (n=6), respectively. A result was determined to be significant if the p-value was $< 0.05$. The null

hypothesis is that there is no significant difference between the observed and expected values in the categories of the independent variables.

To test which category(ies) of the independent variables caused the significant p-values (which results in rejection of the null hypothesis), the data was subdivided and retested [211] (pp. 466-467). This was done by studying Table 2.2 to identify the category(ies) that appeared to differ the most from the values in the other categories. The category that differed most was removed from the dataset and the appropriate statistical test was run in the R program on the remaining dataset. Again, the R program automatically determined whether Yates Correction for Continuity was needed for independent variables (Sex/Age and Species) that were left with 2 categories. As long as the resulting p-value was not significant, the category that was removed was put back into the dataset exactly as it was before and all other independent variable categories were combined into one category by changing the names to 'Other'. The dataset was tested again. If the resulting p-value was significant, it can be determined that the category that was subdivided from the rest of the data was causing the original significant result. If subdividing the data did not produce either a non-significant or significant result where needed, a different category was used to subdivide the dataset or a second category was chosen to subdivide the dataset and the appropriate statistical tests were run again in the R program until the significant category(ies) were identified.

## Results

A contingency table of the prevalences for the three haemosporidian genera is shown in Table 2.1. There was a significant difference in prevalence between the three genera ($\chi^2 = 22.2$, df = 2, $P < 0.0001$). *Haemoproteus* was removed and the dataset retested (Yates $\chi^2 = 0$, df = 1, P = 1) then put back in the dataset and all other categories were combined together and retested

(Yates $\chi 2 = 20.9388$, df = 1, $P < 0.0001$). This shows that the original significance was caused by *Haemoproteus* infections being significantly lower than that of *Plasmodium* or *Leucocytozoon*.

Table 2.1: Contingency table showing the infected and uninfected status of 95 infections for each parasite genus.

| | *Haemoproteus* | *Plasmodium* | *Leucocytozoon* | Totals |
|---|---|---|---|---|
| Infected | 14 | 40 | 41 | 95 |
| Uninfected | 81 | 55 | 54 | 190 |
| Totals | 95 | 95 | 95 | 285 |

Table 2.2 shows the numbers and percentages of *Haemoproteus*, *Plasmodium*, *Leucocytozoon* and dual infections found in each month and year, at each study site, in the three most sampled bird species and in different sex/age classes of bobolinks.

Table 2.3 shows the p-values for the initial statistical tests on the datasets. There were no significant differences in parasite prevalence between years or study sites. The independent variables called Month, Species and Sex/Age all produced two significant p-values each so the null hypothesis was rejected in these six cases. Rejection indicates that the observed value of one or more categories of each dependent variable was significantly different from what was to be expected.

The p-value results after subdividing the data are shown in Table 2.4. The subsequent conclusions are made based on this table. Clay-colored sparrows had significantly higher *Haemoproteus*/*Plasmodium* infections than bobolinks or savannah sparrows. High infection in clay-colored sparrows was driven by the significantly high infection rate of *Haemoproteus* in clay-colored sparrows. Adult male bobolinks had significantly higher *Leucocytozoon* and dual infections than either adult female or juvenile bobolinks. *Haemoproteus*/*Plasmodium* infections

Table 2.2: The number of samples belonging to each dependent and independent variable. Percentages of samples follow in parentheses.

| Independent Variables | Categories | Total Sampled | Dependent Variables | | | | |
|---|---|---|---|---|---|---|---|
| | | | Infected with *H./P.* | Infected with *H.* | Infected with *P.* | Infected with *L.* | Dual Infected |
| Month[a] | | | | | | | |
| | May | 18 | 13 (72%) | 4 (22) | 7 (39) | 4 (22) | 4 (22) |
| | Jun. | 61 | 41 (67) | 8 (13) | 20 (33) | 23 (38) | 17 (28) |
| | Jul. | 34 | 8 (24) | 1 (3) | 6 (18) | 9 (26) | 3 (9) |
| | Aug. | 32 | 8 (25) | 1 (3) | 7 (22) | 5 (16) | 2 (6) |
| | Sept. | 5 | | | | | |
| Year[a] | | | | | | | |
| | 2009 | 6 | 1 (17) | | 1 (17) | 3 (50) | 1 (17) |
| | 2010 | 68 | 35 (51) | 10 (15) | 23 (34) | 18 (26) | 10 (15) |
| | 2011 | 76 | 34 (45) | 4 (5) | 16 (21) | 20 (26) | 15 (20) |
| Study Site[a] | | | | | | | |
| | 1 | 24 | 12 (50) | 4 (17) | 6 (25) | 6 (25) | 4 (17) |
| | 2 | 36 | 17 (47) | 2 (6) | 12 (33) | 9 (25) | 6 (17) |
| | 3 | 22 | 12 (55) | 1 (5) | 7 (32) | 11 (5) | 7 (32) |
| | 4 | 19 | 12 (63) | 4 (21) | 5 (26) | 4 (21) | 3 (16) |
| | 5 | 49 | 17 (35) | 3 (6) | 10 (20) | 11 (22) | 6 (12) |
| Bird Species[b] | | | | | | | |
| | SAVS | 73 | 37 (51) | 4 (5) | 25 (34) | 23 (32) | 16 (22) |
| | BOBO | 32 | 11 (34) | | 7 (22) | 10 (31) | 5 (16) |
| | CCSP | 17 | 14 (82) | 8 (47) | 4 (24) | 5 (29) | 4 (24) |
| Sex/Age[c] | | | | | | | |
| | Adult Male | 14 | 6 (43) | | 4 (29) | 9 (64) | 5 (36) |
| | Adult Female | 9 | 3 (33) | | 1 (11) | 1 (11) | |
| | Juvenile | 9 | 2 (22) | | 2 (22) | | |

[a]= Includes all 150 samples
[b]= Includes only the 3 most sampled bird species
[c]= Includes only the 32 bobolink samples

Table 2.3: P-values of initial statistical tests. The unshaded cells contain p-value results from Pearson's chi-squared tests and shaded cells contain p-value results from Fisher's exact test.

| | Month[a] | Year[a] | Study Site[a] | Species[b] | Sex/Age[c] |
|---|---|---|---|---|---|
| Infected with *H./P.* | < 0.0001* | 0.2593 | 0.2346 | 0.0060* | 0.5945 |
| Infected with *H.* | 0.1025 | 0.1763 | 0.1706 | < 0.0001* | 0.4578 |
| Infected with *P.* | 0.2153 | 0.1812 | 0.7112 | 0.3722 | 0.6132 |
| Infected with *L.* | 0.1278 | 0.4932 | 0.1456 | 0.9859 | 0.0016* |
| Dual Infected | 0.0357* | 0.7466 | 0.3852 | 0.7216 | 0.0222* |

* indicates a significant p-value.
[a]= includes all 150 samples, [b]= includes 73 SAVS, 32 BOBO and 17 CCSP samples, [c]= includes 32 BOBO samples.

Table 2.4: P-values for the chi-squared tests run on the subdivided data. A superscript of either 1, 2, 3 or 4 after each p-value indicates the category(ies) used to subdivide the dataset (see list below). The top p-values were calculated after the category(ies) used to subdivide the data was removed from the dataset completely. The bottom p-value were calculated after the category(ies) were put back in the dataset and all other category names changed to 'Other'.

| | Month | Species | Sex/Age |
|---|---|---|---|
| Infected with *H./P.* | 0.742 [1]<br>1.319e-07 *, [1] | 0.183 [3]<br>0.01103 *, [3] | |
| Infected with *H.* | | 0.4258 [3]<br>3.118e-07 *, [3] | |
| Infected with *L.* | | | 1 [4]<br>0.001518 *, [4] |
| Dual Infected | 0.3316 [2]<br>0.009255 *, [2] | | 1 [4]<br>0.02323 *, [4] |

* = significant p-value
1 = May and Jun. categories used to subdivide dataset
2 = Jun. category used to subdivide dataset
3 = CCSP category used to subdivide dataset
4 = Adult Male category used to subdivide dataset

were significant higher in May and Jun. compared to Jul., Aug. and Sept. Dual infections were significantly higher in Jun. compared to all other collection months.

## Discussion

### Parasite Genera Trend

There was a significant difference in prevalence between the parasite genera in this study (the number of *Haemoproteus* infections [n=14] was significantly lower than *Plasmodium*

[n=40] and *Leucocytozoon* ([n=41]) but since there are so few studies to compare against that do this statistical test, I decided to look at prevalence by genus for other studies in two different ways.

Hundreds of studies on avian Haemosporidia worldwide had been compiled as references for this current study (biased toward USA studies). Table 2.5 was created with 69 of those studies that screened for all three genera (67 other studies were eliminated as references for the table due to various reasons). For each of the 69 studies, the prevalence of the genera was ranked from highest to lowest and the study was referenced in one of the six rankings in Table 2.5. *Haemoproteus* was the most prevalent genus for 45% (31/69) of the studies while *Plasmodium* and *Leucocytozoon* were most prevalent in 25% (17/69) and 30% (21/69) of the studies, respectively.

Though *Haemoproteus* was by far the most prevalent genus worldwide (concurred by [123, 6]), it was the least prevalent genus in this current study. In fact, based on the infections that were identified to genus, this study would belong in the rank of *Leucocytozoon* > *Plasmodium* > *Haemoproteus*, the least common one with only six other studies. It is worth noting that there are sixteen additional infections that were either *Plasmodium* or *Haemoproteus*. Had these infections been identified to genus, it is almost certain that the number of *Plasmodium* infections would have surpassed that of *Leucocytozoon*, placing this study in the rank of *Plasmodium* > *Leucocytozoon* > *Haemoproteus* instead, the second least common rank with only seven other studies. I did not expect to find that, of the twenty studies that sampled birds strictly in the USA, 85% (17/20) of them fell into two rankings (*Haemoproteus* > *Plasmodium* > *Leucocytozoon* and *Leucocytozoon* > *Haemoproteus* > *Plasmodium*); this study does not fall in either one. Thus, based on the large scale, the results presented here appear to be unusual.

Table 2.5: All combinations of highest to lowest rankings of parasite prevalence by genus. Studies compiled as references for the current study were placed in the appropriate ranking in the table according to their prevalence results. The geographic location and bird group was also recorded in the table for each study. The gray highlight indicates a study using only birds sampled in the USA.

| Rank of prevalence by genus | Continent/Region/ Country | Bird Group | Reference |
|---|---|---|---|
| *P.>H.>L.** | USA | Passeriformes | [161] (found no *L*) |
| | North America | Passeriformes | [94] |
| | Galapogos | Passeriformes | [109] (found no *L*) |
| | Nigeria | Non-Passeriformes | [212] |
| | Japan | Passeriformes + others | [213] (found no *L*) |
| | Colombia | Passeriformes + others | [177] |
| | Colombia | Passeriformes + others | [214] |
| | Africa | Passeriformes + others | [144] |
| | Europe | Passeriformes | [110] (found no *L*) |
| | West Africa | Passeriformes + others | [215] |
| *P.>L.>H.* | Canada | Passeriformes | [130] |
| | Russia | Passeriformes | [157] (found no *H*) |
| | Bulgaria | Passeriformes + others | [189] |
| | Spain | Passeriformes | [188](found no *H*) |
| | Switzerland | Passeriformes | [148] |
| | Japan | Passeriformes + others | [47] |
| | Azores | Passeriformes | [178] |
| *H.>P.>L.* | USA | Passeriformes | [81] |
| | USA | Passeriformes | [147] |
| | USA | Passeriformes | [91] (found no *L*) |
| | USA | Passeriformes | [88] (found no *L*) |
| | USA | Passeriformes | [85] |
| | USA | Passeriformes | [84](found no *L*) |
| | USA | Passeriformes + others | [216] |
| | USA | Passeriformes + others | [79] |
| | USA | Non-Passeriformes | [217](found no *L*) |
| | USA | Non-Passeriformes | [57] |
| | Canada | Non-Passeriformes | [142] |
| | Costa Rica | Passeriformes + others | [218] |
| | Colombia | Passeriformes + others | [219] (found no *L*) |
| | Jamaica | Passeriformes + others | [63] |
| | Brazil | Passeriformes + others | [136] |
| | Bulgaria | Passeriformes | [127] (found no *L*) |
| | Czech Republic | Passeriformes | [46] |
| | Subantarctic and tropical sites | Non-Passeriformes | [3] (found no *L*) |
| | Europe and Africa | Passeriformes for sure but not sure about other orders | [164] |
| | Israel | Passeriformes + others | [220] |
| *H.>L.>P.* | USA | Passeriformes + others | [83] |
| | Canada | Non-Passeriformes | [221] |
| | Iran | Non-Passeriformes | [222] (found no *P*) |
| | Madagascar | Passeriformes + others | [171] |
| | Denmark | Passeriformes | [140] (found no *P*) |
| | Curonian Spit | Passeriformes | [190] |
| | Japan | Passeriformes + others | [143] |
| | Spain | Passeriformes | [223] |
| | Finland | Passeriformes | [187] (found no *P*) |

Table 2.5: cont.

| Rank of prevalence by genus | Continent/Region/ Country | Bird Group | Reference |
|---|---|---|---|
| *H.*>*L.*>*P.*, cont. | France | Passeriformes + others | [129] |
| | Philippines | Passeriformes + others | [182] |
| *L.*>*P.*>*H.* | USA | Non-Passeriformes | [131] (found no *H*) |
| | Europe, Africa, North America | Passeriformes + others | [73] |
| | Canada | Passeriformes | [64] |
| | Uganda | Passeriformes + others | [158] |
| | Africa | Passerines | [174] |
| | Japan | Passeriformes + others | [66] |
| *L.*>*H.*>*P.* | USA | Passeriformes | [82] |
| | USA | Passeriformes | [224] (found no *P*) |
| | USA | Passeriformes + others | [80] |
| | USA | Non-Passeriformes | [225] (found no *P*) |
| | USA | Non-Passeriformes | [226] (found no *P*) |
| | USA | Non-Passeriformes | [227] |
| | USA | Non-Passeriformes | [95] (found no *P*) |
| | Europe, USA and Africa | Non-Passeriformes | [145] |
| | Canada | Passeriformes | [228] (found no *P*) |
| | Canada | Passeriformes + others | [78] |
| | Canada | Non-Passeriformes | [184] (found no *P*) |
| | Canada | Non-Passeriformes | [141] |
| | Spain | Passeriformes + others | [229] |
| | Spain | Non-Passeriformes | [194] |
| | Europe | Passeriformes | [126] |

* *P.* = *Plasmodium*, *H.* = *Haemoproteus*, *L.* = *Leucocytozoon*

Parasite genera trends were also compared on a smaller scale by creating Figure 2.1 using 36 studies on birds from the contiguous USA (citations provided in Appendix A). For each study, prevalence results (the number of infected birds/number of birds sampled) were separated by parasite genus then separated further based on which state(s) the birds were from. Two tables were made with this information (Appendix A). For each cell of the table with results from more than one study, the data were added together.

Figure 2.1 presents a pictographic version of the table by showing the proportion that each of the 3 genera comprise of the total infections for 34 states. The proportion of the infections caused by *Haemoproteus*, *Plasmodium* and *Leucocytozoon* in the current study is also shown and is most similar to the proportions in Minnesota (MN) but *Haemoproteus* was higher

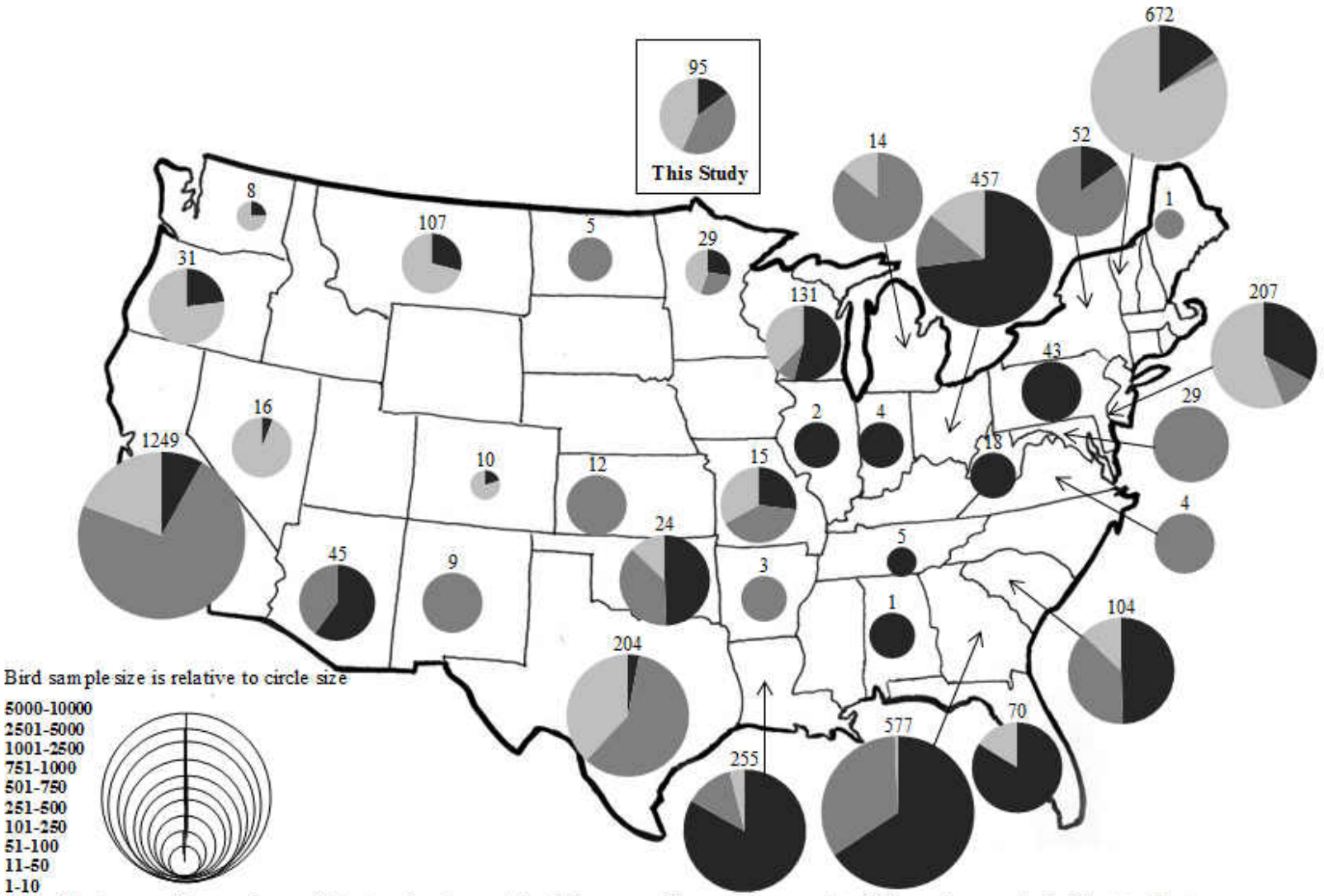



Figure 2.1: A map of the contiguous USA showing the combined Haemosporidia prevalence results of 36 previous works for 34 states. Each circle shows the proportion of infections that were identified as *Haemoproteus* (■), *Plasmodium* (■) and *Leucocytozoon* (■). The number above each circle is the number of infections found. The size of the circle is indicative of the sample size of birds. The proportions of infections by genus from the current study is also shown above the map.

and *Plasmodium* lower in MN by comparison. Missouri was the second closest match. This indicates that although Table 2.5 shows a marked difference in the parasite genera trend in this study compared to the larger scale (worldwide and USA), perhaps the prevalence for each genus in this study is not unusual for the midwest.

