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Investigating Phylogenetic Relationships of Mosquito-Borne Avian Malaria in Mississippi

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By

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Investigating phylogenetic relationships of mosquito-borne avian malaria in Mississippi

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The vectors of avian malaria (Haemosporida) are an understudied component of wildlife disease ecology. Most studies of avian malaria have focused on the secondary bird hosts. This imbalance leaves a significant gap in our knowledge and understanding of the insect hosts. This study investigates the diversity of malaria parasites carried by mosquitoes (Diptera, Culicidae) in the state of Mississippi. Using PCR techniques, haemosporidian infection rates were determined and parasites were identified in a phylogenetic context to those previously annotated. A total of 27,157 female mosquitoes representing 15 species were captured. Five of those species tested positive for malaria parasites with an overall infection rate of 4 per 1000 mosquitoes infected. Mosquitoes were shown to harbor *Plasmodium* and *Haemoproteus* parasites. Surprisingly, a unique lineage of parasites was discovered in *Anopheles* mosquitoes potentially representing a new genus of haemosporidian parasites, reinforcing the need to continue investigating this diverse group of parasites.

DEDICATION

I dedicate this work to my family, Alan, Peggy, and Rebecca Larson, back home in Texas. Without their encouragement and frequent phone calls, I probably would have fled to Old Mexico long ago.

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CHAPTER I

INTRODUCTION

Malaria Parasites

Overview of Haemosporidian Parasites

Malaria parasites (Phylum: Apicomplexa, Order: Haemosporida) are single-celled protozoans of the phylum Apicomplexa and are known to infect every major lineage of terrestrial vertebrates. Malaria parasites have a complex, multi-stage life cycle, completing asexual development within a vertebrate host and a sexual phase within a blood-sucking dipteran fly (Insecta: Diptera) host.

Apicomplexans are obligate single-celled, intracellular parasites characterized by the apicoplast organelle (Escalante and Ayala 1995, Morrison 2009, Lim and McFadden 2010). The organelle harbors DNA, has various functions in molecular synthesis and is hypothesized to have originated from an endosymbiotic event approximately 800 million years ago. The apicoplast is thought to have been a chloroplast-like organelle since haemosporidians are very susceptible to herbicidal treatments, although the presumed ability to photosynthesize has since been lost (Escalante and Ayala 1995, Lim and McFadden 2010).

The Apicomplexa are comprised of four major groups: the coccidians, the piroplasmids, the gregarines, and the haemosporidians. Only recently has extensive molecular work been done to resolve the relationships within the phylum, challenging

many of the conclusions reached by morphological trait-based phylogenies (Morrison 2009). Apicomplexans are thought to be most closely related to the ciliates and dinoflagellates (Escalante and Ayala 1995, Morrison 2009). However, the placement of the phylum in a broader phylogenetic context is otherwise difficult to resolve (Morrison 2009, Bensch et al. 2013). Haemosporidians, the focus of this study, are a group that undergoes merogony in a vertebrate host and sporogony in an insect host. The entirety of the life cycle of haemosporidians occurs in a living host (as reviewed and detailed in Garnham 1966, as reviewed and detailed in Valkiūnas 2005, Morrison 2009).

The relationships within Haemosporida are complex and understudied. The major classifications of these parasites were created before the advent of molecular techniques; these parasites are currently classified into four families: Plasmodiidae, Haemoproteidae, Leucocytozoidae, and Garniidae. Garniid parasites primarily infect reptiles but have only occasionally been found in birds (Gabaldon et al. 1985, as reviewed and detailed in Valkiūnas 2005). The other three families have been better characterized and are much more common in birds. Three major genera within those families are the primary malaria parasites of birds: *Haemoproteus* (Haemoproteidae), *Leucocytozoon* (Leucocytozoidae), and *Plasmodium* (Plasmodiidae) (Corradetti et al. 1963, Levin and Campbell 1971, Hsu et al. 1973, as reviewed and detailed in Valkiūnas 2005, Martinsen et al. 2008, Outlaw and Ricklefs 2011). *Haemoproteus* parasites are further broken down into the subgenera *Haemoproteus* and *Parahaemoproteus* based on their hosts/vectors in addition to deep phylogenetic divergence (Levine and Campbell 1971, as reviewed and detailed in Valkiūnas 2005, Martinsen et al. 2008). The genus *Plasmodium* was defined based on morphology but is now known to be paraphyletic, with avian/reptilian *Plasmodium* being

more closely related to *Haemoproteus* parasites (Martinsen et al. 2008). Furthermore, Outlaw and Ricklefs (2011) demonstrated evidence that all mammalian parasites save one, *Polychromophilus*, a haemosporidian of bats, likely originated from an avian/reptilian *Plasmodium* parasite via a recent host-switching event (Witsenburg et al. 2012). Current evidence suggests that the extant malaria parasites radiated around 20 million years ago, coinciding with a major radiation in passerine birds (Outlaw and Ricklefs 2011). However, due to the lack of sampling and fossil calibration points, the haemosporidian phylogeny is in a state of flux and the subject of much debate (Bensch et al. 2013).

It has been extremely difficult to tease apart what factors drive speciation in avian haemosporidians (as reviewed and detailed in Valkiūnas 2005, Bensch et al. 2013). Since sexual stages occur in the insect hosts, they should play an important role in sexual isolation (Gager et al. 2008, Clark and Clegg 2015). However, current evidence suggests that the vertebrate hosts play a bigger role in predicting geographic diversity of these parasites (Martinez de la Puente et al. 2011, Medeiros et al. 2013). Many bird species migrate long distances, and this may play a role in sexually isolating parasites and introducing them to new hosts (as reviewed and detailed in Valkiūnas 2005, Ramey et al. 2015, von Rönn et al. 2015), although geographic isolation does not seem to play a major role in the diversification of haemosporidians. Vectors may carry and transmit a variety of parasites that may or may not be competent in a specific bird host, but vectors seem to act like way-stations between secondary hosts (Gager et al. 2008, Ishtiaq et al. 2008). Recent evidence suggests that host-switching by parasites drives speciation; parasites

being forced to adapt to the novel hosts as new environments (Lauron et al. 2014, Ricklefs et al. 2014, Santiago-Alarcon et al. 2014).

Haemosporidian parasites are globally distributed with the exception of Antarctica (as reviewed and detailed in Valkiūnas 2005). Like other groups of organisms, haemosporidian species richness decreases with increasing distance from a continental landmass in accordance with the Island Theory of Biogeography (Perez-Rodriguez et al. 2013). In migratory birds, parasite infections are acquired throughout the migratory range, making it difficult to ascertain the geographic distributions of these parasites without studying the insect vectors (Waldenström et al. 2002, Ricklefs et al. 2003, Ramey et al. 2015, von Rönn et al. 2015). Every major group of birds sampled has been shown to harbor haemosporidians, with the exception of permanent Antarctic residents. However, the diversity of haemosporidians is highly biased towards sampling certain bird groups (especially Passeriforms) in geographic localities where research is undertaken, e.g. North America and Europe (as reviewed and detailed in: Valkiūnas 2005, Santiago-Alarcon et al. 2012, Clark et al. 2014).

A Brief History of Avian Malaria

Malaria of humans is the number one vector-borne disease of people worldwide, killing 1.2 million people annually (WHO 2012). It has caused immeasurable suffering and countless deaths throughout human history. Originally limited to the Old World tropics, trade and human migration brought malaria to temperate regions, and ultimately, it was brought to the New World with European and African migrants (Wood 1975). This devastating disease has been the focus of intense investigation since ancient times;

however, until light microscopy was sufficiently advanced, malaria was attributed often to 'swamp vapors.'

The Russian protozoologist V. Ya. Danilewski was the first to qualitatively describe morphology of the various stages of the development malaria parasites in birds in 1884. He described haemosporidioses in wild birds accompanied by anemia, enlargement and whitening of the spleen and liver, with associated accumulation of pigment. The following year, Italian scientists characterized the first human malaria parasite, correlating their findings to that of Danilewski. After fevered work throughout the world, the major genera of haemosporidians were described, culminating in the identification of the mosquito as the vector of *Plasmodium* by Ronald Ross in 1898. For this breakthrough, Ross was awarded the Nobel Prize in Medicine in 1902. Birds were the primary source of information on the life-history, distribution, and treatment of malaria until the Second World War, when effective rodent and primate substitutes were discovered for human malaria studies. However, most of the famous early antimalarial medications were first developed in bird models (as reviewed and detailed in Valkiūnas 2005, Santiago-Alarcon et al. 2012).

There has been renewed interest in avian malaria in recent years due to the interest in disease modeling, ecosystem functions, and generally elucidating the tree of life. The vast majority of studies centered on avian haematozoa have focused on the parasite's secondary hosts, birds. These studies have investigated host-switching, biogeography, phylogenetics, phylogeography, etc. (as reviewed in Clark et al. 2014).

Life Cycle of Avian Malaria

The life cycles of the various avian malaria parasites are complicated and highly variable throughout the order. When looking across Haemosporida, these life stages can be highly variable. Some species have as few as seven life stages, while some have as many as thirty five (as reviewed and detailed in Valkiūnas 2005). Haemosporidians spend their life cycle alternating between two hosts, a vertebrate and blood-sucking dipteran, changing form to invade various host tissues and shifting modes of reproduction (sexual/asexual) between vertebrate and fly hosts. The sexual stage of malaria parasites occurs in the insect hosts which are considered the primary (or definitive host). The vertebrate hosts are referred to as the secondary (or intermediate) hosts (MacCallum 1898, as reviewed and detailed in Garnham 1966, as reviewed and detailed in Valkiūnas 2005). While the insect is the primary host, it is considered the vector of haemosporidians to the bird hosts.

While taking a blood meal, the insect host inoculates sporozoites into vertebrate hosts that move to various fixed tissues and undergo asexual divisions. There are usually several generations of these exoerythrocytic stages of development (known as schizonts or meronts) during which the parasite gradually adjusts to the host. Meronts undergo asexual reproduction and develop into merozoites that continue to develop and eventually invade the erythrocyte (or skeletal muscle tissue in the case of Haemoproteid parasites). It has been hypothesize that haemosporidians somehow cause red blood cells to phagocytize parasite cells, but the mechanism is unknown (Breuer 1985, as reviewed and detailed in Valkiūnas 2005). In human malaria, it has been hypothesized that *Plasmodium* uses cell-surface ion channels to increase erythrocyte membrane

permeability, allowing the invasion of red blood cells (as reviewed in Desai 2014). Within the red blood cell, *Plasmodium* meronts complete development into gametocytes (microgametocytes and macrogametocytes). The gametocytes rupture the erythrocyte, causing the anemia traditionally associated with malaria, move to the peripheral blood vessels, and are ingested when a dipteran host takes a blood meal. Fertilization of the macrogamete by the microgamete occurs outside of any cells, forming the zygote. The zygote is the only diploid form of haemosporidian parasites. The zygote transforms via meiosis into a motile ookinete that then leaves the midgut and matures into the oocyst in the basal lamina of the fly's haemocoele (as reviewed and detailed in Garnham 1966, as reviewed and detailed in Valkiūnas 2005). The ookinete absorbs carbohydrates, disrupts amino acid metabolism, and can cause necrosis of the epithelial cells of the insect host (Alekseev 1986, as reviewed and detailed in Valkiūnas 2005). The oocyst releases sporozoites that penetrate the salivary glands of the insect. Haemosporidians are transmitted when the dipteran host takes a bloodmeal and sporozoites are injected with the saliva of the fly (as reviewed and detailed in Garnham 1966, Clements 1992, as reviewed and detailed in Valkiūnas 2005). The parasites must be able to complete the entirety of its life cycle and can be passed on for either dipteran or vertebrate host to be considered competent (as reviewed and detailed in Garnham 1966, as reviewed and detailed in Valkiūnas 2005).

