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Malaria Parasitemia and Prevalence in the Tufted Titmouse (Baeolophus Bicolor)

Kayla Marie Fast

Malaria parasitemia and prevalence in the Tufted Titmouse (*Baeolophus bicolor*)

By

Kayla Marie Fast

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By

Kayla Marie Fast

Approved:

_____

Diana C. Outlaw
(Major Professor)

_____

Brian A. Counterman
(Committee Member)

_____

Andrea S. Varela-Stokes
(Committee Member)

_____

Mark E. Welch
(Graduate Coordinator)

_____

R. Gregory Dunaway
Dean
College of Arts & Sciences

Name: Kayla Marie Fast

Date of Degree: December 11, 2015

Institution: Mississippi State University

Major Field: Biological Sciences

Major Professor: Diana C. Outlaw

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Candidate for Degree of Master of Science

Haemosporidians, including the avian malaria parasites, are a diverse group of blood parasites that infect terrestrial vertebrates worldwide. There is variability in parasite prevalence (presence) and parasitemia (infection intensity); infections range from virtually inconsequential to lethal. Prevalence and parasitemia of avian malaria in the Tufted Titmouse (*Baeolophus bicolor*) were determined (n=81). The genera *Plasmodium* and *Parahaemoproteus* were detected and quantified from bird blood using microscopy, polymerase chain reaction (PCR), and quantitative PCR (qPCR). Thirteen lineages of malaria parasites were found. Sequence data from the parasite's mitochondrial cytochrome *b* gene indicate that prevalence is 69.1% (*Plasmodium*-89.3%; *Parahaemoprotues*-7.1%; double infection-3.6%). Parasitemia was low in all infected birds. Seasonally, parasite prevalence varied significantly. Avian malaria prevalence and parasitemia were not associated with host sex, age, or health. Observations of infection in this naturally infected bird provide details on host susceptibility that are applicable to the understanding of malaria parasites in other avian hosts.

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DEDICATION

This thesis is dedicated to my family—my parents Joe and Shelly and my sisters Sara, Emily, and Shelby.

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CHAPTER I

BACKGROUND

1.1 Introduction

Haemosporidian malaria parasites affect avian hosts with various intensities; some birds are asymptomatic and carry chronic infections while others are critically infected (as reviewed in Valkiūnas 2005). Acute infections (high parasite load) can severely affect the health of the host and in some cases can be fatal (as reviewed in Valkiūnas 2005). In chronic infections, parasites dwell at low frequencies and often cause no negative effects on the host (Dawson & Bortolotti 2000, Westerdahl et al. 2012). Vulnerability to parasitism is influenced by host body condition and immunocompetence, and there are differences in haemosporidian prevalence (presence/absence) and parasitemia (infection intensity) across avian taxa and parasite lineages (Greiner et al. 1975, Ricklefs et al. 2005, Astudillo et al. 2013). Evidence suggests that different bird taxa are differentially parasitized, but there is a paucity of information as to why (as reviewed in Valkiūnas 2005, Ricklefs et al. 2005, Astudillo et al. 2013).

I have explored the prevalence and parasitemia of malaria parasites in the Tufted Titmouse (*Baeolophus bicolor*). This species has been examined very little for infection with malaria parasites. Prevalence records indicate that the Tufted Titmouse displays a low frequency of infection relative to other birds (Greiner et al. 1975, Kirkpatrick &

Suthers 1988, Astudillo et al. 2013). I have used descriptive measurements (e.g., body condition, stress, sex, and age) to examine patterns of infection (i.e., prevalence and parasitemia) in individual titmice. I used three different methods to detect and quantify haemosporidian parasites in the blood of sampled birds: 1) microscopy, 2) polymerase chain reaction (PCR), and 3) quantitative PCR (qPCR). Novel quantitative techniques in the form of qPCR have recently become an increasingly sensitive method for measuring parasite infection intensity (Zehtindjiev et al. 2008, Christie et al. 2012, Friedl & Groscurth 2012).

This thesis project addressed specific questions about the consequences of infection with avian haemosporidian parasites as well as the techniques used to detect infection status and intensity. The primary objective of this thesis was to determine the prevalence and parasitemia of haemosporidians in the Tufted Titmouse. The second objective was to evaluate the significance of various avian life cycle characteristics with respect to differences in avian malaria prevalence and parasitemia. Several life history traits (e.g., body condition, stress, sex, and age) have previously been investigated for association with host susceptibility and resistance to avian malaria, and I have utilized them here.

My third objective was to evaluate and compare techniques used to detect the presence and intensity of infection with malaria parasites. Prevalence and parasitemia have traditionally been determined using microscopy. PCR has proven to be a more sensitive indicator of infection, or prevalence, in a population (Fallon et al. 2003b, Davis et al. 2013). Chronic haemosporidian infections in particular are more accurately detected by PCR versus microscopy (Nayel et al. 2012). In cases of extremely low

parasitemia, microscopy may fail to indicate the presence of parasites where PCR methods prevail (Fallon et al. 2003b). Bentz et al. (2006) found that up to 80% of European blackbirds (*Turdus merula*) in their study deemed free of parasites by microscopic analysis were actually infected once submitted to molecular procedures. Furthermore, microscopic detection of infected erythrocytes was restricted to those hosts parasitized at high intensities (Bentz et al. 2006). Parasites are also more reliably identified by molecular methods (Fallon et al. 2003b, Waldenstrom et al. 2004, Bentz et al. 2006).

Parasitemia determined by qPCR is a powerful tool for studying epidemiology (Fallon et al. 2003b, Friedl & Groscurth 2012). The effect of haemosporidian infection on avian hosts may be better indicated through measurements of parasitemia, not prevalence (Knowles et al. 2009, Friedl & Groscurth 2012). Indeed, some infection levels are so low and inconsequential to the host that it may not even be logical to consider them as parasitized individuals (Davis et al. 2013). Although microscopy is a low cost method for evaluating prevalence and parasitemia, molecular methods repeatedly show evidence for increased accuracy, reliability, and time conservation. I have explored these alternative methods to understand the effects of avian malaria on a population of locally common birds.

My hypotheses are three-fold: 1) healthy birds will be parasitized at lower frequencies and intensities than less healthy birds; 2) prevalence and parasitemia will be associated with life history characters (i.e., age, sex, and season); and 3) the specific lineage of avian malaria parasite will affect the intensity of infection.

1.2 Avian Haemosporidians

Avian haemosporidian parasites, or the avian malaria parasites, are protozoa from the Phylum Apicomplexa. They are found worldwide and affect all terrestrial vertebrates including a wide range of bird families (Waldenstrom et al. 2002, as reviewed in Valkiūnas 2005). The four genera of malaria parasites that infect birds include *Plasmodium*, *Parahaemoproteus*, *Haemoproteus*, and *Leucocytozoon*, each of which is transmitted by a different blood-sucking, dipteran insect group (Table 1.1; as reviewed in Valkiūnas 2005, Martinsen et al. 2007, Outlaw & Ricklefs 2011, Perkins 2014). *Parahaemoproteus* and *Haemoproteus* were previously considered subgenera of the genus *Haemoproteus*; hereafter, generalizations about pathogenicity, fitness effects, and infection patterns of *Haemoproteus* include *Parahaemoproteus* (Valkiūnas 2005, Outlaw & Ricklefs 2011, Perkins 2014). There are exceptions to the simplification of insect families transmitting specific parasite genera; for example, the species *Leucocytozoon caulleryi* is transmitted by biting midges, not simuliid flies (as reviewed in Valkiūnas 2005). *Plasmodium*, *Parahaemoproteus*, and *Leucocytozoon* are known to parasitize the Tufted Titmouse (Kirkpatrick & Suthers 1988, Ricklefs et al. 2005, Astudillo et al. 2013).

Table 1.1 Avian haemosporidian genera and their vectors

Parasite Genus	Vector Family
Plasmodium	Culicidae
Parahaemoproteus	Ceratopogonidae
Haemoproteus	Hippoboscidae
Leucocytozoon	Simuliidae

One species of *Leucocytozoon* is transmitted by biting midges from the Ceratopogonidae.

The extent to which the health of an avian host is affected varies with parasite genus, and as a general rule, *Plasmodium* and *Leucocytozoon* pose the greatest risk to the vertebrate host (as reviewed in Valkiūnas 2005, Atkinson et al. 2008). Chronic infections of malaria seem to inflict little to no harm on the host while acute infections may be lethal (Dawson & Bortolotti 2000, Westerdahl et al. 2012). The historic extirpation of many endemic Hawaiian birds following novel contact with malaria parasites is a remarkable instance that shows the devastation of acute infection (van Riper et al. 1986). The geographic range of certain haemosporidian genera, including *Plasmodium*, is extremely widespread, and these lineages show evidence of high prevalence (Hellgren et al. 2009).

1.2.1 Life cycle

These protozoa undergo development in two different host groups. Asexual reproduction takes place in the intermediate host—the bird. The definitive host and

location of sexual reproduction is the vector—dipteran insects. While taking a blood meal, an infected insect injects the parasite in its saliva into the bird host (Figure 1.1). Sporozoites, the parasitic stage that enters the bird's body at the bite site, then replicate asexually inside the host's tissues as meronts. Merogony occurs in the meronts to form merozoites that spread to other tissues and replicate or enter host erythrocytes and mature into gametocytes.

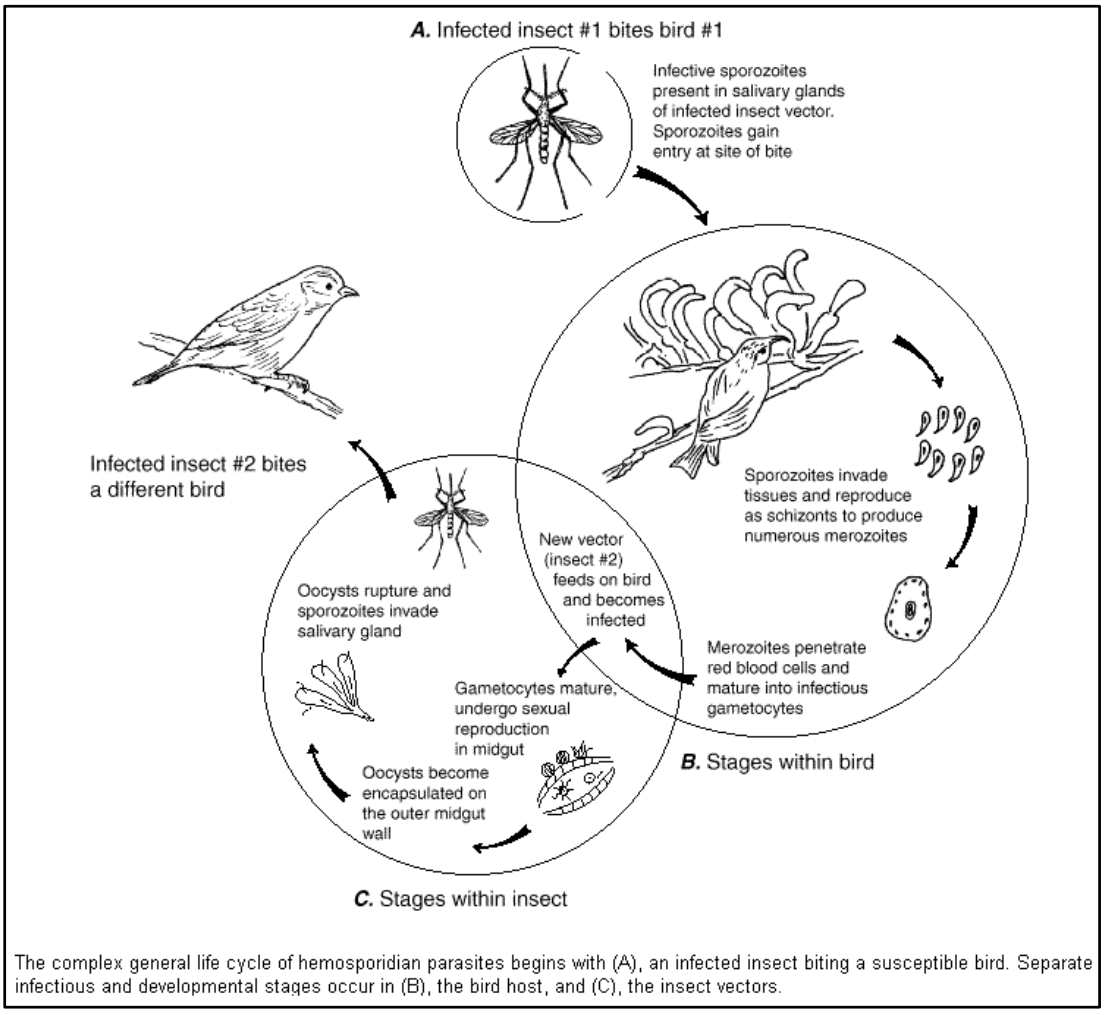

Figure 1.1 Life cycle of avian haemosporidians within the vertebrate and invertebrate host

(Friend and Cole 1999)

In haemoproteids (*Parahaemoproteus* and *Haemoproteus*), merogony occurs in exoerythrocytic cells including the endothelial cells, lungs, liver, kidneys, spleen, heart, and skeletal muscle, but not the blood. In the Leucocytozoidae, merogony does not occur in the blood, but in the liver, spleen, and endothelial cells of blood vessels. In species of *Plasmodium*, merogony occurs in phases of exoerythrocytic and erythrocytic division. Here, exoerythrocytic merogony may occur in the spleen, skin, endothelial cells, and other organs.

Infective gametocytes are then ingested by a second, blood-sucking vector. Within the midgut of the dipteran fly, mature gametocytes undergo sexual reproduction to form a zygote called the ookinete. This parasitic stage moves through the wall of the midgut to produce an oocyst filled with infectious sporozoites. Eventually, the mature oocyst ruptures and the sporozoites move to the vector's salivary glands. From here, sporozoites are transmitted to another avian host through the vector's saliva.

The invasion of host cells is complicated, but follows the same general scheme in all haemosporidians (Valkiūnas 2005). Sporozoites and merozoites are the stages capable of invading host cells. As an example, merozoites penetrate host erythrocytes through induced phagocytosis. The merozoite prompts phagocytosis by placing its apical end against the cell membrane of the host erythrocyte and pressing into the cell. The erythrocyte cell membrane eventually completely encloses the merozoite and forms a parasitophorous vacuole. The foreign merozoite or sporozoite develops into a trophozoite which grows within the parasitophorous vacuole while feeding on components of the host cell’s cytoplasm including hemoglobin; other parasite life stages

also feed on hemoglobin and other nutrients. Mature trophozoites develop into meronts. The meronts undergo merogony as previously stated.

1.2.2 Stages of Infection

The malaria parasite's infection in birds consists of five stages: prepatent stage—when parasites undergo development outside the blood, acute stage—when parasites appear in the blood and reach high parasitemia, crisis stage—when parasitemia is the highest, chronic stage—when parasitemia drops, and latent stage—when parasitemia drops significantly and parasites may be cleared from the blood, but persist in the organs (as reviewed in Valkiūnas 2005). Most infected birds are unable to entirely eliminate malaria parasites from their system or are at least infected for several years (Garnham 1966, Fallis et al. 1974, as cited and reviewed in Valkiūnas 2005). The prepatent period, the interval of time between infection and when evidence of the parasite is first detectable in the host, varies between families of haemosporidians. Parasites can be detected in the Plasmodiidae and Leucocytozoidae in less than five days and between eleven days and three weeks in the Haemoproteidae (as reviewed in Valkiūnas 2005). In *Plasmodium*, the incubation period, or the time before signs or symptoms are recognized, is between five and seven days (Valkiūnas 2005, Atkinson et al. 2008).

1.2.3 Relapse

Relapse is a significant increase in parasitemia after the latent stage or during the chronic stage (as reviewed in Valkiūnas 2005). Cases of relapse are caused by exoerythrocytic merogony, which produces merozoites that infect blood cells (as reviewed in Valkiūnas 2005). Recrudescence occurs when erythrocytic merogony (not

exoerythrocytic merogony) recommences and causes high parasitemia, specifically in the Plasmodiidae (as reviewed in Valkiūnas 2005). Relapse (i.e., exoerythrocytic merogony) in avian *Plasmodium* is induced by erythrocytic merogony rather than sporozoites as in mammalian *Plasmodium* (Applegate 1971).

There is not a mechanistic consensus for relapse among haemosporidian lineages, but relapses can be classified as either seasonal or non-seasonal (as reviewed in Valkiūnas 2005). Seasonal relapse is stimulated by abiotic factors associated with the bird's breeding season and the presence of feeding insect vectors (Applegate 1971, Marzal et al. 2005, as reviewed in Valkiūnas 2005). Spring is the most common period for seasonal relapse and is in part initiated by a rise in sexual hormones within the bird and the increase of day length (Applegate & Beaudoin 1970, as reviewed in Valkiūnas 2005). Relapses in the spring are significant sources for new infections (as reviewed in Valkiūnas 2005). Non-seasonal relapses may occur at any point in the year and have no clear connection with the time of year (as reviewed in Valkiūnas 2005). Much less is known about this type of relapse compared to the seasonal variety, but it is thought that increased stress and changes in immune status may cause relapse (Valkiūnas et al. 2004, as reviewed in Valkiūnas 2005).