**Temporal Trends**

The temporal trends in this study were not a surprise having read what other authors have found. *Haemoproteus*/*Plasmodium* prevalence being highest early in the breeding season (average of 69.72% between May and Jun. in this study) compared to later (average of 16.18% between Jul.-Sept.) is a common trend. An accepted explanation for this trend is that many birds have a relapse of their chronic infections after the stress of migrating to the breeding grounds [78, 125] and the stress of reproductive efforts. This makes it easier to come across the parasites during screening but also shows that the lower prevalence later in the season is likely caused by missing infections in birds that have recovered from the relapse. This relapse could also increase the chances that the parasites will be spread at the breeding site after migration. This may be especially true for *Plasmodium* parasites as this study confirmed that this genus undergoes active transmission within the study area. Since a peak in prevalence later in the breeding season is usually caused by active transmission, especially for juveniles, its absence in this study is likely due to sampling so few juveniles and/or not many birds got infected at the sampling sites during the study.

*Haemoproteus* and *Plasmodium* prevalences by themselves were not significantly different by month though prevalence was highest in May (22.22%) and Jun. (38.89%), respectively. Prevalence of one or both of the genera separately may have been significant had there been a larger sample size or genus-specific primers been used.

*Leucocytozoon* showed no significant difference in prevalence between collection months though it was highest in June (37.70%). The lack of significance may be due to the absence of a possible relapse, so the chance of finding the infections through screening is not significantly different no matter which month the blood was collected. Based on my observations, *Leucocytozoon* parasites are easier to find than *Haemoproteus* or *Plasmodium*, so even if *Leucocytozoon* does undergo relapse, the chance of finding the infection may not change significantly from month to month.

Dual infections were significantly higher in June (27.87%) than any other collection month. This observation is probably a consequence of the prevalence for each genera being highest or second highest during this month.

The temporal trend that looked at differences in prevalence between collections years was never significant. This lack of significance can be interpreted as a good sign. It could indicate that the dynamics of hosts-parasites-vectors are temporally stable between years within the study area. Sample sizes per year were quite low in this study, especially for 2009, so it is possible that even if a significant trend was present the sample sizes were too low to accurately represent it.

An important reason for surveying Haemosporidia for several years is to detect introductions of novel parasite species that easily infects hosts and vectors, and to document if that species or genus would quickly increase in prevalence in successive years. A decrease in prevalence in years could be caused by decrease in precipitation.

**Spatial Trends**

There was no significant difference in prevalences between the study sites. This was not a surprise because the five sites were very similar habitats and only spanned three counties. Study sites 1 and 3 were the furthest away from each other, at about 31 miles in a straight line. I doubt

there would have been a difference even if the sample sizes were larger at each site. There probably would have only been a significant difference if very similar habitats were chosen between several states.

Essentially, the presence of spatial trends among very similar habitats are caused by variations in compositions of vector families and bird species at each site resulting from uncontrollable variables (rainfall, temperature, humidity etc), the differences between which get more substantial with increased distance. It would have been interesting to know if there could have been a difference in prevalence between sites that are not similar in habitat; perhaps the prevalence of Haemosporidia would have been different between forest and grassland sites if samples had also been collected from birds at forest sites within the same study area.

**Bird Species Trends**

Trends of prevalence by bird species are likely caused by the susceptibility of a bird species to infection and the exposure of the birds to infective vectors. Of the three species that were sampled most often, clay-colored sparrows had significantly more *Haemoproteus* infections than bobolinks or savannah sparrows. It is unknown exactly what is causing this trend but it is possible that clay-colored sparrows have more exposure to *Haemoproteus*. It can be assumed that if the birds were being infected with *Haemoproteus* primarily at the breeding grounds, then there would not be a significant difference in prevalence for this genus, so clay-colored sparrows are most likely getting infected at the wintering grounds. As shown in Figure 2.1a the wintering grounds of clay-colored sparrows are concentrated in Mexico so perhaps this is an area where active transmission is occurring via biting midges and/or hippoboscid flies. Savannah sparrows winter distribution (Figure 2.1b) does overlap that of clay-colored sparrows but it is also much more widespread. Perhaps the savannah sparrows that breed in northwest MN do not winter in

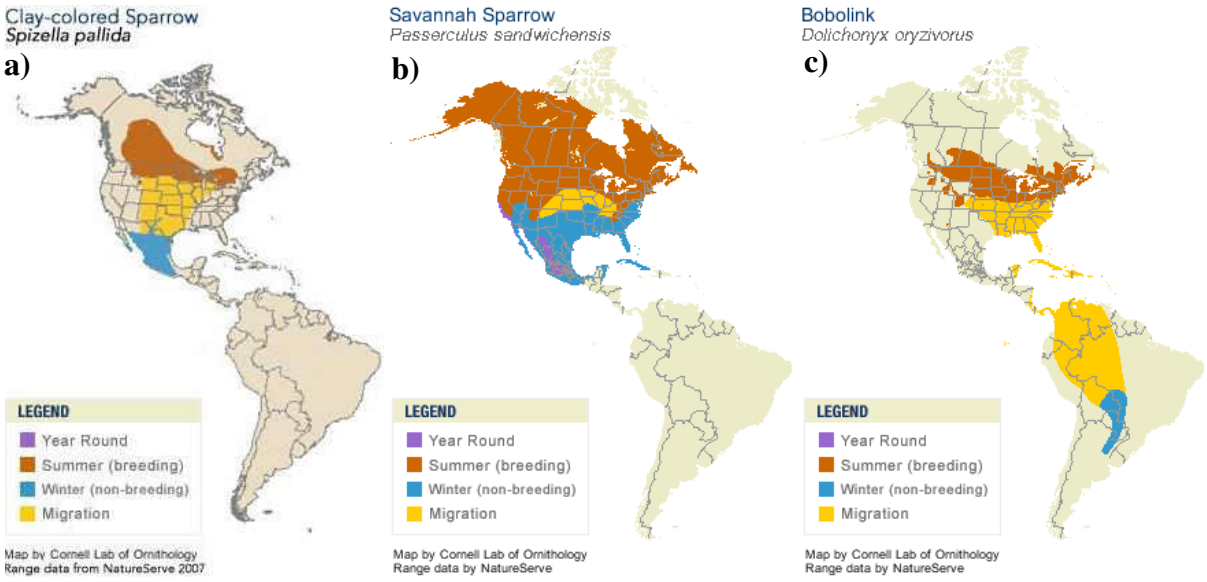



Figure 2.2: Distribution maps of the three most sampled bird species [87].
(a) Clay-colored sparrows
(b) Savannah sparrows
(c) Bobolinks

Mexico but a different area where competent vectors are limited. Even if savannah sparrows and clay-colored sparrows from northwest MN do winter in the same geographic location they may prefer habitats that are just different enough for savannah sparrows to limit exposure to *Haemoproteus* vectors. Bobolinks wintering grounds (Figure 2.2c) are very different as they migrate much further south to South America, some as far as the Galapagos Islands. No infections were identified as *Haemoproteus* in bobolinks, so perhaps they are least exposed to these parasites.

It may also be possible that clay-colored sparrows are more susceptible to *Haemoproteus* or perhaps the high infection rate can be partially explained by high parasitemia. Even if clay-colored sparrows, savannah sparrows and bobolinks are equally exposed to *Haemoproteus*, there

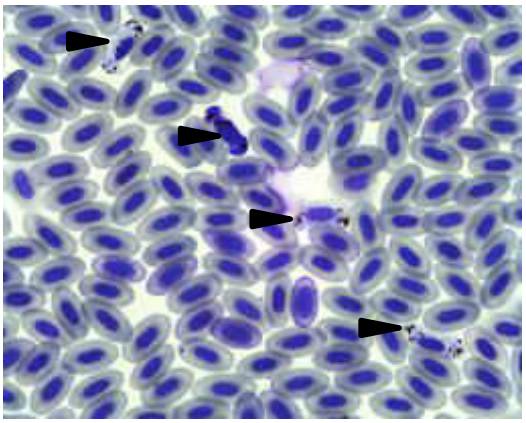


Figure 2.3: Example of high parasitemia in a blood smear from a clay-colored sparrow. Four erythrocytes are infected with *Haemoproteus* in this field of view. Black arrow = parasite

is the chance that this genus causes infection much easier in clay-colored sparrows and/or savannah sparrows and bobolinks are not as competent of a host. A trend that became obvious during slide screening is the parasitemia in clay-colored sparrows was much higher on average than in any other bird species. It was not uncommon to see two to four *Haemoproteus* parasites per field of view in blood smears of clay-colored sparrows (Figure 2.3). This trend of high parasitemia by *Haemoproteus* compared to *Plasmodium* and/or *Leucocytozoon* is a common one [80, 122, 177, 163, 144, 190]. Studies by Murata [143] and Kirkpatrick and Suthers [82] were the only exceptions found.

**Bird Sex/Age Trends**

Adult male bobolinks had significantly more *Leucocytozoon* and dual infections than adult females or juveniles. They also had a higher prevalence of *Haemoproteus* and *Plasmodium* though it was not significant. Sample size was low so it is very possible that a higher sample size would have produced different results. If this is indeed an accurate representation of the trend for bobolinks in the study area, then this could be due to the appearance of the adult males being different from adult females or juveniles. Figure 2.4 shows the coloration differences between breeding adult males (Figure 2.4a) and females (Figure 2.4b) and juveniles (Figure 2.4c). Nonbreeding adult males look like females at the wintering grounds, so if black flies are

preferentially feeding on adult male bobolinks based on color then they must be doing so at the breeding grounds where sexual dimorphism is obvious. Though the current study did not demonstrate active transmission of *Leucocytozoon*, one study did after screening bald eagles from MN [56].

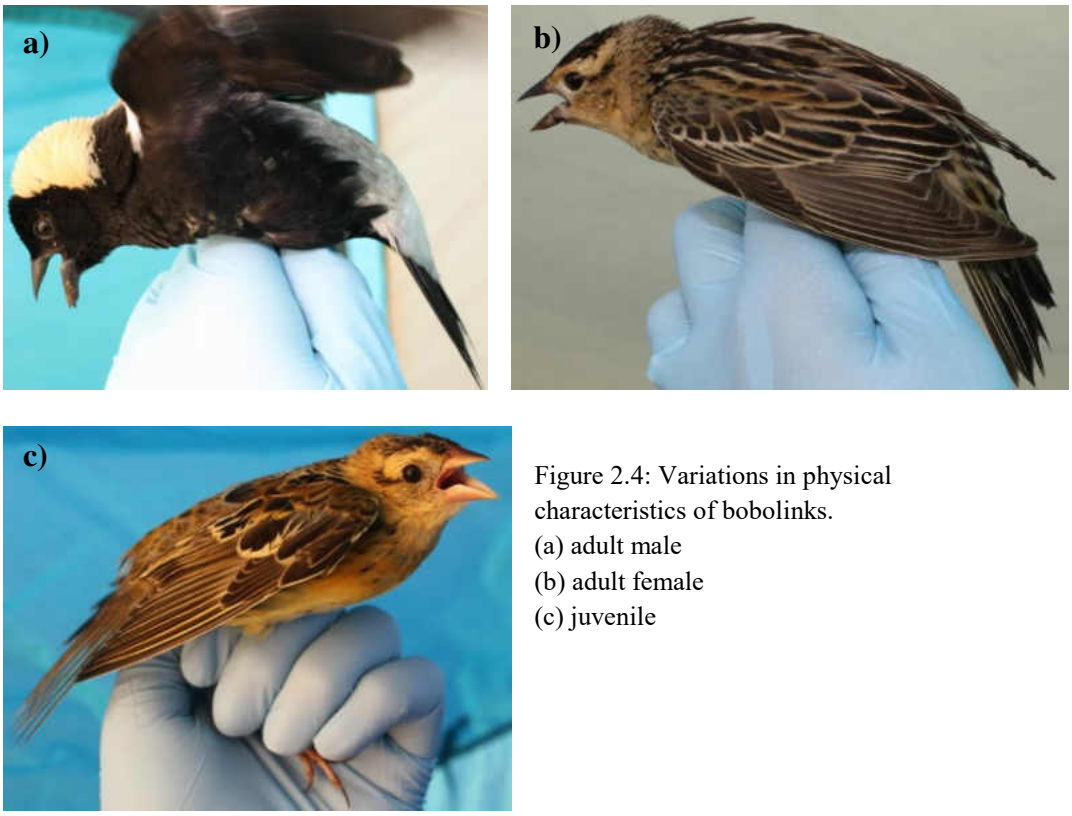



Figure 2.4: Variations in physical characteristics of bobolinks.
(a) adult male
(b) adult female
(c) juvenile

The behavioral differences between age and sex classes of bobolinks may also be to blame for the higher prevalences in males. During the breeding season, the polygymous males compete vigorously for the best terrirory that attracts the most females. They are usually much more visible than females or juveniles, perching on the tallest vegetation and flying short distances frequently, while the polyandrous females build nests. The juveniles obviously had less than one breeding season of exposure to vectors so it is not surprising that parasite prevalence was low.

# CHAPTER III

# PHYLOGENETICS OF BLOOD PARASITES (HAEMOSPORIDIA: *HAEMOPROTEUS*, *PLASMODIUM* AND *LEUCOCYTOZOON*) IN SONGBIRD PASSERINES FROM GRASSLANDS OF NORTHWEST MINNESOTA

## Introduction

### Pre-molecular Phylogeny

The earliest phylogenies of Haemosporidia were primarily created using vector competency, life history traits and/or morphological data from microscopy prior to the 1990s. These original methods helped to determine how to classify the different apicomplexan parasites taxonomically. The *Haemoproteus*, *Plasmodium* and *Leucocytozoon* genera were all established by the late 1800s and the characteristics used to classify parasites into these genera have changed very little. The various subgenera of *Haemoproteus* (n=2), avian *Plasmodium* (n=5) and *Leucocytozoon* (n=2), were established between 1890 and 1997 using non-molecular methods [6]. Several other original phylogenetic goals regarding haemosporidian genera and species were to define their origins and relatedness and determine which are ancestral. A simplified tree is shown in Figure 3.1a to illustrate the classic idea of Haemosporidia phylogeny. The addition of molecular data has helped to alter and clarify this original phylogeny and determine whether current naming conventions for genera and subgenera are supported.

### Phylogeny Based on Molecular Data

The earliest phylogenies on Haemosporidia that included molecular data were produced in the 1990s and were based on DNA sourced primarily from ribosomes (rDNA) [230, 28, 27,

26] and the circumsporozoite protein gene [231]. *Plasmodium* sequences from humans, primates, rodents and birds (only two sequences) were available at the time (no *Haemoproteus* or *Leucocytozoon* infections had been sequenced). One interesting debate that came about due to early molecular phylogeny was the relationship of the two avian *Plasmodium* sequences to *P. falciparum*, which infects humans. One study concluded that *P. falciparum* and avian *Plasmodium* shared a recent common ancestor [28] while another found that they share a much more distant common ancestor [27].

By 1998, rDNA proved to be inadequate for constructing Haemosporidia phylogeny. Attention instead turned to DNA sourced from the mitochondria, mt-DNA [232]. Some evidence suggests that mt-DNA lineages may be reproductively distinct and therefore a better source for phylogenetic analysis at this taxonomic level [233]. In 2000, Bensch et al. [30], published primers designed to screen for a fragment of a highly diverse mitochondrial gene called cytochrome b (mt-cytb) from avian *Haemoproteus* and *Plasmodium.* Many other authors followed suit with new primers for a wide variety of RNA and DNA fragments. By 2004, Hellgren et al. [31] designed primers to amplify a homologous fragment of *Leucocytozoon*, allowing simultaneous phylogenetic analysis with all three genera. Today, dozens of primer pairs exists to amplify RNA or DNA fragments of one, two or all three target genera infecting avians and some primer pairs will also amplify other Haemosporidia genera such as *Hepatocystis*, *Nycteria* and *Polychromophilus*; all are useful in obtaining sequences to be used in phylogeny. A fragment of the mt-cytb gene was obtained from avian Haemosporidia infections found in the current study and phylogenetic analyses were used to find the relationships between all of the infections.

**Comprehensive Molecular Phylogenies**

Three of the most comprehensive phylogenetic studies analyzed sequences from three of the major Haemosporidia genera: *Hepatocystis* (mammal hosts), *Haemoproteus* (bird and lizard hosts) and *Plasmodium* (mammal, bird and lizard hosts). Perkins and Schall (2002) [234] analyzed the mt-cytb gene and the tree they created was rooted with *Theileria annulata*. The two included *Leucocytozoon* sequences were found to be basal in the tree as a close sister outgroup to the ingroup. Martinsen et al. (2008) [235] (who analyzed mt-cytb and three other genes from more samples) used *Leucocytozoon* both as the outgroup and to root the tree. Outlaw and Ricklefs (2011) [236] analyzed the same data as Martinsen et al., [235] using an alternate rooting method.

Simplified versions of the three comprehensive phylogenies are shown in Figure 3.1b-d. In Figure 3.1c-d, the two subgenera of *Haemoproteus* (*Parahaemoproteus* and *Haemoproteus*: Kruse, 1890) are phylogenetically distinct. These subgenera are found between *Plasmodium* and *Leucocytozoon*, and of the two subgenera, *H*. *Parahaemoproteus* (which was found in the current study) is more closely related to *Plasmodium* in birds.

Figure 3.1b-c indicates that *Plasmodium* is not monophyletic due to *Hepatocystis* being found within the genus (the location of *Hepatocystis* was not specified by Outlaw and Ricklefs [236] for Figure 3.1d). In all three comprehensive phylogenies, *Plasmodium* is divided into two clades based on vertebrate hosts (one clade with mammal hosts and the other with lizard/bird hosts).

Evidence has been mounting that suggests there is a need for a revision of Haemospordia taxonomy. Specifically, there has been a request for reassignment of bird and reptile

Haemosporidia that are currently considered to belong to *Plasmodium* [236, 237], since *Plasmodium* is not monophyletic and clearly forms separate clades, divided by vertebrate hosts.

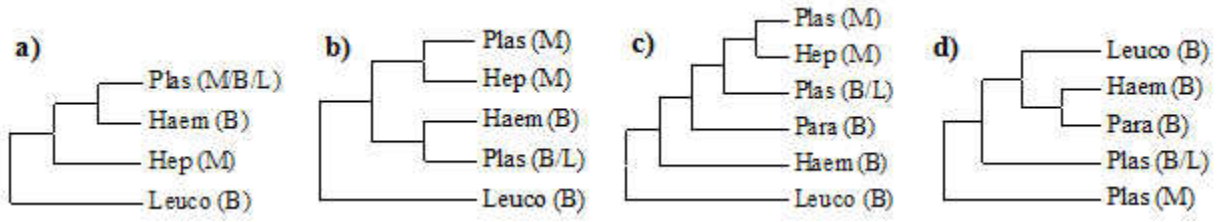



Figure 3.1: Four hypotheses for phylogenies of several haemosporidian genera with the vertebrate hosts they infect noted. The trees are (a) based on classic life history traits and morphology, (b) based on analysis of one gene and the originally published tree was rooted with *Theileria annulata* [234], (c) based on analysis of four genes and *Leucocytozoon* was used as an outgroup and to root the tree [235] and (d) based on the same data that [235] used, except the data was analyzed using an outgroup-free rooting method [236]. Plas= *Plasmodium*, Haem= *Haemoproteus* (genus or subgenus), Para= *Parahaemoproteus* (*Haemoproteus* subgenus), Hep= *Hepatocystis*, Leuco= *Leucocytozoon*, M= mammals, B= birds, L= lizards.

Most malaria trees contain an outgroup from the genus *Leucocytozoon*, which is closely related to, but not usually considered to be one of the malaria genera, since *Leucocytozoon* gametocytes lack hemozoin and meronts are not present in the blood of the vertebrate host. Because of this, together with a high divergence rate, *Leucocytozoon* is often assumed to be ancestral to *Hepatocystis*, *Haemoproteus* and *Plasmodium*.

However, the notion that *Leucocytozoon* is the ancestral genus and therefore represents a good outgroup is controversial. Using the same data as Martinsen et al., [235], Outlaw and Ricklefs (2011) [236] created a tree of Haemosporidia that infers the root based on the data provided [238] rather than establishing *a priori* by specifying *Leucocytozoon* as the root and/or outgroup. A simplified version of their tree is shown in Figure 3.1d and it suggests that *Leucocytozoon* is actually the most derived genus and is a sister group to *Haemoproteus* while *Plasmodium* is ancestral to both (species infecting mammals being most ancestral). This is the opposite tree configuration from what was previously found.