Pathology of Avian Malaria

Malaria parasite infections may present with an array of clinical signs that range from essentially unnoticed to extreme morbidity and mortality in birds. Birds infected with haemosporidians display a variety of signs: ruffled feathers, lethargy, myopathy,

anemia, debilitation, diarrhea, hemorrhages, loss of appetite, breathing difficulty, convulsions, paralysis, and death. The sites of maximum erythrocyte density, the spleen, liver, and blood tissues are most commonly infected. The primary sign of avian malaria in birds is anemia associated with the interruption of cell processes and destruction of erythrocytes. However, a handful of other signs have been known to occur. The exoerythrocytic meronts are generally too small in size and number to cause pronounced pathogenicity, rarely even presenting with inflammatory response (as reviewed and detailed in Garnham 1966, as reviewed and detailed in Valkiūnas 2005, Williams 2005). However, a large number of these meronts can clog vital organs. *Plasmodium*, *Haemoproteus*, and *Parahaemoproteus* meronts have been shown to block the capillaries of the lungs causing pneumonia-like symptoms (Atkinson et al. 1986). Bangkok Hemorrhagic Disease, a noteworthy example from Southeast Asia, involves *Leucocytozoon caulleryi* megalomeronts rupturing in brain tissues causing cerebral paralysis and brain hemorrhages (Kahn and Fallis 1970).

The blood stages of malaria parasites present the most common pathogenic signs (reviewed and detailed in Valkiūnas 2005). The spleen and liver may swell up to twenty times larger than normal and may rupture. Development of numerous erythrocytic meronts causes the destruction of red blood cells and leads to anemia. Acute anemia develops in cases in which birds cannot compensate for loss of erythrocytes. The chemical composition of the blood plasma changes with the development of *Plasmodium* gametocytes, which enhances the effect of red blood cell destruction. The change in pH and addition of toxins decreases the oxygen binding capacity of hemoglobin and lessens effective capillary circulation (Seed and Manwell 1977). *Leucocytozoon* parasites

contain an undescribed 'anti-erythrocyte factor' protein that increases the osmotic fragility of uninfected red blood cells possibly explaining why leucocytozoonosis has much more pronounced hemolysis than other malarias (Kocan 1968, Santiago-Alarcon et al. 2012). *Leucocytozoon* gametocytes also may form long host-parasite cell complexes that can block alveoli and fill up the lungs with fluid during heavy parasitemia. These cell chains cause respiratory problems and other pneumonia-like signs (Siccardi et al. 1974).

Most data about the pathogenicity of avian malaria draft from domesticated birds. Mortality can be extremely high (50-100%) in juvenile birds less than one month old (as seen in human malaria infections as well). *Leucocytozoon* is the primary haemosporidian parasite of domestic fowl. It has led to massive losses at poultry farms. In the 1950s in Quebec, Canada, domestic Pilgrim Geese were introduced at Fort Chimo in an effort to provide a cheap source of meat to Inuit and Eskimo peoples, but their efforts were thwarted when the birds were decimated by leucocytozoonosis (Laird and Bennet 1970). Williams (2005) experimentally infected domestic chickens with *Plasmodium gallinaceum* and observed 10-90% mortality in study groups. However, domestic pigeons infected with *Haemoproteus* parasites may show no signs of disease even with high parasitemia (Ahmed and Mohammed 1978, as reviewed and detailed in Valkiūnas 2005). Malaria parasites can cause measurable effects on the health of their vertebrate hosts, although it seems that morbidity and mortality of the haemosporidians is correlated with parasites infecting naïve hosts and novel species. In naive populations, malaria parasites can cause extensive mortality in birds. Haemosporidians often sicken naïve

exotic birds: penguins have been frequently wiped out by malaria parasites at zoos (Scott 1927, Stoskopf and Beyer 1979, Fix et al. 1988, Cranfield et al. 1990, Ejiri et al. 2009).

Perhaps most famously, the avian malaria parasite, *Plasmodium relictum*, decimated Hawaiian honeycreepers when the vector mosquito *Culex quinquefaciatus* was introduced to the islands by whalers and other traders in the 1700s. Parasites wiped out many endemic birds, allowing introduced birds to gain a foothold on the island and displace the local birds (Warner 1968, van Riper et al. 1986, van Riper 1991, as reviewed and detailed in Valkiūnas 2005). Endemic bird species now occur at elevations higher than 1000 meters, the lowest point at which the mosquitoes do not occur. Since introduction of the parasites, the native Honeycreepers have developed a measure of resistance to the parasites (Atkinson et al. 2013). These birds also have altered their behavior dramatically, foraging in the mosquito zones during the day and retreating above 1000 meters during the nighttime, the period of highest mosquito activity (van Riper 1991, Atkinson and LaPointe 2009).

Wild birds are hard to study in nature, compared to aviaries, in part because predators likely take many of the sick birds in any population (Møller and Nielsen 2007). This has led to debate within the community as to whether avian malaria most strongly affects domesticated and naïve populations of birds (e.g. Hawaiian Honeycreepers), or whether this pattern is due to the difficulty of sampling infected birds. It has been therefore suggested that many haemosporidians may exist as commensals (Sorci 2013, Asghar et al. 2015, Vivedall et al. 2015). Most wild birds infected are juveniles, and they have a similarly high morbidity and mortality to their domestic counterparts. Recent research has shown that these parasites can have pronounced effects on the immune

response of seemingly asymptomatic birds (Atkinson et al. 2013, Ghosh et al. 2014, Vivedall et al. 2015). Additionally, infected birds are generally less fecund, producing fewer eggs, than their uninfected counterparts (Atkinson et al. 1988, as reviewed and detailed in Valkiūnas 2005). Asghar et al. (2015) demonstrated that asymptomatic birds infected with lineages of *Plasmodium* and *Haemoproteus* exhibit significant telomere shortening, a sign of premature aging. Since birds breed throughout their lives, this could have significant impacts on their lifetime fitness. These costs to birds indicate that haemosporidians are indeed parasites.

Vectors of Avian Malaria

Haemosporidians are transmitted to birds by blood-sucking dipteran flies. Four families within the Diptera are vectors for the major lineages of avian haemosporidians. Mosquitoes generally carry parasites belonging to the paraphyletic genus *Plasmodium*, both of mammalian and saurian hosts (as reviewed in Valkiūnas 2005, Martinsen et al. 2008, Santiago-Alarcon et al. 2012). Hippoboscid flies transmit parasites of the subgenus *Haemoproteus*, but are relatively understudied compared to other avian malaria vectors. Black flies (Simuliidae) convey parasites of the genus *Leucocytozoon*. The affinity of simuliids for poultry has led to extensive research into Leucocytozoonosis. Biting midges of the family Ceratopogonidae transmit parasites of the subgenus *Parahaemoproteus*. The majority of ceratopogonids characterized belong to the genus *Culicoides* (as reviewed in Santiago-Alarcon et al. 2012). It is suggested that the importance of ceratopogonids as vectors is often overlooked because they may exhibit vertical stratification, allowing better access to birds in tree canopies but making them more difficult to study (Santiago-Alarcon et al. 2012).

Despite what we do know about relationships between the bird and insect hosts, the vectors of avian malaria are far less studied than their avian counterparts. Recent research has shown that vectors may have a significant role in limiting what birds are potential hosts of haemosporidians through the flies' host preferences (Malmqvist et al. 2004, Hellgren et al. 2008). Clark et al. (2014) performed a survey of the research conducted on bird haemosporidians and showed that 86% of studies focused on the secondary bird hosts, while a mere 14% investigated the dipteran vectors. This potentially leaves a huge imbalance in the understanding of the natural history of these widespread blood parasites.

Mosquitoes

Definition and Classification

Mosquitoes are two-winged flies belonging to the order Diptera and the family Culicidae. They are considered one the more basal families in the order, and are divided into three subfamilies: Toxorhynchitinae, Anophelinae, and Culicinae. The basal placement of mosquitoes in the Dipteran phylogeny has been supported for some time (Bertone et al. 2008, Lambkin et al. 2013). However, the ordering and description of tribes and genera is more contentious. For example, the creation of the genus *Ochlerotatus* from within the genus *Aedes* has led to vigorous debate (Reinert et al. 2008). For the purposes of this study, the rules of generic abbreviation will conform to Reinert et al. (2001).

Adding to the already complex taxonomic relationships within Culicidae, many mosquito morphospecies exist as complexes of several morphologically indistinguishable forms. Perhaps the most studied mosquito genus in the world, *Anopheles*, contains

several species that exist as complexes of several species. The *An. funestus* and *An. triannulatus* complexes exhibit deep genetic divergence between complex member species (Logue et al. 2013, Moreno et al. 2013, Guelbogo et al. 2014). The *An. gambiae* complex furthers these confused relationships by exhibiting evidence for continuing ecological speciation (as reviewed in Severson and Behura 2012).

Worldwide there are over 3,500 mosquito species described. As a general rule, the majority of knowledge of the Culicidae originates from and is promulgated by mosquitoes' role in disease transmission, focusing around 200 medically important species (Reidenbach et al. 2009, Santiago-Alarcon et al. 2012). This has created a boon of information on taxonomy, life history, ecology, etc., causing this group to be easy to work with both in the field and in the laboratory (as reviewed and detailed in: Valkiūnas 2005, Santiago-Alarcon et al. 2012, Clark et al. 2014).

Culicids are found everywhere on the planet, with the exception of areas that are permanently frozen. Like many taxa, most mosquitos are most diverse in the subtropics and tropics. The warm, moist climate of the lower latitudes allows the mosquitoes to maximize survival and development. Mosquitoes found in the arctic are not diverse but are known to block out the sun when they emerge in the summer months (Clements 1992). Mosquitoes in the tropics have life spans ranging from a few days to several weeks. However, in temperate zones, they tend to have live longer and in extreme cases may undergo diapause during winter and live up to a year (Clements 1992, Becker et al. 2003, Denlinger and Armbruster 2014).

Mosquitoes possess an elongate body with long wings and legs. Adult mosquitoes' mouthparts have evolved into a needle-like proboscis, often as long as the

body. The mouthparts are well suited to their fluid feeding nature, able to probe flower nectaries for their primary carbohydrate source. In the case of females, the proboscis is adapted to piercing the epidermis of their vertebrate host and taking in a bloodmeal (Clements 1992, Becker et al. 2003).

Mosquito Life Cycle

Mosquitoes exhibit complete metamorphosis wherein juvenile mosquitoes go through four larval instars, pupating, and finally emerging as adults, the details of which depend on the species. Females generally lay 50 to 500 eggs in rafts or singly either on sites that will be flooded or directly on water. Eggs incubate for as little as a day to several weeks before larvae emerge, usually depending on temperature (or the aforementioned presence of water). Mosquito larval habitats tend to be shallow and/or small bodies of water with little or no water flow. These habitats are generally water-filled treeholes, man-made containers, shallow pools, sheltered stream edges, marshes, etc. The majority of mosquito larvae develop in freshwater, but some are adapted for life in saline or brackish water.

