UNDERSTANDING AVIAN PLASMODIUM DISTRIBUTION: THE ROLE OF VECTOR AND HOST

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UNDERSTANDING AVIAN PLASMODIUM DISTRIBUTION: THE ROLE OF VECTOR AND HOST

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Malaria parasites have a complex life cycle involving sexual reproduction in the mosquito vector and asexual proliferation in the vertebrate host. Mosquito vectors are therefore the definitive host of the malaria parasite. The literature on avian malaria parasites remains biased towards bird-parasite associations, and avian malaria vectors are not well studied in this system. My dissertation fills a gap in the current body of avian malaria research by using molecular techniques to document 1) patterns in phylogeographic structure of avian *Plasmodium* across geographic regions; 2) absence of vector specificity in two common mosquito species in Ithaca, New York; and 3) evidence that local vectors amplify a local avian *Plasmodium* lineage.

In my first chapter, I review our current understanding about the associations between avian malaria vectors and avian *Plasmodium*. I synthesize this with literature on human malaria-mosquito interactions and mosquito feeding preferences to argue that it is crucial to study vector ecology to understand host-parasite dynamics. Variation in mosquito ecology could help explain patterns observed in avian malaria parasite infection. In chapter 2, I document patterns of phylogeographic structure in *Plasmodium* parasites sampled across the range of a single bird species. I demonstrate that geographic patterns in parasite lineage distribution are not solely attributable to differences in the parasites found in different bird species. In chapter 3, I describe the avian *Plasmodium* lineages found in two abundant, local ornithophilic mosquito

species and show that parasites sampled from mosquitoes are represented by many diverse cytochrome *b* haplotypes. The most common of these haplotypes are shared between mosquito species, and overlap only slightly with those previously isolated directly from numerous bird species. In chapter 4, I report results from a feeding experiment using laboratory-reared mosquitoes and wild-caught birds naturally infected with *Plasmodium*. I examine the variation within *Cx. pipiens* in its ability to be invaded by the parasite by conducting PCR on individual mosquitoes following incubation with sufficient time to allow the complete digestion of the blood meal and development of sporozoites in the salivary glands.

BIOGRAPHICAL SKETCH

Mari Kimura was born in New York, NY and spent the first 22 years of her life in the city. She studied English literature at Columbia University as an undergraduate from 1984-1988. She chose not to pursue a career in this field, so she decided to move out west in search of adventure and something more satisfying to do with her life. She arrived in San Francisco two weeks before the 1989 earthquake and stayed until 2002. During this time she had many outdoor adventures, worked at the Natural Resources Defense Council, and became interested in biology. Her first field assistant job involved camping on an island in British Columbia conducting orca surveys. A few years later, she assisted in hornbill research at a remote rainforest site in the Dja Reserve, Cameroon. She decided to pursue a graduate degree in biology and spent several years taking prerequisite science courses in the Bay Area while moonlighting at corporate law firms. She received her master's degree in Ecology and Systematics in 2002 from San Francisco State University under Dr. Thomas B. Smith. Her master's research explored the phylogeography of Wilson's warblers across both their breeding and wintering areas. It was at this time that she started to work with Dr. Irby Lovette, who was a postdoc while she was a master's student. She was so impressed with Irby that she followed him to Cornell as his first graduate student. She originally thought that she would continue studying migratory warblers when she embarked on her doctoral research. Research tends to take on a life of its own, however, and she soon found herself fascinated by the mystery of avian *Plasmodium* and its mosquito vectors, which eventually became the topic of her dissertation.

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My three committee members contributed equally to my dissertation research and development as a scientist. I thank them for many stimulating discussions and for being completely available to me at all times. Dr. Laura Harrington introduced me to the field of medical entomology and taught me everything I know about mosquitoes. Her wealth of knowledge and natural ability to motivate students inspired me to continue with my research, especially during the difficult times. She was my true thesis supervisor as my project moved farther away from birds and closer to mosquitoes. Dr. André Dhondt has an amazing, creative mind. He taught me how to think like a scientist and steered me to locate gaps in our current knowledge and to try to fill those gaps by asking well-framed questions. Dr. Irby Lovette raised the bar for the quality of my work and relentlessly kept me on schedule. I am grateful for the lightning speed with which he returned comments on my drafts, and for his heartfelt encouragement when I did a good job. His pushiness and rigor, while maddening at times, ultimately made me a stronger and more responsible student and may have cured me of my chronic procrastination habits forever.

Many other people contributed to my research over the years: Ravinder Sehgal for intellectual camaraderie, Ellis Greiner for parasitological expertise, Wesley Hochachka for great feedback delivered with dry wit, the Evo Bio Big Kids and the Dhondt lab group for bouncing ideas around, and Keila Seidenstrycker for guidance on captive birds. For help and guidance in the lab and with samples: Amanda Talaba, Laura Stenzler, Caitlin McKenna, Becky Poulson, Rachel Hathaway, Dana Hawley, Jon Darbro. My parents were constantly supportive. Finally, I could not have asked for a more amazing circle of graduate students who will remain lifelong friends. I thank them for their support and for countless fun-filled times over the last six years.

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PREFACE

Malaria parasites (Apicomplexa: Plasmodiidae) have a complex life cycle involving sexual reproduction in the mosquito vector and asexual proliferation in the vertebrate host. Mosquito vectors are therefore the definitive host of the malaria parasite. Although most work on malaria parasites has focused on their interactions with their vertebrate hosts, increasing attention has been placed on vector-parasite interactions, particularly in evolutionary studies of mammalian malarias. The literature on avian malaria parasites, however, remains biased towards bird-parasite associations, and very little is known about the vector-parasite associations in this system.

The advent of molecular techniques has recently revealed lineage diversity and possible cryptic species in avian malaria parasites, but most molecular studies to date have sampled parasites only from the blood of the vertebrate host. Because the sexual phase of the parasite's life cycle is more important to lineage diversification than is the asexual blood phase, it is crucial to elucidate mosquito-parasite interactions to understand the ecological and evolutionary processes that lead to observed patterns in parasite associations with hosts and across geographic regions.

Recent research on mammalian vector-parasite interactions has examined non-human malaria parasite effects on human malaria vector species in the genus *Anopheles*. There are two ways in which these laboratory studies differ from natural conditions: 1) mosquitoes maintained in colony over many generations are inbred, which may affect the mosquito's immune response to parasite infection; and 2) in the wild, *Anopheles* species would not feed on the rodents typically used in the laboratory, and this artificial vector-host combination thus overrides the potential effects of a shared evolutionary history between host and vector. Avian malaria provides the

opportunity to pursue evolutionary questions in a natural transmission system with local birds, mosquitoes and parasites. My dissertation begins to fill a gap in the current body of avian malaria research by using molecular techniques to document 1) patterns in phylogeographic structure of avian *Plasmodium* across geographic regions; 2) absence of vector specificity in two common mosquito species in Ithaca, NY; and 3) evidence that local vectors can mount an effective immune response against avian *Plasmodium* infection.

In my first chapter, I review the literature about associations between known avian malaria vector species and *Plasmodium*, and associations between vectors and their avian hosts. I use these examples to argue that it is crucial to study vector ecology to understand host-parasite dynamics. Consideration of ecological factors such as changes in vector abundance over time and the behavior of mosquitoes in the wild, including host feeding preferences, could help explain patterns see in avian malaria parasite infection that cannot be explained by differences in host-parasite interactions.

My second two chapters describe patterns in the lineage distribution of avian malaria parasites. In chapter 2, I document patterns of phylogeographic structure in *Plasmodium* parasites sampled from recently separated populations of a single avian species, the house finch (*Carpodacus mexicanus*). By focusing on native and recently introduced populations of the house finch, I demonstrate that geographic patterns in parasite lineage distribution are not solely attributable to differences in the parasites found in different bird species.