If the interpretation by Outlaw and Ricklefs [236] is correct, it implies that the two important traits of merogony in blood and hemozoin production are primitive; i.e. these traits evolved once in Haemosporidia and were then subsequently lost in some genera. *Plasmodium* and *Haemoproteus* both retain the hemozoin trait, only *Plasmodium* retains the merogony trait and *Leucocytozoon*, as the most derived genus, has lost both traits. *Plasmodium* retains the merogony trait; this assertion was the basis for creating this genus when merogony in blood was considered a recent acquisition to Haemosporidia. It remains to be seen which theory of Haemosporidia evolution is correct (whether *Leucocytozoon* is the ancestral or derived genus).

The controversy of which haemosporidian genus is ancestral, where the root of the phylogenetic trees should be placed and which genus should be used as the outgroup is why an outgroup was not specified for the phylogenies in the current study and the resulting trees were unrooted.

**Missing Phylogenetic Information**

Phylogenetic parameters that are still lacking are: 1) a molecular clock for Haemosporidia and 2) rates of extinction for Haemosporidia [239]. Thus, these parameters were not specified while performing phylogenetic analyses in the current study. The problem in determining these parameters is the lack of fossil DNA. There are only two existing fossil records; *Paleohaemoproteus* from a biting midge preserved in 100 million year old (myo) amber [240] and *Plasmodium* from a *Culex* mosquito preserved in 35 myo amber [241]. Unfortunately, it is not conclusive whether these fossils can be used as a calibration point to define the clock or extinction rate for avian malaria since present-day *Plasmodium* is not monophyletic [236].

Using cospeciation to find the clock and extinction rate has been shown to be a poor method. For example, it was previously assumed that when humans split from chimpanzees 4-7

mya, *P. falciparum* and *P. reichenowi* (which differ by 3.3% in the cyt b gene) cospeciated respectively [232, 27, 231, 242]. This idea that the two species diverged at the same time as their hosts 4-7 mya was used as the calibration point for the molecular clock. This was challenged by the recent discovery that *P. falciparum* actually falls within the clade of gorilla *Plasmodium* [243] instead of within the clade containing other species of human malaria (e.g. *P. vivax*, *P. ovale* and *P. malariae*). Similar approaches have been tried with malaria in other animals, but using cospeciation to determine the parameter settings has not been helpful thus far.

There are molecular clocks for most vertebrate hosts like birds and mammals. The rate of mt-dna divergence between species is about 2% per million years [244, 245, 246]. Many studies suggest a slower clock for Haemosporidia than their vertebrate hosts (e.g., [232, 247, 248]); it is just unclear just how much slower this clock would be. A slower haemosporidian clock is surprising since generation time is much shorter for Haemosporidia than vertebrates. Though they made several assumptions, Ricklefs and Outlaw (2010) [248] found a divergence rate of 1.3% per million years for Haemosporidia (highest rate being in *Leucocytozoon* and *Hepatocystis*), lower than that of their avian hosts but higher than former estimates based on cospeciation. If a 1.3% rate of divergence is correct, then it is amazing that some haemosporidian genera could have diversified to infect so many hosts in under 20 million years [239].

**Divergence Between Species**

There is some debate as to how divergent two or more sequences need to be in order to be considered different species. Perkins (2000) [249] sequenced twelve mt-cytb lineages of *P. azurophilum* (identified morphologically) from lizards and found that the sequences differed by a maximum of 3.1%. Hellgren et al. (2007) [250] analyzed fragments of the mt-cytb gene from six morphospecies of avian *Haemoproteus* and the average divergence between the morphospecies

was 5.5%. This suggests that, in most cases, separate morphospecies can be expected for sequences with a genetic difference >5% [251]; however, two morphospecies differed by only 0.7%. Križanauskienė et al. (2010) [117] found that a divergence of 2% was enough for species to be morphologically distinct. Closely related *P. falciparum* and *P. reichenowi* diverge by 2.3% across their mitochondrial DNA. Beadell and Fleischer (2005) [32] found that sequences from three infections identified as *P. relictum* differed by 3.4-3.9%. It seems safest to declare that two Haemosporidia sequences belong to separate species if they are diverged by at least 5%. If the divergence is less than that, morphological data from both infections should be gathered to support or disprove species status.

Since there are many hypotheses as to how divergent particular gene sequences need to be in order to assign them to separate species, broader measurements of divergence within and between *Haemoproteus*, *Plasmodium* and *Leucocytozoon* were performed for this study, rather than between sequences.

**Three Species Concept**

An interesting idea exists called a three species concept [249, 220] that defines a species based on morphological, genetic, or phylogenetic characteristics. The morphological species concept is a classical method that distinguishes parasite species through differences or similarities in morphology as seen in blood smears using microscopy. The genetic species concept is a molecular method that distinguishes parasites species based on similarities or divergence of genetic (DNA, RNA, amino acid) sequences. The phylogenetic species concept requires that defined species are monophyletic. In 2006, Martinsen et al. [220] found that fourteen of fifteen *Haemoproteus*, *Plasmodium* and *Leucocytozoon* parasites species identified using morphology were supported by genetic and phylogenetic analyses. The exception was *H.*

*belopolskyi* which was identified using morphology but fell into two separate phylogenetic clades.

In another 2006 study by Martinsen et al. [252], the three species concept was used to molecularly test the validity of the five *Plasmodium* subgenera that infect birds (as mentioned previously, these subgenera were originally created and defined based on microscopy data). Analysis of the coI and mt-cytb genes indicated that monophyly was supported for *Haemamoeba*, *Huffia* and *Bennettinia*. While most (twelve of the fourteen) *Novyella* sequences formed a clade, the two remaining *Novyella* samples were outside the clade, forming the most basal branches of the tree. The subgenus *Giovannolaia* did not form a monophyletic group.

**Avian Malaria in Galapagos Penguins**

In 2009, Levin et al. [21] found that the introduction of *Plasmodium* to the Galapagos Islands (located >600 miles off the west coast of South America) posed a threat to the endangered Galapagos penguin (*Spheniscus mendiculus*) and other endemic birds. The sequences obtained from the penguins closely match lineages that are known to cause severe morbidity and mortality in captive penguins. More recently in 2013, Levin et al. [253] suspected that a bird species capable of migrating vast distances could have introduced *Plasmodium* which was then transmitted to the endangered Galapagos penguins. Levin et al. [253] specifically cite the bobolink as one of the few species with the ability to transmit *Plasmodium* to Galapagos bird species.

This study confirmed active *Plasmodium* transmission in two juvenile bobolinks from northwest Minnesota (MN). Phylogenetic methods were used to determine if the haemosporidian sequences found in these bobolinks matched haemosporidian sequences obtained from Galapagos penguins. If so, then MN could be a source of the penguin infections.

## Materials and Methods

Of the total 111 Haemosporidia infections, 99 have molecular evidence in the form of mitochondrial cytochrome b (mt-cytb) sequences. The lack of twelve sequences was caused by molecular false negatives: eleven were *Haemoproteus* or *Plasmodium* infections and one was *Leucocytozoon*. All sequences were edited using MEGA version 5.2 [254] to delete the primer sequences and the 5' and 3' ends for which nucleotides could not be distinguished and to fix any nucleotide mistakes such as missed bases or multiple base callings. While editing, it was noticed that two of the *Leucocytozoon* and five of the *Haemoproteus*/*Plasmodium* sequences had multiple double base callings, indicating that there were at least two different parasite species in those particular samples. These were eliminated from further phylogenetic analysis. Although as many sequences as possible were retained for analysis, two *Haemoproteus* (there were enough bases to determine genus) and one *Leucocytozoon* sequences were determined to be too short (<100 bp) to be useful and were eliminated as well

A blastn was done in NCBI's GenBank and MalAvi [71] with the remaining 89 sequences to find the closest related sequences, species and lineages. This was useful because it further confirmed that my identification of the genera were correct.

The 89 sequences were also grouped by genus in MEGA. The mean distances between and within the three groups was determined in the form of the number of nucleotide differences after pairwise deletion.

### Creating Two Alignments

The 89 edited sequences were loaded into MEGA to create an alignment. Since the first alignment was the small one, the shortest sequence (*Haemoproteus*) was removed but it was

unique and would instead be used in the larger alignment. The remaining 88 sequences comprised the final small alignment.

A public database called MalAvi [71] contains *Haemoproteus*, *Plasmodium* and *Leucocytozoon* mt-cytb sequences collected from various avian hosts. Each unique sequence is called a lineage. All lineages from North America (n=138) were downloaded from MalAvi on 6 August 2015 and added to a build in MEGA. The 89 sequences (this time including the short *Haemoproteus* sequence) from this study were added to the alignment for total of 227 sequences comprising the larger alignment.

Both alignments were performed with Muscle using default settings. No gaps were present. Though the target sequence that the primers amplified was 479 bases long, all of the sequences from this study were shorter after editing while most of the sequences from MalAvi were complete. The alignments were exported from MEGA in fasta format and loaded into jModelTest version 2.1.7 [255]. The program determined that, with 4 gamma categories, the best nucleotide substitution model to use for phylogenetic analysis of both alignments was General Time Reversal with a discrete gamma distribution and the presence of invariant sites (GTR + G + I).

The haemosporidian lineages from the MalAvi database that were closely related to haemosporidian sequences found in this study were kept in the larger alignment. This was done by creating a Maximum Likelihood (ML) tree in MEGA of the 227 sequences. Settings of GTR + G + I with four gamma categories on all 479 sites were used. The large alignment of 227 sequences was also exported in PAUP format and the file was uploaded into MrBayes version 3.2.2 [256, 257]. A Bayesian analysis was performed using GTR + G + I with four gamma categories for two runs of one cold and three heated markov chain monte carlo (mcmc) searches

for 3 million generations, sampling one in every 500 trees with a 25% burnin of trees being discarded. The resulting ML tree and Bayesian tree were examined in MEGA and FigTree (http://tree.bio.ed.ac.uk/software/ figtree/), respectively, to find the lineages from MalAvi that were most closely related to the infections from the current study. Only the closely related lineages (n=53) and the 89 sequences from this study were kept in the build, which was aligned with Muscle again. The larger alignment was further reduced to 142 sequences and jModelTest indicated that GTR + G + I was still the best model.

**Maximum Likelihood (ML) Methods**

MEGA was used to create an ML tree for each alignment of 88 and 142 sequences. Both trees were created using GTR + G + I with four gamma categories on all 479 sites and 2000 Bootstrap replications were run. Both unrooted trees were exported in Newick format and edited in FigTree.

**Bayesian Methods**

Both MEGA alignments were exported in PAUP format and the files were uploaded into MrBayes version 3.2.2. Each of the two Bayesian analyses were performed using GTR + G + I with four gamma categories for two runs of one cold and three heated markov chain monte carlo (mcmc) searches for either 5,300,000 (the smaller alignment of 88 sequences) or 2,200,000 (the larger alignment of 142 sequences) generations, sampling one in every 500 trees with a 25% burnin of trees being discarded. The standard deviation of split frequencies indicated good convergence with values of 0.0094 and 0.0095 for the 88 and 142 sequence analyses, respectively. Each of the two runs of the analysis of the 88 sequences produced 10,601 trees (21,202 total), of which 7,951 were sampled (15,902 total) after the 25% burnin to elucidate the best tree. Each run for the 142 sequences produced 4,401 trees (8,802 total), of which 3,301

(6,602 total) were sampled to create the best tree. Both unrooted, consensus trees were edited in FigTree.

### Comparison with Avian Malaria from Galapagos Penguins

As of 6 August 2015, NCBI's GenBank contained 63 haemosporidian sequences from Galapagos penguins (35 *Haemoproteus* and 28 *Plasmodium*). These were loaded into a MEGA build and the 12 *Haemoproteus* and 40 *Plasmodium* sequences from this study were also added and the build was aligned using Muscle's default settings. Some of the 115 sequences extended beyond the target 479 bases of the mt-cytb so they were trimmed. The pairwise distances (with pairwise deletion) were computed in MEGA to see how many nucleotides were different between the *Haemoproteus* and *Plasmodium* sequences from this study and those from penguins. Two of the 40 *Plasmodium* sequences from the current study were from the two juvenile bobolinks, proof of active transmission in northwest Minnesota. Since the bobolink is one of the few species capable of migrating to the Galapagos Islands, the resulting pairwise distances were used to find out which of the 63 penguin sequences best matched the sequences from the juvenile bobolinks. All of the other *Plasmodium* and *Haemoproteus* sequences from this study were included in the phylogeny just in case any of them matched a sequence from a penguin.

## Results

### Lineages

Since none of the 89 electropherograms were produced with enough clarity to determine all of the 479 nucleotides, it is impossible to determine if any match a haemosporidian lineage in MalAvi, or determine how many lineages are present in northwest Minnesota (MN). Fourteen sequences had at least 455 (95%) of the target 479 bases so the best analysis of lineages that could be done was to study the MalAvi blastn results of these sequences. The nine

*Leucocytozoon* sequences most closely matched the lineages called CNEORN01 and CISPAL01 while the five *Plasmodium* sequences most closely matched TRPIP2, ZEMAC01, BT7 and SEIAUR01.

**Evolutionary Divergence**

Estimates of evolutionary divergence give a limited idea about the diversity of the Haemosporidia in northwest MN. The calculations are based on the 89 sequences that have been grouped into the three genera. The mean distances within the genera were 6.7, 18.8 and 13.5 (the numbers of nucleotide differences) for *Haemoproteus*, *Plasmodium* and *Leucocytozoon*, respectively. *Haemoproteus* was the least diverse group but this may be misleading because it also contained the fewest and shortest sequences while *Plasmodium* was the most diverse and had the most sequences. The mean distances between the three genera are shown in Table 3.1.

Table 3.1: Mean evolutionary distances between the three genera. The number of nucleotide differences between *Haemoproteus* (*H.*), *Plasmodium* (*P.*) and *Leucocytozoon* (*L.*) are shown.

| | *H.* | *P.* | *L.* |
|---|---|---|---|
| *H.* | | | |
| *P.* | 34.833 | | |
| *L.* | 60.610 | 67.045 | |

The table suggests that perhaps *Plasmodium* and *Leucocytozoon* are the least related and that *Haemoproteus* is more closely related to *Plasmodium* than to *Leucocytozoon*.

**Phylogenetic Trees**

Figures 3.2-3.5 show the resulting Maximum Likelihood (ML) and Bayesian trees for 88 and 142 haemosporidian sequences. In the ML tree of only the 88 sequences (Figure 3.2), *Plasmodium* was polyphyletic, with the top clade being comprised of what is most likely *P.*

*cathemerium* and all other *Plasmodium* species are grouped into the bottom clade. The monophyletic clades of *Leucocytozoon* and *Haemoproteus* fall in the middle. By comparison, in the ML tree of 142 sequences (Figure 3.4), *Plasmodium* was monophyletic due to the additional support of the 53 *Plasmodium* from MalAvi. *Haemoproteus* and *Leucocytozoon* remained monophyletic with *Haemoproteus* being a sister clade to both *Plasmodium* and *Leucocytozoon* but more closely related to *Plasmodium*. The conclusions based on the Bayesian trees of 88 (Figure 3.3) and 142 (Figure 3.5) sequences were similar to eachother. The additional support of the 53 sequences from MalAvi did not change the tree as was the case with the ML analyses. Again, each genus was monophyletic and *Haemoproteus* was a sister clade to *Plasmodium* and *Leucocytozoon* but was more closely related to *Plasmodium* than to *Leucocytozoon*.

In all of the phylogenies, the bird host species were scattered throughout with no obvious trend of them associating with specific haemosporidian clades.

**Comparison with Avian Malaria from Galapagos Penguins**

Based on the comparison of *Haemoproteus* and *Plasmodium* from this study and Galapagos penguins, none of the sequences between the two groups matched perfectly. The two sequences from juvenile bobolinks tentatively identified as *P. homopolare* and *P. cathemerium* were most closely related to accessions GQ395670 and GQ395679, respectively, being different by five and three nucleotides, respectively. There was one *Plasmodium* sequence (from an adult bobolink) and three *Haemoproteus* sequences (one from a gray catbird and two from savannah sparrows) from MN that were different from several Galapagos penguin sequences by only one nucleotide.

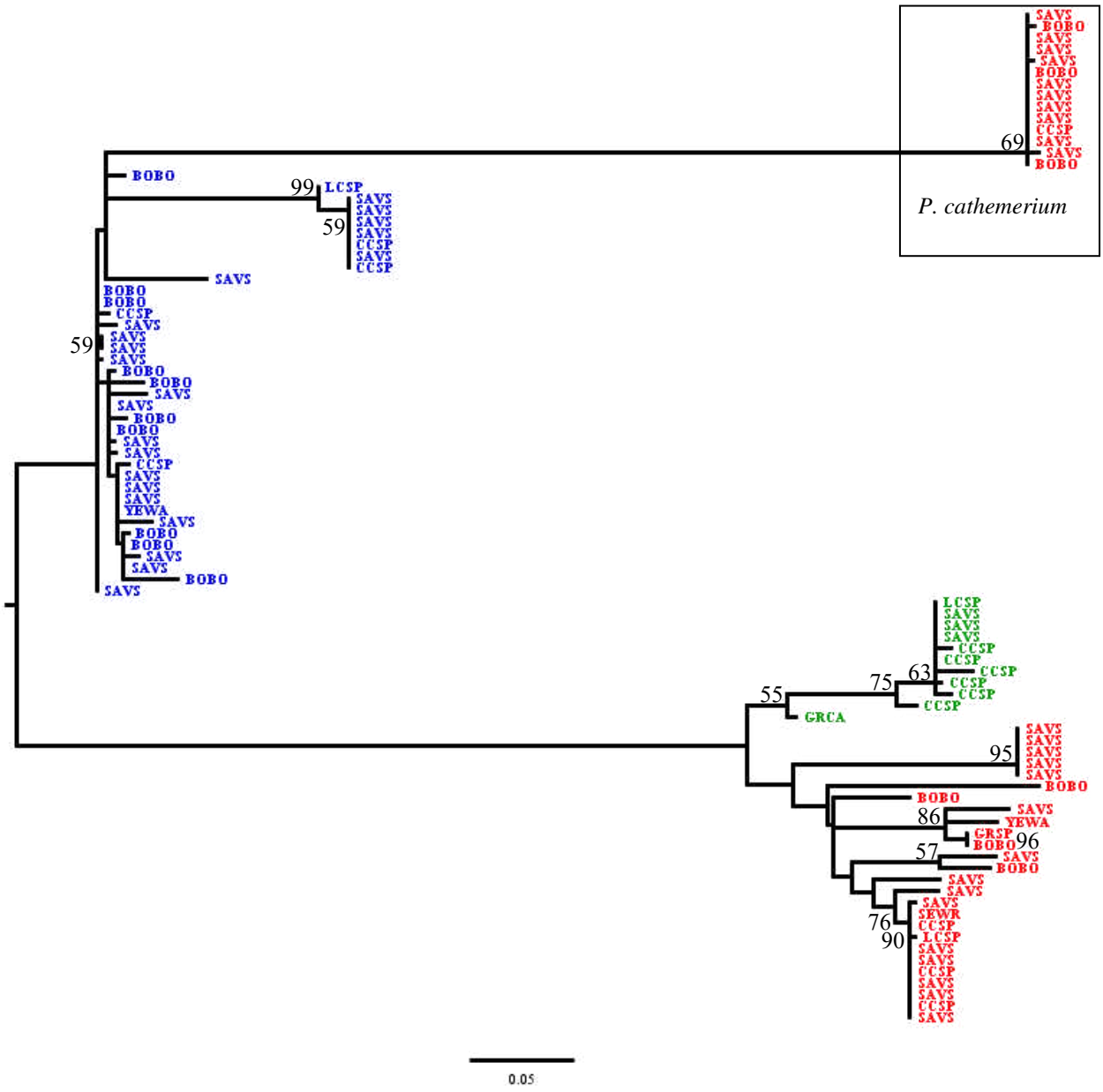



Figure 3.2: Unrooted Maximum Likelihood tree based on 88 cytochrome b sequences from Haemosporidia found in the current study. The branch tip labels are four letters corresponding to the bird host species and are colored as follows: *Haemoproteus*, *Plasmodium* and *Leucocytozoon*. The black numbers are the Bootstrap values in percentages; only those ≥ 50% are shown.