1.2.4 Host-parasite Specificity

Haemosporidians are both host generalists and specialists with regard to avian and dipteran hosts. *Plasmodium* parasites tend to act as host generalists in the avian host, with single parasite lineages infecting many different bird species (Martinez-de la Puente et al. 2011, Szymanski & Lovette 2005). Within the mosquito vector, malaria parasites act as host generalists; for example, there are at least 26 different vectors for *Plasmodium*

relictum (Valkiūnas 2005). In contrast, there is some evidence that phylogenetically related *Parahaemoproteus* lineages infect closely related birds (Martinez-de la Puente et al. 2011). Evidence suggests that there is variation in the specificity of which *Parahaemoproteus* lineages may be transmitted by biting midges (Bobeva et al. 2014). The diversity of malaria parasites transmitted by different biting midge lineages is under question and may vary due to different feeding behaviors (e.g., time of day, habitat, and canopy stratum; Santiago-Alarcon et al. 2012). Still, these statements are not always true and do not hold up for every host-parasite interaction. A lack of specificity has been found for several hosts and parasites (Bensch et al. 2000, Ricklefs & Fallon 2002, Waldenstrom et al. 2002, Szymanski & Lovette 2005), but evidence for host specificity does exist (Ricklefs & Fallon 2002, Waldenstrom et al. 2002, Fallon et al. 2003a).

1.3 Avian Host

1.3.1 Tufted Titmouse

The Tufted Titmouse (*Baeolophus bicolor*) is a common, sedentary songbird in the United States (Passeriformes: Paridae). The songbirds carry the highest prevalence and diversity of haemosporidian parasites; haemosporidians are also highly prevalent in the tits and chickadees of the Paridae (as reviewed in Valkiūnas 2005). The titmouse is distributed from the eastern coast of the continental United States into Texas, Oklahoma, Kansas, and Nebraska. This bird is relatively easy to capture with mist nets and seems unaffected after blood sampling. Previous studies that examined titmice suggest a low prevalence of haemosporidians relative to other bird species (Greiner et al. 1975, Kirkpatrick & Suthers 1988, Astudillo et al. 2013). However, most of these studies determined prevalence using only microscopy and may have underestimated parasite

presence (Fallon et al. 2003b, Davis et al. 2013). Avian haemosporidians have not been examined extensively in this host species; however, closely related birds also from the family Paridae have been tested (Norris et al. 1994, Oppliger et al. 1997, Wood et al. 2007, van Rooyen et al 2013a, van Rooyen et al. 2013b).

1.3.2 Effect on the Avian Host

1.3.2.1 Domestic Birds

The presence of haemosporidians in domestic birds is of particular importance because of the major economic losses farmers suffer when livestock are infected. *Leucocytozoon* is the causative agent of the most severe haemosporidian diseases of domestic birds and often infect poultry (as reviewed in Valkiūnas 2005). Mortality is especially high in young birds. Adults experience parasite manifestation but are more resilient after infection. Domestic birds that suffer from leucocytozoonosis exhibit difficulty breathing, loss of appetite, anemia, and diarrhea among other symptoms. Not all species of *Leucocytozoon* are lethal; some lineages are relatively trivial (Lee et al. 1969, as cited and reviewed in Valkiūnas 2005). Cases of *Haemoproteus* may be lethal, and the locomotor skills are impeded and growth is stunted (Atkinson et al. 1988b, Valkiūnas 2005). *Plasmodium* are known for producing epidemics that kill both juvenile and adult birds (Garnham 1980, Huchzermeyer 1993, as cited and reviewed in Valkiūnas 2005).

1.3.2.2 Wild Birds

Haemosporidian infections of wild birds are more difficult to monitor because sick birds often perish from their infections or are killed because of the disease before

scientists can capture them (Holmes 1982, as cited in Valkiūnas 2005). Captive birds in zoos offer some information about the course of infection in wild birds. Birds in zoos suffer from severe forms of malaria because haemosporidians are often not present in the bird's native habitat and the host is immunologically unprepared (Valkiūnas 2005). Mortality is high in introduced birds, especially when vector populations are not controlled (Valkiūnas 2005).

Plasmodium relictum and its vector *Culex quinquefasciatus* were introduced to the Hawaiian Islands in 1826 (Warner 1968, van Riper et al. 1986, van Riper 1991, as cited and reviewed in Valkiūnas 2005). Haemosporidians were likely present on the islands prior to this time given the abundance of migratory birds visiting the Hawaii Islands (Warner 1968, van Riper et al. 1986, van Riper 1991, as cited and reviewed in Valkiūnas 2005). However, the vector needed to transmit the disease was absent (Warner 1968, van Riper et al. 1986, van Riper 1991, as cited and reviewed in Valkiūnas 2005). This novel parasite drastically altered the distribution of many endemic birds and wiped out some species (Warner 1968, van Riper et al. 1986, van Riper 1991, as cited and reviewed in Valkiūnas 2005). Mortality among wild juveniles may be high, and chronic infections are common in adults, but high parasitemia may occur (Garnham 1950, Herman et al. 1975, Gabaldon & Ulloa 1980, Gabaldon et al. 1985, as cited and reviewed in Valkiūnas 2005).

1.3.2.3 Pathogenicity

The spleen and liver of infected birds may become enlarged and darkened in color due to insoluble pigment buildup in macrophages (Garnham 1966, Seed and Manwell 1977, Gabaldon et al. 1985, Atkinson et al. 1988b, as cited and reviewed in Valkiūnas

2005). During the erythrocytic stages of the haemosporidian parasite's lifecycle, the avian host may become anemic, especially in cases of high parasitemia (Paulman & McAllister 2005, Williams 2005, Palinauskas et al. 2008, Christe et al. 2012). Anemia is brought about by erythrocyte destruction, which is a combination of direct and indirect effects of erythrocytic parasites. The mechanism of parasite development within host erythrocytes directly causes cell death (Valkiūnas 2005). Erythrocyte numbers also decrease because parasite infected erythrocytes are removed from the blood stream by the host's body in an attempt to eliminate the pathogen, but the body cannot produce enough erythroblasts to replace this loss (Valkiūnas 2005). Chemical alteration of blood plasma also contributes to low erythrocyte numbers (Seed & Manwell 1977, as cited in Valkiūnas 2005). The circulation of oxygen in capillaries is inhibited by the change in plasma at high parasitemia because hemoglobin cannot bind to oxygen efficiently (Seed & Manwell 1977, as cited in Valkiūnas 2005). In *Leucocytozoon*, uninfected erythrocytes are especially prone to destruction (Kocan 1968, Seed & Manwell 1977, as cited and reviewed in Valkiūnas 2005). *Leucocytozoon* may also cause severe damage to the lungs as gametocytes form complexes that block airflow in the lungs (Siccardi et al. 1974, as cited in Valkiūnas 2005).

Exoerythrocytic meronts may cause pathology in birds, but usually not as first generation meronts (Valkiūnas 2005). Some species of *Plasmodium* cause severe damage to the host's body when meronts block the capillaries of the brain and other organs (Valkiūnas 2005). The blockage eventually leads to necrosis of the tissues surrounding the meronts and eventual cerebral paralysis when within the brain (Valkiūnas 2005). Megalomeronts of *Leucocytozoon* may cause hemorrhaging within

organs including the brain, spleen, liver, lungs, and heart (Wingstrand 1948, Cowan 1957, Newberne 1957, Desser and Fallis 1967, Akiba 1970, Miller et al. 1983, as cited and reviewed in Valkiūnas 2005). Infections caused by *Haemoproteus* present pneumonia-like symptoms when exoerythrocytic meronts block the capillaries of the lungs and are potentially deadly (Mohammed 1967, Garnham 1966, Bradbury & Gallucci 1972, Ahmed & Mohammed 1977, as cited and reviewed in Valkiūnas 2005). *Haemoproteus* megalomeronts are associated with damage of skeletal and heart muscle (Miltgen et al. 1981, Atkinson et al. 1986, 1988b, Earle et al. 1993, as cited and reviewed in Valkiūnas 2005).

1.3.2.4 Reproductive Success

Haemosporidian infected birds suffer a reduction in reproductive success in the wild. Reproductive activity may be decreased by inhibiting the likelihood of finding a mate. Diseased males attend leks less frequently; perform less successful copulation; mate with young, undesirable females; and breed later in the reproductive season (Johnson & Boyce 1991, as cited in Valkiūnas 2005). Even if a male is able to successfully copulate, those caring for larger clutches experience a higher parasitemia of haemosporidians (Norris et al. 1994). When females are infected and food is scarce, they produce smaller clutches of eggs (Korpimaki et al. 1993, Asghar et al. 2015).

1.3.2.5 No Negative Fitness Effect

In contrast to the literature discussed above, there is also a sizeable amount of evidence to suggest wild birds suffer no negative fitness effects when parasitized by haemosporidians (Peirce 1989, Bennett & Bishop 1990, Bennett et al. 1993a, Desser &

Bennett 1993, as cited and reviewed in Valkiūnas 2005). Although the evidence for benign haemosporidian infections is mounting, researchers have found a hidden consequence. Lifespan, reflected in telomere length, is decreased in chronically infected birds when compared to uninfected birds (Asghar et al. 2015). Infected mothers have fewer offspring and those offspring have shorter telomeres (Asghar et al. 2015). Intensity of infection also contributes to the rate of telomere degradation (Asghar et al. 2015). Contradictions concerning fitness effects in infected birds are abundant in the literature. This may be attributed to the high diversity of haemosporidian parasites as well as their vertebrate and invertebrate hosts. The worldwide distribution of haemosporidian parasites opens numerous avenues for variation which could be rooted in geographical differences, host-parasite interactions, or human error caused by biologists following different standards for data collection internationally.

1.3.2.6 Heterophil/Lymphocyte Ratio

The heterophil/lymphocyte (H/L) ratio is a direct indicator of stress (Al-Murrani et al. 2006, Davis et al. 2008, Krams et al. 2013). In response to stress, heterophil numbers increase and lymphocytes decrease resulting in a higher H/L ratio (Davis et al. 2008, Krams et al. 2013). Heterophils are phagocytes that respond to infections, inflammation, and stress while lymphocytes play a role in immunoglobulin production (Jain 1993, Campbell 1995, Campbell 1996, Rupley 1997, Harmon 1998, Thrall 2004, as cited and reviewed in Davis et al. 2008). Birds may be stressed by parasitic infection and become immunologically compromised (Krams et al. 2013). A greater number of circulating leucocytes act as a predictor of an effective adaptive immune response (Krams et al. 2013). Krams et al. found in 2013 that the H/L ratio does not always mimic

significant changes in white blood cell concentrations and should be interpreted with caution. The H/L ratio can increase in as quickly as 60-120 minutes in captured birds (Cirule et al. 2012). Thus, the H/L ratio may indicate acute stress and be the result of anthropogenic handling (Cirule et al. 2012).

1.3.2.7 Body Condition Index

Body condition reflects the energetic state of an individual, which is defined as the amount of energy stored as fat and protein (Krebs and Singleton 1993, Gosler 1996, Schulte-Hostedde et al. 2001, Schulte-Hostedde et al. 2005, as cited in Peig & Green 2009). Animals with larger quantities of energy reserves are healthier with an increased likelihood of survival (Millar & Hickling 1990, as cited in Schulte-Hostedde et al. 2005, Peig & Green 2009). Body condition indices of birds can be calculated from body mass and measurements of wing chord and tarsus length (Schulte-Hostedde et al. 2005, Labocha & Hayes 2012).

The evidence is not conclusive about associations between body condition and haemosporidian prevalence and parasitemia. At least during the breeding season, birds infected with haemosporidians have a lower body condition and there is a negative association between parasitemia and body condition (Dawson & Bortolotti 2000). Migratory birds exhibit a drastic loss of migratory fat when haemosporidian parasite intensity is high (Valkiūnas 1983, 1993, as cited and reviewed in Valkiūnas 2005), but Ashford (1971, as cited in Bennett et al. 1988) found no association between migratory fat loss and blood parasite presence. Mass is reduced in some infected birds (Peirce 1984, as cited in Bennett et al. 1988). Many studies have found no evidence for an association between body condition and parasite presence or parasitemia (Smith & Cox 1972,

Bennett et al. 1988, Weatherhead & Bennett 1992, Davidar & Morton 1993, Korpimaki et al. 1995, Shutler et al. 1996, as cited and reviewed in Dawson & Bortolotti 2000).

1.3.2.8 Season

Infection frequency and intensity may also vary seasonally as necessary energetic tradeoffs are made for reproduction. During the breeding season, immune defense resources are reallocated to mate competition, egg laying, and parental care (Zuk & McKean 1996). Geographic areas that undergo seasonal changes in climate and environmental conditions offer further sources of temporal change in parasite numbers (Valkiūnas 2005). In addition to a rise in adult susceptibility to haemosporidians, the introduction of naïve juvenile birds annually causes an increase in successful parasite manifestations (as reviewed in Valkiūnas 2005). There are seasonal peaks in the activity of dipteran vectors, which are associated with the rise in haemosporidian transmission (as reviewed in Valkiūnas 2005). These factors often overlap temporally: breeding adult birds, introduction of naïve birds, and active vectors. This intersection as well as the arrival of suitable environmental conditions (e.g., temperature and humidity) allow for increased prevalence and parasitemia in birds (Bennett et al. 1966, Bennett & Cameron 1974, Baker 1975, Herman & Bennett 1976, Burtikashvili 1978, Greiner & Mundy 1979, Valkiūnas 1987a, Valkiūnas 1987b, Atkinson et al. 1988a, Earle et al. 1991, Young et al. 1993, as cited and reviewed in Valkiūnas 2005). Seasonal relapse also adds to the increase in transmission rates during a portion of the year. However, year-round transmission does occur in subtropical and tropical climates where vectors are active throughout the year (Markus & Oosthuizen 1972, Noblet et al. 1975, Ayala et al. 1977,

Gabaldon & Ulloa 1980, Atkinson 1988, Atkinson et al. 1988a, as reviewed in Valkiūnas 2005).

1.3.2.9 Sex

Varied results describe which sex typically is more immunocompetent. Behavioral trends, hormone levels, and reproductive, energetic output are possible drivers behind infection patterns between the sexes. Some evidence suggests that female birds are more capable of mounting a cell-mediated immune response to parasitic infection, produce greater amounts of immunoglobulins, and maintain a healthier body condition index (Moreno et al. 2001, Fargallo et al. 2002, Tschirren et al. 2003b, Arriero et al. 2015). Potti and Merino (1996) saw a similar trend with male birds exhibiting more severe negative fitness effects when infected with parasites (as cited and reviewed in Tschirren et al. 2003b). Body size, behavioral, and ecological differences may render males more susceptible to infection because they are easier targets for transmission by insect vectors (Clutton-Brock et al. 1985; Clutton-Brock 1991, Zuk & McKean 1996, Reimchen 2001, as cited and reviewed in Tschirren et al. 2003b).

Evidence has been found for a hormonal role in the difference between immune responses of the sexes. Testosterone, corticosterone, and other sex hormones have been pinned as culprits in modulating the immune response—usually with females being more immunocompetent (Grossman 1989; Olsen & Kovacs 1996; Gaillard & Spinedi 1998, as cited and reviewed in Tschirren et al. 2003b). Carotenoids are another group of compounds that are of importance to the immune response of birds. Carotenoids are needed for proper immune function, but also for feather coloration used in mate selection (Bendich & Olson 1989, Tschirren et al. 2003b). Internal competition for the pigment

may lead to a trade-off, leaving the host immunologically impaired, especially in males that rely more heavily on feather coloration (Olson & Owens 1998, von Schantz et al. 1999, Tschirren et al. 2003a).

Higher prevalence and parasitemia of parasites has been recorded more often in males than in females (Addis 1946, Solomon 1966, Alexander & Stimson 1988, as cited and reviewed in Tschirren et al. 2003b). On the other hand, higher parasitemia of avian haemosporidians in females has been recorded (Asghar et al. 2011), but other authors saw no significant difference in infection prevalence (Ricklefs et al. 2005, Cloutier et al. 2011), the immune response (Saino et al. 2002) or mortality for either sex (Tschirren et al. 2003b).