In chapter 3, I describe the avian *Plasmodium* lineages found in two abundant, local ornithophilic mosquito species, *Culex pipiens* and *Cx. restuans*, and show that parasites sampled from mosquitoes are represented by many diverse cytochrome *b* haplotypes. The most common of these haplotypes are shared between mosquito

species, and overlap only slightly with those already published for numerous bird species. These patterns of avian *Plasmodium* distribution across geographic regions and among vector species could be driven by either intrinsic evolutionary interactions, such as genetic or biochemical incompatibilities between vector and parasite, or ecological factors, such as avian host feeding preferences.

To begin to tease apart these intrinsic and extrinsic factors, in chapter 4, I conduct a feeding experiment using *Cx. pipiens* and wild-caught red-winged blackbirds (*Agelaius phoeniceus*) naturally infected with *Plasmodium*. I examine the variation within *Cx. pipiens* in its permissiveness (ability to be invaded by the parasite) by conducting PCR on individual mosquitoes following incubation with sufficient time to allow dissemination (complete digestion of the blood meal and development of sporozoites in the salivary glands). My findings confirm that *Cx. pipiens* is able to amplify a *Plasmodium* lineage common in local birds and mosquitoes.

An important advantage of using molecular methods in the study of avian malaria is that they enable identification of parasites in their mosquito stages. Malaria parasite taxonomy is based on the morphology of the parasite as it appears in vertebrate blood, *i.e.*, asexual stages. Thus far it has only been possible to score the presence or absence of *Plasmodium* in mosquitoes via dissection of midgut and salivary glands, but it has not been possible to identify parasite genus or species in the mosquito stages. Identifying *Plasmodium* found in mosquitoes was previously accomplished only by injecting a slurry of ground mosquito directly into naïve birds, waiting for the infection to become patent, and examining stained bird blood cells. Besides being arduous and time-consuming, this method was often unsuccessful because the bird could either die from the infection or eliminate the infection by mounting a strong immune response. Molecular methods therefore provide a much

more tractable route to identifying parasite taxa present in a sample of wild mosquitoes whose feeding history is otherwise not known.

A second advantage of using molecular techniques is that they enable detection of parasites in light infections in bird blood. Identifying parasites in blood using traditional microscopy techniques relies on the morphology of both schizonts and gametocytes, two of the distinctive life stages found in blood. It is difficult to identify morphospecies in a light infection, because many infected cells representing different blood stages must be examined. Furthermore, wild birds may be infected with multiple parasite species or, conversely, have so few cells infected that no distinguishing features are captured. Molecular techniques, especially PCR, are more sensitive than traditional microscopy and provide information about parasites present in submicroscopic (low parasitemia) levels. Such light infections are not biologically insignificant, since it is possible for the parasite to successfully infect a mosquito even when gametocytes are subpatent, *i.e.*, at low density such that they cannot be either seen in a blood smear under a microscope, or detected by PCR.

CHAPTER 1

THE IMPORTANCE OF VECTOR ECOLOGY IN AVIAN MALARIA HOST-PARASITE DYNAMICS

Introduction

Understanding the drivers of avian malaria host-parasite dynamics requires dissecting the complex three-way interaction among protozoan parasite (Haemosporida: Plasmodiidae), avian host and mosquito vector (Diptera: Culicidae) within their ecological contexts. Avian malaria parasites are often regarded as parasites of birds that rely on a mosquito vector for transmission. As a consequence, most research on this system has focused on bird-parasite interactions. In actuality, however, the mosquito is the definitive host and the vertebrate an intermediate host, as sexual reproduction of *Plasmodium* occurs in the mosquito. Despite increasing recent interest in avian malaria in wild birds (Figure 1.2) and the important implications of these parasites for avian ecology, evolution and conservation, the natural vectors for most avian malaria parasites remain largely unknown. The ecology of mosquito vectors could have direct impacts on avian malaria host-parasite dynamics.

The ecology of arthropod vectors of avian malaria and other related parasites may contribute to patterns of avian malaria infection seen among bird families and species across broad geographic regions (White et al., 1978; Peirce, 1981), among bird populations (Bennett et al., 1995; Merilä et al., 1995; Bensch and Åkesson, 2003) and within populations (Wood et al., 2007). In one study, vector abundance was positively correlated with blood parasite prevalence in pigeons among sampling locations (Sol et al., 2000). Moreover, the absence of suitable vectors has been implicated (but not explored) as a factor responsible for the absence of haematozoan parasites in avian

hosts inhabiting tundra (Bennett et al., 1992) and wintering areas of long-distance migratory bird species (Bennett et al., 1980; Bennett et al., 1991; Garvin et al., 2004). Despite the potential for the abundance or absence of vectors to explain patterns of parasite prevalence in birds, data on vectors are sparse. In this review, I synthesize current findings on vector-parasite and host-vector associations to explore the ways in which interactions with vectors could ultimately influence avian malaria host-parasite dynamics. This synthesis highlights the importance of studying mosquito vectors in concert with parasites and their avian hosts to explain the patterns in avian malaria infection often seen across host taxa, across geographic regions, and over time.

Background

The haemosporidian avian blood parasites are assigned to three genera: *Plasmodium, Haemoproteus* and *Leucocytozoon* whose Dipteran vectors belong to the families Culicidae (mosquitoes), Hippoboscidae and Ceratopogonidae (louse flies and midges) and Simuliidae (blackflies), respectively (Valkiunas, 2005). Only the genus *Plasmodium* includes species infecting non-avian vertebrate hosts, including mammals and reptiles. The term "malaria" technically applies only to human disease caused by genus *Plasmodium*, but its common usage has expanded to include *Plasmodium* infection in other vertebrate hosts, including birds. Avian *Plasmodium* species were historically named for their host species. Consequently, every time a parasite was identified from a new avian host it was given a new species name. Current molecular studies have shown that some *Plasmodium* lineages are shared across avian genera (Bensch et al., 2000) and families (Waldenström et al., 2002; Szymanski and Lovette, 2005; Beadell et al., 2006), and some morphospecies are shared across orders, e.g., *P. elongatum* in passerines (Valkiunas et al., 2008), raptors (Telford et al., 1997; Nayar et al., 1998) and penguins (Herman et al., 1968; Stoskopf and Beier, 1979). About 200

avian *Plasmodium* species are defined by their blood stage morphology (Valkiunas, 2005); in contrast, just 4 species infect humans (Warrell and Gilles, 2002). Recently, results of DNA sequencing of avian malaria parasites sampled from bird blood (primarily the mitochondrial cytochrome *b* gene but in some studies also independent nuclear loci), suggested that many more unique DNA sequences exist than morphospecies named (Bensch et al., 2004). The question of whether these lineages defined by unique DNA sequences represent reproductively isolated entities, however, remains largely untested. *Plasmodium* parasites enter their vertebrate host via the infective saliva of a blood feeding mosquito. The parasites then undergo asexual proliferation in the vertebrate host until ingested by another mosquito that feeds on infected blood. The parasites then enter the sexual stage of their life cycle, completing development until they reach their infective (sporozoite) stage in the mosquito salivary glands (Figure 1.1). The fact that the parasite's sexual reproduction occurs within the mosquito, not the vertebrate host, makes the mosquito the primary host for *Plasmodium* (Garnham, 1966). Thus, *Plasmodium* relies on two hosts to complete its development and therefore has opportunities to coevolve with both bird and mosquito. For simplicity hereafter I will refer to the mosquito host as the vector to distinguish it from the avian host.