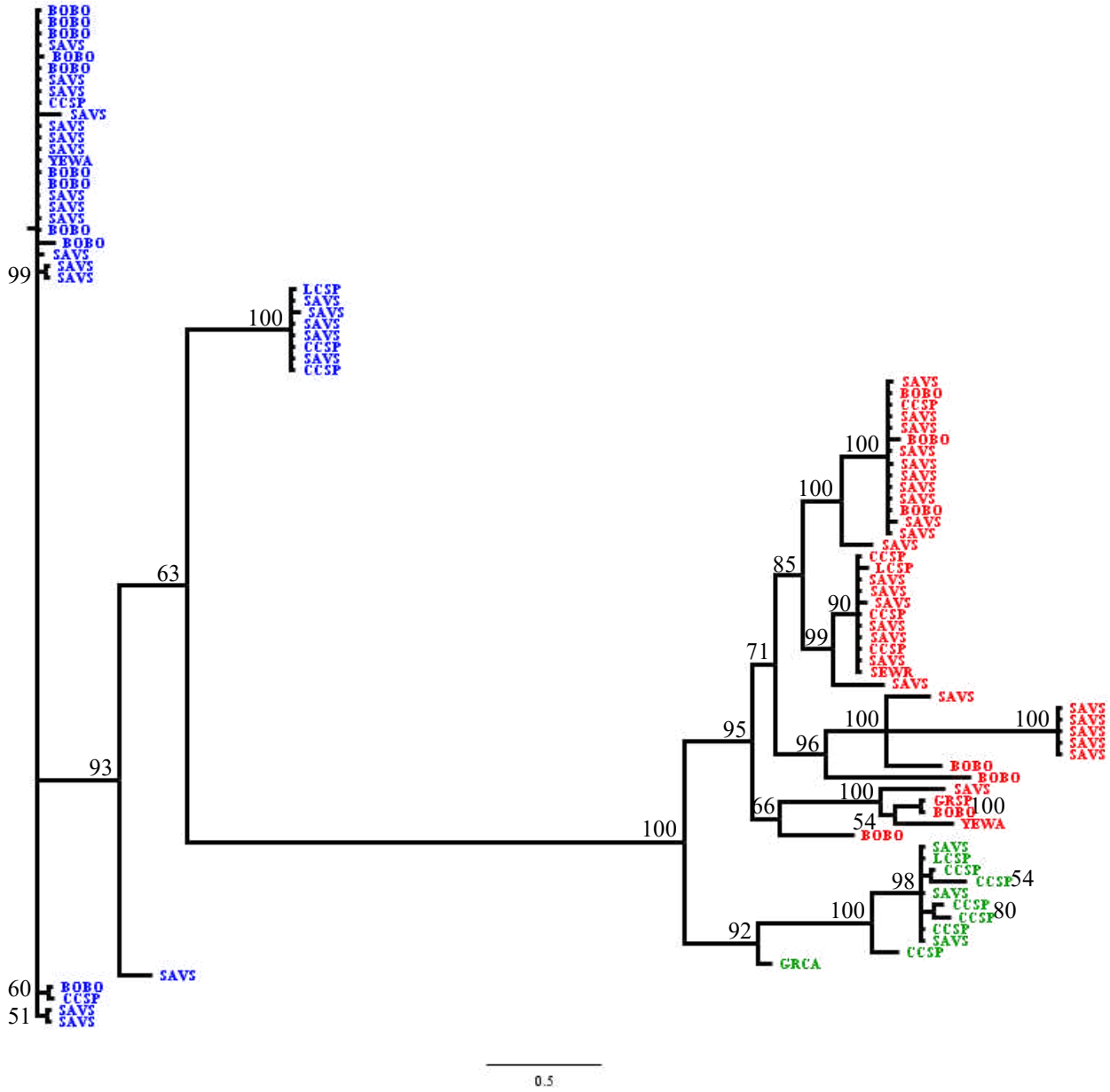
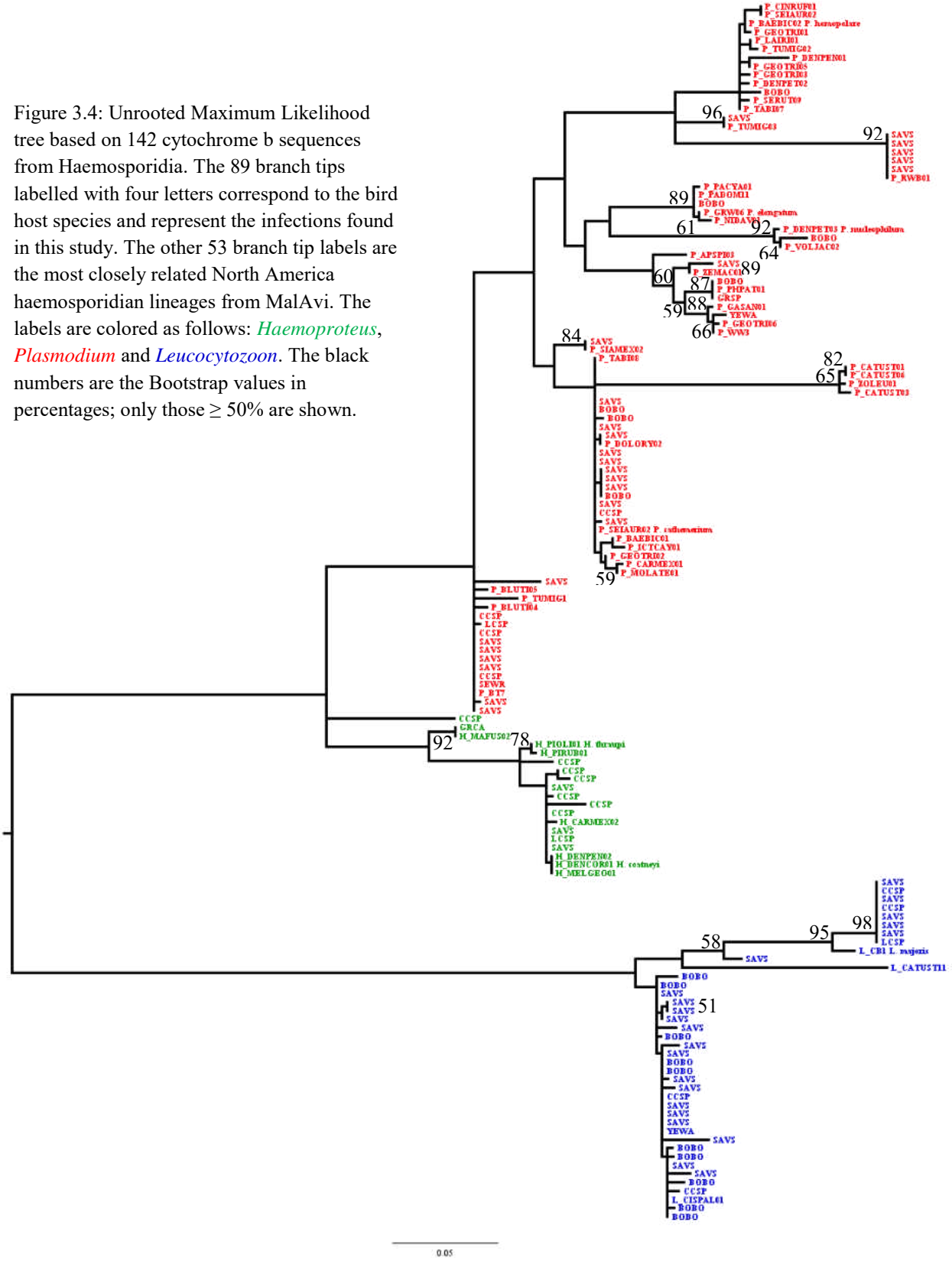



Figure 3.3: Unrooted, consensus tree resulting from Bayesian anaylsis of 88 cytochrome b sequences from Haemosporidia found in the current study. The branch tip labels are four letters corresponding to the bird host species and are colored as follows: *Haemoproteus*, *Plasmodium* and *Leucocytozoon*. The black numbers are the support values in percentages; only those ≥ 50% are shown.

Figure 3.4: Unrooted Maximum Likelihood tree based on 142 cytochrome b sequences from Haemosporidia. The 89 branch tips labelled with four letters correspond to the bird host species and represent the infections found in this study. The other 53 branch tip labels are the most closely related North America haemosporidian lineages from MalAvi. The labels are colored as follows: *Haemoproteus*, *Plasmodium* and *Leucocytozoon*. The black numbers are the Bootstrap values in percentages; only those ≥ 50% are shown.



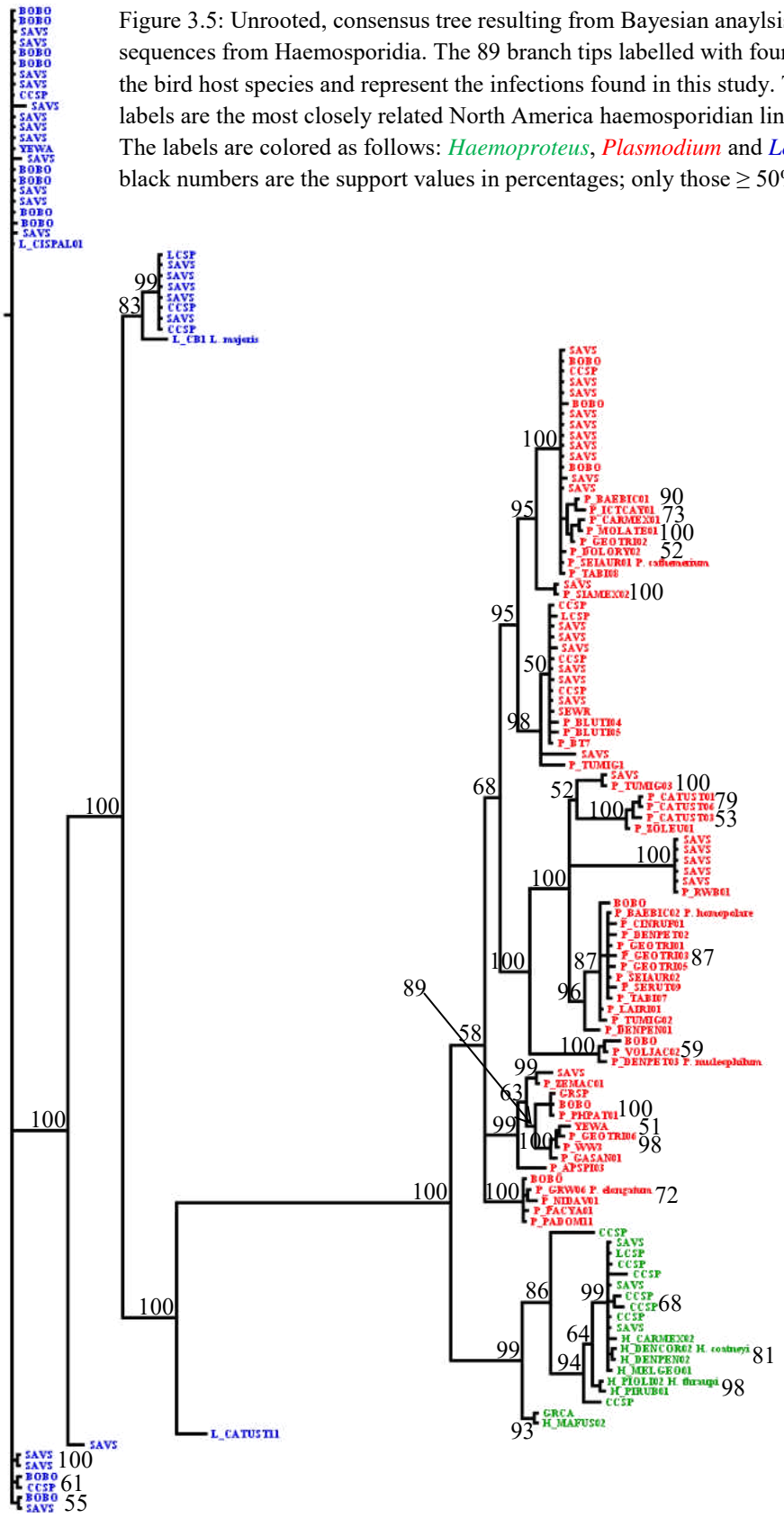


Figure 3.5: Unrooted, consensus tree resulting from Bayesian anaylsis of 142 cytochrome b sequences from Haemosporidia. The 89 branch tips labelled with four letters correspond to the bird host species and represent the infections found in this study. The other 53 branch tip labels are the most closely related North America haemosporidian lineages from MalAvi. The labels are colored as follows: *Haemoproteus*, *Plasmodium* and *Leucocytozoon*. The black numbers are the support values in percentages; only those ≥ 50% are shown.

## Discussion

### Relatedness and Divergence of the Genera

The findings that *Plasmodium* and *Leucocytozoon* are the least related genera and *Haemoproteus* is more close related to *Plasmodium* than to *Leucocytozoon* was supported by all of the ML and Bayesian trees and by the calculations of divergence between the genera. This is supported by many other phylogenetic studies as well, though not all.

The divergence within the three genera indicated that *Haemoproteus* and *Plasmodium* were the least and most diverse genus, respectively. Of the 89 sequences, the number identified as *Haemoproteus* (n= 12) was much fewer than the number of sequences identified as *Plasmodium* (n=40) and *Leucocytozoon* (n= 37). *Haemoproteus* sequences were much shorter on average (388 bases) compared to the average length of a *Plasmodium* (428 bases) and *Leucocytozoon* (444 bases) sequence (the maximum length of a sequence was 479 bases). Fewer and shorter *Haemoproteus* sequences are likely the reasons why this genus appeared to be the least diverse. Initially, it was surprising that *Leucocytozoon* was not the least diverse genus because of the 37 sequences, only two species were identified. To understand why, the divergence within each of the two *Leucocytozoon* species was calculated. Divergence (*i.e.* number of nucleotide differences after pairwise deletion) was only 1.9 for *L. majoris* and 5.5 for *L. fringillinarum*. Since the divergence for the entire *Leucocytozoon* genus is 13.5, this suggests that the relatively low values were primarily due to the two species being very genetically different. Apparently, this is not unusual as many other studies have found substantial genetic diversity between species in this genus [87, 220, 258, 145, 259, 260].

In terms of species richness, *Leucocytozoon* is probably the least diverse genus because between the 37 sequences, only two species were tentatively identified, while *Haemoproteus*

may be the most diverse (five species were identified between twelve sequences). In addition, eight *Plasmodium* species were identified between 40 sequences.

**Lineages**

The 53 lineages from MalAvi that were most closely related to the 89 sequences from this study have a wide range in the USA. The three North American *Haemoproteus* lineages from MalAvi were originally taken from birds in Alaska, Missouri, New York and Vermont. The 43 most closely related *Plasmodium* lineages from MalAvi were reportedly first taken from birds in Alaska, Arizona, California, Colorado, Florida, Georgia, Idaho, Kentucky, Michigan, Missouri, New Hampshire, New York, Oregon, Texas, Vermont and Wisconsin. The three *Leucocytozoon* lineages have only been found previously in birds from Alaska.

It is unfortunate that the exact lineages could not be determined due to all of the 89 sequences being shorter than the target sequence, and the lineages in MalAvi are based on the full 479 nucleotides. However, 14 of the 89 sequences contained 455 bases or more (95% of 479) so for these sequences, the possible lineages could be greatly narrowed down. The nine long *Leucocytozoon* sequences were nearly identical and matched lineages CNEORN01 and CISPAL01 equally well. Both have only been found in passerines from Alaska [261]. The five long *Plasmodium* sequences were more variable and most closely matched lineages BT7, SEIAUR01, TRPIP2 and ZEMAC01. Lineages BT7 and ZEMAC01 are found worldwide, including in the USA, but BT7 is found more frequently. SEIAUR01 is not as widespread but is common within the USA and has even been found in bobolinks. TRPIP2 is least common, having been found once in Africa and twice in Alaska. When lineages CISPAL01, BT7, SEIAUR01 and ZEMAC01 were included in the trees, they fell in with clades tentatively identified as *L. majoris*, *P. circumflexum*, *P. cathemerium* and *P. relictum*, respectively.

**Evidence of Generalist Vectors and Haemosporidia**

There does not appear to be any trend in the phylogenetic trees related to bird host species. This indicates that infective mosquitoes feed opportunistically, rather than specializing in feeding on select bird species. It also indicates that the lineages found in the study area are generalists, having the capability of infecting a wide range of bird species that were sampled.

**Comparison with Avian Malaria from Galapagos Penguins**

The two *Plasmodium* sequences taken from juvenile bobolinks proved active transmission in Minnesota (MN), but did not match the *Plasmodium* infections in Galapagos penguins. Pairwise analysis showed that the two sequences from juvenile bobolinks differed by a minimum of three nucleotides compared to the sequences from Galapagos penguins. Another pairwise analysis of all *Haemoproteus* and *Plasmodium* from this study and those from penguins showed that none matched exactly but three sequences differed by only one nucleotide compared to three sequences from penguins. Though this study failed to support the idea that MN may be a source of malaria in Galapagos penguins, it did not completely rule it out either as more intensive sampling of long distance migrants for malaria could produce a matching lineage.

**Ancestral Genus**

Though the current study does not elucidate the subject, one aspect of phylogeny that is still being debated is which of the three genera is the most ancestral. For the vast majority of time that Haemosporidia phylogeny has been studied, *Leucocytozoon* has been considered to be the most ancestral of the three genera due to its lack of merogony in blood and lack of hemozoin. If this hypothesis of Haemosporidia evolution is true, the fact that *Plasmodium* is virulent and widespread (both geographically and among vertebrates) could mean that vertebrates have not yet developed a defense to it like they could have for a much older genus such as *Leucocytozoon*

(which only infects avians). Perhaps Valkiūnas's [6] finding that *Leucocytozoon* is found in phylogenetically derived birds and but is rarely found in phylogenetically basal birds supports this hypothesis of evolution. It suggests there is a possibility that primitive bird species have evolved with *Leucocytozoon* (indicating that *Leucocytozoon* may be primitive as well) and developed a defense to the genus.

The results of a recent study has rivaled the classic view of phylogeny and concluded that mammalian *Plasmodium* was ancestral [236]. This suggests that the production of hemozoin and erythrocytic merogony may actually be ancestral and that one or both of these traits were lost over time in *Haemoproteus* and *Leucocytozoon*, respectively. If this hypothesis of phylogeny turns out to be true and considering that *Plasmodium* infects such a wide range of vertebrate hosts such as mammals, birds and reptiles, then perhaps this genus has been around much longer than the other genera and has had more time to radiate (especially considering the hypothesized slow divergence rate). *Haemoproteus* is slightly more specialized with regard to the variety of vertebrate hosts it can infect (mostly birds, some reptiles and amphibians, but no mammals). *Leucocytozoon* is the most specialized of all and infects only birds. Perhaps *Leucocytozoon* has had the least amount of time to evolve, radiate and infect other vertebrate classes.

Which mode of evolution is correct remains to be seen as the methods with which to analyze Haemosporidia data (genetic, life history traits, morphology etc) are clarified and more sequences are amassed.