1.3.2.10 Age

Host age may be associated with parasite prevalence and parasitemia, but a definitive answer for which age group (hatch-year or after-hatch-year) is more susceptible is unclear. Hatch-year (HY) birds include juvenile birds approximately one year old and younger, and after-hatch-year (AHY) birds are the adults that makeup the remainder. AHY birds have a higher probability of being infected simply because they have been alive longer and therefore offer more opportunities for transmission, but HYs are susceptible to novel pathogens (as reviewed in Valkiūnas 2005). Most records indicate that AHYs are infected with haemosporidians more often than HYs (Lyubinsky et al. 1940, Bauer 1941, Levine & Hanson 1953, Glushchenko 1963, Subkhonov 1973, Bennet et al. 1974, Musaev & Zeiniev 1974, Greiner 1975, Burtikashvili 1976, Peirce & Marquiss 1983, Valkiūnas 1987a, Kirkpatrick & Suthers 1988, Davidar & Morton 1993, as cited and reviewed in Valkiūnas 2005, Ricklefs et al. 2005). Others saw the opposite

trend with HY birds being more likely to be infected (Levine & Hanson 1953, Kobyshev & Chashina 1972, Yakunin 1972, Kucera 1979, Thul et al 1980, Davidar & Morton 1993, as cited and reviewed in Valkiūnas 2005). Some authors found no significant difference in the prevalence or parasitemia of haemosporidians between bird age groups (Oliger 1940, Bennet et al. 1976, Bennet et al. 1980, Asghar et al. 2011, as cited and reviewed in Valkiūnas 2005).

1.3.2.11 Other Blood Parasites/Double Infection

The presence of other blood parasites may also lower the bird's immune system and leave it vulnerable to further infection (Zehtindjiev et al. 2008). These additional blood parasites may include trypanosomes, filarial nematodes, and multiple avian malaria parasites (Deviche et al. 2001, Garvin et al. 2006). Infection status and level of infection also are parasite-specific as some parasite lineages occur at high frequencies in the blood while others are extremely low (Bentz et al. 2006).

1.4 Insect Vector

In the realm of malaria research, significantly less is known about the dipteran insect vector than the vertebrate host. Precisely which species of biting fly transmits each parasite lineage is unknown (Valkiūnas 2005). In fact, some parasite lineages are transmitted by many different vector species (Valkiūnas 2005). Haemosporidian parasite sexual reproduction within the invertebrate vector can cause mortality. Biting midges and black flies suffer increased death rate and shortened life span when infected at high parasitemia (Davies 1953, Desser & Yang 1973, Allison et al. 1978, as cited and reviewed in Valkiūnas 2005). Casualties are significantly lower at low parasitemia. Low

mortality is also evident in mosquitoes harboring a low intensity of parasites (Freier & Friedman 1987, as cited in Valkiūnas 2005). Mortality is higher in malaria infected mosquitoes than uninfected mosquitoes (Maier 1973, as cited in Valkiūnas 2005).

The mechanism that haemosporidian parasites apply to live off of the invertebrate host is best understood in mosquitoes. The most likely explanation for mosquito mortality is the tissue destruction caused by the parasite while within the midgut (Maier 1973, as cited in Valkiūnas 2005). Epithelial cells of the midgut are warped by parasite presence (Alekseev 1986, as cited in Valkiūnas 2005). Parasites also drain the mosquito of energy in the form of carbohydrates and alter amino acid metabolism (Alekseev 1986, as cited in Valkiūnas 2005). Infected female mosquitoes suffer decreased viability and motility (Alekseev 1986, as cited in Valkiūnas 2005).

CHAPTER II

MATERIALS AND METHODS

2.1 Field Work

I captured Tufted Titmice in Starkville, Mississippi with mist nets from 2012 through 2015 (n=49). Additional samples were acquired from Decatur, Illinois from 2011 through 2013 (n=32). Netting locations are detailed in Table A.1. I made collections year-round to acquire representatives from different points in the study species' lifetime (e.g., breeding/non-breeding seasons and HY/AHY birds). I used brachial venipuncture to collect blood. Blood was then stored in one of two fashions. Under the primary method, blood was flash frozen in liquid nitrogen and then stored at -80°C. Alternatively, blood was preserved in lysis buffer when liquid nitrogen was not available. In the field, I prepared two thin blood smears for each bird by spreading a drop of blood on a clean, glass slide (fewer than two blood smears are available for some birds). Each blood smear was air dried and then fixed with absolute methanol. Each bird was aged based on its plumage. I also collected morphometric data including mass, tarsus length, and wing chord length. Tufted Titmice are not sexually dimorphic so I used molecular methods to determine the sex of each individual. Each bird was marked before release so that the same titmouse was not sampled more than once in the same season.

2.2 Molecular Analyses

2.2.1.1 DNA Extraction

I first extracted whole genomic DNA from whole blood using the DNeasy Blood and Tissue kit (QIAGEN). I then conducted three forms of molecular analyses: 1) determined the sex of individual birds using polymerase chain reaction (PCR) to amplify the chromo-helicase-DNA-binding (CHD) gene, 2) detected parasite presence through PCR amplification of the cytochrome *b* (cyt *b*) gene, and 3) quantified parasitic infections using quantitative PCR (qPCR).

2.2.1.2 Sex Determination

The Tufted Titmouse is sexually monomorphic, and I determined the sex of each bird by amplifying portions of the CHD gene located on the sex chromosomes of birds. Female birds are the heterogametic sex with chromosomes ZW and males homogametic with two Z chromosomes (ZZ). I amplified both the CHD-W and CHD-Z genes using the protocol suggested by Griffiths et al. (1998). Reactions were performed at a final volume of 25 µl containing 2.5 µl 10× *Ex Taq* Buffer, 2.0 µl dNTP Mixture, 0.5 µl bovine serum albumin (BSA), 0.5 µl of each primer, 0.125 µl *TaKaRa Ex Taq* DNA Polymerase, 1.0 µl DNA template, and 17.875 µl ultra pure water. PCR was performed on a MultiGene Gradient PCR Thermal Cycler (Labnet). Transillumination of PCR products revealed two bands in female birds (one CHD-Z band and one CHD-W band) and only one band in males (one CHD-Z band). Electrophoresis was conducted using 1% agarose gels stained with ethidium bromide.

2.2.1.3 Parasite Detection

I detected haemosporidian parasites using PCR amplification of the parasite's mitochondrial cyt *b* gene. PCR is considered the primary method of parasite detection here, and microscopy and qPCR are secondary approaches. The cyt *b* gene is a useful marker for determining the presence or absence of parasites as well as for reconstructing phylogenies (Bensch et al. 2000, Perkins et al. 2007). A segment of the cyt *b* gene 618 base pairs (bp) long including primers was amplified using cyt *b* initial primers from Perkins et al. (2007) and nested primers HaemNF1 and HaemNR3 (Hellgren et al. 2004). Initial reactions were performed at a final volume of 25 µl containing 2.5 µl 10× *Ex Taq* Buffer, 2.0 µl dNTP Mixture, 0.5 µl bovine serum albumin (BSA), 0.5 µl of each primer, 0.125 µl *TaKaRa Ex Taq* DNA Polymerase, 1.0 µl DNA template, and 17.875 µl ultra pure water. Nested reactions were performed with identical volumes of reagents except used 0.5 µl DNA template, and 18.735 µl ultra pure water instead. Initial reactions were run at a thermal cycler regime of 4 min at 94°C, 35 cycles of 20 sec at 94°C, 20 sec at 56°C, 1 min at 68°C, an additional 10 min at 68°C, and a final hold of 4°C. Nested PCR was performed using a thermal profile of 3 min at 94°C, 35 cycles of 30 sec at 94°C, 30 sec at 55°C, 45 sec at 72°C, an additional 10 min at 72°C, and a final hold of 4°C.

Birds were considered to be infected with malaria parasites if a band was observed following electrophoresis of the PCR product. Positive and negative controls were included in every PCR run. Positive PCR products were cleaned using either the QIAquick PCR Purification kit or the QIAquick Gel Extraction kit (QIAGEN). I outsourced clean PCR products to the Arizona State University DNA Lab to sequence both forward and reverse strands on an Applied Biosystems 3730 capillary sequencer

(Life Technologies). A consensus sequence was constructed after aligning forward and reverse strands using Sequencher v. 4.9 (Gene Codes Corporation). I determined the specific lineages of parasites through Basic Local Alignment Service Tool (BLAST) searches against the National Center for Biotechnology Information (NCBI) and MalAvi (Bensch et al. 2009) databases. I finalized groupings of lineages by comparing sequence alignments by eye; a single base pair difference between sequences constitutes different lineages. My primers amplify both *Plasmodium* and *Parahaemoproteus* lineages, which are known to infect the Tufted Titmouse (Kirkpatrick & Suthers 1988, Ricklefs 2005, Astudillo et al. 2015).

2.2.1.4 Parasite Quantification

I quantified parasite numbers in the blood of birds using the TaqMan qPCR system. I created a custom TaqMan probe and primers using Primer3Plus which amplified a target region 121 bp in length (Rozen & Skaletsky 2000). I used forward primer (5'- GGGGTCAAATGAGTTTCTGG-3'), reverse primer (5' AAGAATCTTTTTAAGGTTGGGTCA3') and probe (5'-CCTGGACTTGTTTCATGGATATGTGG-3'). The probe was labeled with a 6-carboxy-fluorescein (FAM) reporter at the 5' end and Black Hole Quencher Amidites (BHQ1) quencher at the 3' end. Each reaction was run in triplicate at a final volume of 20 µl containing 10 µl 2× TaqMan Gene Expression Master Mix, 1 µl 20× TaqMan primer/probe assay, 5 µl genomic DNA template (100 ng μl^{-1}), and 4 µl ultra pure water. Reactions were performed on a StepOnePlus real-time PCR System (Applied Biosystems) with a thermal profile of 2 min at 50°C, 10 min at 95°C, and 48 cycles of 15 sec at 95°C and 1 min at 58°C.

DNA standards were created from the parasite cyt *b* segment amplified by the nested PCR protocol used for parasite detection described previously. A six step, 10-fold dilution series was created using Qubit Fluorometric Quantitation (Life Technologies) to determine total DNA concentration. The standard dilutions ranged from 5.0×10^{-4} ngµl^{-1} (standard 1) to 5.0×10^{-9} ngµl^{-1} (standard 6) which corresponds to 1.91×10^{7} to 1.91×10^{2} copies of the cyt *b* gene. For consistency between plates, the threshold was manually set with standard 1 at a Ct value of 20. Samples that amplified after our Ct value cutoff of 38 were considered to be free of parasites (Caraguel et al. 2011). Individual qPCR runs were accepted if the slope of the standard curve was between -3.3 and -3.8, the R^2 value was greater than 0.99, and efficiency was within 83-100%. These specifications ensured that amplification was acceptable (Smith et al. 2015). Samples that yielded a standard deviation greater than one between the three replicates were performed again and omitted if the standard deviation was still too high.

2.3 Microscopy Analyses

Fixed blood smears were stained in solutions of Wright-Giemsa Stain (ScyTek Laboratories) and phosphate buffer solution (pH 6.8) following the manufacturer's instructions. Using 1,000X oil emersion, I inspected stained blood smears for the presence of malaria parasites, intensity of parasite infection, and H/L ratio. I also noted the prevalence of other blood parasites. I established the parasitemia of malaria parasites including *Parahaemoproteus* and *Plasmodium* by counting the number of parasite infected erythrocytes out of an estimated 10,000 erythrocytes following the protocol of Godfrey et al. (1987). To construct a leukocyte profile and estimate the H/L ratio, I categorized 100 leukocytes as heterophils, eosinophils, basophils, lymphocytes, and

monocytes (Ots & Horak 1998, Krams et al. 2013). The final calculation for parasitemia and H/L ratio were taken to be the average between estimates made for the two slides available for each bird. Parasite presence was determined by combining the results of parasite detection between duplicate slides for each individual.

2.4 Phylogenetic Analyses

Three phylogenies were built: one with 530 *Plasmodium* sequences; one with 750 *Parahaemoproteus* sequences; and one with lineages found exclusively in the Tufted Titmouse. These sequences include those from this study and cyt *b* sequences acquired from MalAvi and Genbank. Cyt *b* sequences were aligned in BioEdit using ClustalW (v. 7.2.5; Hall 1999). A Bayesian analysis was performed in BEAUTi and BEAST (v. 1.8.1; Drummond et al. 2012) using the following parameters: substitution model-GTR, base frequencies-estimated, site heterogeneity model-Gamma, clock model-strict clock, and tree prior-yule process. Tree annotation was performed in Tree Annotator (v. 1.8.1; Drummond et al. 2012) the final tree configured in FigTree (v. 1.4.2; Rambaut 2007).

2.5 Statistical Analyses

All statistical analyses were performed using the software R (v. 3.2.0; R Core Team 2015). Associations between prevalence data and year, season, locality, host sex, and age were determined using Fisher's Exact Tests. ANOVA and Tukey's HSD test were employed for further investigation of seasonal differences in prevalence. The H/L ratio and body condition index were compared with parasite presence, sex, and age using a Mann-Whitney U Test. The body condition index was estimated from the residuals of an Ordinary Least Squares Regression of host body mass on the linear measurements of

tarsus and wing chord (Schulte-Hostedde et al. 2005). Here, a positive residual is indicative of a bird in good condition while a negative residual equals poor condition (Jakob et al. 1996, Schulte-Hostedde et al. 2001, as cited in Schulte-Hostedde et al. 2005). A Fisher's Exact Test was employed to check if estimates of prevalence were similar between detection methods. The reliability of estimating parasitemia by microscopy was evaluated by calculating Pearson's Correlation coefficient for parasite counts between viewers of the same blood smears and between blood smears from the same bird. Estimates of parasitemia between methods and parasitemia between years were compared using ANOVA. This approach was also used to determine if specific lineages caused different intensities of infection. Parasitemia of each parasite genus was compared using a Mann-Whitney U Test. This test was also used to determine if host age and sex were important determining factors for parasitemia. The Kruskal-Wallis Rank Sum Test was applied when dividing trends of parasitemia by season. The body condition index and H/L ratio were submitted to linear regressions to explore associations with parasitemia. A logistic regression was used to analyze the relationship between the H/L ratio and parasitemia estimated from qPCR.

CHAPTER III

RESULTS

3.1 Prevalence

A total of 81 birds were captured, 49 in Starkville, Mississippi and 32 in Decatur, Illinois. The prevalence of avian haemosporidians in these birds was 69.1%. The number of infected birds was statistically similar between sampling localities with 69.4% of birds infected in Starkville, Mississippi and 68.8% in Decatur, Illinois (p=0.8083; n=81; Table 3.1). There was no difference in prevalence between years of collection so all years were analyzed together (p=0.441; n=81).

Table 3.1 Prevalence of haemosporidians in the Tufted Titmouse

	Plasmodium (%)	*Parahaemoproteus* (%)	Double Infection (%)	Total Prevalence (%)	Total Birds
Starkville, MS	30 (88.2)	2 (5.9)	2 (5.9)	34 (69.4)	49
Decatur, IL	20 (90.9)	2 (9.1)	0 (0)	22 (68.8)	32
Both	50 (89.3)	4 (7.1)	2 (3.6)	56 (69.1)	81

The prevalence of haemosporidian parasites was determined using PCR. The proportion of birds infected was similar between geographic locations (p=0.8083; n=81).

Of those infections 89.3% (n=50) were caused by the genus *Plasmodium* and 7.1% (n=4) by *Parahaemoproteus*. Double infections were observed in 2 birds and were excluded from phylogenetic analyses. Individual cases were considered to be true double

infections when chromatograms showed double peaks at multiple positions within the cyt *b* sequence. The distribution of parasite genera and double infections were statistically similar between localities (p=0.6537; n=56). Bird sex (p=0.1848; n=56), age (p=1; n=53), and specific net location (p=0.5631; n=52) did not show any association with parasite genus. Trypanosomes (8.6%) and microfilariae (4.3%) were observed in stained blood smears in low numbers (n=70).

No characteristics of the avian host played a significant role in the likelihood of parasite presence. Neither sex was more likely to be infected with haemosporidian parasites (p=0.134; n=81; Figure 3.1). Likewise, neither AHY nor HY birds were infected more frequently than the other (p= 0.5418; n=75; Figure 3.1). The body condition index did not vary between infected and uninfected birds (tarsus: p=0.9282; n=75; Figure 3.2; wing chord: p=0.9856; n=75; Figure 3.3). Body condition was also similar between the sexes (tarsus: p= 0.9863; n=75; wing chord: p=0.977; n=75) and ages (tarsus: p=0.9628; n=75; wing chord: p=0.9203; n=75). Stress also was not associated with the probability of infection (p=0.4121; n= 68; Figure 3.4). There was no difference in the H/L ratio between birds grouped by sex (p=0.7629; n=62) or age (p=0.5321; n=62). Seasonally, there was a statistically significant difference in infection status between seasons and between the breeding season (March-May) and nonbreeding seasons (p=0.0137; p=0.0282; n=75; Figure 3.5). Prevalence between the spring and fall was the most significant source of seasonal differences (p=0.021, n=75).