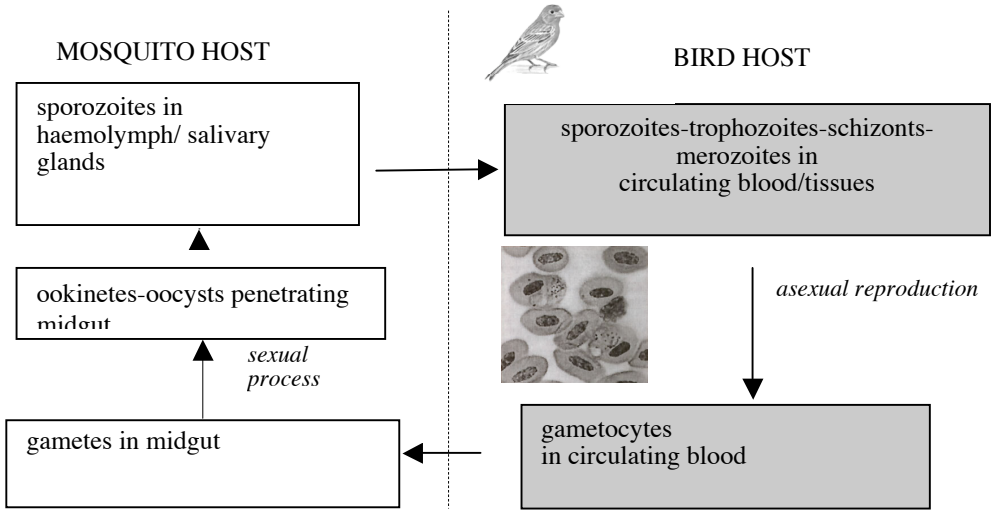

Figure 1.1 Avian malaria life cycle.

Based on the cosmopolitan distribution of avian *Plasmodium* parasites, and their natural presence in nearly all avian taxa, we would predict that many bird-feeding mosquito species should be vectors for avian malaria. To date, however, only 15 mosquito species representing 6 genera have been identified as competent vectors, mostly in the genus *Culex* (Table 1). This small number is more likely a reflection of the low number of mosquito species sampled rather than true rarity of competent vectors. *Culex* species tend to feed on birds, but some species in that genus feed on both birds and mammals. For this reason, some *Culex* species have been the subject of studies focusing on vectors of zoonotic (animal-to-human) infectious diseases. The efficiency with which *Culex* feeds on birds is evidence for the hypothesis that members of this genus have only recently adapted to feeding on mammals; according to one source, *Culex* species take a much longer time to locate a blood vessel on a mammalian host than on a bird host (Marquardt et al., 2005).

Table 1.1 Mosquito species incriminated as avian malaria vectors. "Field" indicates that avian *Plasmodium* was either 1) recovered from wild-caught mosquitoes or 2) transmitted by mosquitoes reared from field-collected eggs. "Lab" includes studies with mosquitoes or parasites obtained from colonies of unknown geographic origin. Species indicated with an asterisk are known mammal feeders and do not feed on birds in the wild.

Species	Type of study
Culex p. quinquefasciatus	lab (Huff, 1931) field (Reeves et al., 1954; LaPointe et al., 2005)
Culex p. pipiens	lab (Huff, 1929; Huff, 1934; Hunninen, 1953)
Culex stigmatosoma	field (Reeves et al., 1954)
Culex nigripalpus	lab (Nayar et al., 1982) field (Forrester et al., 1980)
Culex restuans	lab (Nayar et al., 1981) field (Beier and Trpis, 2001)
Culex tarsalis	lab (Chao and Ball, 1962; Work et al., 1990) field (Reeves et al., 1954)
Culex salinarius	lab (Nayar et al., 1981)
Culex (Melanoconion) ocossa	field (Gager et al., 2008)
Culex saltanensis	field (Grossman and Lourenco-De-Oliveira, 1996)
*Aedes aegypti**	lab (Kelly and Edman, 1992)
Aedes albimanus	lab (Hunninen, 1953)
*Anopheles freeborni**	lab (Mok, 1951)
Culiseta morsitans	lab (Meyer and Bennett, 1976)
Aedeomyia squamipennis	field (Gager et al., 2008)
*Mansonia perturbans**	lab (Meyer and Bennett, 1976)

Only a handful of candidate vector species have been formally tested as avian *Plasmodium* vectors. More surveys of ornithophagic mosquito species for avian *Plasmodium* infection are necessary to evaluate the degree of coevolution between parasite and vector. Such surveys are rare compared to the numerous surveys of avian hosts for blood parasite infection (Figure 1.2).

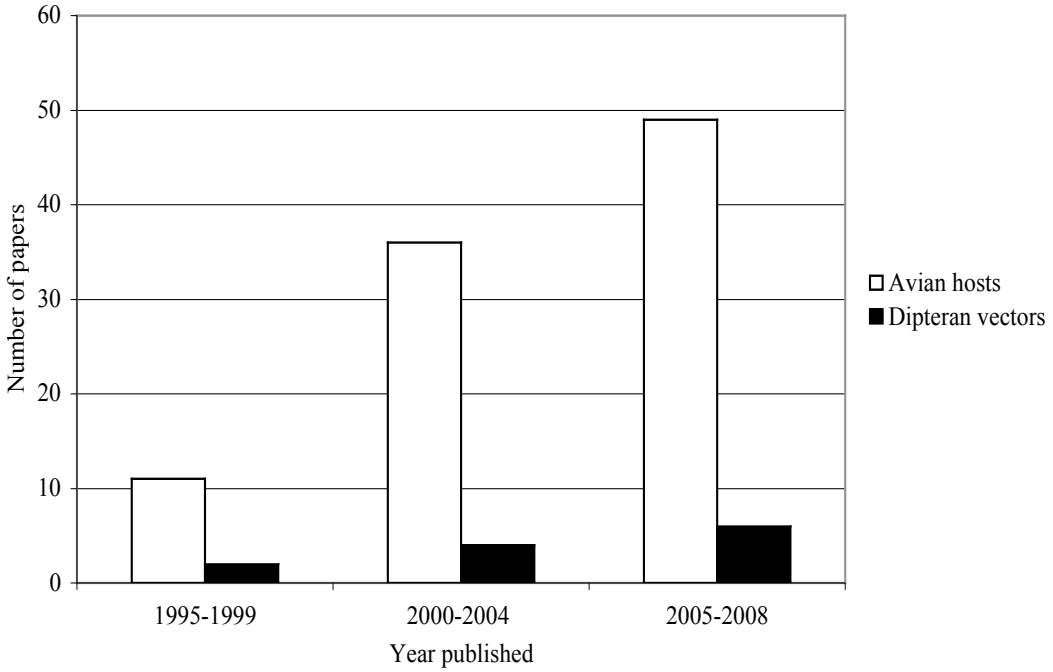

Figure 1.2. Studies found in BIOSIS database search using keywords "avian malaria" between 1995 and 2008, showing 1) increase in total number of studies between 1995 and 2008 and 2) consistently low proportion of studies focusing on vectors relative to hosts.

Evidence for **Plasmodium** *coevolution with hosts*

Avian malaria and blood parasites often exert demonstrable fitness costs on birds. Captive birds without previous exposure are at great risk for malaria infection when they are brought into zoos and other settings where they are maintained in proximity to infected hosts and exposed to appropriate vectors (Fix et al., 1988; Schrenzel et al., 2003). Both correlative and experimental studies of wild birds have shown that blood parasites result in reduced reproductive success in infected individuals (Merino et al., 2000; Marzal et al., 2005). Others have demonstrated negative effects of infection on sexually selected traits such as bird song; for example, blood parasite infection correlates with reduced paternity in sparrows singing nonlocal songs (MacDougall-Shackleton et al., 2002), disrupts the development of the brain

region that is involved in song complexity (Spencer et al., 2005), and reduces song complexity (Gilman et al., 2007). The immune response induced by blood parasite infection in birds is thought to be energetically costly and may adversely effect birds' timing of reproduction (Allander and Bennett, 1995) and parental effort (Richner et al., 1995; Ilmonen et al., 1999; Tomás et al., 2007). Despite the documented negative fitness effects of *Plasmodium* infection, chronic infections are common and widespread. In Hawaii, lowland populations of native honeycreeper species that were once scarce as a result of malaria infection have rebounded but maintain high rates of chronic *Plasmodium* infection, suggesting that they have evolved resistance (Woodworth et al., 2005).