# APPENDIX

# Appendix A

## Tables of Results from 36 USA Studies

Table 4.1: Prevalence of *Haemoproteus*, *Plasmodium* and *Leucocytozoon* by 34 USA states, separated by reference. The prevalence is presented as 'number of birds infected/number of birds screened'.

| | *Haemoproteus* | | *Plasmodium* | | *Leucocytozoon* | |
|---|---|---|---|---|---|---|
| | Prevalence | Reference | Prevalence | Reference | Prevalence | Reference |
| Alabama | 1/30 | [161] | | | | |
| Arizona | 23/60 | [162] | 16/33 | [161] | | |
| | 4/10 | [16]* | 2/10 | [16] * | | |
| Arkansas | | | 3/18 | [55] | | |
| California | 3/9 | [226]* | 11/26 | [73] * | 6/9 | [226] * |
| | 8/26 | [73]* | 10/305 | [83] | 177/591 | [258]* |
| | 95/305 | [83] | 880/8176 | [7] | 23/26 | [73] * |
| | | | | | 36/305 | [83] |
| Colorado | 2/10 | [226] * | | | 8/10 | [226] * |
| Florida | 10/114 | [89]* | | | 11/114 | [89] * |
| | 49/196 | [134]* | | | | |
| Georgia | 61/757 | [91] | 40/757 | [91] | 4/1047 | [79] |
| | 136/1047 | [79] | 154/1097 | [84] | | |
| | 182/1097 | [84] | | | | |
| Illinois | 2/14 | [152] | | | | |
| Indiana | 4/19 | [152] | | | | |
| Kansas | | | 8/48 | [92] | | |
| | | | 4/41 | [55] | | |
| Louisiana | 213/934 | [85] | 32/934 | [85] | 10/934 | [85] |
| Maine | | | 1/10 | [55] | | |
| Maryland | | | 29/210 | [55] | | |
| Michigan | | | 9/350 | [93] | 2/9 | [56]* |
| | | | 2/44 | [146] | | |
| | | | 1/19 | [55] | | |
| Minnesota | 8/18 | [57]* | 5/17 | [57] * | 12/12 | [56]* |
| | | | 3/11 | [55] | 1/18 | [57]* |
| Missouri | 4/50 | [57]* | 6/137 | [57]* | 5/136 | [57]* |
| Montana | 31/82 | [226] * | | | 76/82 | [226] * |
| Nevada | 1/13 | [226] * | | | 12/13 | [226] * |
| | | | | | 3/71 | [131]* |

Table 4.1, cont.

| | *Haemoproteus* | | *Plasmodium* | | *Leucocytozoon* | |
|---|---|---|---|---|---|---|
| | Prevalence | Reference | Prevalence | Reference | Prevalence | Reference |
| New Jersey | 69/697 | [82] | 21/697<br>1/25 | [82]<br>[55] | 116/697 | [82] |
| New Mexico | | | 4/32<br>5/23 | [262]*<br>[55] | | |
| New York | 8/282 | [91] | 5/282<br>39/135 | [91]<br>[135] | | |
| North Dakota | | | 2/2<br>3/15 | [57] *<br>[55] | | |
| Ohio | 40/98<br>295/1106 | [65]<br>[81] | 57/1106<br>1/17 | [81]<br>[55] | 64/1106 | [81] |
| Oklahoma | 12/175 | [147] | 4/175<br>5/99 | [147]<br>[263] (subset of passeriformes only) | 3/175 | [147] |
| Oregon | 7/8 | [226] * | | | 8/8<br>16/125 | [226] *<br>[131]* |
| Pennsylvania | 2/17<br>41/69 | [152]<br>[137]* | | | | |
| South Carolina | 1/33<br>51/565 | [90]*<br>[216] | 37/565<br>2/5 | [216]<br>[55] | 13/565 | [216] |
| Tennessee | 5/10 | [152] | | | | |
| Texas | 7/580 | [227]* | 1/580<br>118/282<br>1/9 | [227]*<br>[133]<br>[55] | 77/580 | [227]* |
| Vermont | 98/1520 | [80] | 15/1520 | [80] | 559/1520 | [80] |
| Virginia | | | 4/53 | [55] | | |
| Washington | 2/6 | [226] * | | | 6/6 | [226] * |
| West Virginia | 18/22 | [152] | | | | |
| Wisconsin | 42/125<br>29/80 | [57] *<br>[95]* | 10/154 | [57]* | 3/125<br>47/80 | [57] *<br>[95]* |

*= Non-Passeriformes birds

Table 4.2: Proportions (percentages) of total infections caused by *Haemoproteus*, *Plasmodium* and *Leucocytozoon* for 34 USA states based on 36 studies.

| | *Haemoproteus* (%) | *Plasmodium* (%) | *Leucocytozoon* (%) | # of infections | N (sample size) |
|---|---|---|---|---|---|
| Alabama | 100 | | | 1 | 30 |
| Arizona | 60 | 40 | | 45 | 103 |
| Arkansas | | 100 | | 3 | 18 |
| California | 8 | 72 | 19 | 1249 | 9107 |
| Colorado | 20 | 0 | 80 | 10 | 10 |
| Florida | 84 | 0 | 16 | 70 | 310 |
| Georgia | 66 | 34 | 0.6 | 577 | 2901 |
| Illinois | 100 | | | 2 | 14 |
| Indiana | 100 | | | 4 | 19 |
| Kansas | | 100 | | 12 | 89 |
| Louisiana | 84 | 13 | 4 | 255 | 934 |
| Maine | | 100 | | 1 | 10 |
| Maryland | | 100 | | 29 | 210 |
| Michigan | | 86 | 14 | 14 | 422 |
| Minnesota | 28 | 28 | 45 | 29 | 41 |
| Missouri | 27 | 40 | 33 | 15 | 137 |
| Montana | 29 | | 71 | 107 | 82 |
| Nevada | 6 | | 94 | 16 | 84 |
| New Jersey | 33 | 11 | 56 | 207 | 722 |
| New Mexico | | 100 | | 9 | 55 |
| New York | 15 | 85 | | 52 | 417 |
| North Dakota | | 100 | | 5 | 17 |
| Ohio | 73 | 13 | 14 | 457 | 1221 |
| Oklahoma | 50 | 38 | 13 | 24 | 274 |
| Oregon | 23 | | 77 | 31 | 133 |
| Pennsylvania | 100 | | | 43 | 84 |
| South Carolina | 50 | 38 | 13 | 104 | 603 |
| Tennessee | 100 | | | 5 | 10 |
| Texas | 3 | 59 | 38 | 204 | 871 |
| Vermont | 15 | 2 | 83 | 672 | 1520 |
| Virginia | | 100 | | 4 | 53 |
| Washington | 25 | | 75 | 8 | 6 |
| West Virginia | 100 | | | 18 | 22 |
| Wisconsin | 54 | 8 | 38 | 131 | 234 |

## REFERENCES


[1] S. Merino, A. Barbosa, J. Moreno and J. Potti, "Absence of haematozoa in a wild chinstrap penguin Pygoscelis antarctica population.," *Polar Biology,* vol. 18, no. 3, pp. 227-228, 1997.

[2] H. I. Jones, J. M. Gallagher and G. D. Miller, "Survey of south polar skuas (Catharacta maccormicki) for blood parasites in the Vestfold Hills region of Antarctica.," *Journal of Wildlife Diseases,* vol. 38, no. 1, pp. 213-215, 2002.

[3] P. Quillfeldt, J. Martínez, J. Hennicke, K. Ludynia, A. Gladbach, J. F. Masello, S. Riou and S. Merino, "Hemosporidian blood parasites in seabirds—a comparative genetic study of species from antarctic to tropical habitats.," *Naturwissenschaften,* vol. 97, no. 9, pp. 809-817, 2010.

[4] G. Valkiūnas, "Haemosporidian vector research: marriage of molecular and microscopical approaches is essential.," *Molecular Ecology,* vol. 20, no. 15, pp. 3084-3086, 2011.

[5] R. E. T. Vanstreels, F. R. Miranda, V. Ruoppolo, A. O. de Almeida Reis, E. S. Costa, A. Pessôa, J. Torres, L. Cunha, R. da Piuco, V. Valiati, D. Gonzalez-Acuna, M. Labruna, M. Petry, S. Epiphanio and J. Catão-Dias, "Investigation of blood parasites of pygoscelid penguins at the King George and Elephant Islands, South Shetlands Archipelago, Antarctica.," *Polar Biology,* vol. 37, no. 1, p. 135–139, 2014.

[6] G. Valkiūnas, Avian malaria parasites and other Haemosporidia., Boca Raton, Florida: CRC Press, 2005, p. 932.

[7] C. M. Herman, W. C. Reeves, H. E. McClure, E. M. French and W. M. Hammon, "Studies on avian malaria in vectors and hosts of encephalitis in Kern County, California. I. Infections in avian hosts.," *American Journal of Tropical Medicine and Hygiene,* vol. 3, no. 4, pp. 676-695, 1954.

[8] R. Ross, "On some peculiar pigmented cells found in two mosquitoes fed on malarial blood.," *The British Medical Journal,* vol. 2, no. 1929, pp. 1786-1788, 1897.

[9] R. Ross, "The role of the mosquito in the evolution of the malarial parasite.," *The Lancet,* vol. 152, no. 3912, pp. 488-490, 1898.

[10] C. T. Atkinson and C. van Riper III, "Pathogenicity and epizootiology of avian haematozoa: Plasmodium, Haemoproteus, and Leucocytozoon.," in *Bird-parasite interactions: Ecology, evolution, and behavior*, J. E. Loye and M. Zuk, Eds., England, Oxford University Press, 1991, pp. 19-48.

[11] E. Korpimäki, H. Hakkarainen and G. F. Bennett, "Blood parasites and reproductive success of Tengmalm's owls: detrimental effects on females but not on males?.," *Functional Ecology,* vol. 7, no. 4, pp. 420-426, 1993.

[12] C. J. Cardona, A. Ihejirika and L. McClellan, "Haemoproteus lophortyx infection in bobwhite quail.," *Avian Diseases,* vol. 46, no. 1, pp. 249-255, 2002.

[13] S. T. Ferrell, K. Snowden, A. B. Marlar, M. Garner and N. P. Lung, "Fatal hemoprotozoal

infections in multiple avian species in a zoological park.," *Journal of Zoo and Wildlife Medicine,* vol. 38, no. 2, pp. 309-316, 2007.

[14] T. A. Donovan, M. Schrenzel, T. A. Tucker, A. P. Pessier and I. H. Stalis, "Hepatic hemorrhage, hemocoelom, and sudden death due to Haemoproteus infection in passerine birds: eleven cases.," *Journal of Veterinary Diagnostic Investigation,* vol. 20, no. 3, pp. 304-313, 2008.

[15] P. Olias, M. Wegelin, W. Zenker, S. Freter, A. D. Gruber and R. Klopfleisch, "Avian malaria deaths in parrots, Europe.," *Emerging Infectious Diseases,* vol. 17, no. 5, pp. 950-952, 2011.

[16] M. A. Pacheco, A. A. Escalante, M. M. Garner, G. A. Bradley and R. F. Aguilar, "Haemosporidian infection in captive masked bobwhite quail (Colinus virginianus ridgwayi), an endangered subspecies of the northern bobwhite quail.," *Veterinary Parasitology,* vol. 182, pp. 113-120, 2011.

[17] V. Palinauskas, T. A. Iezhova, A. Križanauskienė, M. Y. Markovets, S. Bensch and G. Valkiūnas, "Molecular characterization and distribution of Haemoproteus minutus (Haemosporida, Haemoproteidae): a pathogenic avian parasite.," *Parasitology International,* vol. 62, p. 358–363, 2013.

[18] G. Valkiūnas, A. M. Anwar, C. T. Atkinson, E. C. Greiner, I. Paperna and M. A. Peirce, "What distinguishes malaria parasites from other pigmented haemosporidians?.," *Trends in Parasitology,* vol. 21, no. 8, pp. 357-358, 2005.

[19] C. T. Atkinson, K. L. Woods, R. J. Dusek, L. S. Sileo and W. M. Iko, "Wildlife disease and conservation in Hawaii: pathogenicity of avian malaria (Plasmodium relictum) in experimentally infected iiwi (Vestiaria coccinea).," *Journal of Parasitology,* vol. 111, pp. S59-S69, 1995.

[20] C. T. Atkinson, R. J. Dusek, K. L. Woods and W. M. Iko, "Pathogenicity of avian malaria in experimentally-infected Hawaii amakihi.," *Journal of Wildlife Diseases,* vol. 36, no. 2, pp. 197-204, 2000.

[21] I. I. Levin, D. C. Outlaw, F. H. Vargas and P. G. Parker, "Plasmodium blood parasite found in endangered Galapagos penguins (Spheniscus mendiculus).," *Biological Conservation,* vol. 142, pp. 3191-3195, 2009.

[22] G. F. Bennett, M. A. Peirce and R. W. Ashford, "Avian haematozoa: mortality and pathogenicity.," *Journal of Natural History,* vol. 27, no. 5, pp. 993-1001, 1993.

[23] M. A. Peirce, A. G. Greenwood and K. Swinnerton, "Pathogenicity of Leucocytozoon marchouxi in the pink pigeon (Columba mayeri) in Mauritius.," *Veterinary Record,* vol. 140, no. 6, pp. 155-156, 1997.

[24] M. M. Szymanski and I. J. Lovette, "High lineage diversity and host sharing of malarial parasites in a local avian assemblage.," *Journal of Parasitology,* vol. 91, no. 4, pp. 768-774, 2005.

[25] S. Merino and J. Potti, "High prevalence of haematozoa in nestlings of a passerine species, the pied flycatcher (Ficedula hypoleuca).," *The Auk,* vol. 112, no. 4, pp. 1041-1043, 1995.

[26] A. A. Escalante and F. J. Ayala, "Evolutionary origin of Plasmodium and other apicomplexa based on rRNA genes.," *PNAS,* vol. 92, no. 13, pp. 5793-5797, 1995.

[27] A. A. Escalante and F. J. Ayala, "Phylogeny of the malarial genus Plasmodium, derived

from rRNA gene sequences.," *PNAS,* vol. 91, no. 24, pp. 11373-11377, 1994.

[28] A. P. Waters, D. G. Higgins and T. F. McCutchan, "Plasmodium falciparum appears to have arisen as a result of lateral transfer between avian and human hosts.," *PNAS,* vol. 88, pp. 3140-3144, 1991.

[29] T. F. McCutchan, F. Vidal, A. A. Lal, J. H. Gunderson, H. J. Elwood and M. L. Sogin, "Primary sequences of two small subunit ribosomal RNA genes from Plasmodium falciparum.," *Molecular and Biochemical Parasitology,* vol. 28, no. 1, pp. 63-68, 1988.

[30] S. Bensch, M. Stjernman, D. Hasselquist, Ö. Östman, B. Hannson, H. Westerdahl and R. T. Pinheiro, "Host specificity in avian blood parasites: a study of Plasmodium and Haemoproteus mitochondrial DNA amplified from birds.," *Proceedings of the Royal Society of London B: Biological Sciences,* vol. 267, no. 1452, pp. 1583-1589, 2000.

[31] O. Hellgren, J. Waldenström and S. Bensch, "A new PCR assay for simultaneous studies of Leucocytozoon, Plasmodium, and Haemoproteus from avian blood.," *Journal of Parasitology,* vol. 90, no. 4, pp. 797-802, 2004.

[32] J. S. Beadell and R. C. Fleischer, "A restriction enzyme-based assay to distringuish between avian hemosporidians.," *Journal of Parasitology,* vol. 91, no. 3, pp. 683-685, 2005.

[33] C. T. Atkinson, R. J. Dusek and J. K. Lease, "Serological responses and immunity to superinfection with avian malaria in experimentally-infected Hawaii amakihi.," *Journal of Wildlife Diseases,* vol. 37, no. 1, pp. 20-27, 2001.

[34] V. Palinauskas, O. V. Dolnik, G. Valkiūnas and S. Bensch, "Laser microdissection microscopy and single cell PCR of avian hemosporidians.," *Journal of Parasitology,* vol. 96, no. 2, pp. 420-424, 2010.

[35] A. B. Gager, J. Del Rosario Loaiza, D. C. Dearborn and E. Bermingham, "Do mosquitoes filter the access of Plasmodium cytochrome b lineages to an avian host?.," *Molecular Ecology,* vol. 17, no. 10, pp. 2552-2561, 2008.

[36] F. Ishtiaq, L. Guillaumot, S. M. Clegg, A. B. Phillimore, R. A. Black, I. P. F. Owens, N. I. Mundy and B. C. Sheldon, "Avian haematozoan parasites and their associations with mosquitoes across Southwest Pacific Islands.," *Molecular Ecology,* vol. 17, no. 20, pp. 4545-4555, 2008.

[37] K. S. Kim, Y. Tsuda and A. Yamada, "Bloodmeal identification and detection of avian malaria parasite from mosquitoes (Diptera: Culicidae) inhabiting coastal areas of Tokyo Bay, Japan.," *Journal of Medical Entomology,* vol. 46, no. 5, pp. 1230-1234, 2009.

[38] T. Hughes, P. Irwin, E. Hofmeister and S. M. Paskewitz, "Occurrence of avian Plasmodium and west nile virus in Culex species in Wisconsin.," *Journal of the American Mosquito Control Association,* vol. 26, no. 1, pp. 24-31, 2010.

[39] K. S. Kim and Y. Tsuda, "Seasonal changes in the feeding pattern of Culex pipiens pallens govern the transmission dynamics of multiple lineages of avian malaria parasites in Japanese wild bird community.," *Molecular Ecology,* vol. 19, no. 24, pp. 5545-5554, 2010.

[40] M. Kimura, J. M. Darbro and L. C. Harrington, "Avian malaria parasites share congeneric mosquito vectors.," *Journal of Parasitology,* vol. 96, no. 1, pp. 144-151, 2010.

[41] K. Y. Njabo, A. J. Cornel, C. Bonneaud, E. Toffelmier, R. N. M. Sehgal, G. Valkiūnas, A. F. Russell and T. Smith, "Nonspecific patterns of vector, host and avian malaria parasite

associations in a central African rainforest.," *Molecular Ecology,* vol. 20, no. 5, pp. 1049-1061, 2011.

[42] A. Inci, A. Yildirim, K. Y. Njabo, O. Duzlu, Z. Biskin and A. Ciloglu, "Detection and molecular characterization of avian Plasmodium from mosquitoes in central Turkey.," *Veterinary Parasitology,* vol. 188, no. 1-2, pp. 179-184, 2012.

[43] K. S. Kim and Y. Tsuda, "Avian Plasmodium lineages found in spot surveys of mosquitoes from 2007 to 2010 at Sakata wetland, Japan: do dominant lineages persist for multiple years?.," *Molecular Ecology,* vol. 21, no. 21, pp. 5374-5385, 2012.

[44] M. Ferraguti, J. Martínez-de la Puente, J. Muñoz, D. Roiz, S. Ruiz, R. Soriguer and J. Figuerola, "Avian Plasmodium in Culex and Ochlerotatus mosquitoes from southern Spain: effects of season and host-feeding source on parasite dynamics.," *PLOS ONE,* vol. 8, no. 6, p. e66237, 2013.

[45] F. Lalubin, A. Delédevant, O. Glaizot and P. Christe, "Temporal changes in mosquito abundance (Culex pipiens), avian malaria prevalence and lineage composition.," *Parasites & Vectors,* vol. 6, no. 1, p. 307, 2013.

[46] P. Synek, P. Munclinger, T. Albrecht and J. Votypka, "Avian haemosporidians in haematophagous insects in the Czech Republic.," *Paraitology Research,* vol. 112, pp. 839-845, 2013.

[47] M. Tanigawa, Y. Sato, H. Ejiri, T. Imura, R. Chiba, H. Yamamoto, M. Kawaguchi, Y. Tsuda, K. Murata and M. Yukawa, "Molecular Identification of Avian Haemosporidia in Wild Birds and Mosquitoes on Tsushima Island, Japan.," *Journal of Veterinary Medical Science,* vol. 75, no. 3, pp. 319-326, 2013.

[48] O. Hellgren, S. Bensch and B. Malmqvist, "Bird hosts, blood parasites and their vectors - associations uncovered by molecular analyses of blackfly blood meals.," *Molecular Ecology,* vol. 17, no. 6, pp. 1605-1613, 2008.

[49] Y. Sato, A. Tamada, Y. Mochizuki, S. Nakamura, E. Okano, C. Yoshida, H. Ejiri, S. Omori, M. Yukawa and K. Murata, "Molecular detection of Leucocytozoon lovati from probable vectors, black flies (Simuliudae) collected in the alpine regions of Japan.," *Parasitology Research,* vol. 104, no. 2, pp. 251-255, 2009.