Particulate matter is the primary food resource for most species, including detritus, phytoplankton, etc. (Clements 1992, Becker et al. 2003). Recent research suggests that the microbiota of larval habitats can have significant effects on a mosquito's life and their ability to transmit diseases down the road (Jupatanakul et al. 2014). Mosquitoes with similar larval habitats share similar microbial flora rather than closely related mosquito species (Coon et al. 2014).

All mosquito larvae require atmospheric oxygen, although the manner in which they access air varies. Anopheline larvae take in air by two spiracles at the end of the

abdomen, and they float lengthwise at the water's surface. Most mosquitoes of the subfamily Culicinae, and all the Toxorhynchitinae, have spiracles at the end of a siphon at the posterior end of the insect. These larvae rest at an angle to the water's surface to respire through their siphons. Within the Culicinae, the genera *Mansonia* and *Coquillettidia* are able to remain fully submerged. These genera possess modified siphons that pierce into the air-filled tissues that fill the stems and roots of certain aquatic plants. Larvae pass through four instars before pupation (Clements 1992), and mosquito pupae emerge from the fourth larval molt. The head and thorax of the pupa form a cephalothorax. The abdomen now terminates in two 'paddles' that are used for propulsion, and pupae now respire from 'trumpets' located in the middle of the thorax rather than from the abdomen. Development continues, utilizing fats stored from the larval stages. Some larval organs are eliminated, replaced by adult organs derived from undifferentiated stem cells.

The fully formed adult forces the pupal cuticle to split open, emerges, and steps onto the water. Once the wings are dry, the mosquito takes flight looking for sugar sources, usually from flowers, but also from honeydew and rotting fruit. After gaining the energy needed for extended flight, mosquitoes proceed to find a mate (Clements 1992, Becker et al. 2003).

Once the adult mates, females of most mosquito species (anautogenous species) begins to seek a vertebrate host to take a bloodmeal. The bloodmeal contains proteins needed for egg development, primarily yolk production (Clements 1992).

When a female mosquito lands on a vertebrate host, the styletized components of the mouthparts are driven into the skin, injecting saliva. The saliva has anti-hemostatic

properties, preventing the aggregation of platelets at the bite site. Immunogens in the saliva are responsible for the characteristic skin reaction to mosquito bites. If left interrupted, the female will feed until abdominal stretch receptors signal a stop. Eggs are produced in batches from the blood meal rather than continuously. Once eggs have been produced, the female mosquito seeks a suitable ovipositing site (Clements 1992).

Mosquito Host Seeking

Carbon dioxide is the primary attractant mosquitoes detect to locate hosts (Webster et al. 2015). Females fly upwind towards the host, and various host-mediated stimuli (convection currents, volatile compounds, etc.) provide additional cues (Clements 1992, Becker et al. 2003). In *An. gambiae*, Webster et al. (2015) indicated that mosquitoes respond more to the presence of CO_2 rather than human scents alone, suggesting that mosquitoes use CO_2 as an indication that living hosts are nearby. Mosquitoes use highly complex sense organs on their antennae and palps to detect CO_2 and host-specific volatiles (Clements 1992, Santiago-Alarcon et al. 2012, Rinker et al. 2013).

Some mosquito species are highly host specific, mainly feeding on one or few host species while other mosquitoes are considered host generalists (Clements 1992, Becker et al. 2003, Kilpatrick et al. 2006, Santiago-Alarcon et al. 2012, Mehus and Vaughan 2013). Anthropophilic members of the *An. gambiae* complex exhibited subtle differences in antennal transcriptome responses to the presence of human scents when compared to another member of the complex that is a mammal generalist, indicating evolutionarily rooted host preferences (Rinker et al. 2013). If the preferred vertebrate

host is unavailable, mosquitoes shift their feeding patterns in order to complete egg development (Clements 1992, Becker et al. 2003, Kilpatrick et al. 2006).

Haemosporidians and Mosquitoes

Malaria parasites have many pronounced effects on their mosquito hosts. Malaria infections can have significant effects on the ability of mosquitoes to maintain normal flight speeds and flight times (Santiago-Alarcon et al. 2012). They have also been shown to have negative effects on mortality and morbidity (Maier 1973, as reviewed and detailed in Valkiūnas 2005, Valkiūnas et al. 2013, Lalubin et al. 2014, but see Ferguson 2003a). The ookinete absorbs carbohydrates, disrupts amino acid metabolism and may cause necrosis of the epithelial cells of the insect host (Alekseev 1986, Maier 1973, as reviewed and detailed in Valkiūnas 2005). Malaria parasites cause physical damage when penetrating the epithelium of the mosquito midgut (Clements 1992). Mosquito immune responses are elevated significantly. *P. gallinaceum* was found to inhibit the immune response of *Ae. aegypti* by suppressing its encapsulation response (Boete et al. 2004). While infected, female mosquitos have a more difficult time acquiring a blood-meal, and thus make more attempts to feed, inoculating more vertebrate hosts (Rossignol et al. 1984, Rossignol et al. 1986, and Li et al. 1992, Ferguson et al. 2003b). Infected mosquitoes require more nutrients to sustain themselves during infection (Cornet et al. 2014b, Nyasembe et al. 2014), and while infected, mosquito fecundity is generally reduced (Ferguson et al. 2003b).

Mosquito susceptibility to haemosporidian infection is governed by a variety of factors. The age, nutritional status, ambient temperature, and gut microbiota may all play a role in the susceptibility of infection by malaria parasites as well as the mosquito's

ability to deal with the infection (Boete et al. 2004, Santiago-Alarcon et al. 2012, Lefevre et al. 2013).

Mosquitoes are thought to drive many of the cycles of infection, relapse, and spread of haemosporidian parasites. Mosquitoes tend to be attracted to vertebrate hosts that are experiencing a chronic haemosporidian infection (Lacroix et al. 2005, Cornet et al. 2013a, Cornet et al. 2013b, De Moraes et al. 2014). In avian malaria, the traditional view has been that parasitemia in the vertebrate host coincides with the emergence of mosquitoes (Beaudin et al. 1971, Atkinson et al. 2014). However, other evidence shows the prevalence of infected birds lagged behind the prevalence of mosquitoes in South Africa (Okanga et al. 2013a). Historical records show that malaria relapses coincided with the emergence of mosquitoes (Hulden et al. 2008). Cornet et al. (2014a) recently showed that the presence of mosquitoes to canaries experiencing a chronic *P. relictum* infection induced higher parasitemia than control birds. Although the mechanism is unknown, this result suggests that haemosporidians may respond to mosquito presence and increase in number to increase the possibility of ingestion by the insect. Similarly, Paul et al. (2004) suggested that mosquitoes may 'kick-start' the cycle of malaria infection in humans.

Mosquitoes of Mississippi

There are currently records for 61 species of mosquitoes in 12 genera in Mississippi, only a handful of which are considered major human pests. In Mississippi, human malaria, transmitted primarily by *An. quadrimaculatus*, was rampant until it was eradicated in the 1950s (Goddard and Hattaway 2011). There has never been a systematic statewide survey, some species (*Cx. coronator*, *Mn. titillans*, etc.) being

recently discovered in the state (Goddard and Harrison 2005, Varnardo et al. 2005). As with the majority of mosquito research, Mississippi surveys of mosquitoes have primarily focused on those of medical importance (Goddard et al. 2010).

Study Aims

As previously described, investigations into haemosporidian parasites' primary hosts, have been much less common than studies of their secondary bird hosts, and this imbalance leaves a significant disparity in our knowledge and understanding of the other half of malaria parasite life cycles and ecology. To close a portion of this gap of haemosporidian natural history, this study focused on potential mosquito vectors of avian malaria in the state of Mississippi. A statewide survey of mosquitoes was performed in order to maximize the diversity of mosquitoes captured. Upon capture, DNA screening techniques were used to identify the presence of malaria parasites and estimate mosquito infection rates in the state. Those malaria parasites detected were related to existing parasites in a phylogenetic context.

CHAPTER II

METHODS

Statewide Survey

In order to maximize the potential haemosporidian diversity sampled using geographic breadth as a proxy, mosquitoes were collected from 11 sites across Mississippi. Preliminary sampling occurred at Noxubee US National Wildlife Refuge (USNWR). Using shapefiles acquired from the Mississippi Automated Resource Information System (MARIS), the remaining 10 points were randomly plotted within the state's borders in ArcGIS® version 10.3 (ESRI Inc.). These points were placed randomly in order to maximize the potential extent of the geographic survey. All randomized points were then snapped to the nearest state-owned property due to permitting restraints. In ArcGIS, those points automatically snap to the nearest edge of the state-owned plot. To prevent a land-use bias by collecting on the edges of differentially managed land, I randomly plotted one point within each of the 10 state-owned properties selected by the snapping step. The GPS points generated by the final plotting step were used for mosquito collection (see Table 1 and Figure 1). All sites occurred in state parks or wildlife management areas (WMA) and are as follows:

- Bienville WMA: A part of the Bienville National Forest characterized by mixed hardwood-pine. It was a relatively openly spaced forest with a

minimal understory. Recently felled trees were abundant near the trapping site.

- Buccaneer State Park: A highly managed site that is heavily frequented by visitors. Traps were placed at the edge of a brackish marsh dominated by cordgrass, live oaks, and palmettos.
- Caney Creek WMA: This site is also a part of the Bienville National Forest. Unlike Bienville WMA, hardwoods dominated the trapping location. The trees were much more closely spaced and the leaf litter was extremely pronounced.
- Chickasawhay WMA: This site is a part of the De Soto National Forest and is a fire maintained pine savannah. Longleaf pine was the dominant plant species, knee high grasses were also abundant.
- Legion State Park: This site is maintained for use by campers and hikers. The trapping site was located next to a lake surrounded by upland hardwoods.
- Malmaison WMA: This site was characterized by densely distributed hardwoods. During trapping, standing water was abundant throughout the area.
- Old River WMA: Traps were set in a bottomland hardwood forest with a dense understory near the bank of the Pearl River. The soil was noticeably silty.

- Pearl River WMA: This site is adjacent to the northwest portion of Ross Barnett Reservoir. Traps were placed in a stand of trees across a maintenance road from a cattail marsh.
- Stoneville WMA: This site was characterized by a bottomland hardwood forest with much standing water in the area near the traps.
- Upper Sardis WMA: Traps were placed in a bottomland hardwood forest with interspersed loblolly pines near the Little Tallahatchie River.
- Noxubee US National Wildlife Refuge: Habitat has a relatively flat topography and is characterized as bottomland hardwood forests, periodically inundated by water due to natural and managed flooding (MacGown et al. 2012).