Evidence for **Plasmodium** *coevolution with vectors*

Plasmodium infects both male and female birds. However, *Plasmodium* can only infect female mosquitoes, because males do not feed on blood. Therefore, the fitness costs to mosquitoes are quantified as direct costs on fecundity, longevity, behavior relating to host seeking, blood feeding and oviposition. The mosquito immune response to *Plasmodium* infection and its genetic basis is a rapidly growing area of research (Dimopoulos et al., 1997; Hill et al., 2005; Tripet et al., 2008). There are three poorly understood physical barriers to *Plasmodium* infection within the mosquito, known as the midgut infection, midgut escape and salivary gland barriers. The midgut infection barrier is comprised of the peritrophic membrane, which is made up of chitins, proteins and proteoglycans. This membrane matrix is secreted within minutes of blood meal ingestion and completely encloses the blood bolus (Marquardt et al., 2005). The interaction between the time to completion of the peritrophic matrix with the time to ookinete formation by the parasite (both of which vary among vector and parasite species) could play an important role in maintaining the reproductive

isolation of multiple malaria species within the gut (Alavi et al., 2003), but some parasites use chitinase to dissolve and break through the peritrophic membrane (Huber et al., 1991). The second barrier is the midgut itself, which the developing oocyst must penetrate before releasing sporozoites into the mosquito haemolymph. The salivary gland is the third barrier, through which sporozoites must enter in order to be injected into a new host when the mosquito feeds (Marquardt et al., 2005). To successfully invade a mosquito the parasite must penetrate all three physical barriers in turn and overcome the innate immune system of the mosquito.

The fitness costs of avian malaria infection to vectors are not as well studied as fitness costs to birds. Vector-parasite coevolution can be inferred by variation in the rate at which different mosquito species are infected by avian *Plasmodium* species. Many early experiments found that different avian *Plasmodium* morphospecies varied in their ability to successfully infect and reach the salivary glands in different mosquito species that fed on experimentally infected birds (Hunninen, 1951; Hunninen, 1953). Mosquitoes were believed to be specific to parasite species whereas it was known that birds could host multiple parasite species simultaneously; passaging parasites through a mosquito was suggested as a method for isolating a parasite species of interest in a multiply infected avian host (Garnham, 1966).

After feeding on an infected bird in the laboratory, mosquito species vary in their ability to be infected and in their parasite loads (Huff, 1930; Telford et al., 1997). Early studies therefore concluded that vector competence (the ability of a vector to harbor complete development of the parasite and to transmit the parasite successfully to a new host) had a genetic basis (Huff, 1935; Manwell, 1938). Vector competence of individuals also varied within mosquito species (Huff, 1930), and lines of competent and refractory female mosquitoes could be created by artificial selection

(Huff, 1927; Huff, 1929). All these experiments suggested that competence had a genetic basis, but did not quantify fitness costs *per se.*

Our knowledge of fitness costs of malaria infection to mosquitoes comes primarily from work with human malaria vector species in the genus *Anopheles*. Infection with *Plasmodium* reduces fecundity in female *Anopheles* mosquitoes (Ferguson and Read, 2002; Hurd et al., 2005). Parasites also manipulate vector behavior to enhance transmission. Parasite strategies include increasing vector longevity and increasing probing time (the amount of time females search for blood vessels under the host's skin). The latter strategy increases the probability of the parasite being injected into a new host (Hurd, 2003). The ecological and evolutionary similarities between avian and human malaria species would predict that they share similar a coevolutionary history with their vectors. The exclusivity of human malaria species to members of the *Anopheles* genus suggest a coevolutionary history (Warrell and Gilles, 2002), and a similar history may have led to members of the *Culex* genus becoming dominant avian malaria vectors.

Importance of vectors in an ecological context

It is important to study mosquitoes in the field because laboratory competence tests do not address true competence in the wild. The probability of transmission among hosts in nature, however, depends on several other ecological factors, particularly vector abundance and host preference (Eldridge, 2000). Vector abundance could affect parasite prevalence in birds by increasing the probability of contact and eventually parasite transmission. In addition, vector species that are not highly competent in the lab can cause disease epidemics in a host population if vectors become extremely abundant (Miller et al., 1989). Another ecological factor, host

preference, varies among mosquito species, and could result in higher parasite prevalence in preferred or available host species.

The importance of coevolution between host and parasite is illustrated by the avian malaria epidemic in Hawaii that has been implicated in large-scale extinctions of the native avifauna (Warner, 1968; Van Riper III et al., 1986) and continues to threaten native bird species (Freed et al., 2005). The Hawaiian islands have no native mosquito species. The accidental human introduction in the early 1800s of a competent vector, *Culex quinquefasciatus*, therefore had an enormous impact on native birds that had not coevolved with *Plasmodium*. Unlike most other regions where avian malaria is endemic, Hawaii represents a situation where the vector was introduced relatively recently, probably multiple times (Fonseca et al., 2000), enabling the parasite, *P. relictum*, to jump from introduced birds to native birds. Hawaii currently has only one dominant avian *Plasmodium* vector species, *Cx. quinquefasciatus*; two other abundant forest-dwelling mosquito species in Hawaii were shown to be refractory to *Plasmodium* infection (LaPointe et al., 2005).

Many populations of Hawaiian birds perished from acute malaria infections at lower elevations but some highland populations survived. The low malaria prevalence in the highlands has been attributed to the scarcity of vectors at higher elevations. This scarcity could result from the lower temperatures at high elevations that limited either the rate of development of the mosquito larvae or of the parasite in the mosquito to the infectious stage (Freed et al., 2005). Vector scarcity could also result from the scarcity of larval habitat at high elevations, which was limited to water holding tanks on the roofs of houses (Adak et al., 2005). Recent investigations show that larval habitat availability is more important than elevation *per se* in predicting vector abundance in Hawaii (Aruch et al., 2007). Mathematical models suggest that abiotic factors such as rainfall and temperature interact with biotic factors such as larval

density dependence to predict different responses in vector abundance to environmental change at different elevations (Ahumada et al., 2004). Results from these and other studies on the Hawaiian system suggest that vector abundance directly influences parasite prevalence in Hawaiian birds. Hawaii, however, is unusual in that it involves only one parasite and one mosquito vector species. In most places, where avian *Plasmodium* is endemic, multiple mosquito species frequently come into contact with multiple parasite species, and thus these systems are potentially more ecologically complex.

There are three reasons that it is important to study vectors in their ecological context. The first is that lab systems often involve unnatural parasite-vector combinations, which differ from natural parasite-vector combinations because they lack a shared coevolutionary history. For example, the melanization response, which blocks oocyst development, is induced in *Anopheles* by infection with a rodent-specific *Plasmodium* species (*P. chabaudi*), but not by infection with the human-specific *Plasmodium* species that it encounters in nature (Aguilar et al., 2005). The discovery that natural vector-parasite combinations induced a stronger immune response than unnatural combinations (e.g. rodent parasites with human-feeding vectors) has moved malaria vector-parasite evolution research forward towards field-based rather than lab-based approaches (Cohuet et al., 2006; Tripet et al., 2008). The second reason to study vectors in an ecological context is that lab experiments often use inbred mosquitoes or parasite lines. Inbreeding could affect the expression of the immune response, sometimes resulting in an overestimation of competence (Niare et al., 2002). Finally, lab studies often utilize model hosts, such as chickens, canaries or ducks, and do not reflect the interactions that may occur based on the broader host feeding preference of vector species.