[50] J. Martínez-de la Puente, J. Martínez, J. Rivero-de Aguilar, J. Herrero and S. Merino, "On the specificity of avian blood parasites: revealing specific and generalist relationships between haemosporidians and biting midges.," *Molecular Ecology,* vol. 20, no. 15, pp. 3275-3287, 2011.

[51] D. Santiago-Alarcon, V. Palinauskas and H. M. Schaefer, "Diptera vectors of avian Haemosporidian parasites: untangling parasite life cycles and their taxonomy.," *Biological Reviews,* vol. 87, no. 4, pp. 928-964, 2012.

[52] M. Ferraguti, J. Martínez-de la Puente, S. Ruiz, R. Soriguer and J. Figuerola, "On the study of the transmission networks of blood parasites from SW Spain: diversity of avian haemosporidians in the biting midge Culicoides circumscriptus and wild birds.," *Parasites & Vectors,* vol. 6, no. 1, p. 208, 2013.

[53] G. Valkiūnas, R. Kazlauskienė, R. Bernotienė, V. Palinauskas and T. A. Iezhova, "Abortive long-lasting sporogony of two Haemoproteus species (Haemosporida, Haemoproteidae) in the mosquito Ochlerotatus cantans, with perspectives on haemosporidian vector research.," *Parasitology Research,* vol. 112, no. 6, pp. 2159-2169,

2013.

[54] W. C. Reeves, B. C. Herold, L. Rosen, B. Brookman and W. M. Hammon, "Studies on avian malaria in vectors and hosts of encephalitis in Kern County, California. II. Infections in mosquito vectors.," *American Journal of Tropical Medicine and Hygiene,* vol. 3, no. 4, pp. 696-703, 1954.

[55] D. W. Micks, "Malaria in the English Sparrow.," *Journal of Parasitology,* vol. 35, no. 5, pp. 543-544, 1949.

[56] J. N. Stuht, W. W. Bowerman and D. A. Best, "Leucocytozoonosis in nestling bald eagles in Michigan and Minnesota.," *Journal of Wildlife Diseases,* vol. 35, no. 3, pp. 608-612, 1999.

[57] M. D. Castle and B. M. Christensen, "Hematozoa of wild turkeys from the midwestern United States: translocation of wild turkeys and its potential role in the introduction of Plasmodium kempi.," *Journal of Wildlife Diseases,* vol. 26, no. 2, pp. 180-185, 1990.

[58] P. S. Wetmore, "A species of Plasmodium from the sharp-tailed grouse infective to other birds.," *The Journal of Wildlife Management,* vol. 3, no. 4, pp. 361-365, 1939.

[59] R. M. Stabler, N. J. Kitzmiller and C. E. Braun, "Plasmodium in a Darwin's tinamou from Colorado.," *Journal of Parasitology,* vol. 59, no. 2, p. 395, 1973.

[60] F. L. Knopf, "Avian assemblages on altered grasslands.," *Studies in Avian Biology,* vol. 15, pp. 247-257, 1994.

[61] B. Peterjohn and J. R. Sauer, "Population status of North American grassland birds from the North American breeding bird survey. In PD Vickery, JR Herkert (Eds.), Ecology and conservation of grassland birds of the western hemisphere.," *Studies in Avian Biology,* vol. 19, pp. 27-44, 1999.

[62] "Minnesota's Biomes," 2015. [Online]. Available: http://www.dnr.state.mn.us/biomes/tallgrass.html.

[63] G. F. Bennett, H. Witt and E. M. White, "Blood parasites of some Jamaican birds.," *Journal of Wildlife Diseases,* vol. 16, no. 1, pp. 29-38, 1980.

[64] P. J. Weatherhead and G. F. Bennett, "Ecology of red-winged blackbird parasitism by haematozoa.," *Canadian Journal of Zoology,* vol. 69, no. 9, pp. 2352-2359, 1991.

[65] M. C. Garvin, J. P. Basbaum, R. M. Ducore and K. E. Bell, "Patterns of Haemoproteus beckeri parasitism in the gray catbird (Dumatella carolinensis) during the breeding season.," *Journal of Wildlife Diseases,* vol. 39, no. 3, pp. 582-587, 2003.

[66] T. Imura, Y. Suzuki, H. Ejiri, Y. Sato, K. Ishida, D. Sumiyama, K. Murata and M. Yukawa, "Prevalence of avian haematozoa in wild birds in a high-altitude forest in Japan.," *Veterinary Parasitology,* vol. 183, no. 3, pp. 244-248, 2012.

[67] P. Synek, T. Albrecht, M. Vinkler, J. Schnitzer, J. Votýpka and P. Munclinger, "Haemosporidian parasites of a European passerine wintering in South Asia: diversity, mixed infections and effect on host condition.," *Parasitology Research,* vol. 112, p. 1667–1677, 2013.

[68] J. van Rooyen, F. Lalubin, O. Glaizot and P. Christe, "Avian haemosporidian persistence and co-infection in great tits at the individual level.," *Malaria Journal,* vol. 12, no. 1, p. 40, 2013.

[69] J. L. Longmire, A. K. Lewis, N. C. Brown, J. M. Buckingham, L. M. Clark, M. D. Jones,

L. J. Meincke, J. Meyne, R. L. Ratliff, F. A. Ray, R. P. Wagner and R. K. Moyzis, "Isolation and molecular characterization of a highly polymorphic centromeric tandem repeat in the family Falconidae.," *Genomics,* vol. 2, no. 1, pp. 14-24, 1988.

[70] V. Tkach and J. Pawlowski, "A new method of DNA extraction from the ethanol-fixed parasitic worms.," *Acta Parasitologica,* vol. 44, no. 2, pp. 147-148, 1999.

[71] S. Bensch, O. Hellgren and J. Pérez-Tris, "MalAvi: a public database of malaria parasites and related haemosporidians in avian hosts based on mitochondrial cytochrome b lineages.," *Molecular Ecology Resources,* vol. 9, no. 5, pp. 1353-1358, 2009.

[72] L. A. Freed and R. L. Cann, "DNA quality and accuracy of avian malaria PCR diagnostics: a review.," *Condor,* vol. 108, no. 2, pp. 459-473, 2006.

[73] G. Valkiūnas, T. A. Iezhova, A. Križanauskienê, V. Palinauskas, R. N. M. Sehgal and S. Bensch, "A comparative analysis of microscopy and PCR-based detection methods for blood parasites.," *Journal of Parasitology,* vol. 94, no. 6, pp. 1395-1401, 2008.

[74] C. L. Cosgrove, K. P. Day and B. C. Sheldon, "Coamplification of Leucocytozoon by PCR diagnostic tests for avian malaria: a cautionary note.," *Journal of Parasitology,* vol. 92, no. 6, pp. 1362-1365, 2006.

[75] C. L. Cosgrove, S. C. L. Knowles, K. P. Day and B. C. Sheldon, "No evidence for avian malaria infection during the nestling phase in a passerine bird.," *Journal of Parasitology,* vol. 92, no. 6, pp. 1302-1304, 2006.

[76] E. Szöllősi, O. Hellgren and D. Hasselquist, "A cautionary note on the use of nested PCR for parasite screening - an example from avian blood parasites.," *Journal of Parasitology,* vol. 94, no. 2, pp. 562-564, 2008.

[77] E. C. Greiner, G. F. Bennett, E. M. White and R. F. Coombs, "Distribution of the avian hematozoa of North America.," *Canadian Journal of Zoology,* vol. 53, no. 12, pp. 1762-1787, 1975.

[78] G. F. Bennett and A. M. Fallis, "Blood parasites of birds in Algonquin Park, Canada, and a discussion of their transmission.," *Canadian Journal of Zoology,* vol. 38, no. 2, pp. 261-273, 1960.

[79] G. J. Love, S. A. Wilkin and M. H. Goodwin, "Incidence of blood parasites in birds collected in southwestern Georgia.," *The Journal of Parasitology,* vol. 39, no. 1, pp. 52-57, 1953.

[80] W. H. Barnard and R. D. Bair, "Prevalence of avian haematozoa in central Vermont.," *Journal of Wildlife Diseases,* vol. 22, no. 3, pp. 365-374, 1986.

[81] L. W. DeGroote and P. G. Rodewald, "Blood parasites in migrating wood-warblers (Parulidae): effects on refueling, energetic condition, and migration timing.," *Journal of Avian Biology,* vol. 41, no. 2, pp. 147-153, 2010.

[82] C. E. Kirkpatrick and S. H. B., "Epizootiology of blood parasite infections in passerine birds from central New Jersey.," *Canadian Journal of Zoology,* vol. 66, no. 11, pp. 2374-2382, 1988.

[83] E. S. Martinsen, B. J. Blumberg, R. J. Eisen and J. J. Schall, "Avian hemosporidian parasites from northern California oak woodland and chaparral habitats.," *Journal of Wildlife Diseases,* vol. 44, no. 2, pp. 260-268, 2008.

[84] H. B. Jordan, "Blood protozoa of birds trapped in Athens, Georgia.," *Journal of*

*Parasitology,* vol. 29, no. 4, pp. 260-263, 1943.

[85] M. C. Garvin, J. V. Remsen, M. A. Bishop and G. F. Bennett, "Hematozoa from passeriform birds in Louisiana.," *Journal of Parasitology,* vol. 79, no. 3, pp. 318-321, 1993.

[86] S. F. Ribeiro, F. Sebaio, F. C. S. Branquinho, M. Â. Marini, A. R. Vago and É. M. Braga, "Avian malaria in Brazilian passerine birds: parasitism detected by nested PCR using DNA from stained blood smears.," *Journal of Parasitology,* vol. 130, pp. 261-267, 2005.

[87] O. Hellgren, "The occurrence of haemosporidian parasites in the Fennoscandian bluethroat, (Luscinia svecica) population.," *Journal of Ornithology,* vol. 146, pp. 55-60, 2005.

[88] M. C. Garvin and E. C. Greiner, "Epizootiology of Haemoproteus danilewskyi (Haemosporina: Haemoproteidae) in blue jays (Cyanocitta cristata) in southcentral Florida.," *Journal of Wildlife Diseases,* vol. 39, no. 1, pp. 1-9, 2003.

[89] R. J. Dusek, M. G. Spalding, D. J. Forrester and E. C. Greiner, "Haemoproteus balearicae and other blood parasites of free-ranging Florida sandhill crane chicks.," *Journal of Wildlife Diseases,* vol. 40, no. 4, pp. 682-687, 2004.

[90] S. L. Webb, A. M. Fedynich, S. K. Yeltatzie, T. L. DeVault and O. E. Rhodes, "Survey of blood parasites in black vultures and turkey vultures from South Carolina.," *Southeatern Naturalist,* vol. 4, no. 2, pp. 355-360, 2005.

[91] B. K. Hartup, A. Oberc, B. Stott-Messick, A. K. Davis and E. C. H. Swarthout, "Blood parasites of house finches (Carpodacus mexicanus) from Georgia and New York.," *Journal of Wildlife Diseases,* vol. 44, no. 2, pp. 469-474, 2008.

[92] J. J. Janovy, "Epidemiology of Plasmodium hexamerium Huff, 1935, in meadowlarks and starlings of the Cheyenne Bottoms, Barton County, Kansas.," *Journal of Parasitology,* vol. 52, no. 3, pp. 573-578, 1966.

[93] L. R. Gogolin and J. E. Freier, "Avian malaria among house sparrows: a survey of disease and mosquito vectors (Diperta: Culicidae) in Reed City, Michigan.," *The Great Lakes Entomologist,* vol. 19, no. 1, pp. 43-50, 1986.

[94] K. M. Pagenkopp, J. Klicka, K. L. Durrant, J. C. Garvin and R. C. Fleischer, "Geographic variation in malarial parasite lineages in the common yellowthroat (Geothlypis trichas).," *Conservation Genetics,* vol. 9, no. 6, pp. 1577-1588, 2008.

[95] S. J. Taft, R. N. Rosenfield and J. Bielefeldt, "Avian hematozoa of adult and nestling cooper's hawks (Accipiter cooperii) in Wisconsin.," *Helminthological Society of Washington,* vol. 61, no. 1, pp. 146-148, 1994.

[96] G. Valkiūnas, "Blood parasites of birds: some obstacles in their use in ecological and evolutionary biology studies.," *Avian Ecology and Behavior,* vol. 7, pp. 85-98, 2001.

[97] S. I. Jarvi, J. J. Schultz and C. T. Atkinson, "PCR diagnostics underestimate the prevalence of avian malaria (Plasmodium relictum) in experimentally-infected passerines.," *Journal of Parasitology,* vol. 88, no. 1, pp. 153-158, 2002.

[98] F. A. Richard, R. N. M. Sehgal, H. I. Jones and T. B. Smith, "A comparative analysis of PCR-based detection methods for avian malaria.," *Journal of Parasitology,* vol. 88, no. 4, pp. 819-822, 2002.

[99] H. Nagata, "Reevaluation of the prevalence of blood parasites in Japanese passerines by

using PCR based molecular diagnostics.," *Ornithological Science,* vol. 5, no. 1, pp. 105-112, 2006.

[100] S. M. Fallon and R. E. Ricklefs, "Parasitemia in PCR-detected Plasmodium and Haemoproteus infections in birds.," *Journal of Avian Biology,* vol. 39, pp. 514-522, 2008.

[101] J. Martínez, J. Martínez-de la Puente, J. Herrero, S. del Cerro, E. Lobato, J. Rivero-de Aguilar, R. A. Vásquez and S. Merino, "A restriction site to differentiate Plasmodium and Haemoproteus infections in birds: on the inefficiency of general primers for detection of mixed infections," *Parasitology,* vol. 136, pp. 713-722, 2009.

[102] R. Guthrie and A. Susi, "A simple phenylalanine method for detecting phenylketonuria in large populations of newborn infants.," *Pediatrics,* vol. 32, pp. 338-343, 1963.

[103] S. Bereczky, A. Mårtensson, J. P. Gil and A. Färnert, "Short report: rapid DNA extraction from archive blood spots on filter paper for genotyping of Plasmodium falciparum.," *American Journal of Tropical Medicine and Hygiene,* vol. 72, no. 3, pp. 249-251, 2005.

[104] S. I. Jarvi, M. E. M. Farias, H. Baker, H. B. Freifeld, P. E. Baker, E. Van Gelder, J. G. Massey and C. T. Atkinson, "Detection of avian malaria (Plasmodium spp.) in native land birds of American Somoa.," *Conservation Genetics,* vol. 4, pp. 629-637, 2003.

[105] S. M. Fallon, R. E. Ricklefs, B. L. Swanson and E. Bermingham, "Detecting avian malaria: an improved polymerase chain reaction diagnostic.," *Journal of Parasitology,* vol. 89, no. 5, pp. 1044-1047, 2003.

[106] S. Bentz, T. Rigaud, M. Barroca, F. Martin-Laurent, D. Bru, J. Moreau and B. Faivre, "Sensitive measure of prevalence and parasitaemia of haemosporidia from European blackbird (Turdus merula) populations: value of PCR-RFLP and quantitative PCR.," *Parasitology,* vol. 133, no. 6, pp. 685-692, 2006.

[107] K. L. Durrant, J. S. Beadell, F. Ishtiaq, G. R. Graves, S. L. Olson, E. Gering, M. A. Peirce, C. M. Milensky, B. K. Schmidt, C. Gebhard and R. C. Fleischer, "Avian haematozoa in South America: a comparison of temperate and tropical zones.," *Ornithological Monographs,* vol. 60, pp. 98-111, 2006.

[108] N. O. Belo, R. T. Pinheiro, E. S. Reis, R. E. Ricklefs and É. Braga, "Prevalence and lineage diversity of avian haemosporidians from three distinct cerrado habitats in Brazil.," *PLOS ONE,* vol. 6, no. 3, p. e17654, 2011.

[109] E. H. Sari, H. Klompen and P. G. Parker, "Tracking the origins of lice, haemosporidian parasites and feather mites of the Galapagos flycatcher (Myiarchus magnirostris).," *Journal of Biogeography,* vol. 40, no. 6, pp. 1082-1093, 2013.

[110] P. Zehtindjiev, A. Križanauskienė, S. Scebba, D. Dimitrov, G. Valkiūnas, A. Hegemann, B. I. Tieleman and S. Bensch, "Haemosporidian infections in skylarks (Alauda arvensis): a comparative PCR-based and microscopy study on the parasite diversity and prevalence in southern Italy and Netherlands.," *European Journal of Wildlife Research,* vol. 58, no. 1, pp. 335-344, 2012.

[111] L. Z. Garamszegi, "The sensitivity of microscopy and PCR-based detection methods affecting estimates of prevalence of blood parasite in birds.," *Journal of Parasitology,* vol. 96, no. 6, pp. 1197-1203, 2010.

[112] G. Valkiūnas, C. T. Atkinson, S. Bensch, R. N. M. Sehgal and R. E. Ricklefs, "Parasite misidentifications in GenBank: how to minimize their number?.," *Trends in Parasitology,*

vol. 24, no. 6, pp. 247-248, 2008.

[113] G. Valkiūnas, P. Zehtindjiev, D. Dimitrov, A. Križanauskienê, T. A. Iezhova and S. Bensch, "Polymerase chain reaction-based identification of Plasmodium (Huffia) elongatum, with remarks on species identity of haemosporidian lineages deposited in GenBank.," *Parasitology Research,* vol. 102, no. 6, pp. 1185-1193, 2008.

[114] V. Palinauskas, V. Kosarev, A. Shapoval, S. Bensch and G. Valkiūnas, "Comparison of mitochondrial cytochrome b lineages and morphospecies of two avian malaria parasites of the subgenera Haemamoeba and Giovannolaia Haemosporida Plasmodiidae.," *Zootaxa,* vol. 1626, pp. 39-50, 2007.

[115] G. Valkiūnas, T. A. Iezhova, C. Loiseau, T. B. Smith and R. N. M. Sehgal, "New malaria parasites of the subgenus Novyella in African rainforest birds, with remarks on their high prevalence, classification and diagnostics.," *Parasitology Research,* vol. 104, pp. 1061-1077, 2009.

[116] T. A. Iezhova, G. Valkiūnas, C. Loiseau, T. B. Smith and R. N. M. Sehgal, "Haemoproteus cyanomitrae sp. Nov. (Haemosporida: Haemoproteidae) from a widespread African songbird, the olive sunbird, Cyanomitra olivacea.," *Journal of Parasitology,* vol. 96, no. 1, pp. 137-143, 2010.

[117] A. Križanauskienė, J. Pérez-Tris, V. Palinauskas, O. Hellgren, S. Bensch and G. Valkiūnas, "Molecular phylogenetic and morphological analysis of haemosporidian parasites (Haemosporida) in a naturally infected European songbird, the blackcap Sylvia atricapilla, with description of Haemoproteus pallidulus sp. Nov.," *Journal of Parasitology,* vol. 137, no. 2, pp. 217-227, 2010.

[118] G. Valkiūnas, S. Bensch, T. A. Iezhova, A. Križanauskienė, O. Hellgren and C. V. Bolshakov, "Nested cytochrome b polymerase chain reaction diagnostics underestimate mixed infections of avian blood haemosporidian parasites: microscopy is still essential.," *Journal of Parasitology,* vol. 92, no. 2, pp. 418-422, 2006.

[119] G. Valkiūnas, T. A. Iezhova, C. Loiseau, A. Chasar, T. B. Smith and R. N. M. Sehgal, "New species of haemosporidian parasites (haemosporida) from African rainforest birds, with remarks on their classification.," *Parasitology Research,* vol. 103, pp. 1213-1228, 2008.

[120] C. Loiseau, T. Iezhova, G. Valkiūnas, A. Chasar, A. Hutchinson, W. Buermann, T. B. Smith and R. N. M. Sehgal, "Spatial variation of haemosporidian parasite infection in African rainforest bird species.," *Journal of Parasitology,* vol. 96, no. 1, pp. 21-29, 2010.

[121] A. Silva-Iturriza, V. Ketmaier and R. Tiedemann, "Profound population structure in the Philippine bulbul Hypsipetes philippinus (Pycnonotidae, Aves) is not reflected in its Haemoproteus haemosporidian parasite.," *Infection, Genetics and Evolution,* vol. 12, no. 1, pp. 127-136, 2012.