Mosquito Collection

Mosquitoes were collected with three Centers for Disease Control (CDC) Miniature Light Traps (Sudia and Chamberlain 1962), each powered by a Genesis™ NP10-6 6 volt, 10 Ampere-hour rechargeable lead-acid batteries. Traps were placed within 10 meters of the GPS point suspended from 64-inch metal garden hooks using nylon cordage at approximately 1.5 meters above the ground. Batteries were covered with plastic bags to protect the terminals from precipitation or condensation. Additionally, each trap was baited with CO_2 in the form of dry ice to maximize the attraction of mosquitoes to the traps (Clements 1992). Approximately 1 kilogram dry ice was placed in each of three 1-quart Igloo® Red Legend™ flip spout beverage coolers to facilitate slow release of CO_2 overnight. Light trap samples were gathered overnight for

at least twelve hours (6:00pm-6:00am). Additionally, mosquitoes were also collected via aspiration at resting sites and sweep netting of areas proximal to the light traps in the mornings and evenings for approximately 30 minutes. Traps, netting, and aspiration occurred over the course of two nights at each site. The sites were sampled between 6 July 2013 and 8 September 2013 (Table 1). Mild drought-like conditions were present throughout sampling (NOAA GMD).

Mosquito Preparation

Mosquitoes were sacrificed via freezing and permanently stored at -20°C to await identification and genetic analysis (Gager et al. 2008, Njabo et al. 2009, Kimura et al. 2010, Njabo et al. 2011). Mosquitoes were separated from the bycatch and identified using a dissecting microscope. Female mosquitoes were identified to morphospecies using a dichotomous key specialized for use in Mississippi (Varnardo et al. 2012). New mosquito records for the state or counties were stored as voucher specimens at the Clay Lyle Entomology Museum at Mississippi State University. After identification and segregation by species, mosquitoes were pooled. Mosquitoes were divided evenly based upon the number captured at each site and placed into 1.5mL microcentrifuge tubes. Initial trial samples included pools of up to 50 mosquitoes, but pool size was reduced to no more than 25 (R. Sehgal personal correspondence). This was due to mosquito eye proteins interfering with the DNA extraction process (Arez et al. 2000). The pooling technique arose, due to large numbers of mosquitoes captured, from a simple necessity of cost, resource, and time allocation in the field of vector biology (Gager et al. 2008, Ejiri et al. 2009, Njabo et al. 2009, Kimura et al. 2010, Njabo et al. 2011).

This thesis represents a portion of the total number of mosquitoes captured (over 40,000 in 2100 pools). After around half of the study pools (1015) had been processed (DNA extracted and screened via PCR), it was decided that a random subsample of all study pools would be taken in the interests of time and cost allocation. This strategy was used to determine if the subset was reflective of the whole study. This was achieved using a preliminary infection rate of 0.1435 pools positive for haemosporidians. The Power Package in R version 3.1.2 (R Development Core Team 2015) was then used to determine the minimum number of samples required to have a power of 0.8 in a binomial framework. It was concluded that 382 samples would be needed, and samples were then randomly selected in R. These samples included both processed and unprocessed samples. DNA was extracted, and PCR screening protocols were performed to identify the infection status of all unprocessed samples (200) from the subset. The subsample was indeed reflective of the previously processed samples in terms of pools positive for haemosporidians and infection rate estimation, and this study includes all samples processed.

Mosquito pools were homogenized with a Wheaton™ Tenbroeck type 2mL tissue grinder. DNA was extracted from mosquito pools using a DNeasy® kit protocol (Qiagen™, Valencia, California). 15 microliters of the reducing agent diothiothreitol was added to the digestion buffer to aid in dissolving the mosquito's chitinous exoskeleton (Njabo et al., 2011). At least 150µL total DNA was extracted from all pools.

The marker used to screen haemosporidians was the parasite's mitochondrial gene cytochrome *b* (cyt *b*). Haemosporidian parasites have a severely reduced mitochondrial genome with repeats of the same three genes (Roy and Penny 2006, Slamovitz et al.

2007). As a part of the electron transport chain, cyt *b* has been considered a putatively neutral marker. However, recent research suggests that significant selection may be occurring, and hence should be utilized with caution (Outlaw and Ricklefs 2011). Nevertheless, for the purposes of my study, I chose cyt *b* to screen for parasites because it is the most commonly used marker in the literature, with a wide variety of published primers and procedures readily available (Ricklefs and Fallon 2002, Ricklefs et al. 2004, Sehgal et al. 2005, Ishtiaq et al. 2008, Njabo et al. 2009, Kimura et al. 2010, Njabo et al. 2011, Cornuault et al. 2012, Clark et al. 2014).

Parasite infection screening took place through the use of several haemosporidian-specific cyt *b* primers. A nested PCR procedure modifying the protocols of Perkins et al. (2007) and Waldenström et al. (2004) was developed since none of my samples showed positives on any initial amplification. Initial amplification was performed with the primers PerkinsF [5'-TAATGCCTAGACGTATTCCTGATTATCCAG-3'] and PerkinsR [5'-TGTTTGCTTGGGAGCTGTAATCATAATGTG-3'] following the protocol set forth by Perkins et al. (2007). The initial amplification used the following thermocycling parameters: 4 minutes of denaturation at 94C followed by 40 cycles of 94C for 20 seconds, 55C for 20 seconds and 1 minute of 68C, ending with an elongation step at 68C for 10 minutes. After initial amplification, products were screened on 1.5% agarose gels, stained with ethidium bromide, and visualized with a UV light source. Positive/negative infections were indicated by presence/absence of bands on the gel. Infections indicated by initial amplification were then reamplified modifying Waldenström et al.'s (2004) protocol with the primers HaemF [5'-ATGGTGCTTTCGATATATGCATG-3'] and HaemR2 [5'-GCATTATCTGGATGTGATAATGGT-3'] with the following thermal

profile: 4 minutes of denaturation at 94C, followed by 40 cycles at 94C for 30 seconds, 51C for 30 seconds, 72C for 45 seconds, and 72C for 10 minutes in the elongation step. Positive and negative controls were used to avoid false negatives and false positives. Positive parasite infections indicated by the second amplification were used in analysis of infection rates. Positive bands were extracted from the gel using QIAquick® Gel Extraction Kit (Qiagen™) following the manufacturer's protocol. The resultant product infections were cleaned using QIaquick® Gel Extraction Kit (Qiagen™) and amplified products were sequenced in 20 µL ABI BIGDYE® v3 (Applied Biosystems Inc.) sequencing reactions (at Arizona State University). Sequencing reactions were cleaned via ethanol precipitation and run on an ABI 3730 Genetic Analyzer.

Sequences were assembled in Sequencher v4.9 (Gene Codes Corporation, Ann Arbor, MI), aligned in ClustalX, and manually corrected by eye. Alignment of the sequences was straightforward because the sequence is from a coding gene, and the electropherograms were unambiguous. Sequences were then confirmed as malaria parasites in BLASTN.

Infection Rate Determination

Infections (or lack thereof) indicated by the PCR protocol were used to estimate haemosporidian infection rates in mosquitoes. Minimum field infection rates for estimating haemosporidian infection per thousand mosquitoes were calculated using the bias corrected maximum likelihood estimation (MLE) methodology with 95% confidence skewness-corrected score intervals (Njabo 2011, Biggerstaff 2012). Maximum-likelihood estimates of the infection rate in mosquitoes were calculated with the

PoolInfRate (www.cdc.gov), v. 4.0 add-in for Microsoft Excel (Biggerstaff 2012). Species specific infection rates and the overall infection rate were then determined.

Phylogenetic Analysis of Haemosporidian Parasites

To select taxa for phylogenetic analysis, sequences were mined from the nucleotide database of the National Center for Biotechnology Information (NCBI; GenBank), using the BLASTN search function. Using percent identity as the criterion, the top five sequence matches were downloaded and appended to the study samples in Sequencher 4.9.

Phylogenetic relationships were reconstructed in a Bayesian framework using the program BEAST (Drummond et al., 2012). This program does not require the *a priori* selection of an outgroup taxon which is beneficial in our field where sampling is ongoing and the overall phylogeny is in a state of flux (Outlaw and Ricklefs, 2011, Bensch et al., 2013). The Markov Chain was sampled every 1000 generations for 10 million generations. Bayesian posterior branch probabilities were obtained by taking the majority rule consensus of the sampled trees, excluding the first 15,000 trees as burn in. Trees were visualized using FigTree v1.4 (available at http://tree.bio.ed.ac.uk/software/figtree/). Weakly and moderately supported clades were collapsed into triangular polytomies unless strongly supported branches existed within those nodes.

Table 1 Sampling Locations

Location	Latitude	Longitude	Physical Region	County	Dates
Buccaneer State Park	30.263151	-89.408806	Coastal Zone	Hancock	31 July - 1 Aug. 2013
Upper Sardis WMA	34.526417	-89.365273	North Central Hills	Lafayette/Marshall	11-12 July 2013
Pearl River WMA	32.532778	-89.940880	North Central Hills	Madison	4-5 Sept. 2013
Stoneville WMA	33.459544	-90.921965	Delta	Washington	18-19 July 2013
Bienville WMA	32.381944	-89.628405	Jackson Prairie	Scott	12-13 July 2013
Legion State Park	33.155568	-89.048005	North Central Hills	Winston	9-10 July 2013
Old River WMA	30.788472	-89.820313	Pine Belt	Pearl River	29-30 July 2013
Chickasawhay WMA	31.543947	-88.954677	Pine Belt	Jones	2-3 Aug. 2013
Caney Creek WMA	32.194512	-89.608396	South Central Hills	Smith	10-11 July 2013
Malmaison WMA	33.680105	-90.025177	Loess Hills	Grenada	16-17 July 2013
Noxubee US Wildlife Refuge	33.285556	-88.756944	North Central Hills	Noxubee	6-7 July 2013

Locations of mosquito collection sites. Latitude and longitude are presented in decimal degrees.