Transmission intensity is correlated with prevalence in hosts. One study found that the level of population genetic structuring of human malaria parasite *P. falciparum* sampled from their vectors was higher in low transmission sites in South America and Thailand than in high transmission sites in Africa and Papua New Guinea (PNG). This difference was attributed to high levels of outcrossing in Africa and PNG, leading to more recombination, less structuring and higher diversity (Anderson et al., 2000). If this is a general pattern, then we might expect parasite genetic diversity to be maintained in areas with low transmission, as measured by low prevalence in hosts, such as in South America (Valkiunas et al., 2003) and other regions where avian malaria prevalence is low.

The vector-host interaction: host feeding preference

Host feeding preference is an integral part of the avian malaria life cycle. Laboratory experiments demonstrate a mosquito's vector competence by successfully documenting complete development of the parasite life cycle (from gametocyte to sporozoite, Figure 1.1) or successfully transmitting a parasite to a new experimental host. In the wild, however, parasites are transmitted only during a mosquito's successful blood feeding from a wide range of available avian hosts. Blood feeding must last long enough so that the mosquito can successfully inject enough infective sporozoites into the host to cause an infection. The outcome of vector-host interactions could therefore affect the host range and ultimately the geographic range of malaria parasites. Competent mosquito species capable of feeding on a broad range of avian hosts could disseminate parasites farther than those specializing on a narrow range of hosts. Mosquitoes may obtain blood meals from particular bird species for several reasons: 1) mosquito and bird species overlap in space and time;

2) mosquitoes are preferentially attracted to those bird species; or 3) those bird species do not exhibit successful defensive behavior.

The recent emergence of several zoonotic vector-borne pathogens, particularly those originating from birds (*e.g.*, West Nile virus), has stimulated renewed interest into mosquito feeding preferences (Molaei et al., 2006; Molaei et al., 2007; Patrican et al., 2007; Hamer et al., 2008). Most such studies infer host preference by analyzing contents of mosquito blood meals to identify the bird species fed upon. Results from such surveys have shown clearly that certain bird species are better represented in mosquito blood meals than others.

The differences in preferred avian hosts within mosquito species, and among studies, suggest that host preference is variable and depends on ecological factors, including host availability. Mosquito blood meal identification studies with PCR and DNA sequencing often find the most common bird species highly represented in most mosquito blood meals. Abundant bird species such as American robin (*Turdus migratorius*), common grackle (*Quiscalus quiscula*) and Northern cardinal (*Cardinalis cardinalis*) are highly represented in the blood meals of several *Culex* species in North America (Savage et al., 2007); some of the same species were the most common avian hosts found in blood meals of another ornithophilic mosquito species, *Cs. melanura* (Molaei and Andreadis, 2006), while another study found a less abundant woodland bird, wood thrush (*Hylocichla mustelina*), to be the most common host of *Cs. melanura* and *Cs. morsitans* (Molaei et al., 2006). However, another study examining the blood meal contents of three mosquito species found variation in the most common avian host; while *T. migratorius* was the most common host species represented in two of the three mosquito species studied (*Cx. pipiens* and *Cx. restuans*), black-capped chickadees (*Poecile atricapilla*) was the most common in the blood meals of *Cx. salinarius* (Molaei et al., 2006). Another abundant bird species, mourning dove

(*Zenaida macroura*) was the most frequent host of *Cx. quinquefasciatus* (Molaei et al., 2007).

Beyond simply feeding on the most abundant host, however, one study indicated that mosquitoes do preferentially feed on certain bird species over others regardless of relative abundance: Carolina chickadee (*Poecile carolinensis*) and yellow-crowned night heron (*Nyctanassa violacea*) were found in a high proportion of blood meals of *Culex erraticus*, but found in low densities in bird surveys (Hassan et al., 2003). One study of another avian blood parasite genus, *Leucocytozoon*, showed certain bird species represented in high frequency in the blood meals of simuliid vectors, suggesting that vector-host specificity could hinder transmission of parasites among host taxa (Hellgren et al., 2008). Whenever possible, such studies should include the calculation of forage ratios (Ponlawat and Harrington, 2005) by measuring host abundance in order to determine true preferences. Caution must be taken when interpreting the results of blood meal analyses, as these reflect patterns of host feeding rather than the specific attraction between mosquito and bird.

An alternative approach to blood meal analysis for assessing host preference is to quantify the behavior of mosquitoes given a choice of different potential hosts. In host-choice experiments where mosquitoes had equal access to more than one type of host, mosquitoes have discriminated based on species (chickens over quail, (Lord and Day, 2000) and host age (adults over nestlings, (Scott et al., 1990; Griffing et al., 2007). Mosquitoes seek hosts guided by cues such as temperature, particularly in nocturnal host-seeking species (Marquardt et al., 2005), and species-specific volatiles or odors (Takken and Knols, 1999). Some mosquitoes prefer some colors over others in experiments (Brett, 1938), although plumage color failed to predict the likelihood of haematozoan infection at the bird species level (Yezerinac and Weatherhead, 1995). Disease infection status could also affect a bird's attractiveness to mosquitoes. For

example, *Anopheles* mosquitoes flew more often towards the same human hosts when they had elevated gametocytemia, relative to when they had lower gametocytemia (Lacroix et al., 2005). Other studies, however, that measured the attraction of mosquitoes to birds infected with various arboviruses (Scott et al., 1990) or the bacterium *Mycoplasma gallisepticum* (Darbro et al. 2007), did not find any significant difference between attraction or feeding success on infected and uninfected birds. While host preference has been documented in known avian malaria vector species, the basis of host choice is not known. Whether or not a mosquito feeds successfully on a bird depends on the bird's behavior as well as the mosquito's host seeking cues.

Effects of bird behavior on host preference

The behavior of birds could influence their interactions with vectors, which in turn could influence their probability of infection with blood parasites. For example, birds that form large nocturnal roosts could increase the probability that they are infected by forming a larger source of heat and/or emitting more volatiles that could attract host seeking female mosquitoes more readily than a single roosting individual. This prediction was supported in one study, where mosquito abundance increased with the size of an adjacent cliff swallow colony (Brown and Sethi, 2002). Increased mosquito abundance could increase the probability of blood parasite transmission by increasing the number of potential bird-mosquito encounters, although this was not explicitly tested in the cliff swallow study. Aggregated birds may be more attractive to host-seeking mosquitoes. Common North American birds that form nocturnal roosts are known to have high prevalences or high levels of mortality from *Plasmodium* infection, e.g., *T. migratorius* (Beier et al., 1981) and red-winged blackbird (*Agelaius phoeniceus*, Herman, 1938). In one case, a shift in roosting behavior was hypothesized to occur in response to the foraging height of host-seeking

blackflies, vectors of *Leucocytozoon* species (Rohner et al., 2000). The presence of roosting birds is not always associated with high mosquito densities, however. In one study, mosquito abundance correlated more closely with canopy height than with density of bird hosts, many of which formed large groups on the ground (Lothrop and Reisen, 2001).