[122] R. Ventim, P. Tenreiro, N. Grade, P. Encarnação, M. Araújo, L. Mendes, J. Pérez-Tris and J. A. Ramos, "Characterization of haemosporidian infections in warblers and sparrows at south-western European reed beds.," *Journal of Ornithology,* vol. 153, no. 2, pp. 505-512, 2012.

[123] G. F. Bennett, M. Whiteway and C. Woodworth-Lynas, "A host-parasite catalogue of the avian haematozoa, supplement 1.," *Occasional Papers in Biology,* vol. 15, 1982.

[124] J. E. Applegate, "Population changes in latent avian malaria infections associated with

season and corticosterone treatment.," *Journal of Parasitology,* vol. 56, no. 3, pp. 439-443, 1970.

[125] J. E. Applegate, "Spring relapse of Plasmodium relictum infections in an experimental field population of English sparrows (Passer domesticus).," *Journal of Wildlife Diseases,* vol. 7, pp. 37-42, 1971.

[126] B. J. Hatchwell, M. J. Wood, M. Anwar and C. M. Perrins, "The prevalence and ecology of the haematozoan parasites of European blackbirds, Turdus merula.," *Canadian Journal of Zoology,* vol. 78, no. 4, pp. 684-687, 2000.

[127] P. Shurulinkov, N. Chakarov and G. Daskalova, "Blood parasites, body condition, and wing length in two subspecies of yellow wagtail (Motacilla flava) during migration.," *Parasitology Research,* vol. 110, no. 5, pp. 2043-2051, 2012.

[128] R. M. Stabler, C. E. Braun and T. D. I. Beck, "Hematozoa in sage grouse from Colorado.," *Journal of Wildlife Diseases,* vol. 13, no. 4, pp. 414-417, 1977.

[129] G. F. Bennett, F. Thommes, J. Blancou and M. Artois, "Blood parasites of some birds from the Lorraine region, France.," *Journal of Wildlife Diseases,* vol. 18, no. 1, pp. 81-88, 1982.

[130] P. J. Weatherhead and G. F. Bennett, "Ecology of parasitism of brown-headed cowbirds by haematozoa.," *Canadian Journal of Zoology,* vol. 70, no. 1, pp. 1-7, 1992.

[131] M. R. Dunbar, S. Tornquist and M. R. Giordano, "Blood parasites in sage-grouse from Nevada and Oregon.," *Journal of Wildlife Diseases,* vol. 39, no. 1, pp. 203-208, 2003.

[132] D. Santiago-Alarcon, R. Mettler, G. Segelbacher and H. M. Schaefer, "Haemosporidian parasitism in the blackcap Sylvia atricapilla in relation to spring arrival and body condition.," *Journal of Avian Biology,* vol. 44, no. 6, pp. 521-530, 2013.

[133] E. D. Box, "Blood and tissue protozoa of the English sparrow (Passer domesticus domesticus) in Galveston, Texas.," *Journal of Protozoology,* vol. 13, no. 2, pp. 204-208, 1966.

[134] M. S. Schrader, E. L. Walters, F. C. James and E. C. Greiner, "Seasonal prevalence of a haematozoan parasite of red-bellied woodpeckers (Melanerpes carolinus) and its association with host condition and overwinter survival.," *The Auk,* vol. 120, no. 1, pp. 130-137, 2003.

[135] C. R. Freeman-Gallant, K. D. O'Connor and M. E. Breuer, "Sexual selection and the geography of Plasmodium infection in savannah sparrows (Passerculus sandwichensis).," *Oecologia,* vol. 127, no. 4, pp. 517-521, 2001.

[136] G. F. Bennett and O. de Souza Lopes, "Blood parasites of some birds from Sao Paulo State, Brazil.," *Mem. Inst. Oswaldo Cruz, Rio de Janeiro,* vol. 75, no. 1-2, pp. 117-134, 1980.

[137] J. Wiehn, E. Korpimäki, K. L. Bildstein and J. Sorjonen, "Mate choice and reproductive success in the American kestrel: a role for blood parasites?.," *Ethology,* vol. 103, no. 4, pp. 304-317, 1997.

[138] S. M. Fallon, R. E. Ricklefs, S. C. Latta and E. Bermingham, "Temporal stability of insular avian malarial parasite communities.," *Proceedings of the Royal Society of London B: Biological Sciences,* vol. 271, no. 1538, pp. 493-500, 2004.

[139] C. T. Atkinson, "Epizootiology of Haemoproteus meleagridis (Protozoa: Haemosporina) in Florida: potential vectors and prevalence in naturally infected Culicoides (Diptera:

Ceratopogonidae).," *Journal of Medical Entomology,* vol. 25, no. 1, pp. 39-44, 1988.

[140] A. P. Møller, "Host–parasite interactions and vectors in the barn swallow in relation to climate change.," *Global Change Biology,* vol. 16, no. 4, pp. 1158-1170, 2010.

[141] G. F. Bennett and C. D. MacInnes, "Blood parasites of geese of the McConnell River, N.W.T.," *Canadian Journal of Zoology,* vol. 50, no. 1, pp. 1-4, 1972.

[142] G. F. Bennett, B. Turner and G. Holton, "Blood parasites of trumpeter swans, Olor buccinators (Richardson), from Alberta.," *Journal of Wildlife Diseases,* vol. 17, no. 2, pp. 213-215, 1981.

[143] K. Murata, "Prevalence of blood parasites in Japanese wild birds.," *Journal of Veterinary Medical Science,* vol. 64, no. 9, pp. 785-790, 2002.

[144] R. N. M. Sehgal, H. I. Jones and T. B. Smith, "Blood parasites of some west African rainforest birds.," *Journal of Veterinary Medical Science,* vol. 67, no. 3, pp. 295-301, 2005.

[145] H. D. Ishak, J. P. Dumbacher, N. L. Anderson, J. J. Keane, G. Valkiūnas, S. M. Haig, L. A. Tell and R. N. M. Sehgal, "Blood parasites in owls with conservation implications for the spotted owl (Strix occidentalis).," *PLOS ONE,* vol. 3, no. 5, p. e2304, 2008.

[146] S. Mateyka and J. Mills, "Prevalence of Plasmodium spp. in northern Michigan.," *University of Michigan Biological Station,* 2008.

[147] M. D. Bay and K. D. Andrews, "Prevalence of hematozoan parasites (apicomplexa) in some common passerine birds (Passeriformes) from east-central Oklahoma.," *Texas Journal of Science,* vol. 61, no. 4, 2009.

[148] J. van Rooyen, F. Lalubin, O. Glaizot and P. Christe, "Altitudinal variation in haemosporidian parasite distribution in great tit populations.," *Parasites & Vectors,* vol. 6, p. 139, 2013.

[149] S. Bensch, J. Waldenström, N. Jonzén, H. Westerdahl, B. Hansson, D. Sejberg and D. Hasselquist, "Temporal dynamics and diversity of avian malaria parasites in a single host species.," *Journal of Animal Ecology,* vol. 76, no. 1, pp. 112-122, 2007.

[150] C. T. Atkinson, D. J. Forrester and E. C. Greiner, "Epizootiology of Haemoproteus meleagridis (Protozoa: Haemosporina) in Florida: seasonal transmission and vector abundance.," *Journal of Medical Entomology,* vol. 25, no. 1, pp. 45-51, 1988.

[151] G. F. Bennett, V. D. Stotts and M. C. Bateman, "Blood parasites of black ducks and other anatids from Labrador and insular Newfoundland.," *Canadian Journal of Zoology,* vol. 69, no. 5, pp. 1405-1407, 1991.

[152] C. E. Gibb, J. Jones, M. K. Girvan, J. J. Barg and R. J. Robertson, "Geographic variation in prevalence and parasitemia of Haemoproteus paruli in the cerulean warbler (Dendroica cerulean).," *Canadian Journal of Zoology,* vol. 83, pp. 626-629, 2005.

[153] R. N. M. Sehgal, W. Buermann, R. J. Harrigan, C. Bonneaud, C. Loiseau, A. Chasar, I. Sepil, G. Valkiūnas, T. Iezhova, S. Saaatchi and S. T. B, "Spatially explicit predictions of blood parasites in a widely distributed African rainforest bird.," *Proceedings of the Royal Society B: Biological Sciences,* vol. 278, no. 1708, pp. 1025-1033, 2011.

[154] D. Sol, R. Jovani and J. Torres, "Geographical variation in blood parasites in feral pigeons: the role of vectors.," *Ecography,* vol. 23, pp. 307-314, 2000.

[155] S. M. Fallon, E. Bermingham and R. E. Ricklefs, "Island and taxon effects in parasitism

revisited: avian malaria in the Lesser Antilles.," *Evolution,* vol. 57, no. 3, pp. 606-615, 2003.

[156] R. E. Ricklefs, J. Dodge Gray, S. C. Latta and M. Svensson-Coelho, "Distribution anomalies in avian haemosporidian parasites in the southern Lesser Antilles.," *Journal of Avian Biology,* vol. 42, no. 6, pp. 570-584, 2011.

[157] G. Valkiūnas, T. A. Iezhova, C. V. Bolshakov and V. Kosarev, "Blood parasites of the house sparrow Passer domesticus from northwestern Russia, with remarks on trends of global geographical distribution in this bird.," *Journal of Natural History,* vol. 40, no. 29-31, pp. 1709-1718, 2006.

[158] G. Valkiūnas, R. N. M. Sehgal, T. A. Iezhova and T. B. Smith, "Further observations on the blood parasites of birds in Uganda.," *Journal of Wildlife Diseases,* vol. 41, no. 3, pp. 580-587, 2005.

[159] S. Kleindorfer, S. Lambert and D. C. Paton, "Ticks (Ixodes sp.) and blood parasites (Haemoproteus spp.) in New Holland honeyeaters (Phylidonyris novaehollandiae): evidence for site specificity and fitness costs.," *Emu,* vol. 106, pp. 113-118, 2006.

[160] J. S. Beadell, E. Gering, J. Austin, J. P. Dumbacher, M. A. Peirce, T. K. Pratt, C. T. Atkinson and R. C. Fleischer, "Prevalence and differential host-specificity of two avian blood parasite genera in the Australo-Papuan region.," *Molecular Ecology,* vol. 13, no. 12, pp. 3829-3844, 2004.

[161] A. K. Davis, W. R. Hood and G. E. Hill, "Prevalence of blood parasites in eastern versus western house finches: are eastern birds resistant to infection?.," *EcoHealth,* vol. 10, no. 3, pp. 290-297, 2013.

[162] H. B. Fokidis, E. C. Greiner and P. Deviche, "Interspecific variation in avian blood parasites and haematology associated with urbanization in a desert habitat.," *Journal of Avian Biology,* vol. 39, no. 3, pp. 300-310, 2008.

[163] T. Krama, V. Suraka, M. Hukkanen, S. Rytkönen, M. Orell, D. Cīrule, M. J. Rantala and I. Krams, "Physiological condition and blood parasites of breeding great tits: a comparison of core and northernmost populations.," *Journal of Ornithology,* vol. 154, no. 4, pp. 1019-1028, 2013.

[164] O. Hellgren, J. Waldenström, J. Peréz-Tris, E. Szöllősi, D. Hasselquist, A. Križanauskienė, U. Ottosson and S. Bensch, "Detecting shifts of transmission areas in avian blood parasites- a phylogenetic approach.," *Molecular Ecology,* vol. 16, pp. 1281-1290, 2007.

[165] C. T. Atkinson, J. K. Lease, R. J. Dusek and M. D. Samuel, "Prevalence of pox-like lesions and malaria in forest bird communities on leeward Mauna Loa Volcano, Hawaii.," *The Condor,* vol. 107, no. 3, pp. 537-546, 2005.

[166] C. J. Drakeley, I. Carneiro, H. Reyburn, R. Malima, J. P. A. Lusingu, J. Cox, T. G. Theander, W. M. M. M. Nkya, M. M. Lemnge and E. M. Riley, "Altitude-dependent and-independent variations in Plasmodium falciparum prevalence in northeastern Tanzania.," *Journal of Infectious Diseases,* vol. 191, no. 10, pp. 1589-1598, 2005.

[167] R. Bødker, H. A. Msangeni, W. Kisinza and S. W. Lindsay, "Relationship between the intensity of exposure to malaria parasites and infection in the Usambara Mountains, Tanzania.," *The American Journal of Tropical Medicine and Hygiene,* vol. 74, no. 5, pp. 716-723, 2006.

[168] F. Ishtiaq, E. Gering, J. H. Rappole, A. R. Rahmani, Y. V. Jhala, C. J. Dove, C. Milensky, S. L. Olson, M. A. Peirce and R. C. Fleischer, "Prevalence and diversity of avian hematozoan parasites in Asia: a regional survey.," *Journal of Wildlife Diseases,* vol. 43, no. 3, pp. 382-398, 2007.

[169] P. Shurulinkov and N. Chakarov, "Prevalence of blood parasites in different local populations of reed warbler (Acrocephalus scirpaceus) and great reed warbler (Acrocephalus arundinaceus).," *Parasitology Research,* vol. 99, no. 5, pp. 588-592, 2006.

[170] V. Benedikt, V. Barus, M. Capek, M. Havlicek and I. Literak, "Blood parasites (Haemoproteus and microfilariae) in birds from the Caribbean slope of Costa Rica.," *Acta Parasitologica,* vol. 54, no. 3, pp. 197-204, 2009.

[171] A. F. Savage, V. Robert, S. M. Goodman, V. Raharimanga, M. J. Raherilalao, A. Andrianarimisa, F. Ariey and E. C. Greiner, "Blood parasites in birds from Madagascar.," *Journal of Wildlife Diseases,* vol. 45, no. 4, pp. 907-920, 2009.

[172] I. Zamora-Vilchis, S. E. Williams and C. N. Johnson, "Environmental temperature affects prevalence of blood parasites of birds on an elevation gradient: implications for disease in a warming climate.," *PLOS ONE,* no. 7, p. e39208, 2012.

[173] C. Bonneaud, I. Sepil, B. Milá, W. Buermann, J. Pollinger, R. N. M. Sehgal, G. Valkiūnas, T. A. Iezhova, S. Saatchi and T. B. Smith, "The prevalence of avian Plasmodium is higher in undisturbed tropical forests of Cameroon.," *Journal of Tropical Ecology,* vol. 25, no. 4, pp. 439-447, 2009.

[174] A. Chasar, C. Loiseau, G. Valkiūnas, T. Iezhova, T. B. Smith and R. N. M. Sehgal, "Prevalence and diversity patterns of avian blood parasites in degraded African rainforest habitats.," *Molecular Ecology,* vol. 18, pp. 4121-4133, 2009.

[175] S. G. Laurance, D. Jones, D. Westcott, A. Mckeown, G. Harrington and D. W. Hilbert, "Habitat fragmentation and ecological traits influence the prevalence of avian blood parasites in a tropical rainforest landscape.," *PLOS ONE,* vol. 8, no. 10, p. e76227, 2013.

[176] R. E. Ricklefs, "Embryonic development period and the prevalence of avian blood parasites.," *PNAS,* vol. 89, no. 10, pp. 4722-4725, 1992.

[177] A. Londoño, P. C. Pulgarin-R and S. Blair, "Blood parasites in birds from the lowlands of northern Colombia.," *Caribbean Journal of Science,* vol. 43, no. 1, pp. 87-93, 2007.

[178] O. Hellgren, A. Križanauskienė, D. Hasselquist and S. Bensch, "Low haemosporidian diversity and one key-host species in a bird malaria community on a mid-Atlantic island (São Miguel, Azores).," *Journal of Wildlife Diseases,* vol. 47, no. 4, pp. 849-859, 2011.

[179] L. Mendes, T. Piersma, M. Lecoq, B. Spaans and R. E. Ricklefs, "Disease limited distributions? Contrasts in the prevalence of avian malaria in shorebird species using marine and freshwater habitats.," *Oikos,* vol. 109, pp. 396-404, 2005.

[180] E. Yohannes, A. Križanauskienė, M. Valu, S. Bensch and B. Kempenaers, "Prevalence of malaria and related Haemosporidian parasites in two shorebird species with different winter habitat distribution.," *Journal of Ornithology,* vol. 150, pp. 287-291, 2009.

[181] C. Wiley, A. Qvarnström and L. Gustafsson, "Effects of hybridization on the immunity of collared Ficedula albicollis and pied flycatchers F. hypoleuca, and their infection by haemosporidians.," *Journal of Avian Biology,* vol. 40, no. 4, pp. 352-357, 2009.

[182] A. Silva-Iturriza, V. Ketmaier and R. Tiedemann, "Prevalence of avian haemosporidian

parasites and their host fidelity in the central Philippine islands.," *Parasitology International,* vol. 61, no. 4, pp. 650-657, 2012.

[183] M. K. Peters, "Ant-following and the prevalence of blood parasites in birds of African rainforests.," *Journal of Avian Biology,* vol. 41, no. 2, pp. 105-110, 2010.

[184] N. A. Williams, J. L. Mahrt and F. C. Zwickel, "The ecology of blood parasites in blue grouse from Vancouver Island, British Columbia.," *Canadian Journal of Zoology,* vol. 58, no. 12, pp. 2175-2186, 1980.

[185] D. G. McCurdy, D. Shutler, A. Mullie and M. R. Forbes, "Sex-biased parasitism of avian hosts: relations to blood parasite taxon and mating system.," *Oikos,* vol. 82, no. 2, pp. 303-312, 1998.

[186] M. J. Wood, C. L. Cosgrove, T. A. Wilkin, S. C. L. Knowles, K. P. Day and B. C. Sheldon, "Within-population variation in prevalence and lineage distribution of avian malaria in blue tits, Cyanistes caeruleus.," *Molecular Ecology,* vol. 16, no. 15, pp. 3263-3273, 2007.

[187] P. T. Rintamäki, M. Ojanen, H. Pakkala and M. Tynjälä, "Blood parasites of migrating willow warblers (Phylloscopus trochilus) at a stopover site.," *Canadian Journal of Zoology,* vol. 76, pp. 984-988, 1998.

[188] J. Figuerola, E. Muñoz, R. Gutiérrez and D. Ferrer, "Blood parasites, leucocytes and plumage brightness in the cirl bunting, Emberiza cirlus.," *Functional Ecology,* vol. 13, pp. 594-601, 1999.

[189] P. Shurulinkov and V. Golemansky, "Plasmodium and Leucocytozoon (Sporozoa: Haemosporida) of wild birds in Bulgaria.," *Acta Protozoologica,* vol. 42, pp. 205-214, 2003.

[190] G. Valkiūnas, T. A. Iezhova and A. P. Shapoval, "High prevalence of blood parasites in hawfinch Coccothraustes coccothraustes.," *Journal of Natural History,* vol. 37, no. 22, pp. 2647-2652, 2003.

[191] N. Bunbury, E. Barton, C. G. Jones, A. G. Greenwood, K. M. Tyler and D. J. Bell, "Avian blood parasites in an endangered columbid: Leucocytozoon marchouxi in the Mauritian pink pigeon Columba mayeri.," *Parasitology,* vol. 134, pp. 797-804, 2007.

[192] S. C. Wiersch, T. Lubjuhn, W. A. Maier and H. Kampen, "Haemosporidian infection in passerine birds from lower Saxony.," *Journal of Ornithology,* vol. 148, no. 1, pp. 17-24, 2007.

[193] R. O. C. Motta, M. V. R. Marques, F. C. Ferreira, D. de Assis Andery, R. S. Horta, R. B. Peixoto, G. A. Lacorte, P. de Abreu Moreira, F. de Oliveira Paes Leme, M. M. Melo, N. R. da Silva Martins and E. M. Braga, "Does haemosporidian infection affect hematological and biochemical profiles of the endangered black-fronted piping-guan (Aburria jacutinga)?.," *PeerJ,* vol. 2013, no. 1, p. e45, 2013.