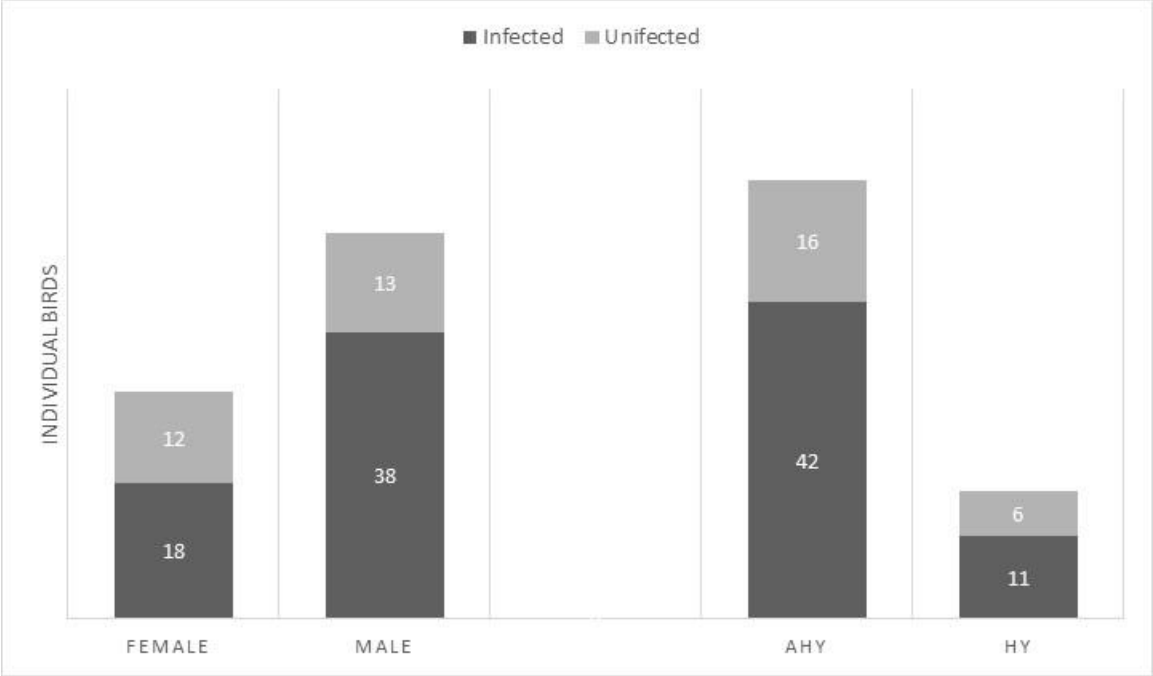

Figure 3.1 Haemosporidian infection status based on bird sex and age.

Neither sex (p=0.134; n=81) nor age group (p= 0.5418; n=75) had a higher prevalence of haemosporidian parasites.

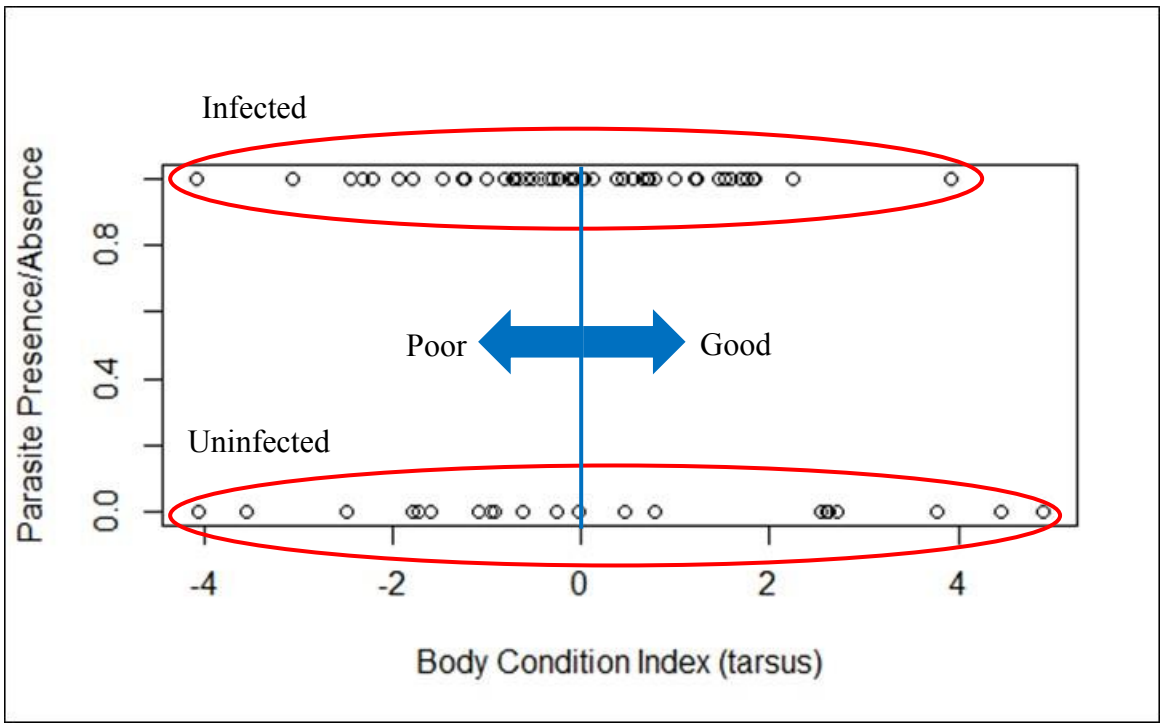

Figure 3.2 Body condition index (tarsus) of infected and uninfected birds

The body condition index was estimated from an ordinary least squares regression of bird body mass on a linear body measurement, in this case tarsus length. Within the index (on the x-axis), positive numbers indicate birds in better body condition while negative numbers are birds in poor condition. On the y-axis, infected birds are given a value of 1 and uninfected birds a value of 0. There was no difference in body condition between infected and uninfected birds ($p=0.9282$; $n=75$).

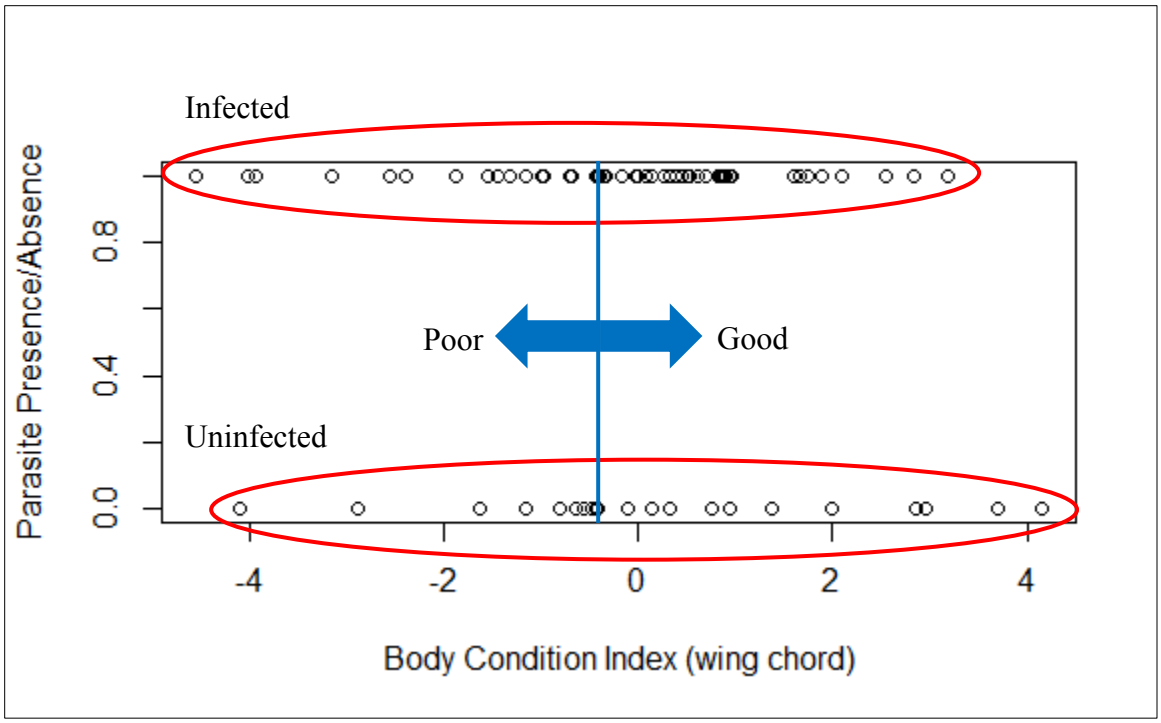

Figure 3.3 Body condition index (wing chord) of infected and uninfected birds

The body condition index was estimated from an ordinary least squares regression of bird body mass on a linear body measurement, in this case wing chord length. Within the index (on the x-axis), positive numbers indicate birds in better body condition while negative numbers are birds in poor condition. On the y-axis, infected birds are given a value of 1 and uninfected birds a value of 0. There was no difference in body condition between infected and uninfected birds ($p=0.9856$; $n=75$).

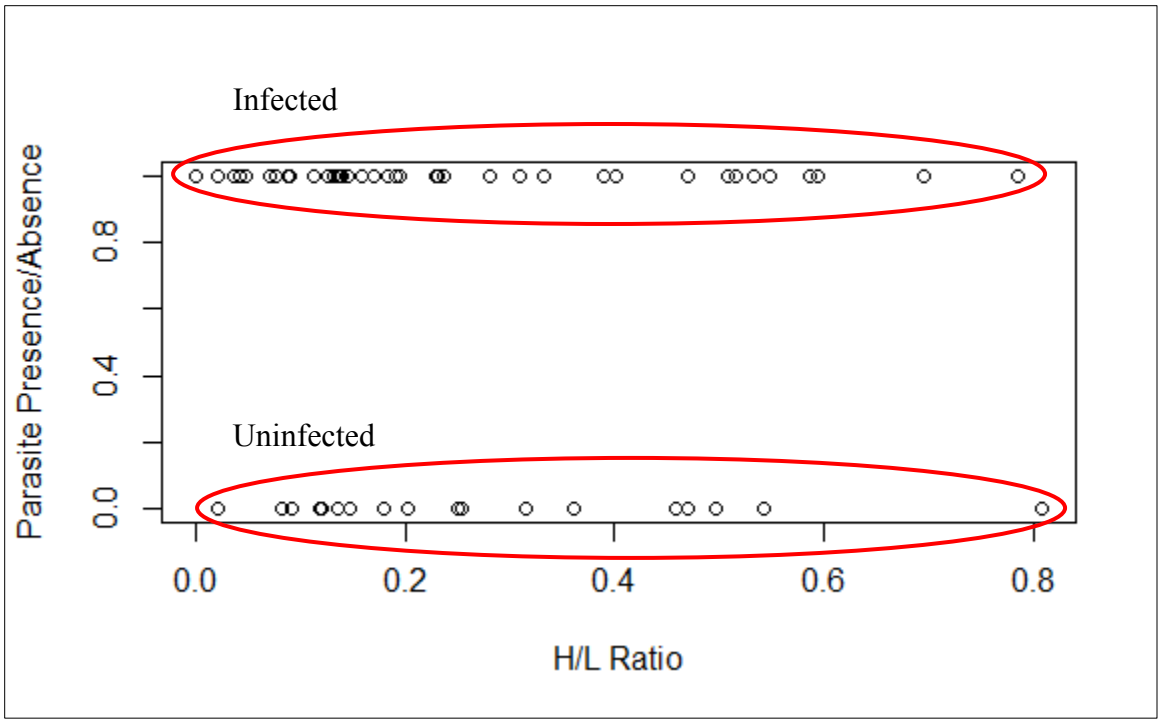

Figure 3.4 H/L ratio of infected and uninfected birds

The H/L ratio is an indicator of stress. There was no association between the H/L ratio and haemosporidian prevalence ($p=0.4121$; $n= 68$).

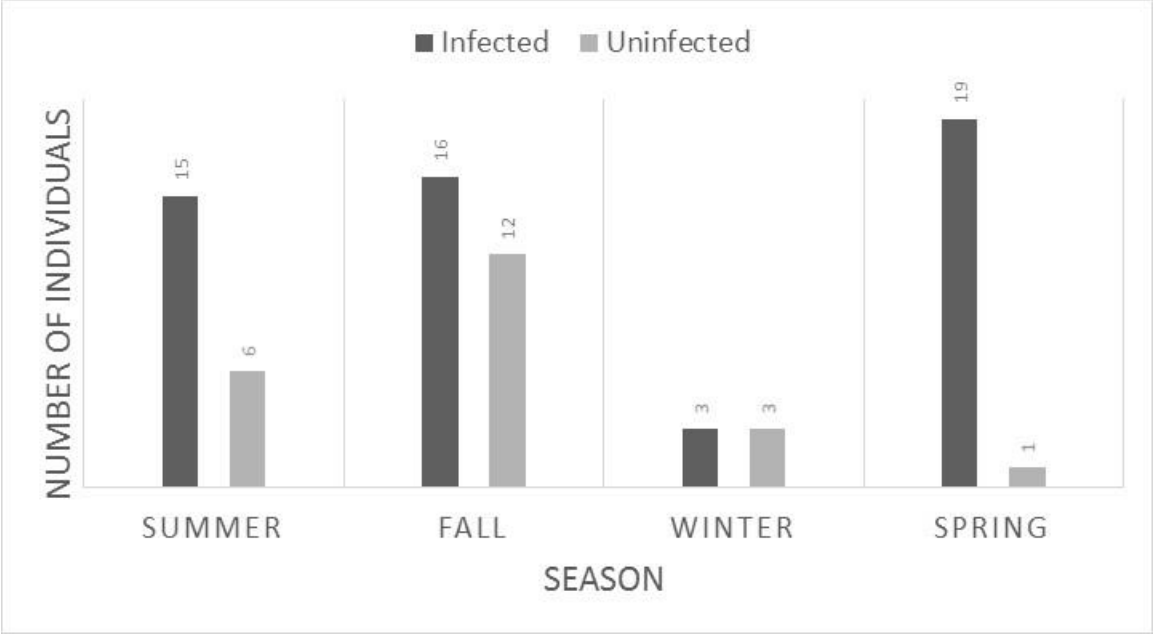

Figure 3.5 Seasonal haemosporidian infection status

Seasonally, there was a significant difference in parasite prevalence ($p=0.0137$; $n=75$). The most significant difference in seasonal prevalence occurred between the spring and fall ($p=0.021$, $n=75$).

3.2 Parasitemia

3.2.1 Microscopy

Using microscopy to determine parasitemia, estimates were similar between blood smears made from the same bird ($p<0.05$; $r=0.80$; $n=43$) and between viewers of the same blood smears ($p<0.05$; $r=0.956$; $n=72$). All birds infected with haemosporidians were infected at low parasitemia with less than 1.0% of erythrocytes infected, and the majority of infections were very low with less than 0.1% of erythrocytes infected (Figure 3.6; see Table A.2 for additional information). Infections of *Parahaemoproteus* showed significantly higher parasitemia than *Plasmodium* ($p<0.05$; $n=47$). *Plasmodium* infections showed parasitemia of less than 0.1% in all birds while *Parahaemoproteus* reached 0.23% (Figure 3.7). Specific lineages of *Parahaemoproteus* or *Plasmodium* did

not influence parasitemia (p=0.113; n=52). Parasitemia was not associated with host sex (p=0.111; n=71; Figure A.1), host age (p=0.9598; n=65; Figure A.2), or season (p=0.2568; n=65; Figure A.3). Body condition of the host did not vary with parasitemia when the index was estimated from tarsus length (R^2=0.02, p=0.3004, n=65; Figure 3.8) or wing chord (R^2=0.0007, p=0.84; n=65; Figure 3.9). The H/L ratio showed no association with parasitemia R^2=0.004, (p=0.2281; n=68; Figure 3.10).

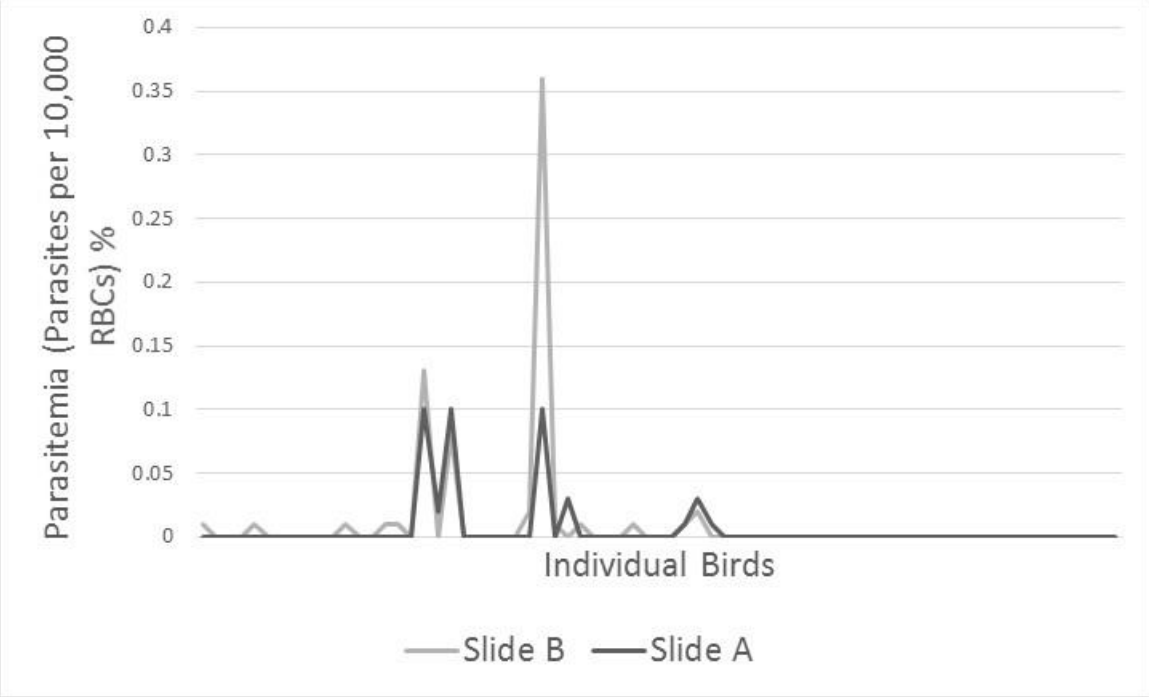

Figure 3.6 Haemosporidian parasitemia estimated from stained blood smears

Two blood smears were viewed for each bird, and the results from both scans were statistically similar (p<0.05; r=0.80; n=43).