Whether or not a bird is able to evade a mosquito while it is attempting to feed also affects how much blood is ingested, which in turn affects the probability of ingestion of parasites. Experimental feeding experiments show that mosquitoes (*Cs. melanura*) obtain smaller blood meals from starlings than from other birds, which was attributed to the tendency of starlings to exhibit defensive behavior resulting in interrupted blood meals (Hodgson et al., 2001). Defensive behavior could lead to more parasites being disseminated because of the mosquito's need to feed on several hosts to obtain a full blood meal. Defensive behavior varies within (Anderson and Brust, 1997) and among (Darbro and Harrington, 2007) bird species. The level of defensive behavior can also be mediated by illness, as ill birds expend less energy on defensive behavior (Darbro et al., 2007). When given a choice between a free and an immobilized bird, mosquitoes choose to feed on the immobilized bird regardless of the species (Edman et al., 1974).

Effects of habitat choice on host preference

Mosquito species may also bite certain bird species more than others because of overlap in habitat, regardless of feeding preference. Sometimes a choice of hosts is not available and mosquitoes may end up feeding on whatever birds are available. Congeneric mosquito species, e.g., in *Anopheles* (Stoops et al., 2007), differ in their habitat preferences. Differences in habitat preferences result from a combination of factors such as direct choice by ovipositing females and passive aggregation (e.g., via

wind) and spatial patterns in adult mortality, as found in a tree-hole breeding mosquito (Ellis, 2008). Variation in mosquito abundance both within and among habitats could in turn affect *Plasmodium* transmission probability to birds utilizing those habitats.

Moreover, mosquito habitat preferences could lead to the spatial structuring of *Plasmodium* lineages if mosquito species were competent for different *Plasmodium* lineages or encountered a different suite of potential avian hosts in their preferred habitat. Local adaptation between parasites and their hosts is well-documented in many host-parasite systems (Lively and Dybdahl, 2000). Local adaptation to two allopatric variants of a human malaria species was seen in two *Anopheles* species that differ in their ecological requirements, where parasite genetic subpopulations were unable to infect allopatric mosquito species (Joy et al., 2008).

Variation in mosquito distribution among microhabitats coupled with parasite-vector specificity may explain fine-scale geographic patterns in *Plasmodium* lineage distribution seen in two bird population studies (Bensch and Åkesson, 2003; Wood et al., 2007). Because these studies focused on single bird populations, it was unlikely that fine-scale host-parasite interactions were causing these patterns. Geographic patterns of *Plasmodium* and *Haemoproteus* lineage distribution was found among bird communities on different Caribbean islands (Fallon et al., 2005), where the absence of particular lineages on islands was attributed to host-parasite coevolution. These patterns might instead be explained by variation in the relative abundance of available vectors among islands.

Mosquito phenology and temporal patterns of **Plasmodium** *lineage distribution*

Temporal patterns in avian host populations could be explained by changes in vector species' relative abundance over time. A long-term study of a population of blue tits in the UK showed that *P. relictum* prevalence stays constant over the season

but *P. circumflexum* exhibits a bimodal peak, and that this peak did not correspond with predicted changes in the birds' immune system based on reproductive effort (Cosgrove et al., 2008) If these patterns are dependent on changing transmission dynamics among seasons, and if *P. relictum* and *P. circumflexum* rely on different vector species, then they could be explained by differences in the phenologies of these vectors. Mosquito species are known vary in their phenology patterns, with some co-occurring species staying uniformly high through the season while others peak and decline (Barker et al., 2003). If mosquitoes were specific to *Plasmodium* lineages, then variation in their phenology could be responsible for some of the temporal patterns seen in parasite lineage composition within populations (Bensch and Åkesson, 2003; Fallon et al., 2004; Bensch et al., 2007) and changes in overall prevalence seen in avian hosts over time (Bennett and Cameron, 1974; Allander and Bennett, 1994).

Conclusions and future directions

The ecology and evolution of vector-borne parasites are driven by complex interactions among parasite, vector and host. Most research on avian blood parasites has focused on the host-parasite association. However, vector-host interactions, such as feeding preference, either innate or apparent, or the reciprocal effect of relative abundances on vector and host populations, can ultimately influence the prevalence and specificity of blood parasites in their hosts. Vector-parasite interactions, particularly vector-specificity, can influence the probability of emergence of parasites into new avian host species, geographic regions, or populations. More research on vector-specificity is needed to assess the importance of vector ecology in the evolution of avian *Plasmodium*; variation in phenology and geographic distribution of avian *Plasmodium* could be more closely linked to variation in vector ecology if parasites were vector-specific. Inferred mechanisms of vector immunity and infection from

rodent *Plasmodium* studies in the lab provide hypotheses that could explain temporal and geographic variation in avian *Plasmodium* prevalence in their hosts in the field. These hypotheses could then be tested with a combination of field sampling of *Plasmodium* in mosquitoes and experiments to determine host feeding preference and vector competence.

Future research should include field sampling of mosquitoes as well as birds to determine which parasites are present in both vectors and hosts. Few studies have sampled diverse mosquito species across habitats to identify natural vectors (but see Gager et al. 2008). Besides identification of vectors, data on their host feeding preferences at the community level are needed to understand how interaction with vectors might facilitate or hinder a parasite's access to new hosts. The increased application of molecular methods to arthropod vectors has revealed some surprising parasite-vector associations as the discovery of a new clade of Trypanosome parasites in *Culex pipiens* (Van Dyken et al., 2006). These new associations will likely accumulate as more vector species are studied. Most of the current literature on vector competence includes incrimination of mosquito vectors for particular parasite species; field sampling of mosquitoes should complement these targeted transmission studies to aid in our understanding of the importance of vector ecology in host-parasite dynamics.

REFERENCES

Adak, T., Singh, O.P., Das, M.K., Wattal, S. and Nanda, N. (2005). Comparative susceptibility of three important malaria vectors *Anopheles stephensi*, *Anopheles fluviatilis*, and *Anopheles sundaicus* to *Plasmodium vivax*. *Journal of Parasitology* 91, 79-82.

Aguilar, R., Dong, Y., Warr, E. and Dimopoulos, G. (2005). *Anopheles* infection responses; laboratory models versus field malaria transmission systems. *Acta Tropica* 95, 285-291.

Ahumada, J.A., Lapointe, D. and Samuel, M.D. (2004). Modeling the population dynamics of *Culex quinquefasciatus* (Diptera: Culicidae), along an elevational gradient in Hawaii. *Journal of Medical Entomology* 41, 1157-1170.

Alavi, Y., Arai, M., Mendoza, J., Tufet-Bayona, M., Sinha, R., Fowler, K., Billker, O., Franke-Fayard, B., Janse, C.J., Waters, A. and Sinden, R.E. (2003). The dynamics of interactions between *Plasmodium* and the mosquito: a study of the infectivity of *Plasmodium berghei* and *Plasmodium gallinaceum*, and their transmission by *Anopheles stephensi, Anopheles gambiae* and *Aedes aegypti*. *International Journal of Parasitology* 33, 933-943.

Allander, K. and Bennett, G.F. (1995). Retardation of breeding onset in great tits (*Parus major*) by blood parasites. *Functional Ecology* 9, 677-682.

Allander, K. and Bennett, G., F. (1994). Prevalence and intensity of haematozoan infection in a population of great tits *Parus major* from Gotland, Sweden. *Journal of Avian Biology* 25, 69-74.

Anderson, R.A. and Brust, R.A. (1997). Interrupted blood feeding by *Culex* (Diptera:Culicidae) in relation to individual host tolerance to mosquito attack. *Journal of Medical Entomology* 34, 95-101.

Anderson, T.J.C., Haubold, B., Williams, J.T., Estrada-Franco, J.G., Richardson, L., Mollinedo, R., Bockarie, M., Mokili, J., Mharakurwa, S., French, N., Whitworth, J., Velez, I., Brockman, A., Nosten, F., Ferreira, M. and Day, K. (2000). Microsatellite markers reveal a spectrum of population structures in the malaria parasite *Plasmodium falciparum*. *Molecular Biology and Evolution* 17(10), 1467-1482.