[194] A. Pérez-Rodríguez, J. de la Puente, A. Onrubia and J. Pérez-Tris, "Molecular characterization of haemosporidian parasites from kites of the genus Milvus (Aves: Accipitridae).," *International Journal for Parasitology,* vol. 43, p. 381–387, 2013.

[195] M. Zuk, "Reproductive strategies and disease susceptibility: an evolutionary viewpoint.," *Parasitology Today,* vol. 6, no. 7, pp. 231-233, 1990.

[196] R. Poulin, "Sexual inequalities in helminth infections: a cost of being a male?.," *American Naturalist,* vol. 147, no. 2, pp. 287-295, 1996.

[197] M. Zuk and A. M. Stoehr, "Immune defense and host life history.," *The American Naturalist,* vol. 160, no. S4, pp. S9-S22, 2002.

[198] I. Folstad and A. J. Karter, "Parasites, bright males, and the immunocompetence handicap.," *American Naturalist,* vol. 139, no. 3, pp. 603-622, 1992.

[199] M. Zuk and K. A. McKean, "Sex differences in parasite infections: patterns and processes.," *International Journal for Parasitology,* vol. 26, no. 10, pp. 1009-1024, 1996.

[200] P. Deviche, E. C. Greiner and X. Manteca, "Seasonal and age-related changes in blood parasite prevalence in dark-eyed juncos (Junco hyemalis, Aves, Passeriformes).," *Journal of Experimental Zoology,* vol. 289, no. 7, pp. 456-466, 2001.

[201] M. L. Roberts, K. L. Buchanan and M. R. Evans, "Testing the immunocompetence handicap hypothesis: a review of the evidence.," *Animal Behaviour,* vol. 68, no. 2, pp. 227-239, 2004.

[202] J. J. Boonekamp, A. H. F. Ros and S. Verhulst, "Immune activation suppresses plasma testosterone level: a meta-analysis.," *Biology Letters,* vol. 4, no. 6, pp. 741-744, 2008.

[203] P. Davidar and E. S. Morton, "Living with parasites: prevalence of a blood parasite and its effect on survivorship in the purple martin.," *The Auk,* vol. 110, no. 1, pp. 109-116, 1993.

[204] J. J. Sanz, E. Arriero, J. Moreno and S. Merino, "Interactions between hemoparasite status and female age in the primary reproductive output of pied flycatchers.," *Oecologia,* vol. 126, pp. 339-344, 2001.

[205] V. Madsen, G. Valkiūnas, T. A. Iezhova, C. Mercade, M. Sanchez and J. L. Osorno, "Testosterone levels and gular pouch coloration in courting magnificent frigatebird (Fregata magnificens): variation with age-class, visited status and blood parasite infection.," *Hormones and Behavior,* vol. 51, no. 1, pp. 156-163, 2007.

[206] G. F. Bennett and M. Cameron, "Seasonal prevalence of avian haematozoa in passeriform birds of Atlantic Canada.," *Canadian Journal of Zoology,* vol. 52, no. 10, pp. 1259-1264, 1974.

[207] C. M. Herman, J. H. Barrow and I. B. Tarshis, "Leucocytozoonosis in Canada geese at the Seney National Wildlife Refuge.," *Journal of Wildlife Diseases,* vol. 11, no. 3, pp. 404-411, 1975.

[208] A. Gabaldon and G. Ulloa, "Holoendemicity of malaria: an avian model.," *Transactions of the Royal Society of Tropical Medicine and Hygiene,* vol. 74, no. 4, pp. 501-507, 1980.

[209] D. Sol, R. Jovani and J. Torres, "Parasite mediated mortality and host immune response explain age-related differences in blood parasitism in birds.," *Oecologia,* vol. 135, no. 4, pp. 542-547, 2003.

[210] C. T. R, "R: A language and environment for statistical computing.," R Foundation for Statistical Computing, Vienna, 2015.

[211] J. H. Zar, Biostatistical analysis., New Jersey: Prentice-Hall, 1999, p. 663.

[212] A. I. Akinpelu, "Prevalence and intensity of blood parasites in wild pigeons and doves (family: Columbidae) from Shasha Forest Reserve, Ile-Ife, Nigeria.," *Asian Journal of Animal and Veterinary Advances,* vol. 3, no. 2, pp. 109-114, 2008.

[213] K. Murata, R. Nii, S. Yui, E. Sasaki, S. Ishikawa, Y. Sato, S. Matsui, S. Horie, K. Akatani, M. Takagi, K. Sawabe and Y. Tsuda, "Avian haemosporidian parasites infection in wild birds inhabiting Minami-Daito Island of the northwest Pacific, Japan.," *Journal of*

*Veterinary Medical Science,* vol. 70, no. 5, pp. 501-503, 2008.

[214] N. Basto, O. A. Rodríguez, C. J. Marinkelle, R. Gutiérrez and N. E. Matta, "Haematozoa in birds from La Macarena National Natural Park (Colombia).," *Caldasia,* vol. 28, no. 2, pp. 371-377, 2006.

[215] J. S. Beadell, R. Covas, C. Gebhard, F. Ishtiaq, M. Melo, B. K. Schmidt, S. L. Perkins, G. R. Graves and R. C. Fleischer, "Host associations and evolutionary relationships of avian blood parasites from west Africa.," *International Journal for Parasitology,* vol. 39, no. 2, pp. 257-266, 2009.

[216] W. E. Collins, G. M. Jeffery, J. C. Skinner, A. J. Harrison and F. Arnold, "Blood parasites of birds at Wateree, South Carolina.," *Journal of Parasitology,* vol. 52, no. 4, pp. 671-673, 1966.

[217] D. Shutler, R. G. Clark, S. T. Rutherford and A. Mullie, "Blood parasites, clutch volume, and condition of gadwalls and mallards.," *Journal of Avian Biology,* vol. 30, no. 3, pp. 295-301, 1999.

[218] G. Valkiūnas, T. A. Iezhova, D. R. Brooks, B. Hanelt, S. V. Brant, M. E. Sutherlin and D. Causey, "Additional observations on blood parasites of birds in Costa Rica.," *Journal of Wildlife Diseases,* vol. 40, no. 3, pp. 555-561, 2004.

[219] O. A. Rodrigues and N. E. Matta, "Blood parasites in some birds from eastern plains of Colombia.," *Mem. Inst. Oswaldo Cruz,* vol. 96, no. 8, pp. 1173-1176, 2001.

[220] E. S. Martinsen, I. Paperna and J. J. Schall, "Morphological versus molecular identification of avian haemosporidia: an exploration of three species concepts.," *Parasitology,* vol. 133, pp. 279-288, 2006.

[221] C. D. Fowle, "The blood parasites of the blue grouse.," *Science,* vol. 103, no. 2685, pp. 708-709, 1946.

[222] H. Borji, E. Moghaddas, G. H. Razmi, M. H. Bami, M. Mohri and M. Azad, "Prevalence of pigeon haemosporidians and effect of infection on biochemical factors in Iran.," *Journal of Parasitic Diseases,* vol. 35, no. 2, pp. 199-201, 2011.

[223] S. Merino, J. Moreno, J. J. Sanz and E. Arriero, "Are avian blood parasites pathogenic in the wild? A medication experiment in blue tits (Parus caeruleus).," *Proceedings of the Royal Society of London B: Biological Sciences,* vol. 267, pp. 2507-2510, 2000.

[224] P. Deviche, E. C. Greiner and X. Manteca, "Interspecific variability of prevalence in blood parasites of adult passerine birds during the breeding season in Alaska.," *Journal of Wildlife Diseases,* vol. 37, no. 1, pp. 28-35, 2001.

[225] L. L. Leppert, A. M. Dufty, S. Stock, M. Oleyar and G. S. Kaltenecker, "Survey of blood parasites in two forest owls, northern saw-whet owls and flammulated owls, of western North America.," *Journal of Wildlife Diseases,* vol. 44, no. 2, pp. 475-479, 2008.

[226] J. L. Mahrt, F. C. Zwickel and T. G. Tessier, "Blood parasites of blue grouse (Dendragapus obscurus) in western North America.," *Journal of Wildlife Diseases,* vol. 27, no. 3, pp. 482-485, 1991.

[227] J. S. Loven, E. G. Bolen and B. W. Cain, "Blood parasitemia in a south Texas wintering waterfowl population.," *Journal of Wildlife Diseases,* vol. 16, no. 1, pp. 25-25, 1980.

[228] G. F. Bennett, "Blood parasites of some birds from Labrador.," *Canadian Journal of Zoology,* vol. 50, no. 3, pp. 353-356, 1972.

[229] S. Merino, J. Potti and J. A. Fargallo, "Blood parasites of passerine birds from central Spain.," *Journal of Wildlife Diseases,* vol. 33, no. 3, pp. 638-641, 1997.

[230] J. R. Barta, M. C. Jenkins and H. D. Danforth, "Evolutionary relationships of avian Eimeria species among other apicomplexan protozoa: monophyly of the apicomplexa is supported.," *Molecular Biology and Evolution,* vol. 8, no. 3, pp. 345-355, 1991.

[231] A. A. Escalante, E. Barrio and F. J. Ayala, "Evolutionary origin of human and primate malarias: evidence from the circumsporozoite protein gene.," *Molecular Biology and Evolution,* vol. 12, no. 4, pp. 616-626, 1995.

[232] A. A. Escalante, D. E. Freeland, W. E. Collins and A. A. Lal, "The evolution of primate malaria parasites based on the gene encoding cytochrome b from the linear mitochondrial genome.," *PNAS,* vol. 95, no. 14, pp. 8124-8129, 1998.

[233] S. Bensch, J. Pérez-Tris, J. Waldenström and O. Hellgren, "Linkage between nuclear and mitochondrial DNA sequences in avian malaria parasites: multiple cases of cryptic speciation?.," *Evolution,* vol. 58, no. 7, pp. 1617-1621, 2004.

[234] S. L. Perkins and J. J. Schall, "A molecular phylogeny of malarial parasites recovered from cytochrome b gene sequences.," *Journal of Parasitology,* vol. 88, no. 5, pp. 972-978, 2002.

[235] E. S. Martinsen, S. L. Perkins and J. J. Schall, "A three-genome phylogeny of malaria parasites (Plasmodium and closely related genera): evolution of life-history traits and host switches.," *Molecular Phylogenetics and Evolution,* vol. 47, no. 1, pp. 261-273, 2008.

[236] D. C. Outlaw and R. E. Ricklefs, "Rerooting the evolutionary tree of malaria parasites.," *PNAS,* vol. 108, no. 32, pp. 13183-13187, 2011.

[237] S. M. Rich and G. Xu, "Resolving the phylogeny of malaria parasites.," *PNAS,* vol. 108, no. 32, pp. 12973-12974, 2011.

[238] J. P. Huelsenbeck, J. P. Bollback and A. M. Levine, "Inferring the root of a phylogenetic tree.," *Systematic Biology,* vol. 51, no. 1, pp. 32-43, 2002.

[239] S. Bensch, O. Hellgren, A. Križanauskienė, V. Palinauskas, G. Valkiūnas, D. Outlaw and R. E. Ricklefs, "How can we determine the molecular clock of malaria parasites?.," *Trends in Parasitology,* vol. 29, no. 8, pp. 363-369, 2013.

[240] G. Poinar and S. R. Telford, "Paleohaemoproteus burmacis gen. n., sp. n. (Haemospororida: Plasmodiidae) from an early cretaceous biting midge (Diptera: Ceratopogonidae).," *Parasitology,* vol. 131, no. 1, pp. 79-84, 2005.

[241] G. Poinar, "Plasmodium dominicana n. sp. (Plasmodiidae: Haemospororida) from tertiary Dominican amber.," *Systematic Parasitology,* vol. 61, no. 1, pp. 47-52, 2005.

[242] B. Ollomo, P. Durand, F. Prugnolle, E. Douzery, C. Arnathau, D. Nkoghe, E. Leroy and F. Renaud, "A new malaria agent in African hominids.," *PLOS Pathogens,* vol. 5, no. 5, p. e1000446, 2009.

[243] W. Liu, Y. Li, G. H. Learn, R. S. Rudicell, J. D. Robertson, B. F. Keele, J.-B. N. Ndjango, C. M. Sanz, D. B. Morgan, S. Locatelli, M. K. Gonder, P. J. Kranzusch, P. D. Walsh, E. Delaporte, E. Mpoudi-Ngole and B. H. Hahn, "Origin of the human malaria parasite Plasmodium falciparum in gorillas.," *Nature,* vol. 467, no. 7314, pp. 420-425, 2010.

[244] R. C. Fleischer, C. E. McIntosh and C. L. Tarr, "Evolution on a volcanic conveyor belt: using phylogeographic reconstructions and K–Ar-based ages of the Hawaiian Islands to estimate molecular evolutionary rates.," *Molecular Ecology,* vol. 7, no. 4, pp. 533-545,

1998.

[245] I. J. Lovette, "Mitochondrial dating and mixed support for the “2% rule” in birds.," *Auk,* vol. 121, no. 1, pp. 1-6, 2004.

[246] J. T. Weir and D. Schluter, "Calibrating the avian molecular clock.," *Molecular Ecology,* vol. 17, no. 10, pp. 2321-2328, 2008.

[247] R. E. Ricklefs and S. M. Fallon, "Diversification and host switching in avian malaria parasites.," *Proceedings of the Royal Society of London B: Biological Sciences,* vol. 269, no. 1494, pp. 885-892, 2002.

[248] R. E. Ricklefs and D. C. Outlaw, "A molecular clock for malaria parasites.," *Science,* vol. 329, no. 5988, pp. 226-229, 2010.

[249] S. L. Perkins, "Species concepts and malaria parasites: detecting a cryptic species of Plasmodium.," *Proceedings of the Royal Society of London B: Biological Sciences,* vol. 267, pp. 2345-2350, 2000.

[250] D. A. Joy, X. Feng, J. Mu, T. Furuya, K. Chotivanich, A. U. Krettli, M. Ho, A. Wang, N. J. White, E. Suh, P. Beerli and X. Z. Su, "Early origin and recent expansion of Plasmodium falciparum.," *Science,* vol. 300, no. 5617, pp. 318-321, 2003.

[251] O. Hellgren, A. Križanauskienė, G. Valkiūnas and S. Bensch, "Diversity and phylogeny of mitochondrial cytochrome b lineages from six morphospecies of avian Haemoproteus (Haemosporida: Haemoproteidae).," *Journal of Parasitology,* vol. 93, no. 4, pp. 889-896, 2007.

[252] E. S. Martinsen, J. L. Waite and J. J. Schall, "Morphologically defined subgenera of Plasmodium from avian hosts: test of monophyly by phylogenetic analysis of two mitochondrial genes.," *Parasitology,* vol. 134, no. 4, pp. 483-490, 2006.

[253] I. I. Levin, P. Zwiers, S. L. Deem, E. A. Geest, J. M. Higashiguchi, T. A. Iezhova, G. Jiménez-Uzcátegui, D. H. Kim, J. P. Morton, N. G. Perlut, R. B. Renfrew, H. E. R. Sari, G. Valkiūnas and P. G. Parker, "Multiple lineages of avian malaria parasites (Plasmodium) in the Galapagos Islands and evidence for arrival via migratory birds.," *Conservation Biology,* vol. 27, no. 6, pp. 1366-1377, 2013.

[254] K. Tamura, D. Peterson, N. Peterson, G. Stecher, M. Nei and S. Kumar, "MEGA5: Molecular Evolutionary Genetics Analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods.," *Molecular Biology and Evolution,* vol. 28, no. 10, pp. 2731-2739, 2011.

[255] D. Darriba, G. L. Taboada, R. Doallo and D. Posada, "jModelTest 2: more models, new heuristics and parallel computing.," *Nature Methods,* vol. 9, no. 8, p. 772, 2012.

[256] J. P. Huelsenbeck and F. Ronquist, "MRBAYES: Bayesian inference of phylogeny.," *Bioinformatics,* vol. 17, pp. 754-755, 2001.

[257] F. Ronquist and J. P. Huelsenbeck, "MRBAYES 3: Bayesian phylogenetic inference under mixed models.," *Bioinformatics,* vol. 19, pp. 1572-1574, 2003.

[258] R. N. M. Sehgal, A. C. Hull, N. L. Anderson, G. Valkiūnas, M. J. Markovets, S. Kawamura and L. A. Tell, "Evidence for cryptic speciation of Leucocytozoon spp. (Haemosporida, Leucocytozoidae) in diurnal raptors.," *Journal of Parasitology,* vol. 92, pp. 375-379, 2006.

[259] O. J. Krone, J. Waldenström, G. Valkiūnas, O. Lessow, K. Müller, T. A. Iezhova, J. Fickel

and S. Bensch, "Haemosporidian blood parasites in European birds of prey and owls.," *Journal of Parasitology,* vol. 94, pp. 709-715, 2008.

[260] G. Valkiūnas, R. N. M. Sehgal, T. A. Iezhova and A. C. Hull, "Identification of Leucocytozoon toddi group (Haemosporidia: Leucocytozoidae), with remarks on the species taxonomy of Leucocytozoids.," *Journal of Parasitology,* vol. 96, no. 1, pp. 170-177, 2010.

[261] K. S. Oakgrove, R. J. Harrigan, C. Loiseau, S. Guers, B. Seppi and R. N. M. Sehgal, "Distribution, diversity and drivers of blood-borne parasite co-infections in Alaskan bird populations.," *International Journal for Parasitology,* vol. 44, no. 10, pp. 717-727, 2014.

[262] B. H. Smith, D. W. Duszynski and K. Johnson, "Survey for coccidia and haemosporidia in the lesser prairie-chicken (Tympanuchus pallidicinctus) from New Mexico with description of a new Eimeria species.," *Journal of Wildlife Diseases,* vol. 39, no. 2, pp. 347-353, 2003.

[263] J. J. Janovy, "A preliminary survey of blood parasites of Oklahoma birds.," *Proceedings of the Oklahoma Academy of Science,* vol. 43, pp. 59-61, 1963.

[264] M. Asghar, D. Hasselquist and S. Bensch, "Are chronic avian haemosporidian infections costly in wild birds?.," *Journal of Avian Biology,* vol. 42, no. 6, pp. 530-537, 2011.

[265] G. F. Bennett and M. Warren, "Biology of the malaysian strain of Plasmodium juxtanucleare Versiani and Gomes, 1941. I. Description of the stages in the vertebrate host.," *The Journal of Parasitology,* vol. 52, no. 3, pp. 565-569, 1966.

[266] A. Oppliger, P. Christe and H. Richner, "Clutch size and malarial parasites in female great tits.," *Behavioral Ecology,* vol. 8, no. 2, pp. 148-152, 1997.

[267] D. C. Outlaw and R. E. Ricklefs, "Comparative gene evolution in haemosporidian (apicomplexa) parasites of birds and mammals.," *Molecular Biology and Evolution,* vol. 27, no. 3, pp. 537-542, 2010.

[268] J. Reullier, J. Pérez-Tris, S. Bensch and J. Secondi, "Diversity, distribution and exchange of blood parasites meeting at an avian moving contact zone.," *Molecular Ecology,* vol. 15, no. 3, pp. 753-763, 2006.

[269] D. Santiago-Alarcon, R. Bloch, G. Rolshausen, H. M. Schaefer and G. Segelbacher, "Prevalence, diversity, and interaction patterns of avian haemosporidians in a four-year study of blackcaps in a migratory divide.," *Parasitology,* vol. 138, no. 7, pp. 824-835, 2011.

[270] B. E. Young, M. C. Garvin and D. B. McDonald, "Blood parasites in birds from Monteverde, Costa Rica.," *Journal of Wildlife Diseases,* vol. 29, no. 4, pp. 555-560, 1993.

[271] M. C. Garvin and J. V. Remsen, "An alternative hypothesis for parasite loads of brightly colored birds: exposure at the nest.," *Auk,* vol. 114, no. 2, pp. 179-191, 1997.

[272] P. Zehtindjiev, K. Ivanova, J. Mariaux and B. B. Georgiev, "First data on the genetic diversity of avian haemosporidians in China: cytochrome b lineages of the genera Plasmodium and Haemoproteus (Haemosporida) from Gansu Province.," *Parasitology Research,* vol. 112, no. 10, 2013.