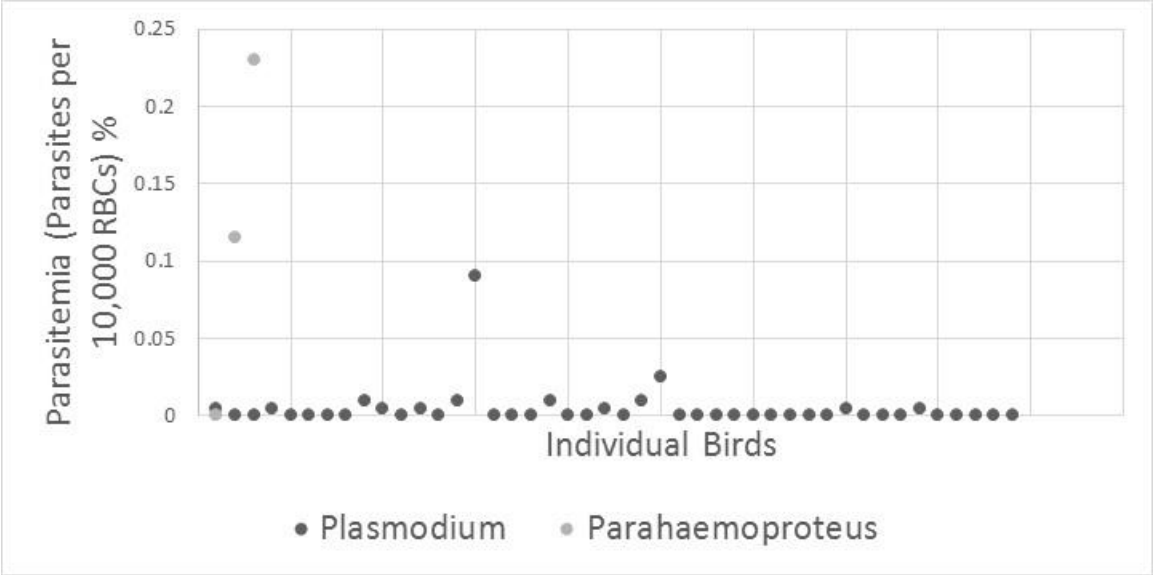

Figure 3.7 Parasitemia of genera *Plasmodium* and *Parahaemoproteus* estimated from microscopy

Parasitemia reported is the average across both blood smears available for each bird. Parasitemia was significantly higher in *Parahaemoproteus* infections that for *Plasmodium* ($p<0.05$; $n=47$). All birds infected with haemosporidians were infected at low parasitemia with less than 1.0% of erythrocytes infected, and the majority of infections were very low with less than 0.1% of erythrocytes infected.

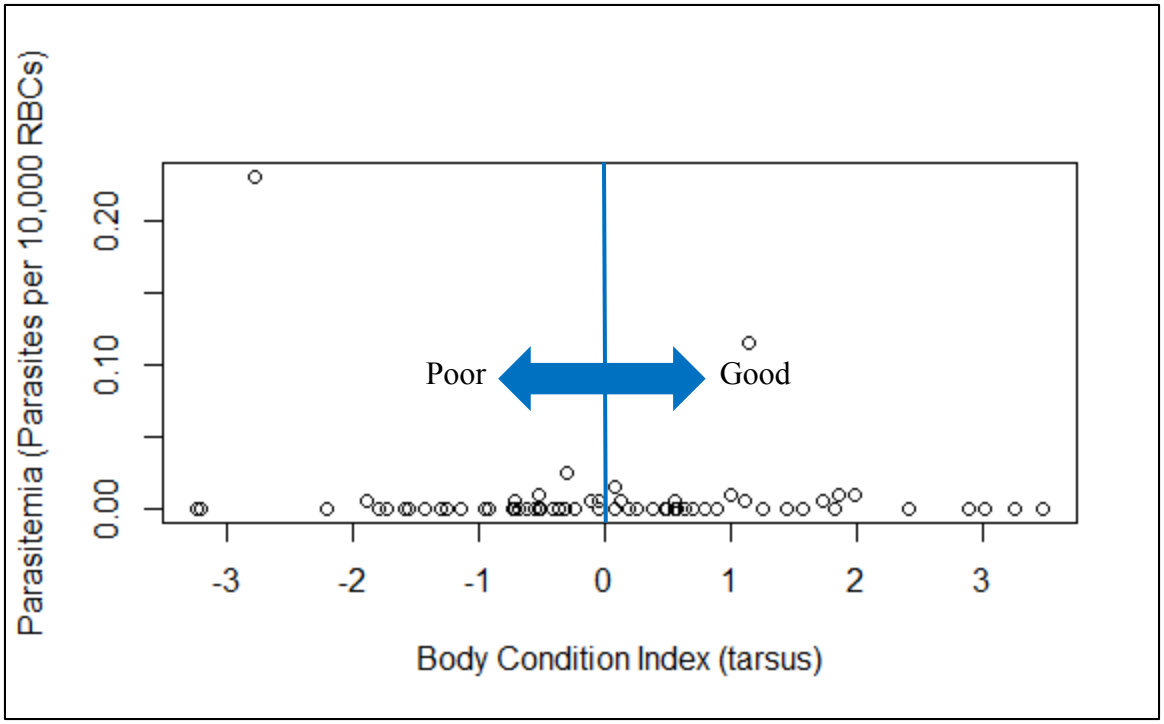

Figure 3.8 Body condition index based on tarsus length and parasitemia estimated from microscopy

The body condition index was estimated from an ordinary least squares regression of bird body mass on a linear body measurement, in this case tarsus length. Within the index (on the x-axis), positive numbers indicate birds in better body condition while negative numbers are birds in poor condition. Parasitemia by microscopy was not associated with this index of body condition (R^2=0.02, p=0.3004, n=65).

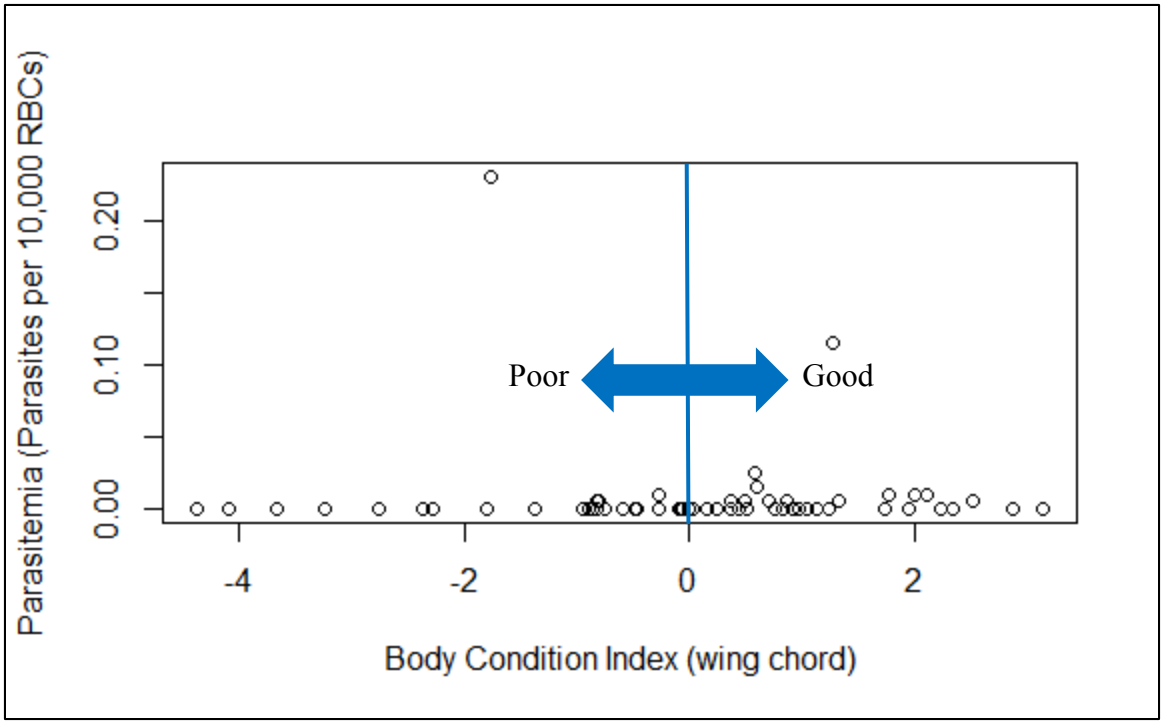

Figure 3.9 Body condition index based on wing chord and parasitemia estimated from microscopy

The body condition index was estimated from an ordinary least squares regression of bird body mass on a linear body measurement, in this case wing chord length. Within the index (on the x-axis), positive numbers indicate birds in better body condition while negative numbers are birds in poor condition. Parasitemia by microscopy was not associated with this index of body condition ($R^2$0.0007, p=0.84; n=65).

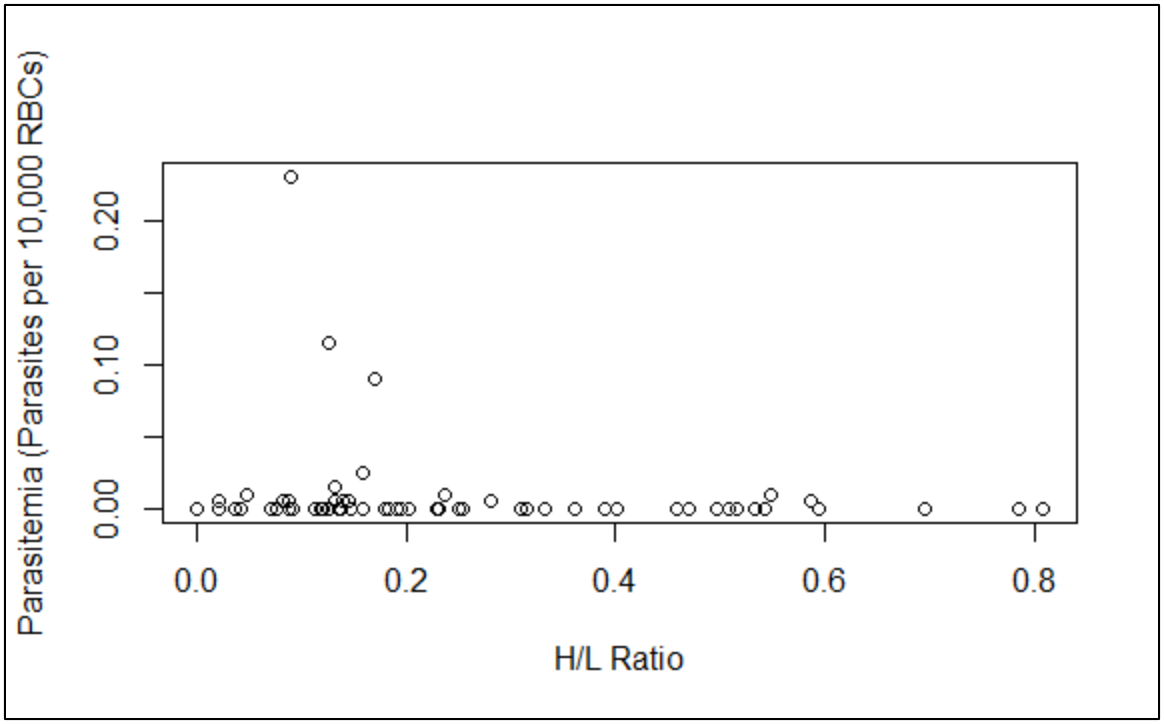

Figure 3.10 H/L ratio and parasitemia estimated from microscopy

The H/L ratio showed no association with parasitemia (R^2=0.004, p=0.2281; n=68).

3.2.2 qPCR

I screened 36 samples using the custom qPCR protocol. From those samples, only one sample was removed from analyses because the standard deviation was greater than one between the three replicates. The Ct values ranged from 28-48, which translates to 0-122,445 copies of the parasite's cyt *b* gene indicating low parasitemia (Table A.3). As with microscopy, the estimate of parasitemia by qPCR was not influenced by parasite lineage (p=0.217; n=13).

Parasitemia did not differ significantly between years (p=0.317; n=29) so all sampling years were analyzed together. Parasitemia was not associated with host sex (p=0.2032; n=34; Figure A.4), host age (p=0.5776; n=28; Figure A.5), or season (p=0.3956; n=28; Figure A.6). Body condition index of the host did not vary with

parasitemia when the index was estimated from tarsus length (R^2=0.02, p=0.534, n=28; Figure 3.11) or wing chord (R^2=0.05, p=0.252; n=28; Figure 3.12). The H/L ratio showed little significant association with parasitemia (R^2=0.09, p=0.10; n=31; Figure 3.13).

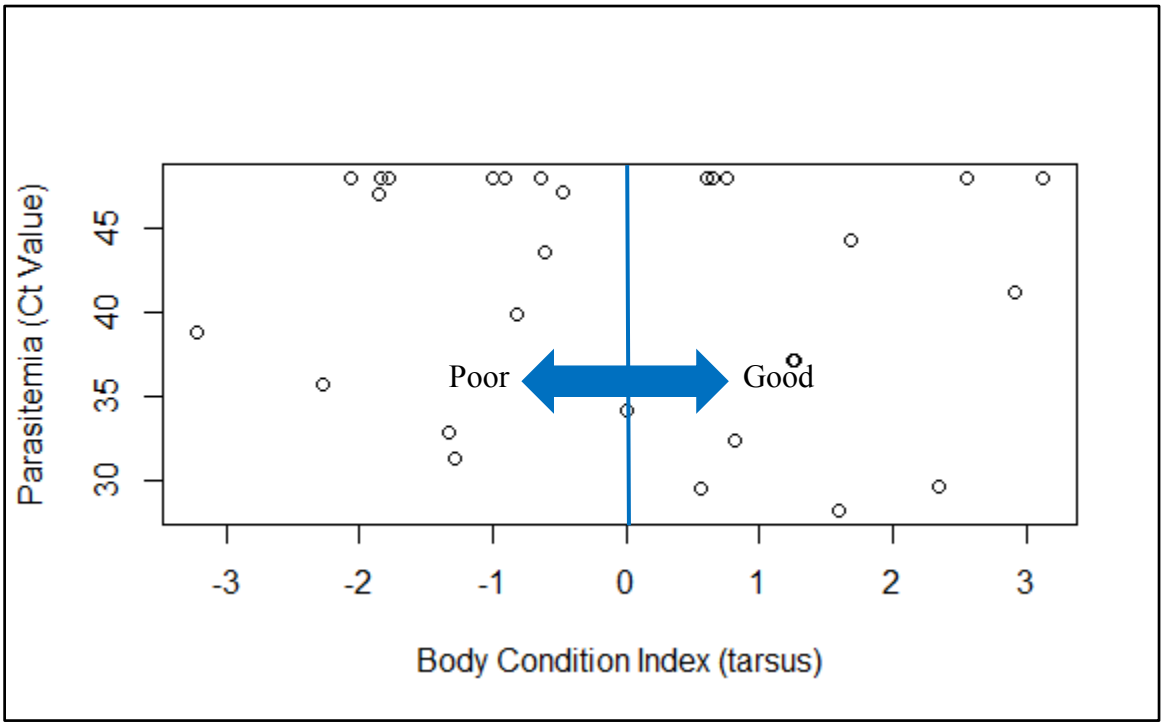

Figure 3.11 Body condition index based on tarsus length and parasitemia estimated from qPCR

The body condition index was estimated from an ordinary least squares regression of bird body mass on a linear body measurement, in this case tarsus length. Within the index (on the x-axis), positive numbers indicate birds in better body condition while negative numbers are birds in poor condition. Parasitemia by qPCR was not associated with this index of body condition (R^2=0.02, p=0.534, n=28).