Aruch, S., Atkinson, C.T., Savage, A.F. and Lapointe, D.A. (2007). Prevalence and distribution of pox-like lesions, avian malaria, and mosquito vectors in Kipahulu Valley, Haleakala National Park, Hawai'i, USA. *Journal of Wildlife Diseases* 43, 567-575.

Barker, C.M., Paulson, S.L., Cantrell, S. and Davis, B.S. (2003). Habitat preferences and phenology of *Ochlerotatus triseriatus* and *Aedes albopictus* (Diptera: Culicidae) in southwestern Virginia. *Journal of Medical Entomology* 40, 403-410.

Beadell, J.S., Ishtiaq, F., Covas, R., Melo, M., Warren, B.H., Atkinson, C.T., Bensch, S., Graves, G.R., Jhala, Y.V., Peirce, M.A., Rahmani, A.R., Fonseca, D.M. and Fleischer, R.C. (2006). Global phylogeographic limits of Hawaii's avian malaria. *Proceedings of the Royal Society Series B-Biological Sciences* 273, 2935-2944.

Beier, J.C. and Trpis, M. (2001). Incrimination of natural culicine vectors which transmit *Plasmodium elongatum* to penguins at the Baltimore Zoo. *Canadian Journal of Zoology* 59, 470-475.

Beier, J.C., Strandberg, J., Stoskopf, M.K. and Craft, C. (1981). Mortality in robins (*Turdus migratorius*) due to avian malaria. *Journal of Wildlife Diseases* 17, 247-250.

Bennett, G.F., Garvin, M. and Bates, J.M. (1991). Avian hematozoa from west-central Bolivia. *Journal of Parasitology* 77, 207-211.

Bennett, G.F., Montgomerie, R. and Seutin, G. (1992). Scarcity of haematozoa in birds breeding on the Arctic tundra of North America. *Condor* 94, 289-292.

Bennett, G.F., Witt, H. and White, E.M. (1980). Blood parasites of some Jamaican birds. *Journal of Wildlife Diseases* 16, 29-38.

Bennett, G.F. and Cameron, M. (1974). Seasonal prevalence of avian hematozoa in passeriform birds of Atlantic Canada. *Canadian Journal of Zoology* 52, 1259-1264.

Bennett, G.F., Squires-Parsons, D., Siikamäki, P., Huhta, E., Allander, K. and Hillström, L. (1995). A comparison of the blood parasites of three Fenno-Scandian populations of the pied flycatcher *Ficedula hypoleuca*. *Journal of Avian Biology* 26, 33-38.

Bensch, S., Waldenstrom, J., Jonzen, N., Westerdahl, H., Hansson, B., Sejberg, D. and Hasselquist, D. (2007). Temporal dynamics and diversity of avian malaria parasites in a single host species. *Journal of Animal Ecology* 76, 112-122.

Bensch, S. and Åkesson, S. (2003). Temporal and spatial variation of hematozoans in Scandinavian willow warblers. *Journal of Parasitology* 89, 388-391.

Bensch, S., Pérez-Tris, J., Waldenström, J. and Hellgren, O. (2004). Linkage between nuclear and mitochondrial DNA sequences in avian malaria parasites: Multiple cases of cryptic speciation? *Evolution* 58, 1617-1621.

Bensch, S., Stjernman, M., Hasselquist, D., Östman, Ö., Hansson, B., Westerdahl, H. and Torres, P.R. (2000). Host specificity in avian blood parasites: A study of *Plasmodium* and *Haemoproteus* mitochondrial DNA amplified from birds. *Proceedings of the Royal Society Series B-Biological Sciences* 267, 1583-1589.

Brett, G.A. (1938). On the relative attractiveness to *Aëdes aegypti* of certain coloured cloths. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 32, 113-124.

Brown, C.R. and Sethi, R.A. (2002). Mosquito abundance is correlated with Cliff Swallow (*Petrochelidon pyrrhonota*) colony size. *Journal of Medical Entomology* 39, 115-120.

Chao, J. and Ball, G.H. (1962). Effect of low temperature on *Plasmodium relictum* in *Culex tarsalis*. *Journal of Parasitology* 48, 252-254.

Cohuet, A., Osta, M.A., Morlais, I., Awono-Ambene, P.H., Michel, K., Simard, F., Christophides, G.K., Fontenille, D. and Kafatos, F.C. (2006). *Anopheles* and *Plasmodium*: from laboratory models to natural systems in the field. *EMBO Report* 7, 1285-1289.

Cosgrove, C.L., Wood, M.J., Day, K.P. and Sheldon, B.C. (2008). Seasonal variation in *Plasmodium* prevalence in a population of blue tits *Cyanistes caeruleus*. *Journal of Animal Ecology* 77, 540-548.

Darbro, J.M., Dhondt, A.A., Vermeylen, F.M. and Harrington, L.C. (2007). *Mycoplasma gallisepticum* infection in house finches (*Carpodacus mexicanus*) affects mosquito blood feeding patterns. *American Journal of Tropical Medicine & Hygiene* 77, 488-494.

Darbro, J.M. and Harrington, L.C. (2007). Avian defensive behavior and blood-feeding success of the West Nile vector mosquito, *Culex pipiens*. *Behavioral Ecology* 18, 750-757.

Dimopoulos, G., Richman, A., Muller, H.M. and Kafatos, F.C. (1997). Molecular immune responses of the mosquito *Anopheles gambiae* to bacteria and malaria parasites. *Proceeding of the National Academy of Sciences USA* 94, 11508-11513.

Edman, J.D., Webber, L.A. and Schmid, A.A. (1974). Effect of host defenses on the feeding pattern of *Culex nigripalpus* when offered a choice of blood sources. *Journal of Parasitology* 60, 874-883.

Eldridge, B.F. 2000. The epidemiology of arthropodborne diseases. *In* Medical Entomology: a textbook on public health and veterinary problems caused by arthropods. *Edited by* Eldridge, B.F., and J.D. Edman. Kluwer Academic Publishers, Norwell, MA, pp 151-164.

Ellis, A.M. (2008). Linking movement and oviposition behaviour to spatial population distribution in the tree hole mosquito *Ochlerotatus triseriatus*. *Journal of Animal Ecology* 77, 156-166.

Fallon, S.M., Ricklefs, R.E., Latta, S.C. and Bermingham, E. (2004). Temporal stability of insular avian malarial parasite communities. *Proceedings of the Royal Society Series B-Biology* 271, 493-500.

Fallon, S.M., Bermingham, E. and Ricklefs, R.E. (2005). Host specialization and geographic localization of avian malaria parasites: A regional analysis in the Lesser Antilles. *American Naturalist* 165, 466-480.

Ferguson, H.M. and Read, A.F. (2002). Why is the effect of malaria parasites on mosquito survival still unresolved? *Trends in Parasitology* 6, 256-261.

Fix, A.S., Waterhouse, C., Greiner, E.C. and Stoskopf, M.K. (1988). *Plasmodium relictum* as a cause of avian malaria in wild-caught magellanic penguins (*Spheniscus magellanicus*). *Journal of Wildlife Diseases* 24, 610-619.

Fonseca, D.M., Lapointe, D.A. and Fleischer, R.C. (2000). Bottlenecks and multiple introductions: population genetics of the vector of avian malaria in Hawaii. *Molecular Ecology* 11, 1803-1814.

Forrester, D.J., Nayar, J.K. and Foster, G.W. (1980). *Culex nigripalpus*: a natural vector of wild turkey malaria (*Plasmodium hermani*) in Florida. *Journal of Wildlife Diseases* 16, 391-394.

Freed, L.A., Cann, R.L., Goff, M.L., Kuntz, W.A. and Bodner, G.R. (2005). Increase in avian malaria at upper elevation in Hawai'i. *Condor* 107, 753-764.