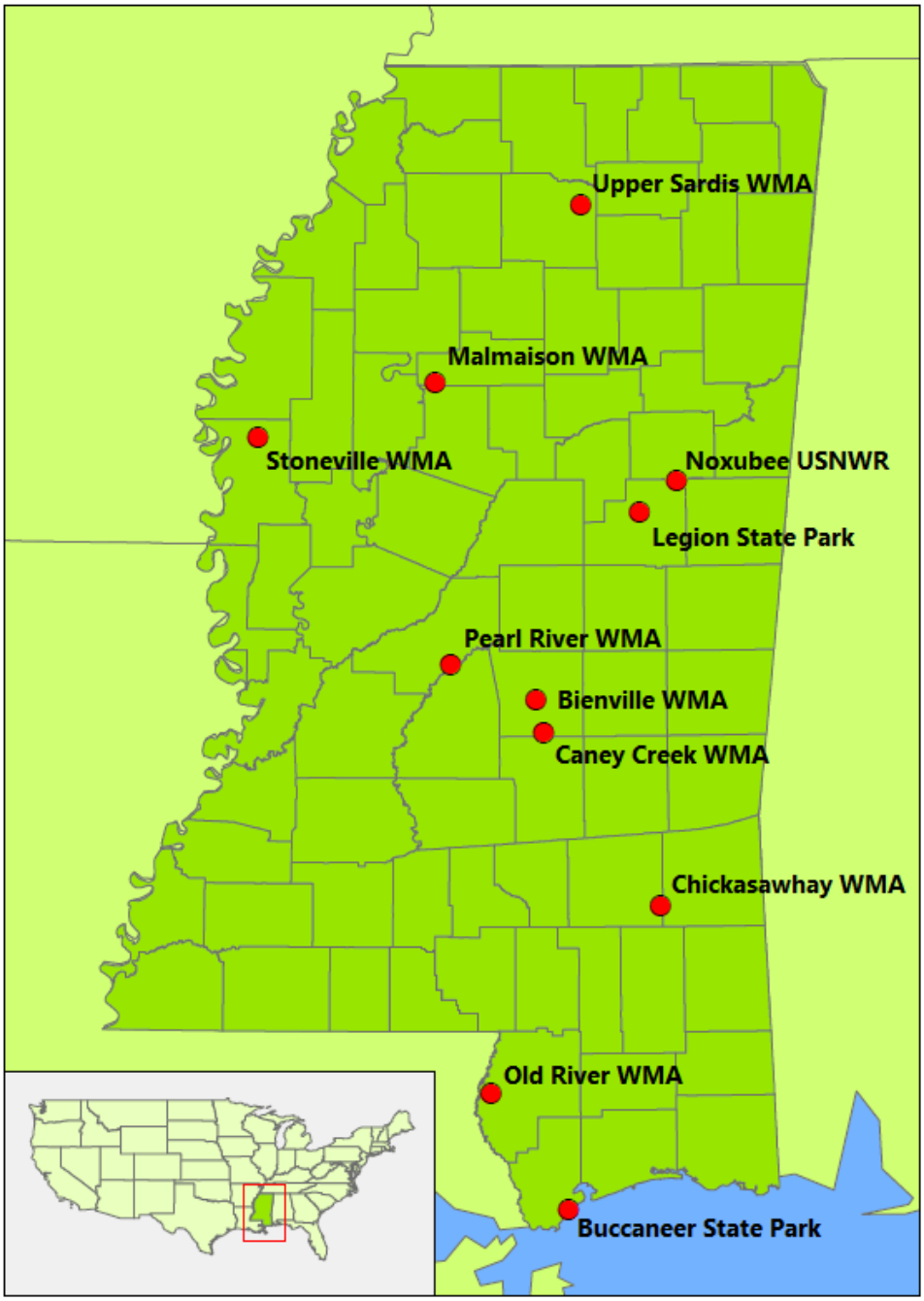

Figure 1 Collection locations

Map of Mississippi with sampling sites indicated by red circles.

CHAPTER III

RESULTS

Mosquitoes Captured

From the subsample described in the methods, a total of 27,157 female mosquitoes representing 15 species were captured and identified to species during the sampling period. The mosquitoes were divided into 1192 sample pools. The following species were captured: *Ae. vexans* (Meigen), *An. crucians* (Complex), *An. punctipennis* (Say), *An. quadrimaculatus* (Complex), *Cq. perturbans* (Walker), *Cx. erraticus* (Dyar and Knab), *Cx. nigripalpus* (Theobald), *Cx. quinquefasciatus* (Say), *Mn. titillans* (Walker), *Oc. atlanticus/Oc. tormentor* (Dyar and Knab), *Oc. fulvus pallens* (Ross), *Oc. taeniorhyncus* (Wiedmann), *Ps. colombiae* (Dyar and Knab), *Ps. ferox* (von Humboldt), and *Ur. sapphirina* (Osten Sacken). *An. quadrimaculatus* is a species complex composed of at least five species, the females of which are nearly impossible to distinguish morphologically (Reinert et al. 1997). They were pooled as one species. The *An. crucians* complex was similarly grouped, as it has the same issue as *An. quadrimaculatus* (Wilkerson et al. 2004). *Oc. atlanticus* and *Oc. tormentor* suffer from the same lack of female distinctiveness and were also pooled together. *Cx. erraticus* and *An. crucians* were the most numerous species sampled. Table 2 and Figure 2 give a full accounting of the each species captured.

Infection Status

Five of the fifteen species were positive for haemosporidian parasites via the PCR protocol: *An. crucians* complex, *An. quadrimaculatus* complex, *Cq. perturbans*, *Cx. erraticus*, and *Ps. ferox* (see Table 2). The MLE estimate of infected mosquitoes overall was 4.22 per 1000 mosquitoes. *An. crucians* had a MLE infection rate of 5.96 per 1000. *An. quadrimaculatus* had a MLE infection rate of 8 per 1000. *Cq. perturbans* had a MLE infection rate of 2.47 per 1000. *Cx. erraticus* had a MLE infection rate of 3.48 per 1000, and *Ps. ferox* had a MLE infection rate of 48.32 per 1000 mosquitoes. Frequency of positive pools and confidence intervals are provided in Table 2.

Diversity of Haemosporidian Lineages

A total of 109 pools tested positive for haemosporidian parasites. 52 samples returned sequence of good enough quality (250 base pairs or greater) for use in a phylogenetic reconstruction. Table 3 lists the mosquito pools used in the reconstruction. No resultant chromatograms contained double peaks in base pair reads indicating that no samples contained mixed infections (multiple parasites in one sample).

The majority of samples mined from GenBank represented avian *Plasmodium*. Parasite sequences from the genera *Hepatocystis*, *Haemoproteus*, *Parahaemoproteus*, and *Polychromophilus* were also downloaded. All GenBank samples had at least 92% sequence identity with the study samples. The GenBank sequence JQ070884, a putative *Hepatocystis* sequence found from the monkey *Cercopithecus nicitans* in Africa, was removed from analysis as it proved to be a misidentified avian *Plasmodium* parasite. Table 4 lists the GenBank derived samples. See Table 5 in Appendix A for a full list of what GenBank samples were similar to the study samples.

Figure 3 illustrates the phylogeny reconstructed for this study. The relationships between the three major genera (*Plasmodium*, *Haemoproteus*, and *Polychromophilus*) are well supported. One sample (DL06 from *Cx. erraticus*) strongly groups within *Haemoproteus*. 22 pools fall within avian *Plasmodium*. Parasite sequence from this clade was derived from *Cq. perturbans*, *Cx. erraticus*, *Ps. ferox*, and *An. quadrimaculatus* samples. No samples fell directly within the genus *Polychromophilus*. However, 29 mosquito pools form a strongly supported clade, the sequences of which are not identical to any known malaria parasites. All the sequences from this clade were derived from *Anopheles* pools. Only two *Anopheles* pools fell within avian *Plasmodium* (sample DLA4 and DL162).

Table 2 Mosquito prevalence and haemosporidian infection status

Species	Total Screened	Number of Pools	Pools Positive for Malaria	Freq. Positive Pools	Infection Rate/1000	Lower Limit	Upper Limit
Ae. vexans	1344	77	0	0	0.00	0.00	2.75
An.crucians	9614	387	53	0.14	5.96	4.51	7.74
An. punctipennis	12	5	0	0	0.00	0.00	199.40
An. quadrimaculatus	1002	39	7	0.18	8.00	3.51	16.11
Cq. perturbans	1654	75	4	0.05	2.47	0.80	5.93
Cx. erraticus	11645	482	39	0.08	3.48	2.52	4.71
Cx. nigripalpus	146	22	0	0	0.00	0.00	23.71
Cx. quinquefasciatus	6	2	0	0	0.00	0.00	300.42
Mn. titillans	18	6	0	0	0.00	0.00	152.06
Oc. atlanticus/tormentor	39	7	0	0	0.00	0.00	72.08
Oc. fulvus pallens	1	1	0	0	0.00	0.00	793.45
Oc. taeniorhyncus	6	3	0	0	0.00	0.00	337.80
Ps. columbiae	14	2	0	0	0.00	0.00	138.84
Ps. ferox	124	26	6	0.23	48.32	21.32	94.83
Ur. sapphirina	1532	58	0	0	0.00	0.00	2.42
Total	27157	1192	109	0.09	4.22	3.49	5.08

Mosquito species captured and analyzed for the study. Infection Rate/1000 indicates the MLE estimate given by PooledInfRate (www.cdc.gov) and upper and lower 95% confidence intervals.

Table 3 Mosquito pools used in phylogeny

Sample	Mosquito	Site	Sample	Mosquito	Site
DL02	*Cx. erraticus*	Noxubee	DL414	*An. crucians*	Pearl River
DL06	*Cx. erraticus*	Noxubee	DL442	*An. crucians*	Pearl River
DL12	*Cx. erraticus*	Noxubee	DL461	*An. crucians*	Pearl River
DL13	*Cx. erraticus*	Noxubee	DL493	*An. crucians*	Pearl River
DL14	*Cx. erraticus*	Noxubee	DL504	*An. crucians*	Pearl River
DL17	*Ps. ferox*	Noxubee	DL506	*An. crucians*	Pearl River
DL19	*Ps. ferox*	Noxubee	DL513	*An. crucians*	Pearl River
DL21	*Ps. ferox*	Noxubee	DL514	*An. crucians*	Pearl River
DL162	*An. crucians*	Pearl River	DL518	*An. crucians*	Pearl River
DL164	*An. crucians*	Pearl River	DL570	*An. crucians*	Pearl River
DL165	*An. quadrimaculatus*	Pearl River	DL581	*An. crucians*	Pearl River
DL169	*An. quadrimaculatus*	Pearl River	DL584	*An. crucians*	Pearl River
DL183	*An. crucians*	Pearl River	DL751	*Cx. erraticus*	Pearl River
DL222	*An. quadrimaculatus*	Pearl River	DL755	*Cx. erraticus*	Pearl River
DL240	*An. crucians*	Pearl River	DL757	*Cx. erraticus*	Pearl River
DL244	*An. quadrimaculatus*	Pearl River	DL764	*Cx. erraticus*	Pearl River
DL256	*An. crucians*	Pearl River	DL775	*Cx. erraticus*	Pearl River
DL264	*An. quadrimaculatus*	Pearl River	DL779	*Cx. erraticus*	Pearl River
DL277	*An. crucians*	Pearl River	DL781	*Cx. erraticus*	Pearl River
DL282	*An. crucians*	Pearl River	DL786	*Cx. erraticus*	Pearl River
DL283	*An. crucians*	Pearl River	DL788	*Cx. erraticus*	Pearl River
DL284	*An. crucians*	Pearl River	DL790	*Cx. erraticus*	Pearl River
DL297	*An. crucians*	Pearl River	DL791	*Cx. erraticus*	Pearl River
DL298	*An. crucians*	Pearl River	DL799	*Cx. erraticus*	Pearl River
DL299	*An. crucians*	Pearl River	DL851	*Cx. erraticus*	Pearl River
DL339	*An. crucians*	Pearl River	DLA4	*An. quadrimaculatus*	Noxubee

List of sample pools used in the phylogenetic reconstruction with species and collecting location.