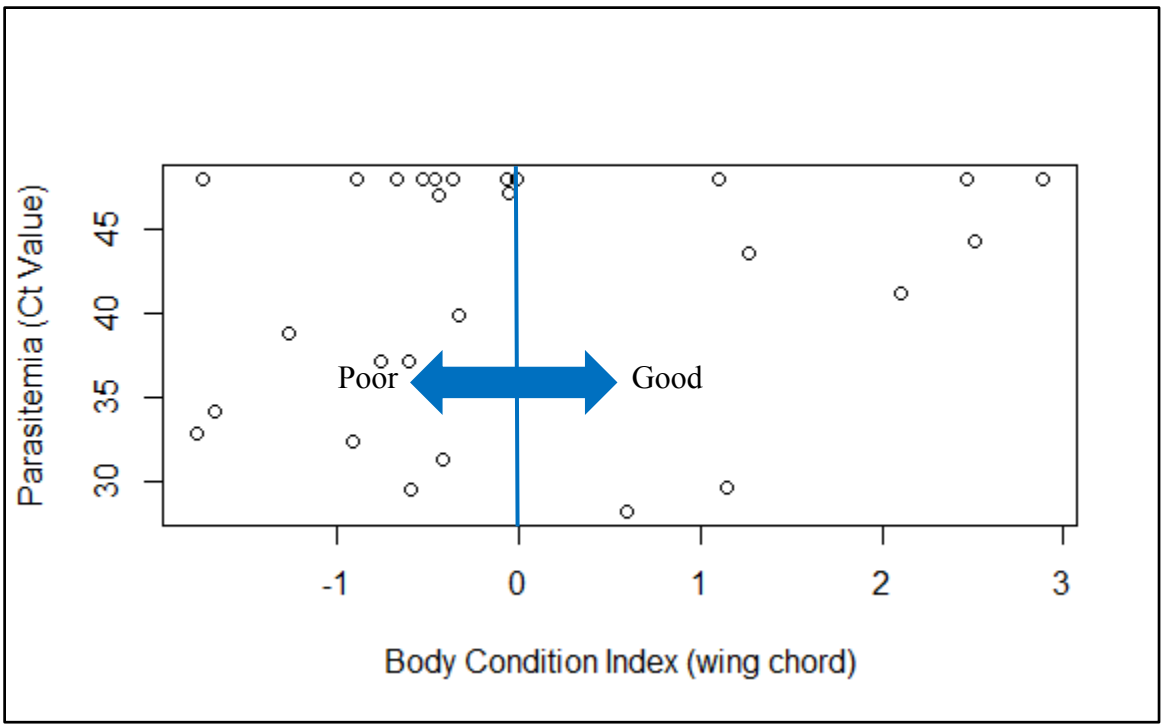

Figure 3.12 Body condition index based on wing chord length and parasitemia estimated from qPCR

The body condition index was estimated from an ordinary least squares regression of bird body mass on a linear body measurement, in this case wing chord length. Within the index (on the x-axis), positive numbers indicate birds in better body condition while negative numbers are birds in poor condition. Parasitemia by qPCR was not associated with this index of body condition (R^2=0.05, p=0.252; n=28).

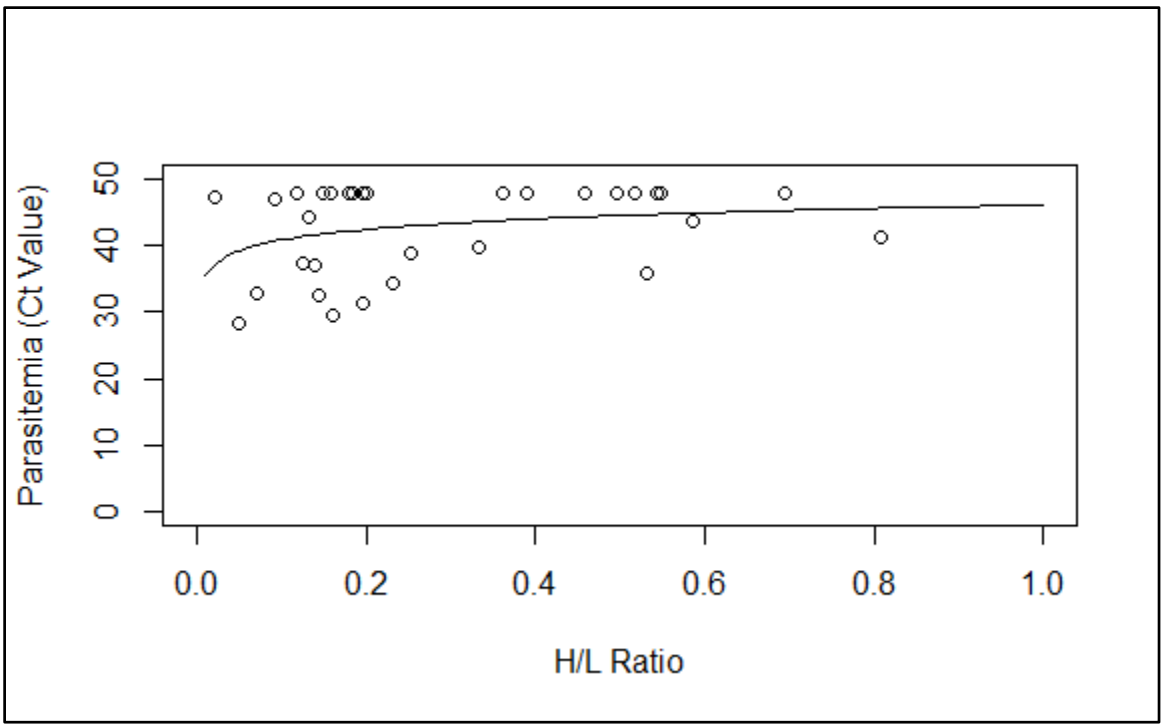

Figure 3.13 H/L ratio and parasitemia estimated from qPCR

The H/L ratio showed no association with parasitemia (R^2=0.09, p=0.1; n=31).

3.3 Phylogenetic Analysis

The Bayesian phylogenies revealed nine *Plasmodium* lineages and four *Parahaemoproteus* lineages. Four *Plasmodium* lineages and one *Parahaemoproteus* had not been recorded yet in Genbank or MalAvi. Here, a one base pair difference between sequences is the cutoff to define lineages. *Plasmodium* lineage 5 (PLAS5), morphospecies *Plasmodium cathemerium*, was highly prevalent in the Tufted Titmouse and other birds and was distributed across the United States and Mexico (Figure A.7). PLAS4 was specific to the Tufted Titmouse. It had previously been detected in four Tufted Titmice and was prevalent in the birds collected for this study. A novel lineage, PLAS3, was present in two titmice. Three novel lineages of *Plasmodium* (PLAS1, PLAS2, and PLAS9) were found in only one titmouse each. PLAS8 was present in two

titmice and one other bird. PLAS8 and PLAS9 are similar to morphospecies *Plasmodium lutzi*. The lineage PLAS8 was present in one titmouse and only one other bird. The lineage PLAS6 infected two titmice and matches the highly widespread *Plasmodium relictum* lineage GRW04. One other lineage infecting the Tufted Titmouse was previously found (lineage BAEBIC02) that was not present in any birds from the current study. One novel *Parahaemoproteus* lineage (PARA4) was present in only one titmouse from Illinois (Figure A.8). Three other *Parahaemoproteus* lineages (PARA1, PARA2, and PARA3) were present and were grouped closely together on the phylogenetic tree. The phylogeny including only parasites found in the Tufted Titmouse (Figure A.9) showed no clear geographic distribution patterns.

3.4 Comparison of Methods

The estimate of haemosporidian prevalence was significantly different based on the detection method (i.e., microscopy, PCR, and qPCR) used ($p<0.05$; $n=32$). Microscopy and qPCR yielded negative results where PCR found the same samples to be positive for malaria parasites. Sequence data was available for all samples that were positive by PCR. All samples negative from PCR were also negative by microscopy. One sample negative by PCR appeared to be positive by qPCR. By scanning blood smears for infected erythrocytes, the prevalence was 23.9% ($n=71$), PCR gave a prevalence of 69.1% ($n=81$), and qPCR 25.0% ($n=32$).

Estimates of parasitemia were not similar between the two methods used ($p=0.175$; $R^2=0.03$; $n=31$; Figure 3.14). There was often agreement on samples negative for parasites. The exceptions are: in four samples where no parasites were seen under the microscope, qPCR detected parasite DNA, and parasites were seen in blood smears in

four samples negative by qPCR. An inverse relationship between Ct value and parasites per 10,000 erythrocytes is expected if both methods make the same estimate of parasitemia. However, that is not the case here.

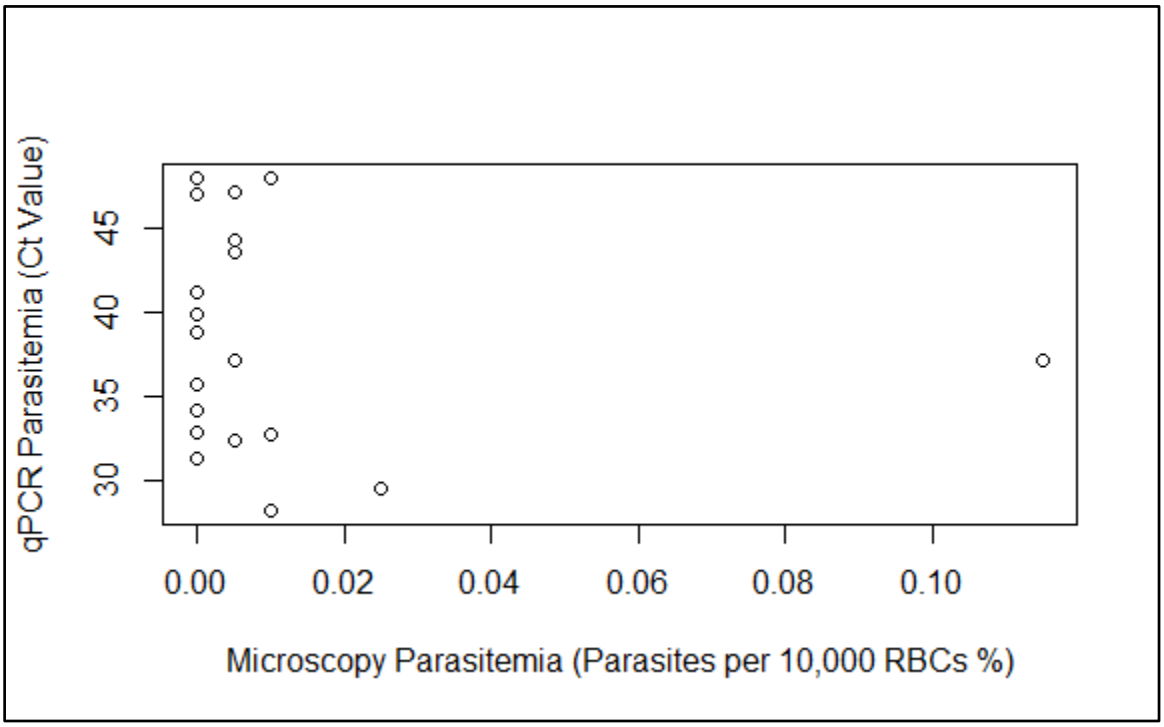

Figure 3.14 Comparison of parasitemia estimation methods

Estimates of parasitemia were not similar between the two methods used ($p=0.175$; $R^2=0.03$; $n=31$).

CHAPTER IV

DISCUSSION

4.1 Season

Tufted Titmice showed a peak in haemosporidian prevalence in the spring. This trend corresponds with the arrival of actively feeding dipteran vectors, naïve juvenile birds, and adult birds stressed by reproduction. Relapse stimulated by onset of the breeding season contributes to the seasonal increase in parasite presence. The lack of seasonality in parasitemia may be attributed to sampling bias. Heavily infected birds may be too sick to be captured under normal circumstances. Potential bias in sampling will be discussed in more detail below.

4.2 Sex

Neither male nor female birds exhibited a higher prevalence or parasitemia of parasites. Prevalence equality between bird sexes has been documented in several bird species (Ricklefs et al. 2005, Cloutier et al. 2011). Carotenoids are essential compounds for the immune response and feather coloration (Bendich & Olson 1989, Tschirren et al. 2003b). Competition for carotenoids in sexually mature males may be less in the Tufted Titmouse because it is a sexually monomorphic species lacking coloration, but this is merely speculation. Both sexes assist in brood rearing; therefore, neither sex is likely to

be significantly more susceptible to feeding vectors because of their behavior or suffer a worse hardship physically.

4.3 Age

There was no significant difference in the proportion of individual birds infected with haemosporidians or their intensity based on the host's age. Haemosporidian parasites may require a presence in both adult and juvenile populations in order to survive (as reviewed in Valkiūnas 2005). A portion of young birds seems to be infected each year because of their naivety and susceptibility to parasites (as reviewed in Valkiūnas 2005). Adult birds are also infected and maintain parasite numbers often in chronic infections. The parasite utilizes the lifespan of adult birds as the probability of transmission increases with the age of the bird (as reviewed in Valkiūnas 2005). The timing of egg hatching as it relates to peak numbers of vectors significantly affects whether or not juvenile birds are infected during a particular season or year (Atkinson & van Riper 1991, as cited and reviewed in Valkiūnas 2005).

4.4 H/L Ratio and Body condition

Infected birds did not suffer from negative fitness effects that can be measured by the H/L ratio and body condition index. The intensity of infection also did not show any relationship with host health. Arriero et al. (2015) also found no association between the H/L ratio and haemosporidian parasite presence in birds. Many other sources have cited the benign nature of malaria parasites in wild birds (Peirce 1989, Bennett & Bishop 1990, Bennett et al. 1993a, Desser & Bennett 1993, as cited and reviewed in Valkiūnas 2005). Perhaps a different measure of host health such as hematocrit, hemoglobin, furcular fat,

or a more detailed leucocyte profile would yield different results (Valkiūnas 2005, Krams et al. 2013, Bairlein 1995).

4.5 Low Parasitemia

Microscopic and molecular methods revealed low parasitemia in all Tufted Titmice infected with avian malaria. Low parasitemia may be the result of low virulence and specialist parasite lineages (Perkins et al. 1998, Richard et al. 2002, Szymanski & Lovette 2005). Although prevalence of haemosporidians was not low in the study population, parasite lineages that are infrequent may exist at low parasitemia and inflict little harm to the host (Ewald 1994, as cited in Perkins et al. 1998). Low parasitemia may also indicate that every bird infected was in the chronic stage of infection and had survived the acute stage.

4.6 Phylogenetic Analyses

Thirteen different haemosporidian lineages were identified using cyt *b* sequence data. The data show evidence of both specialist and generalist parasite lineages. Several lineages were widespread and infected a wide diversity of avian hosts in many different geographic locations. One *Plasmodium* lineage was found exclusively in Tufted Titmice (novel lineages found in only one titmouse are excluded from specialist lineages). Astudillo et al. (2013) reported finding a novel *Plasmodium* lineage in a single titmouse, which they named BAEBIC04. Sequence data for lineage BAEBIC04 was unavailable for use. Five novel lineages including representatives from the genera *Plasmodium* and *Parahaemoproteus* were detected and indicate that the diversity of avian malaria parasites is vast and requires continued sampling to completely uncover the depth of

variation. Samples from the eastern range of the Tufted Titmouse are of particular importance because much of the bird's range is neglected.

4.7 Problems with Methods of Detecting Parasites

The three methods used to estimate parasite prevalence (i.e., microscopy, PCR, and qPCR) and parasitemia (i.e., microscopy and qPCR) did not return similar results. Haemosporidian detection can be problematic because the parasite develops in the blood and tissues of the vertebrate host at different stages (Valkiūnas 2011). For example, some cases of infection cause mortality or severe sickness while in the tissue stages of infection and never reach the blood (Valkiūnas 2011). Also, molecular methods such as PCR amplify parasite DNA regardless of the life stage. Sporozoites that may be present in the host, but cannot actually establish an infection, are detected and signal a positive infection (Valkiūnas 2011). A combination of microscopy with molecular methods is a more reliable tactic to accurately estimate parasite presence (Valkiūnas 2011).

Parasite quantity estimated using microscopy may have been underestimated due to poorly prepared and stained slides. An appropriate number of erythrocytes was counted for the calculation of parasitemia. Increasing the number of erythrocytes counted per slide may increase the accuracy of measuring prevalence and parasitemia. Quantitative PCR was unable to detect parasites in all samples that sequence data was available for. There may have been very few parasites in the blood of those birds, and increasing the amount of template DNA used in each qPCR run may increase reliability. However, the amount of template DNA used was limited by the volume of blood available for each bird. Optimizing the qPCR protocol with more primer/probe combinations is possible, but the cyt *b* DNA segment used for identifying malaria

parasites is limiting because it is AT rich. Quantitative PCR gives an estimate of parasite cyt *b* copies in a blood sample. This number does not necessarily directly correspond to parasite numbers as one erythrocyte can be infected by more than one parasite and cyt *b* copy number varies with life stage of the parasite (Asghar et al. 2011). Finally, the number of samples viable for qPCR was relatively low at 35. Only one blood sample from a bird infected with *Parahaemoproteus* was usable for qPCR, and comparisons between parasitemia of the genera were impossible to perform.

4.8 Sampling Bias

Wild Tufted Titmice were collected using mist nets. This method introduced sampling bias as healthy birds are more likely to be captured than sick birds (as reviewed in Valkiūnas 2005). Sick birds, including those heavily infected with malaria parasites, are not strong enough to take part in the normal behaviors that lead to capture by mist net. In fact, wild birds that are able to perform typical foraging and territory defense activities do not surpass certain levels of parasitemia (4-5 % of erythrocytes infected) and do not usually reach high parasitemia (Valkiūnas 1993, as cited in Valkiūnas 2005). Wild birds that have been found infected at high parasitemia were collected because the bird experienced decreased mobility (e.g., capture by predator or vehicle collision; Bennett et al. 1993b, Valkiūnas 1993, Valkiūnas 1998, as cited and reviewed in Valkiūnas 2005). Birds experiencing the acute phase of avian malaria are often not captured in mist nets; only birds that have survived the acute phase are sampled by scientists (Ahmed & Mohammed 1978, Garnham 1980, Valkiūnas 1998, as cited in Valkiūnas 2005). An alternative method to capture birds is game bird shooting. However, this method also

introduces bias as game bird hunting is commonly restricted to seasons of the year when most birds experience chronic and latent stages of infection (Valkiūnas 2005).

4.9 Role of Vectors

In a study of geographic variation, Sol et al. (2000) found lower prevalence of *Haemoproteus columbae* in regions with a low density of louse flies, the dipteran vector. Vectors play a very important role in parasitism of vertebrate hosts (Anderson and May 1982, Ewald 1983, Piersma 1997, Poulin 1998, as cited in Sol et al. 2000). The vector density to host density ratio is a crucial factor allowing for parasite manifestation (Begon et al. 1996, Hudson & Dobson 1997, as cited in Sol et al. 2000).

Bird feeding behavior heightens the importance of vector distribution. Bird taxa search for various food sources in different foraging guilds (e.g., ground, low-middle, middle-upper canopy), and dipteran vectors are differentially distributed across these strata (Greiner et al. 1975, Kirkpatrick & Suthers 1988, Astudillo et al. 2013). Tufted Titmice typically forage for seeds, fruit, and insects in the middle-upper stratum of the canopy (Kirkpatrick & Suthers 1988, Astudillo et al. 2013). In a study of several bird species, those foraging in the low-middle stratum were more frequently infected with *Haemoproteus*, the middle-upper stratum with *Plasmodium*, and the ground dwellers with *Leucocytozoon* (Astudillo et al. 2013). Kirkpatrick and Suthers (1988) also found ground foraging birds more likely to be infected with *Leucocytozoon*. However, this trend is not supported by all vector vertical distribution data. Mosquitoes, the vector of *Plasmodium*, were found most frequently at ground level; biting midges, the vector of *Parahaemoproteus*, in the canopy; and the black fly, transmitter of *Leucocytozoon*, in the canopy (Cerny et al. 2011, Astudillo et al. 2013). Rohner et al. (2000) saw that Great

Horned Owls (*Bubo virginianus*) nested in the mid-canopy for most of the year, but moved to the ground level when *Leucocytozoon* transmitting black flies became seasonally prevalent in the mid-canopy.

Others have found no link between haemosporidian prevalence and feeding or nesting stratum (Greiner et al. 1975, Rohner et al. 2000, Ricklefs et al. 2005). The lack of vector presence in the expected strata may be due to under-sampling; the lineages of haemosporidians detected may not be transmitted by the specific vector lineages collected (Astudillo et al. 2013). Host specificity comes into play here as different lineages of parasite are transmitted by some lineages of vector and vertebrate host and not by others (Astudillo et al. 2013).

I found a high prevalence of *Plasmodium* and a low prevalence of *Parahaemoproteus*. In a survey of several bird species, Tufted Titmice were infected with *Plasmodium*, but *Parahaemoproteus* prevalence was higher (Astudillo et al. 2013.). Ricklefs et al. (2005) also found two lineages of *Plasmodium*, but no *Parahaemoproteus*. The scheme of infections by different genera is difficult to interpret because only four birds infected with *Parahaemoproteus* were found.

4.10 Conclusion

The prevalence and parasitemia of avian malaria in the Tufted Titmouse were determined using a combination of microscopy, PCR, and qPCR. Malaria parasites were highly prevalent in this bird across all sampling sites and consisted primarily of the genus *Plasmodium*, but *Parahaemoproteus* was also present. Thirteen lineages of malaria parasites were found including five novel ones. Some lineages infected a diverse array of hosts, but host specific lineages for the Tufted Titmouse were also found. All infected

birds carried chronic, low parasite loads. Blood smear analysis indicated that *Parahaemoproteus* parasitemia was significantly higher than *Plasmodium* parasitemia, but qPCR was unable to support that claim due to low sample sizes. The most significant trend in the Tufted Titmouse is a seasonal difference in parasite prevalence. Prevalence and parasitemia showed no association with any other measure of interest (i.e., host sex, age, or health). A clearer story about *Parahaemoproteus* in this host would be told with continued collections. Also, acquiring more DNA from each bird would likely improve parasite detection and quantification by qPCR. Well-developed methods were applied here as well as a new qPCR protocol, which can be extended to use for haemosporidians of other hosts.

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APPENDIX A

SUPPLEMENTAL MATERIAL

A.1 Sampling locations

Table A.1 Sampling locations

Netting Location	Locality	GPS Coordinates
R. R. Foil Plant Science Research Center	Starkville, MS	33°28'27.47"N, 88°47'23.61"W
Aldersgate Church	Starkville, MS	33°28'26.40"N, 88°48'40.74"W
Osborn Prairie	Starkville, MS	33°30'48.93"N, 88°43'44.30"W
Central Avenue Area	Starkville, MS	33°28'18.93"N, 88°48'51.32"W
Sand Creek Conservation Area	Decatur, IL	39°49'25.46"N, 89° 0'42.43"W
Rock Springs Environmental Center	Decatur, IL	39°49'40.98"N, 89° 1'21.74"W
Fort Daniel Conservation Area	Decatur, IL	39°46'33.86"N, 88°49'30.36"W

A.2 Parasitemia

Table A.2 Parasitemia determined using microscopy

Bird ID	Slide A Parasitemia (%)	Slide B Parasitemia (%)	Average Slide Parasitemia (%)
KF001	0	0.01	0.005
KF002	0	0	0
KF003	0	0	0
KF004	0	0	0
KF005	N/A	0.01	0.01
KF006	0	0	0
KF007	0	0	0
KF008	0	0	0
KF009	0	N/A	0
KF010	0	0	0
KF011	0	0	0
KF012	0	0.01	0.005
KF013	0	0	0
KF014	0	0	0
KF015	0	0.01	0.005
KF016	0	0.01	0.005
KF017	0	0	0
KF018	0.1	0.13	0.115
KF019	0.02	0	0.01
KF020	0.1	0.08	0.09

Table A.2 (Continued)

KF021	0	N/A	0
KF022	0	0	0
KF023	0	0	0
KF024	0	0	0
KF025	0	0	0
KF026	0	0.02	0.01
KF027	0.1	0.36	0.23
KF028	0	0.01	0.005
KF029	0.03	0	0.015
KF030	0	0.01	0.005
KF031	0	0	0
KF032	0	0	0
KF033	0	0	0
KF034	0	0.01	0.005
KF035	0	0	0
KF036	0	0	0
KF037	0	0	0
KF038	0.01	0.01	0.01
KF039	0.03	0.02	0.025
KF040	0.01	0	0.005
KF041	0	0	0
KF042	0	0	0
KF043	0	N/A	0
WWT001	0	0	0
WWT002	0	0	0
WWT003	0	0	0
WWT004	0	N/A	0
WWT005	0	N/A	0
WWT006	0	N/A	0
WW047TM	0	N/A	0
WW048TM	0	N/A	0
WW049TM	0	N/A	0
WW050TM	0	N/A	0
TUTI138	0	N/A	0
TUTI175	0	N/A	0
TUTI183	0	N/A	0
TUTI187	0	N/A	0
TUTI198	0	0	0
TUTI199	0	N/A	0

Table A.2 (Continued)

TUTI217	0	N/A	0
TUTI227	0	N/A	0
TUTI229	0	N/A	0
TUTI233	0	N/A	0
TUTI237	0	N/A	0
TUTI260	0	N/A	0
TUTI267	0	N/A	0
TUTI272	0	N/A	0
TUTI277	0	N/A	0
TUTI295	0	N/A	0
TUTI573	0	N/A	0
TUTI735	0	N/A	0
TUTI748	0	N/A	0
TUTI764	0	N/A	0
TUTI771	0	N/A	0
TUTI772	0	N/A	0
TUTI776	0	N/A	0
TUTI779	0	N/A	0
TUTI780	0	N/A	0
TUTI797	0	N/A	0

Parasitemia was determined by counting the number of parasite infected erythrocytes out of 10,000 total erythrocytes on a stained blood smear. Results are expressed as percentages. Cells with the contents "N/A" indicate that blood smears were unavailable.

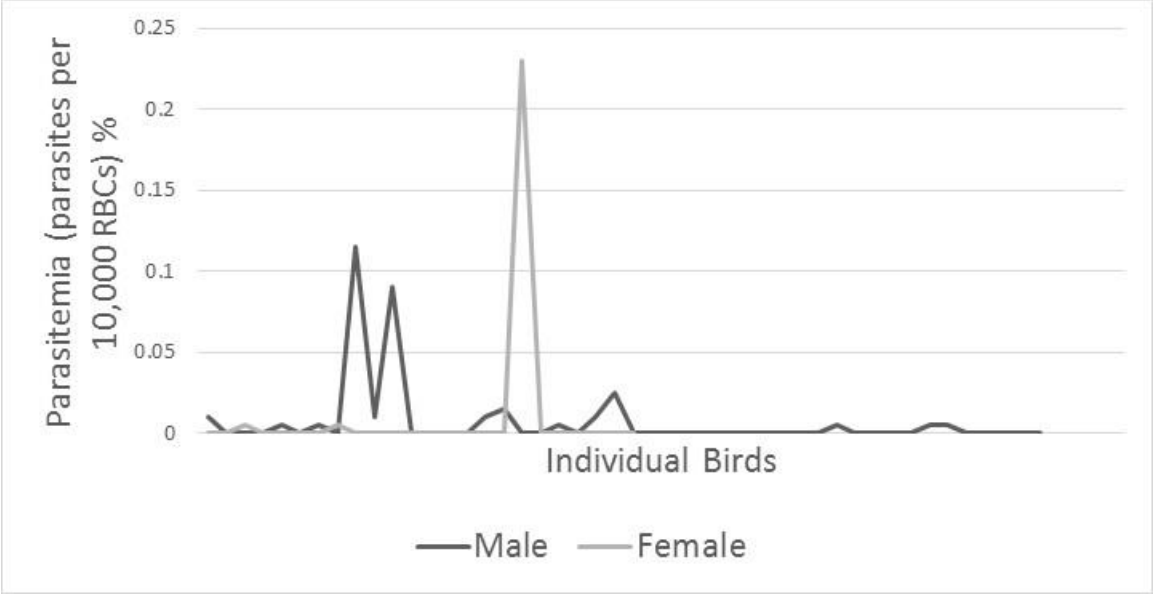


Figure A.1 Haemosporidian parasitemia by microscopy of birds by sex

Parasitemia by microscopy was not associated with the sex of the host (p=0.111; n=71).

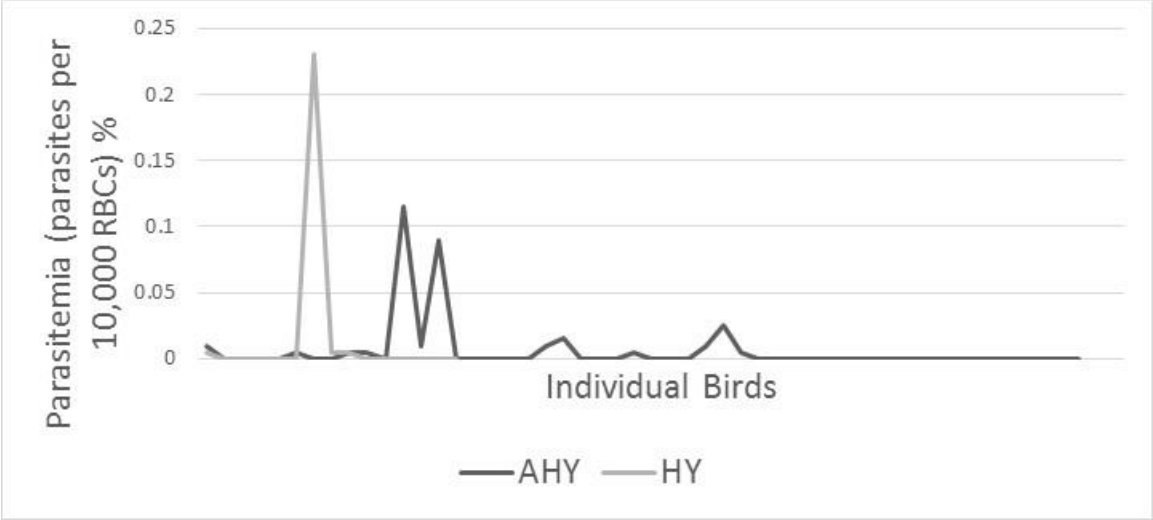


Figure A.2 Haemosporidian parasitemia by microscopy of birds by age

Parasitemia by microscopy was not associated with the sex of the host (p=0.9598; n=65).

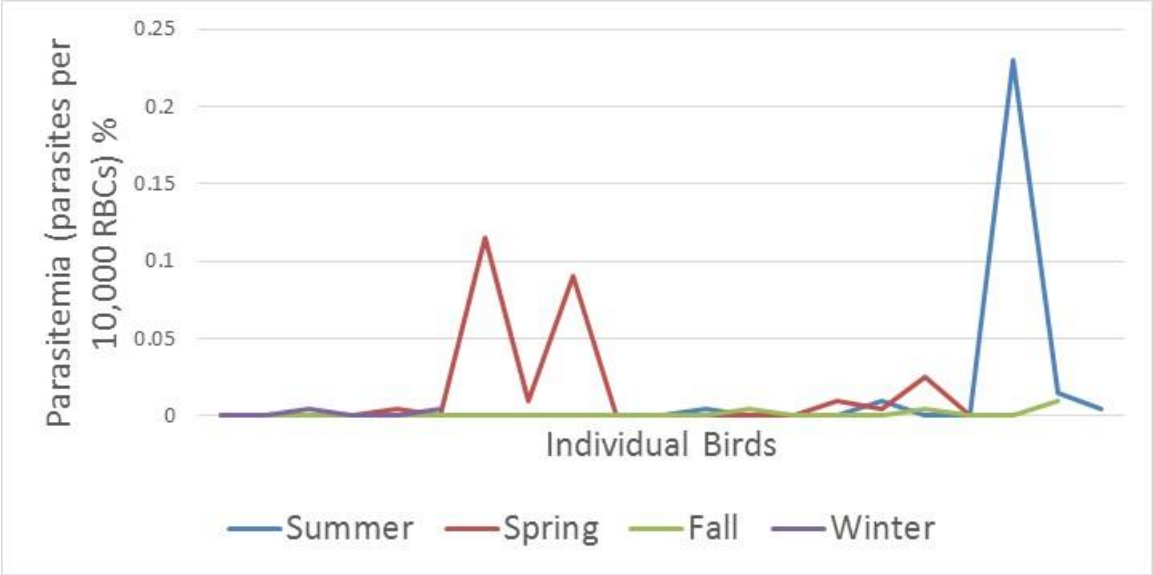


Figure A.3 Haemosporidian parasitemia by microscopy of birds by season

Parasitemia by microscopy was not associated with season (p=0.2568; n=65).

Table A.3 Parasitemia determined using qPCR

Bird ID	Ct Mean	Mean cyt *b* Copy Number
KF002	39.89	69.56
KF003	48.00	0.00
KF004	35.76	745.97
KF005	48.00	0.00
KF006	48.00	0.00
KF007	47.07	0.41
KF008	48.00	0.00
KF009	38.83	105.86
KF010	48.00	0.00
KF011	41.18	20.30
KF012	37.14	399.26
KF013	48.00	0.00
KF014	34.21	2100.75
KF015	47.13	0.39
KF016	44.28	3.91
KF017	48.00	0.00
KF019	37.19	290.44
KF020	28.28	122445.22
KF036	32.41	8281.52

Table A.3 (Continued)

KF037	31.39	15667.74
KF038	48.00	0.00
KF039	32.85	6227.58
KF040	32.76	5780.28
KF041	29.55	54394.48
KF042	43.64	3.69
KF043	48.00	0.00
WW047TM	29.74	45433.06
WW048TM	48.00	0.00
WW049TM	48.00	0.00
WWT001	48.00	0.00
WWT002	48.00	0.00
WWT003	48.00	0.00
WWT004	48.00	0.00
WWT005	48.00	0.00
WWT006	48.00	0.00

Parasitemia is represented as the mean Ct value of three replicates determined by qPCR and the actual parasite copy number.