Table 4 GenBank samples used in phylogeny

GenBank Accession No.	Parasite	Host	Location
AB308051	*Plasmodium*	*Cx. quinquefasciatus*	Japan
AB308052	*Plasmodium*	*Coquillettidia spp.*	Japan
AB601436	*Plasmodium*	*Tarsiger cyanurus*	Japan
AB601445	*Plasmodium*	*Amaurornis phoenicurus*	Japan
AB604306	*Plasmodium*	*Spheniscus humboldti*	Japan
AB604307	*Plasmodium*	*Spheniscus humboldti*	Galapagos
AB608052	*Plasmodium*	*Coquillettidia spp.*	Japan
AB617729	*Plasmodium*	*Cx. nigropunctatus*	japan
AB617730	*Plasmodium*	*Cx. nigropunctatus*	Japan
AB690267	*Plasmodium*	*Cx. inatomii*	Japan
AY377128	*Plasmodium*	*Lab derived*	USA
DP025675	*Plasmodium*	*Gallus*	Thailand
DQ241510	*Plasmodium*	*Cyanocompsa cyanoides*	Guyana
DQ241528	*Plasmodium*	*Butorides striatus*	Guyana
DQ241529	*Plasmodium*	*Cyanocompsa cyanoides*	Guyana
DQ241537	*Plasmodium*	*Cyanocompsa cyanoides*	South America
DQ241538	*Plasmodium*	*numerous birds*	Uruguay
DQ356303	*Plasmodium*	*Cyanocorax yncas glaucenscens*	USA
DQ659538	*Plasmodium*	*Carpodarcus mexicanus*	USA
DQ838988	*Plasmodium*	*Passer domesticus*	USA
DQ847262	*Plasmodium*	*avian blood*	Europe
DQ991068	*Plasmodium*	*Cyanistes caeruleus*	UK
EU328168	*Plasmodium*	*Geothlypis trichas*	USA
EU627827	*Plasmodium*	*Strix varia*	USA
EU810616	*Plasmodium*	*Ploceus melanogaster*	Africa
GQ141560	*Plasmodium*	*Bubo virginianus*	USA
GQ141565	*Parahaemoproteus*	*Coereba flaveola*	Antilles
GQ141585	*Parahaemoproteus*	*Phaenicophilus palmarum*	Antilles
GQ141600	*Plasmodium*	*Margarops fuscus*	USA
GQ395667	*Haemoproteus*	*Spheniscus mendiculus*	Galapagos
GQ395687	*Plasmodium*	*Spheniscus sp.*	Galapagos
GQ395691	*Plasmodium*	*Spheniscus sp.*	Galapagos
GU252011	*Plasmodium*	*Bubo virginianus*	USA
GU252012	*Plasmodium*	*Turdus fumigatus*	Brazil
GU252029	*Plasmodium*	*Coerebra flaveola*	Yucatan
GU395691	*Plasmodium*	*Spheniscus sp.*	Galapagos
HM055583	*Polychromophilus murinus*	*Myotis spp.*	Switzerland
HM055585	*Polychromophilus murinus*	*Myotis spp.*	Switzerland
HM055589	*Polychromophilus murinus*	*Myotis spp.*	Switzerland
HM222481	*Plasmodium*	*Seiurus aurocapillus*	Brazil

Table 4 (continued)

GenBank Accession No.	Parasite	Host	Location
HQ591360	*Plasmodium*	*Spheniscus sp.*	Brazil
HQ591361	*Plasmodium*	*Spheniscus sp.*	Brazil
HQ853668	*Plasmodium*	*Aedes*	Mexico
JF441408	*Plasmodium*	*Cx. theileri*	Turkey
JF833046	*Plasmodium*	*Spheniscus mendiculus*	Galapagos
JN819331	*Plasmodium*	*Tangara icterocephala*	Brazil
JN819333	*Plasmodium*	*Tangara icterocephala*	
JN819400	*Haemoproteus*	*Turdus assimilis*	Costa Rica
JN990712	*Polychromophilus murinus*	*Myotis daubentonii*	Switzerland
JN990713	*Polychromophilus murinus*	*Myotis daubentonii*	Switzerland
JQ070884	*Hepatocystis*	*Cercopithecus nictitans*	Africa
JQ905577	*Plasmodium*	*Ixobrychus flavicollis*	New Zealand
JQ988200	*Plasmodium*	*Myioborus melanocephalus*	Andes
JQ988285	*Plasmodium*	*Coeligena violifer*	Andes
JQ988527	*Parahaemoproteus*	*Synallaxis azarae*	Andes
JQ988547	*Plasmodium*	*Turdus chiguanco*	Andes
JQ988611	*Plasmodium*	*Ramphocelus carbo*	Andes
JQ988687	*Plasmodium*	*Diglossa cyanea*	Andes
JX029894	*Plasmodium*	*Formicarius colma*	Brazil
JX276946	*Plasmodium*	*Zosterops borbonicus*	Mascarene Islands
JX458327	*Plasmodium*	*Cx. modestus*	Spain
JX458328	*Plasmodium*	*Cx. modestus*	Spain
JX458333	*Plasmodium*	*Oc. caspius*	Spain
KC250003	*Plasmodium*	*Aburria jacutinga*	South America
KC867660	*Plasmodium*	*Migratory bird*	Galapagos
KC867661	*Plasmodium*	*Migratory bird*	Galapagos
KC867662	*Plasmodium*	*Migratory bird*	Galapagos
KC867665	*Plasmodium*	*Migratory bird*	Galapagos
KC867666	*Plasmodium*	*Migratory bird*	Galapagos
KC867667	*Plasmodium*	*Migratory bird*	Galapagos
KC867668	*Plasmodium*	*Migratory bird*	Galapagos
KF159681	*Polychromophilus*	*Miniopterus villiersi*	Africa
KF159699	*Polychromophilus*	*Miniopterus villiersi*	Africa
KF537307	*Plasmodium*	*Diglossa cyanea*	Colombia
KF767406	*Plasmodium*	*Troglodytes aedon*	Andes
KF874677	*Plasmodium*	*Aulacorhynchus prasinus*	Peru
KF874681	*Plasmodium*	*Arremon torquatus*	Peru
KF874685	*Plasmodium*	*Mionectes oleagineus*	Peru
KF874687	*Plasmodium*	*Myadestes ralloides*	Peru
KF900267	*Plasmodium*	*Cardinalis*	USA

Table 4 (continued).

GenBank Accession No.	Parasite	Host	Location
KJ131270	*Polychromophilus melanipherus*	*Miniopterus schreibersii*	Switzerland
KJ145056	*Plasmodium*	*Cyornis hainanus*	China
KJ145065	*Plasmodium*	*Stachyris nigriceps*	China
KJ466077	*Haemoproteus*	*Euphonia xanthogaster*	Andes
KJ579150	*Plasmodium*	*Cx. pipiens*	France
KM213976	*Plasmodium*	*Enicurus ruficapillus*	Malaysia
KM396866	*Plasmodium*	*Cx. pipiens*	Spain
KM396867	*Plasmodium*	*Cx. pipiens*	Spain
KM598218	*Plasmodium*	*Melospiza melodia*	Michigan
KP025675	*Plasmodium*	*Gallus*	Thailand

List of samples mined from GenBank. Parasite taxonomic information, along with the host the sequence was derived from, and geographic location are provided.

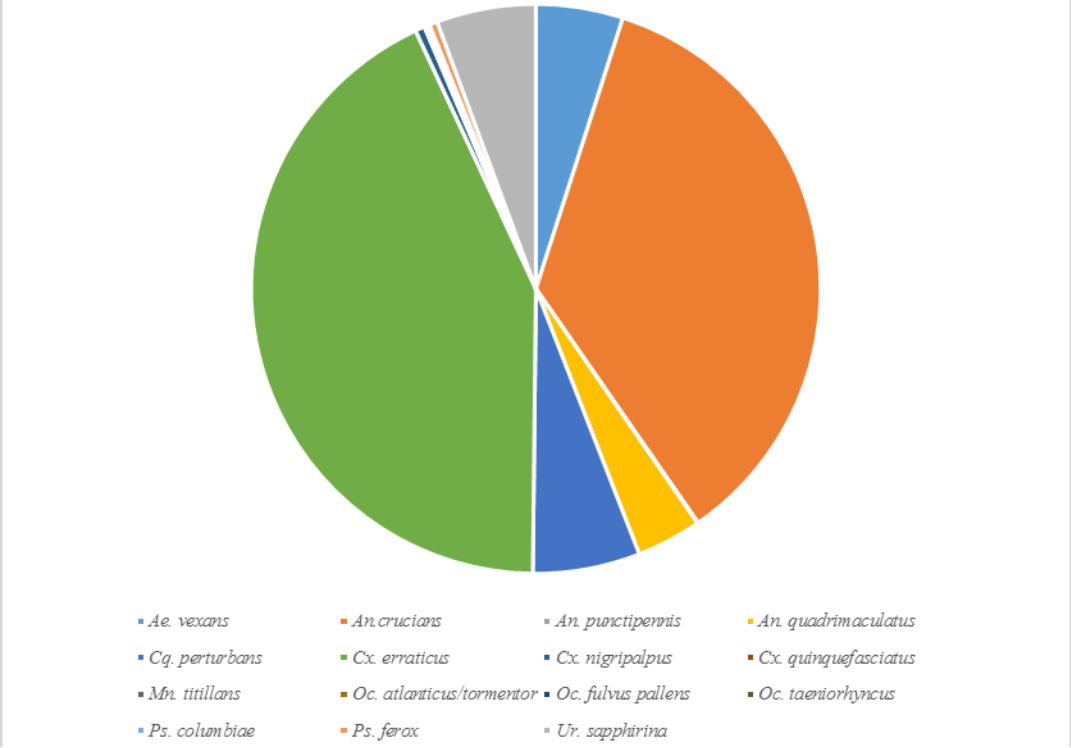

Figure 2 Mosquito diversity

Visual representation of the proportion of mosquitoes captured in the study

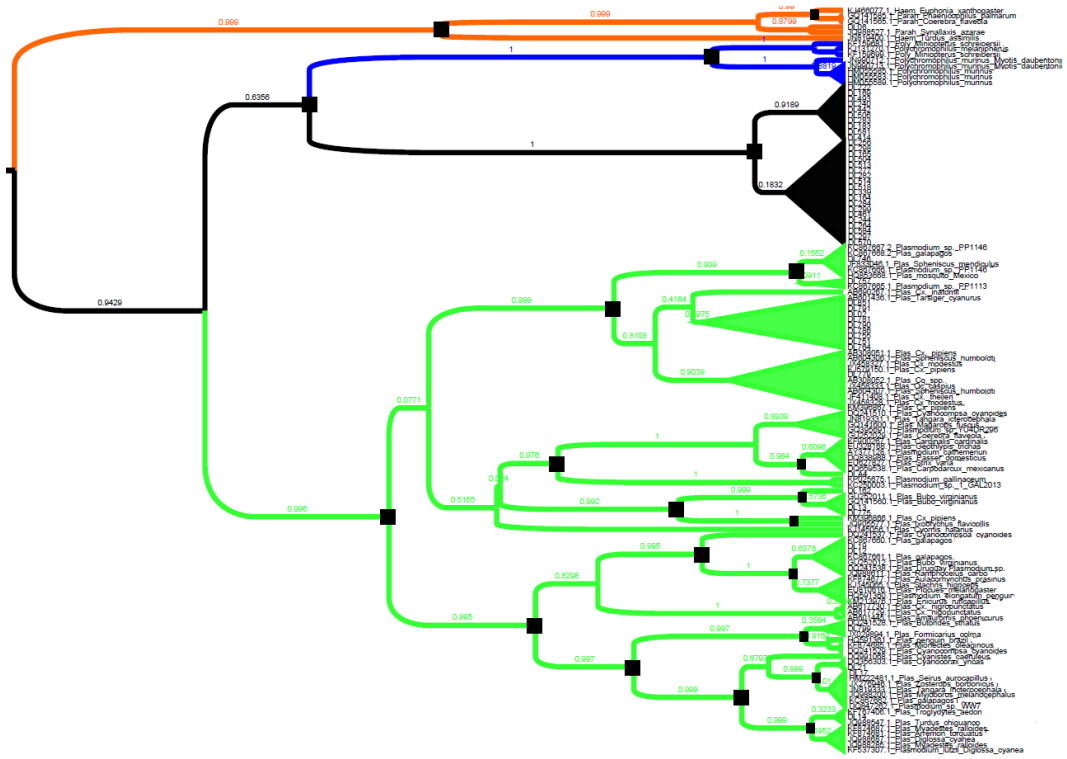

Figure 3 Phylogeny of Mississippi haemosporidians

Bayesian reconstruction of a phylogeny comprised of haemosporidians derived from this study and GenBank samples (see Table 4). The orange represents parasites of the genus *Haemoproteus*. Green portrays *Plasmodium*, and blue shows *Polychromophilus*. The black clade represents a lineage of haemosporidians unique to Mississippi. Black blocks indicate Bayesian posterior support of 0.95 or greater.

CHAPTER IV

DISCUSSION

Observed Infection Rates

This study, the first of its kind in Mississippi, indicates that that haemosporidians are present in the mosquitoes in the state. However, it is important to note that all traps were placed within ten meters of the other traps at each site and collection only occurred over two days. Because mosquitoes can range farther than ten meters and mosquito assemblages fluctuate, the former two factors in sample collection could have limited the diversity of mosquitoes in this study. The overall infection rate of 4 per 1000 mosquitoes estimated in this study is very similar to other studies carried out in temperate latitudes that pool mosquitoes (Carlson et al. 2011, Glaizot et al. 2012, Inci et al. 2012, Loaiza and Miller 2013, Medeiros et al. 2013). However, when mosquitoes are screened individually, mosquito haemosporidian infection rates are very similar to those found in sub-Saharan Africa (Kimura et al. 2010, Zele et al. 2014). This suggests that the common practice of pooling mosquitoes may not give an accurate rate of infection when dealing with the vectors of haemosporidians. However, due to the sheer number of mosquitoes captured in this study, pooling was a logistic necessity.

Interestingly, two of the most common mosquitoes, *Ae. vexans* and *Ur. sapphirina*, were uninfected with malaria parasites. *Ur. sapphirina* tends to feed on amphibians and any haemosporidians they harbor may not be detected by the PCR

protocol used (Goddard et al. 2010, Clark et al. 2014). *Ae. vexans* may suffer from the same PCR issue. However, it has been shown to carry avian malaria in other studies, although there is no evidence it was competent to pass on these parasites (Carlson et al. 2011, Inci et al. 2012). *Ae. vexans* has been shown to consistently feed preferentially on mammals (Mehus and Vaughan 2013).

Potential Vectors of Avian Malaria

There were five potential avian malaria vector species found in this study. *An. crucians* was the second most numerous mosquito species (complex) in the study and had the most pools positive for haemosporidians. The complex prefers habitats along the edges of water bodies with aquatic vegetation (Goddard et al. 2010). All the *An. crucians* positive for malaria parasites were from Pearl River WMA, which exemplifies their habitat preferences, being situated on the edge of a cattail marsh in a manmade lake. These mosquitoes only harbored haemosporidians belonging to the parasite lineage unique to Mississippi. *An. quadrimaculatus* mainly harbored parasites from the Mississippi lineage, but one pool was infected with a *Plasmodium* lineage found throughout the New World. It was surprising how many *Anopheles* pools were positive since members of the genus are generally highly mammalphilic. (Clements 1992, Wilkerson et al. 2004, Goddard et al. 2010). Four *Cq. perturbans* pools tested positive for malaria parasites, but none returned quality sequence for use in analysis. *Cq. perturbans* larvae depend on aquatic plants for air, and thus this species is common along waterfronts, feeding on a mixture of mammals on birds where it would become infected with haemosporidians (Goddard et al. 2010). Nearly half of the mosquitoes trapped were *Cx. erraticus*, supporting its position as one of the most common species in Mississippi

(Cupp et al. 2004, Goddard et al. 2010). *Cx. erraticus* carried the largest diversity of parasites in this study. One pool tested positive for *Haemoproteus*, and the remainder for avian *Plasmodium*. Estep et al. (2011) surveyed *Cx. erraticus* bloodmeals in Alabama and found that they were highly ornithophilic, feeding largely on locally common birds (American Robin, Carolina Chickadee, Northern Mockingbird, etc.). *Ps. ferox* had the highest infection rate in the study at 48 per 1000 mosquitoes positive. This infection rate estimate of *Ps. ferox* is similar to rates found in mosquitoes in Cameroon, a tropical area (Njabo et al. 2011); here it is likely an artifact of sampling very few individuals of this species. By sampling a greater number of *Ps. ferox*, future infection rate estimates would likely be similar to the other mosquito species of this study. This mosquito was not captured in high numbers in this study, contrary to expectations from the literature. *Ps. ferox* may be dependent on rainfall, and the mild drought experienced may have impacted this species' abundance in the study (Goddard et al. 2010, Ruiz et al. 2010).

Diversity of Haemosporidians in Mosquitoes of Mississippi

Unsurprisingly, the haemosporidian lineages found in this study are phylogenetically related to parasites from across the world. On the other hand, the diversity of genera sampled in Mississippi was noteworthy. One single sample was strongly supported to belong to the subgenus *Parahaemoproteus* from South America and the Antilles. It is unlikely that this parasite would be able to complete its development in mosquito and was an incidental infection (Valkiūnas et al. 2013). All parasites derived from *Cx. erraticus*, *Ps. ferox*, and the aforementioned single *An. quadrimaculatus* had strong identity with avian *Plasmodium* in the phylogeny. Not surprisingly, the vast majority of samples had strong identity to avian *Plasmodium* of

New World origin. Some lineages matched those of songbirds from China, mosquitoes from Japan and Turkey. This result conforms to the idea that *Plasmodium* is a generalist with a wide host and geographic breadth (as reviewed in: Valkiūnas 2005, Santiago-Alarcon et al. 2012, Clark et al. 2014, Zhang et al. 2014, Olsson-pons et al. 2015). *Culex* mosquitoes are generally ornithophilic and are consistently shown to be infected with avian malaria parasites (Cupp et al. 2004, Estep et al. 2011, Njabo et al. 2011, Santiago-Alarcon et al. 2012).

Most intriguingly, 29 samples exclusively from *Anopheles* pools formed a strongly supported unique lineage in this phylogenetic reconstruction. This lineage most closely grouped with sequences from *Polychromophilus*, the bat haemosporidian, but was not significantly supported (posterior support of 0.6). *Polychromophilus*, as previously mentioned, likely originated from a host switching event from a reptilian or avian *Plasmodium*. This parasite is transmitted by bat flies (Nycteribiidae, Diptera) although mosquitoes and biting midges may also play a role (Witsenburg et al. 2012). The life cycle, epidemiology and pathology of this haemosporidian genus are similar to parasites that infect birds (Witsenburg et al. 2014, Witsenburg et al. 2015a, Witsenburg et al. 2015b). The posterior support for this clade is identical to that of the *Polychromophilus* samples. This unique lineage of haemosporidians may represent diversity at the generic level. It is suspicious that a mosquito group that heavily favors mammals would harbor a heretofore unidentified avian haemosporidian lineage. It is most likely that this novel lineage infects mammals, but morphological identification in a vertebrate host would be required to confirm this. The mere presence of a novel lineage of haemosporidian in Mississippi suggests that these parasites may have been overlooked in many other hosts

and locales. This lineage is possibly a parasite of deer (S. Perkins, personal correspondence), but sampling from deer hosts is necessary to confirm this (a cooperative agreement with the Mississippi Department of Wildlife, Fisheries and Parks is pending for this investigation).

Cautionary Note

A cautionary note must be sounded to temper the results of this study. Firstly, none of these mosquitoes has ever experimentally been shown to transmit avian malaria. *An. quadrimaculatus* was the major vector of malaria in humans in the United States, and as such, it is not surprising that it also can be infected with other haemosporidian lineages (Goddard and Hattaway 2011). It was recently confirmed that *Haemoproteus* undergoes abortive development throughout the bodies of mosquitoes (Valkiūnas et al. 2013). This study reinforced the need for experimental confirmation of vectorial capacity.

Additionally, PCR techniques are very powerful and quickly let us ascertain a picture of the parasite community without the training investment of microscopy. However, the use of PCR techniques to detect haemosporidians is not fool proof. PCR will amplify the DNA of any parasite life stage, so researchers should not over-interpret their results without the use of microscopy (Valkiūnas 2011, Bobeva et al. 2013, Clark et al. 2014). Only in the last few years, after this study was designed and mostly carried out, was there a reemergence of microscopy, to be combined with PCR-based research. Future studies, building off of this foundational research, will include both microscopy and PCR. In addition, even though PCR is extremely sensitive, it still requires a high enough parasite load to be detected (Lachish et al. 2012). It is entirely possible that a handful of haemosporidian cells were missed by the techniques used in this study.

This study represents a snapshot in time during the late summer of 2013 in Mississippi. Mild, drought-like conditions were present. Precipitation is often the most important variable in predicting mosquito presence in an area (Ruiz et al. 2010, Okanga et al. 2013a). Local temperatures can have a strong effect on mosquito infection rates (Murdock et al. 2014). These conditions could greatly have affected the prevalence and diversity of mosquitoes at sampling locations. Lalubin et al. (2013) showed that *Plasmodium* prevalence peaked from late spring to summer in Switzerland, suggesting that the timing of sampling is critical in evaluating avian malaria lineages in mosquitoes. If parasite lineages are transmitted most frequently during bird breeding season, then mosquitoes collected later in the year would likely have far lower avian malaria estimates (Synek et al. 2013). It is entirely possible that the late summer of 2013 was simply not representative of general parasite prevalence in Mississippi.

Notably, this study did not capture one of the most common mosquitoes of Mississippi. *Ae. albopictus* (Skuse) is the most common mosquito in the southeastern US and most rapidly expanding mosquito on the planet (Bonizonni et al. 2013). It is one of the most important biting mosquitoes in the state (Goddard et al. 2010). However, it is possible that due to the history of surveying medically important mosquitoes in or near urban areas, my widespread geographic survey avoided previous bias towards this anthropophilic mosquito, which regularly inhabits artificial containers (Goddard et al. 2010, Bonizonni et al. 2013). *Ae. albopictus* is highly mammalphilic and has not been shown to carry avian malaria, perhaps subsequent studies of regional mammals will further elucidate host/vector/parasite interactions (LaPointe et al. 2005, Martinez de la Puente et al. 2015).

This first look into the potential vectors of avian malaria in Mississippi raised very interesting possibilities. Several candidate mosquito vectors were identified, and future work can focus on these species to determine vectorial capacity. It is also important that bloodmeals be analyzed to determine on which vertebrates Mississippi mosquitoes are feeding. Caution should be used when looking at bloodmeals, however. Ejiri et al. (2011) found avian malaria in a mosquito engorged with cow blood. Bloodmeal analysis is simply another tool to tease apart the natural history of haemosporidians. This study has shown that there is great potential for the enzootic cycle of avian malaria to perpetuate in Mississippi. Most notably, this study has revealed a novel lineage of haemosporidians in Mississippi, quite possibly a new genus of malaria parasite that is potentially sister to *Polychromophilus*.