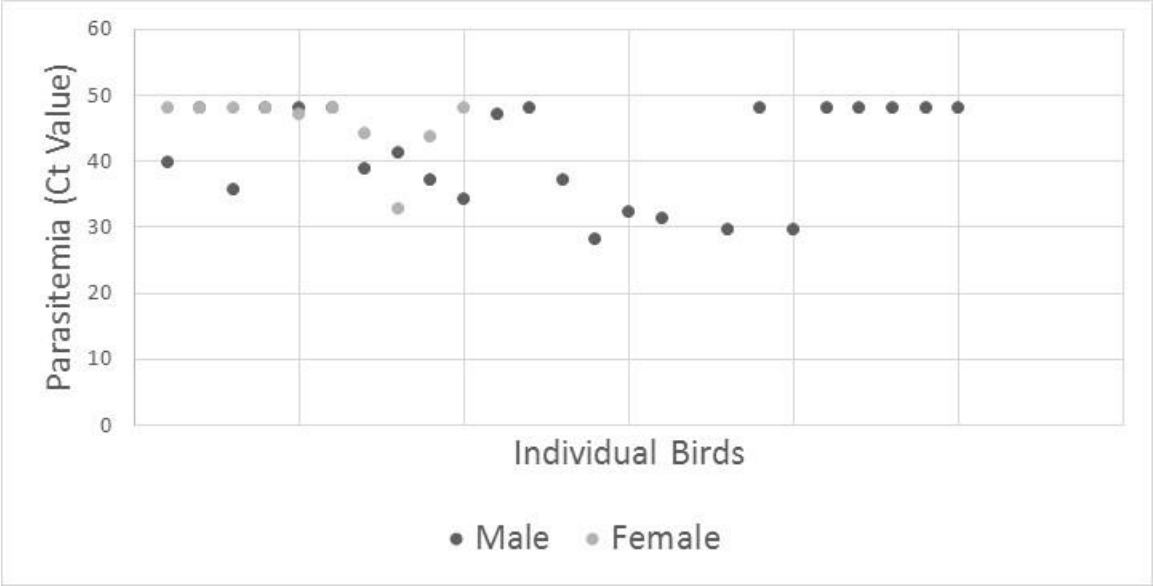


Figure A.4 Haemosporidian parasitemia by qPCR of birds by sex

Parasitemia by qPCR was not associated with the sex of the host (p=0.2032; n=34).

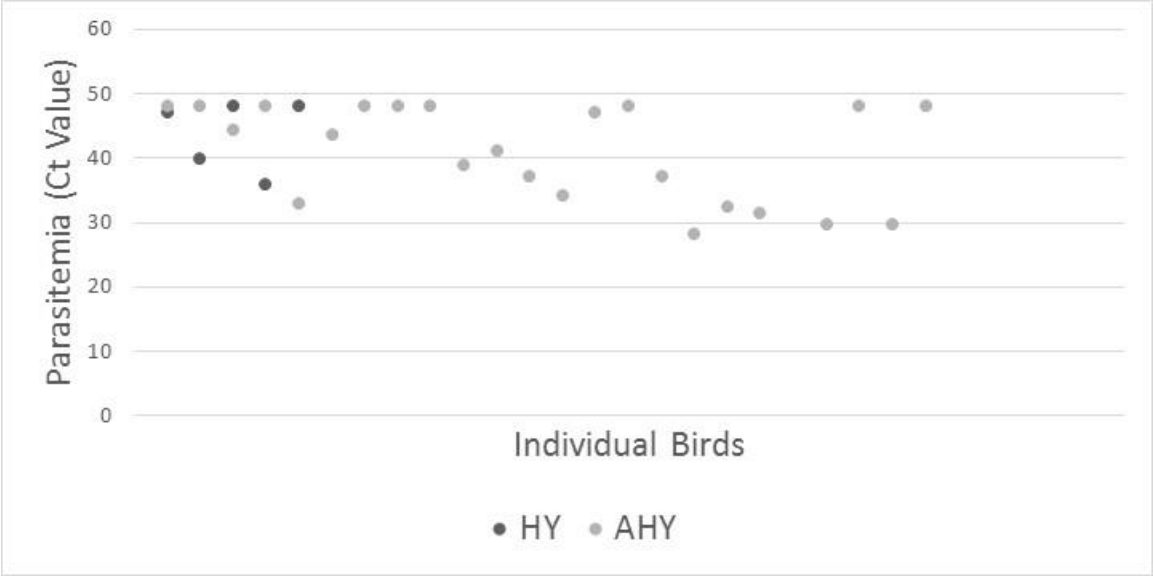


Figure A.5 Haemosporidian parasitemia by qPCR of birds by age

Parasitemia by qPCR was not associated with the age of the host (p=0.5776; n=28).

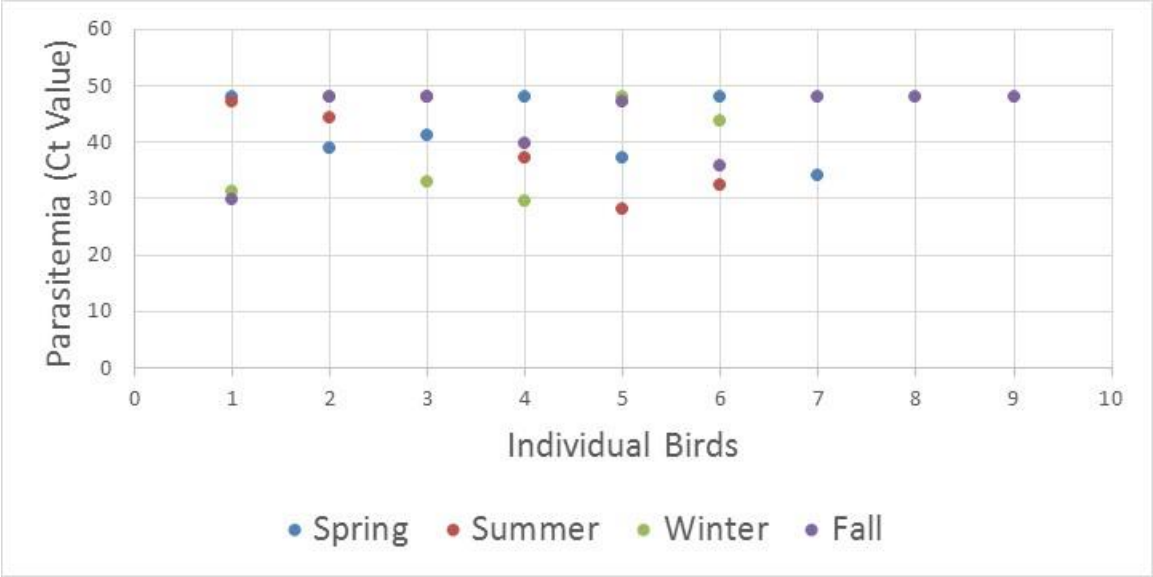


Figure A.6 Haemosporidian parasitemia by qPCR of birds by season.

Parasitemia by qPCR was not associated with season (p=0.3956; n=28).

A.3 Phylogenies

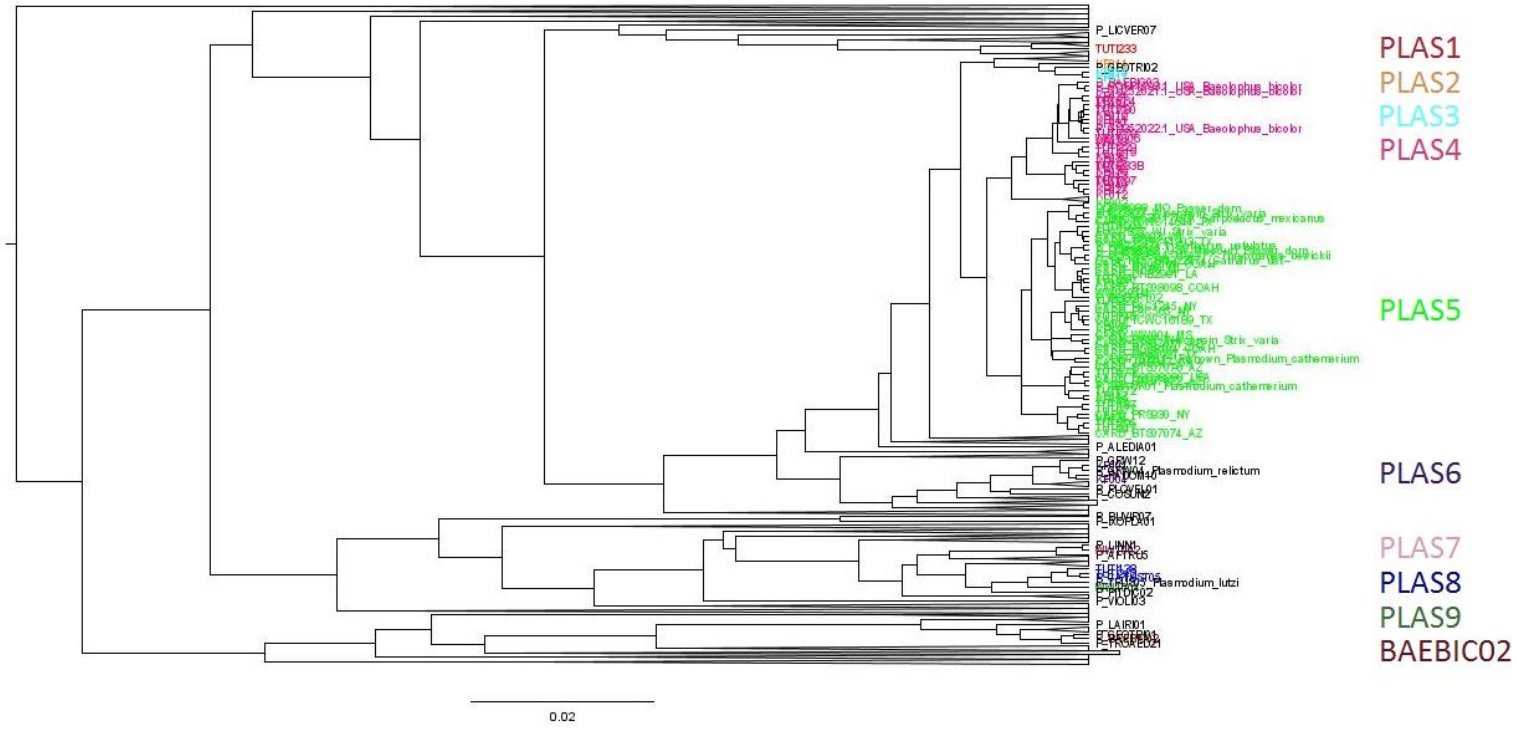


Figure A.7 *Plasmodium* Bayesian phylogeny

The Bayesian phylogeny was built in BEAST using 530 cyt b sequences of *Plasmodium*. Lineages PLAS1-PLAS9 include sequences recovered from Tufted Titmice in the current study. Lineage BAEBIC02 was previously isolated from the Tufted Titmouse. Lineage PLAS4 is specific to the Tufted Titmouse. Four lineages are novel.

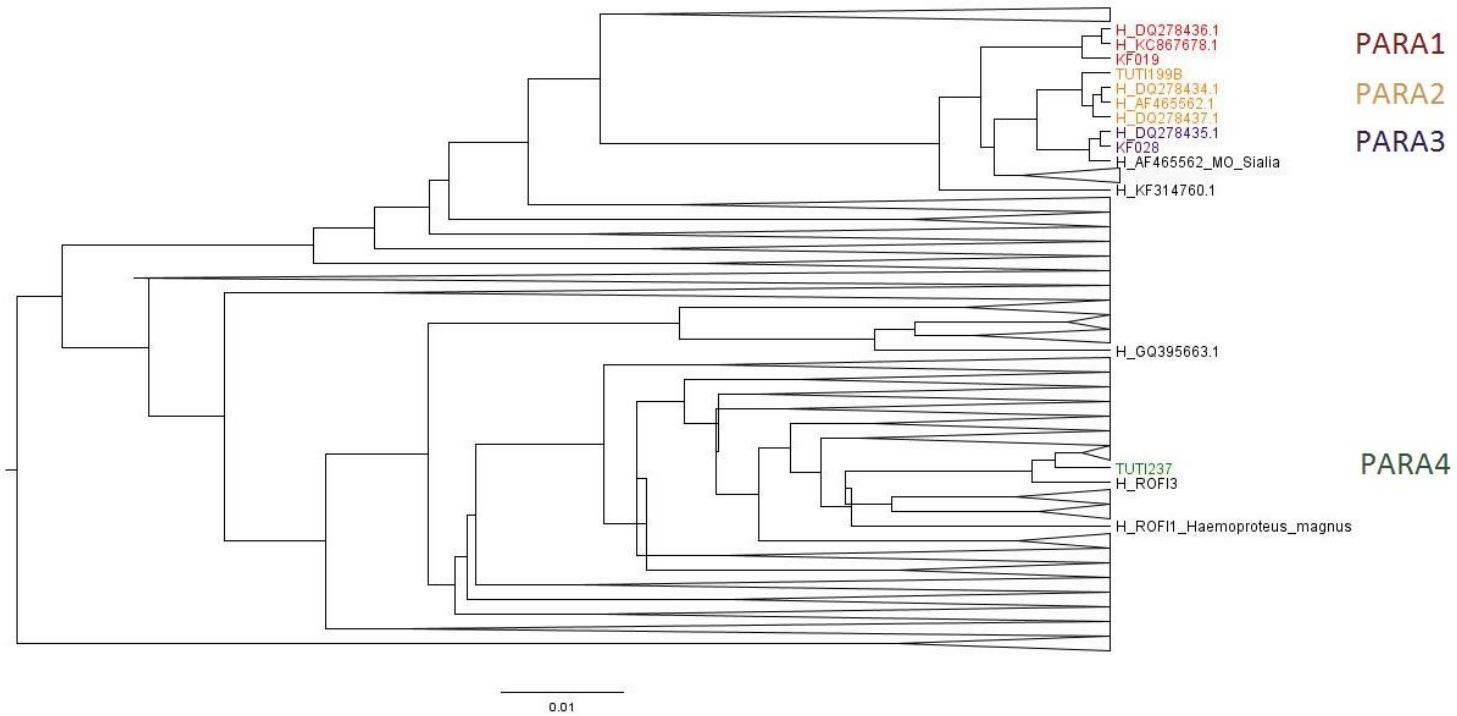
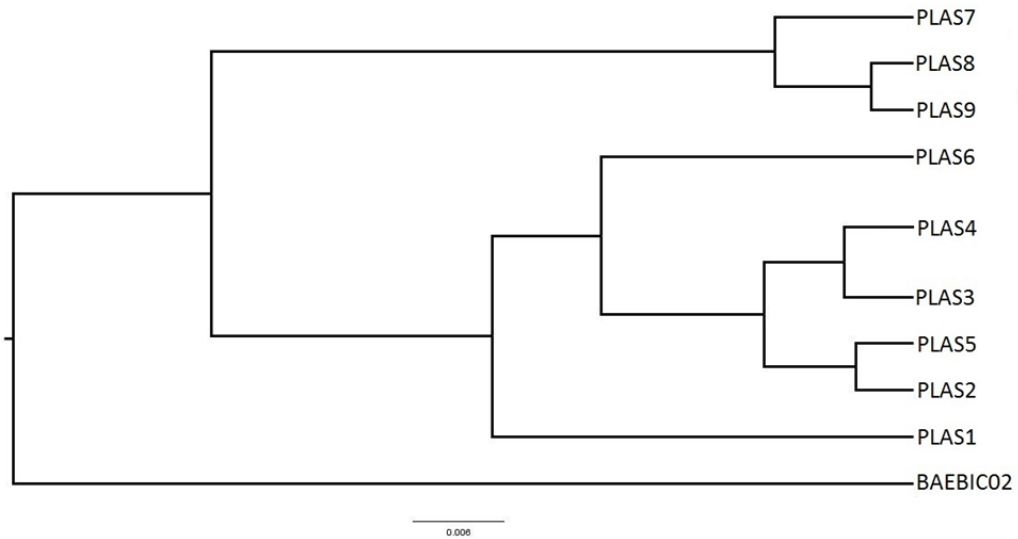


Figure A.8 *Parahaemoproteus* Bayesian phylogeny

The Bayesian phylogeny was built in BEAST using 750 cyt b sequences of *Parahaemoproteus*. Lineages PARA1-PARA4 include sequences recovered from Tufted Titmice in the current study. One lineage, PARA4, is novel.



	Mississippi	Illinois	Missouri
PLAS7	1		
PLAS8		2	
PLAS9	1		
PLAS6	2		
PLAS4	14	7	1
PLAS3	2		
PLAS5	7	11	
PLAS2	1		
PLAS1		1	
BAEBIC02			1

Figure A.9 Phylogenic relationships of titmouse *Plasmodium* parasites and their geographic location

A Bayesian phylogeny was built from the *Plasmodium* sequences found in the Tufted Titmouse.