Concluding Remarks

Had I the opportunity to change my project, a few aspects could be changed. Firstly, I would have used gravid traps in addition to CDC traps. According to anecdotal evidence, mosquitoes captured in gravid traps do not suffer the severe damage to diagnostic scales and setae. Perhaps, I might have spent fewer late nights identifying mosquitoes had they been less damaged. CDC traps also suffer from attracting many medically irrelevant insect species. The bycatch insects inflicted much of the aforementioned damage to the mosquitoes, forcing me to discard countless dismembered mosquitoes. The grating between the CDC trap's light source should be modified to prevent larger arthropods from being sucked into the trap. Additionally, I wish that my sampling had taken place in the late spring and early summer, rather than late summer when much of the literature suggests avian malaria is transmitted. The sheer scale of my

survey made more in depth investigations into these potential vectors difficult. Using the manmade Mississippi state boundaries was probably unwise since organisms are not (usually) restricted by political delineations of the land. A smaller, more localized study might have allowed me to analyze bloodmeals, getting a picture of what vertebrates are part of this enzootic cycle. Having stated that, any changes, focusing on ornithophilic mosquitoes for example, I might now make to my study could have led me to miss out on uncovering a potentially new genus of parasites.

The mosquitoes of Mississippi are varied and diverse, and carry numerous lineages of malaria parasite, one previously unknown to the field. Infection rates here in Mississippi are similar to those across the globe, which suggests that this study captured what was intended. Nevertheless, follow up studies will include analysis of the mosquito bloodmeals and sampling of deer, bats, bears, and wild hogs.

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APPENDIX A

GENBANK SEQUENCE MATCHES

GenBank Sequence Mining Samples

All samples acquired from GenBank used the criteria of percent identity as the criterion. All samples had greater than 91% identity when study samples were BLASTed. Table 5 divulges the study samples and their associated GenBank matches.

Table 5 GenBank sequence matches

GenBank Accession No.	**Parasite**	**Host**	**Location**	**Mosquito Pools**
AB308051	*Plasmodium*	*Cx. quinquefasciatus*	Japan	DL779, DL790, DL851
AB308052	*Plasmodium*	*Coquillettidia spp.*	Japan	DL779
AB601436	*Plasmodium*	*Tarsiger cyanurus*	Japan	DL02
AB601445	*Plasmodium*	*Amaurornis phoenicurus*	Japan	DL164, DL240, DL256, DL414, DL570
AB604306	*Plasmodium*	*Spheniscus humboldti*	Japan	DL02, DL779, DL781, DL786, DL790, DL851
AB604307	*Plasmodium*	*Spheniscus humboldti*	Japan	DL751, DL764
AB608052	*Plasmodium*	*Coquillettidia spp.*	Japan	DL791
AB617729	*Plasmodium*	*Cx. nigropunctatus*	Japan	DL164, DL165, DL169, DL222, DL240, DL256, DL414, DL493, DL504, DL506, DL513, DL514, DL518, DL570

Table 5 (continued)

AB617730	*Plasmodium*	*Cx. nigropunctatus*	Japan	DL164, DL165, DL169, DL183, DL222, DL240, DL256, DL414, DL442, DL493, DL504, DL506, DL513, DL514, DL518, DL570
AB690267	*Plasmodium*	*Cx. inatomii*	Japan	DL02, DL779, DL781, DL786, DL790, DL851, DL854
AY377128	*Plasmodium*	*Lab derived*	USA	DLA4
DP025675	*Plasmodium*	*Gallus gallus*	Thailand	DL165
DQ241510	*Plasmodium*	*Cyanocompsa cyanoides*	Guyana	DL313
DQ241528	*Plasmodium*	*Butorides striatus*	Guyana	DL799
DQ241529	*Plasmodium*	*Cyanocompsa cyanoides*	Guyana	DL799
DQ241537	*Plasmodium*	*Cyanocompsa cyanoides*	South America	DL164, DL165, DL169, DL183, DL222, DL240, DL244, DL256, DL414, DL442, DL461, DL493, DL504, DL506, DL513, DL514, DL518, DL570
DQ241538	*Plasmodium*	*numerous birds*	Uruguay	DL12
DQ356303	*Plasmodium*	*Cyanocorax yncas glaucenscens*	USA	DL21
DQ659538	*Plasmodium*	*Carpodarcus mexicanus*	USA	DLA4

Table 5 (continued)

DQ838988	*Plasmodium*	*Passer domesticus*	USA	DLA4
DQ847262	*Plasmodium*	*avian blood*	Europe	DL21
DQ991068	*Plasmodium*	*Cyanistes caeruleus*	UK	DL13
EU328168	*Plasmodium*	*Geothlypis trichas*	USA	DLA4
EU627827	*Plasmodium*	*Strix varia*	USA	DLA4
EU810616	*Plasmodium*	*Plocues melanogaster*	Africa	DL19
GQ141560	*Plasmodium*	*Bubo virginianus*	USA	DL13, DL162, DL775
GQ141565	*Parahaemoproteus*	*Coerebra flaveola*	Antilles	DL06
GQ141585	*Parahaemoproteus*	*Phaenicophilus palmarum*	Antilles	DL06
GQ141600	*Plasmodium*	*Magarops fuscus*	USA	DL298, DL339
GQ395667	*Haemoproteus*	*Spheniscus mendiculus*	Galapagos	DL06
GQ395687	*Plasmodium*	*Spheniscus sp.*	Galapagos	DL786
GQ395691	*Plasmodium*	*Spheniscus sp.*	Galapagos	DL339
GU252011	*Plasmodium*	*Bubo virginianus*	USA	DL13, DL162, DL775
GU252012	*Plasmodium*	*Turdus fumigatus*	Brazil	DL12

Table 5 (continued)

GU252029	*Plasmodium*	*Coerebra flaveola*	Yucatan	DL298, DL339
GU395691	*Plasmodium*	*Spheniscus sp.*	Galapagos	DL298
HM055583	*Polychromophilus murinus*	*Myotis spp.*	Switzerland	DL244, DL264, DL277, DL283, DL299, DL461, DK581, DL584
HM055585	*Polychromophilus murinus*	*Myotis spp.*	Switzerland	DL244, DL264, DL277, DL283, DL284, DL297, DL298, DL299, DL339, DL461, DK581, DL584
HM055589	*Polychromophilus murinus*	*Myotis spp.*	Switzerland	DL244, DL264, DL277, DL283, DL284, DL297, DL299, DL461, DK581, DL584
HM222481	*Plasmodium*	*Seirus aurocapillus*	Brazil	DL17
HQ591360	*Plasmodium*	*Spheniscus sp.*	Brazil	DL19
HQ591361	*Plasmodium*	*Spheniscus sp.*	Brazil	DL799
HQ853668	*Plasmodium*	*Aedes*	Mexico	DL746
JF441408	*Plasmodium*	*Cx. theileri*	Turkey	DL751, DL764
JF833046	*Plasmodium*	*Spheniscus mendiculus*	Galapagos	DL746
JN819331	*Plasmodium*	*Tangara icterocephala*	Brazil	DL297

Table 5 (continued)

JN819333	*Plasmodium*	*Tangara icterocephala*	Brazil	DL17
JN819400	*Haemoproteus*	*Turdus assimilis*	Costa Rica	DL313
JN990712	*Polychromophilus murinus*	*Myotis daubentonii*	Switzerland	DL329
JN990713	*Polychromophilus murinus*	*Myotis daubentonii*	Switzerland	DL284, DL264, DL277, DL282, DL283, DL297, DL299, DL581, DL584
JQ070884	*Hepatocystis*	*Cercopithecus nictitans*	Africa	DL 162, DL164, DL165, DL169, DL183, DL222, DL40, DL244, DL256, DL264, DL277 DL282, DL283, DL284, DL297, DL298, DL299, DL339, DL414, DL442, DL461, DL493, DL504, DL506, DL513, DL518, DL570, DL581, DL584
JQ905577	*Plasmodium*	*Ixobrychus flavicollis*	New Zealand	DL162, DL775
JQ988200	*Plasmodium*	*Myioborus melanocephalus*	Andes	DL17, DL21
JQ988285	*Plasmodium*	*Coelingena violifer*	Andes	DL14
JQ988527	*Parahaemoproteus*	*Synallaxis azarae*	Andes	DL06
JQ988547	*Plasmodium*	*Turdus chiguanco*	Andes	DL14
JQ988611	*Plasmodium*	*Ramphoceuls carbo*	Andes	DL19

Table 5 (continued)

JQ988687	*Plasmodium*	*Diglossa cyanea*	Andes	DL14
JX029894	*Plasmodium*	*Formicarius colma*	Brazil	DL799
JX276946	*Plasmodium*	*Zosterops borbonicus*	Mascarene Islands	DL17, DL21
JX458327	*Plasmodium*	*Cx. modestus*	Spain	DL02, DL751, DL755, DL764, DL779, DL781, DL786, DL90, DL791, DL851
JX458328	*Plasmodium*	*Cx. modestus*	Spain	DL751, DL755, DL764
JX458333	*Plasmodium*	*Oc. caspius*	Spain	DL751, DL755, DL764
KC250003	*Plasmodium*	*Aburria jacutinga*	South America	DL442
KC867660	*Plasmodium*	*Migratory bird*	Galapagos	DL19
KC867661	*Plasmodium*	*Migratory bird*	Galapagos	DL19
KC867662	*Plasmodium*	*Migratory bird*	Galapagos	DL17, DL21
KC867665	*Plasmodium*	*Migratory bird*	Galapagos	DL762, DL788
KC867666	*Plasmodium*	*Migratory bird*	Galapagos	DL746
KC867667	*Plasmodium*	*Migratory bird*	Galapagos	DL746
KC867668	*Plasmodium*	*Migratory bird*	Galapagos	DL746

Table 5 (continued)

KF159681	*Polychromophilus*	*Miniopterus villiersi*	Africa	DL282, DL284
KF159699	*Polychromophilus*	*Miniopterus villiersi*	Africa	DL282
KF537307	*Plasmodium*	*Diglossa cyanea*	Colombia	DL775
KF767406	*Plasmodium*	*Troglodytes aedon*	Andes	DL14
KF874677	*Plasmodium*	*Aulacorhynchus prasinus*	Peru	DL12
KF874681	*Plasmodium*	*Arremon torquatus*	Peru	DL13
KF874685	*Plasmodium*	*Mionectes oleaginous*	Peru	DL799
KF874687	*Plasmodium*	*Myadestes ralloides*	Peru	DL13, DL14
KF900267	*Plasmodium*	*Cardinalis cardinalis*	USA	DL313
KJ131270	*Polychromophilus melanipherus*	*Miniopterus schreibersii*	Switzerland	DL282
KJ145056	*Plasmodium*	*Cyornis haianus*	China	DL162
KJ145065	*Plasmodium*	*Stachyris nigriceps*	China	DL12
KJ466077	*Haemoproteus*	*Euphonia xanthogaster*	Andes	DL06
KJ579150	*Plasmodium*	*Cx. pipiens*	France	DL755, DL779, DL781, DL786, DL790, DL791, DL851
KM213976	*Plasmodium*	*Enicurus ruficapillus*	Malaysia	DL12

Table 5 (continued)

KM396866	*Plasmodium*	*Cx. pipiens*	Spain	DL162, DL775
KM396867	*Plasmodium*	*Cx. pipiens*	Spain	DL02, DL755, DL781
KM598218	*Plasmodium*	*Melospiza melodia*	Michigan	DL222, DL493, DL504, DL506, DL513, DL514, DL518
KP025675	*Plasmodium*	*Gallus gallus*	Thailand	DL169, DL183, DL442