Gager, A.B., Del Rosario Loaiza, J., Dearborn, D.C. and Bermingham, E. (2008). Do mosquitoes filter the access of *Plasmodium* cytochrome *b* lineages to an avian host? *Molecular Ecology* 17, 2552-2561.

Garnham, P.C.C. (1966) *Malaria parasites and other haemosporidia*. Blackwell Scientific Publications, Oxford.

Garvin, M.C., Marra, P.P. and Crain, S.K. (2004). Prevalence of hematazoa in overwintering American Redstarts (*Setophaga ruticilla*): no evidence for local transmission. *Journal of Wildlife Diseases* 40, 115-118.

Gilman, S., Blumstein, D., T. and Foufopoulos, J. (2007). The effect of hemosporidian infections on white-crowned sparrow singing behavior. *Ethology* 113, 437-445.

Griffing, S.M., Kilpatrick, A.M., Clark, L. and Marra, P.P. (2007). Mosquito landing rates on nesting American robins (*Turdus migratorius*). *Vector Borne Zoonotic Diseases* 7, 437-443.

Grossman, G., A. and Lourenco-De-Oliveira, R. (1996). Laboratory observations of the emergence, longevity, blood feeding and oviposition behaviour of *Culex saltanensis Dyar*. *Revista Brasileira de Entomologia* 40, 357-365.

Hamer, G.L., Kitron, U.D., Brawn, J.D., Loss, S.R., Ruiz, M.O., Goldberg, T.L. and Walker, E.D. (2008). *Culex pipiens* (Diptera: Culicidae): a bridge vector of West Nile virus to humans. *Journal of Medical Entomology* 45, 125-128.

Hassan, H.K., Cupp, E.W., Hill, G.E., Katholi, C.R., Klingler, K. and Unnasch, T.R. (2003). Avian host preference by vectors of eastern equine encephalomyelitis virus. *American Journal of Tropical Medicine and Hygiene* 69, 641-647.

Hellgren, O., Bensch, S. and Malmqvist, B. (2008). Bird hosts, blood parasites and their vectors – associations uncovered by molecular analyses of blackfly blood meals. *Molecular Ecology* 1, 1-9.

Herman, C.M. (1938). Epidemiology of malaria in eastern red-wings (*Agelaius p. phoeniceus*). *American Journal of Hygiene* 28, 232-243.

Herman, C.M., Kocan, R.M., Snyder, E.L. and Knisley, J.O. (1968). *Plasmodium elongatum* from a penguin. *Wildlife Diseases* 4, 132.

Hill, C.A., Kafatos, F.C., Stansfield, S.K. and Collins, F.H. (2005). Arthropod-borne diseases: vector control in the genomics era. *Nature Reviews Microbiology* 3, 262-268.

Hodgson, J.C., Spielman, A., Komar, N., Krahforst, C., F., Wallace, G., T. and Pollack, R., J. (2001). Interrupted blood-feeding by *Culiseta melanura* (Diptera: Culicidae) on European starlings. *Journal of Medical Entomology* 38, 59-66.

Huber, M., Cabib, E. and Miller, L.H. (1991). Malaria parasite chitinase and penetration of the mosquito peritrophic membrane. *Proceedings of the National Academy of Sciences USA* 88, 2807-2810.

Huff, C.G. (1934). Comparative studies on susceptible and insusceptible *Culex pipiens* in relation to infections with *Plasmodium cathemerium* and *P. relictum*. *American Journal of Hygiene* 19, 123-147.

———. (1935). Natural immunity and susceptibility of culicine mosquitoes to avian malaria. *American Journal of Tropical Medicine & Hygiene* 4, 427-434.

Huff, C.G. (1927). Studies on the infectivity of plasmodia of birds for mosquitoes, with special reference to the problem of immunity in the mosquito. *American Journal of Hygiene* 7, 706-734.

———. (1930). Individual immunity and susceptibility of *Culex pipiens* to various species of bird malaria as studied by means of double infectious feedings. *American Journal of Hygiene* 12, 424-441.

Huff, C.G. (1931). The inheritance of natural immunity to *Plasmodium cathemerium* in two species of *Culex*. *Journal of Preventive Medicine* 5, 249-259.

———. (1929). The effects of selection upon susceptibility to bird malaria in *Culex pipiens*. *Linn Ann Trop Med Parasitol.* 23, 427–439.

Hunninen, A.V. (1953). Comparative development of *Plasmodium relictum* oocysts in *Anopheles quadrimaculatus*, *A. albimanus*, and *Culex pipiens*. *Journal of Parasitology* 39, 28-32.

Hunninen, A.V. (1951). Comparative susceptibility of four anopheline mosquitoes to *Plasmodium relictum*. *Journal of the National Malaria Society* 10, 216-223.

Hurd, H. (2003). Manipulation of medically important insect vectors by their parasites. *Annual Review of Entomology* 48, 141-161.

Hurd, H., Taylor, P.J., Adams, D., Underhill, A. and Eggleston, P. (2005). Evaluating the costs of mosquito resistance to malaria parasites. *Evolution* 59, 2560-2572.

Ilmonen, P., Hakkarainen, H., Koivunen, V., Korpimaki, E., Mullie, A. and Shutler, D. (1999). Parental effort and blood parasitism in Tengmalm's owl: Effects of natural and experimental variation in food abundance. *Oikos* 86, 79-86.

Joy, D.A., Gonzalez-Ceron, L., Carlton, J.M., Gueye, A., Fay, M., McCutchan, T.F. and Su, X.Z. (2008). Local adaptation and vector-mediated population structure in *Plasmodium vivax* malaria. *Molecular Biology and Evolution* 25, 1245-1252.

Kelly, R. and Edman, J.D. (1992). Multiple transmission of *Plasmodium gallinaceum* (Eucoccida: Plasmodiidae) during serial probing by *Aedes aegypti* (Diptera: Culicidae) on several hosts. *Journal of Medical Entomology* 29, 329-331.

Lacroix, R., Mukabana, W.R., Gouagna, L.C. and Koella, J.C. (2005). Malaria infection increases attractiveness of humans to mosquitoes. *PLoS Biol* 3, e298.

LaPointe, D.A., Goff, M.L. and Atkinson, C.T. (2005). Comparative susceptibility of introduced forest dwelling mosquitoes in Hawai'i to avian malaria, *Plasmodium relictum*. *Journal of Parasitology* 91, 843-849.

Lively, C.M. and Dybdahl, M.F. (2000). Parasite adaptation to locally common host genotypes. *Nature* 405, 679-681.

Lord, C.C. and Day, J.F. (2000). Attractiveness of chickens and bobwhite quail for *Culex nigripalpus*. *Journal of the American Mosquito Control Association* 16, 271-273.

Lothrop, H.D. and Reisen, W.K. (2001). Landscape affects the host-seeking patterns of *Culex tarsalis* (Diptera: Culicidae) in the Coachella Valley of California. *Journal of Medical Entomology* 38, 325-332.

MacDougall-Shackleton, E.A., Derryberry, E.P. and Hahn, T.P. (2002). Nonlocal male mountain white-crowned sparrows have lower paternity and higher parasite loads than males singing local dialect. *Behavioral Ecology* 13, 682-689.

Manwell, R.D. (1938). Reciprocal immunity in the avian malarias. *American Journal of Hygiene* 27, 196-211.

Marquardt, W.C., W.C. Black, J.E. Freier, H.H. Hagedorn, J. Hemingway, S. Higgs, A.A. James, B. Kondratieff, and C.G. Moore. (2005) *Biology of Disease Vectors*. Elsevier Academic Press, Burlington, MA.

Marzal, A., de Lope, F., Navarro, C. and Møller, A.P. (2005). Malarial parasites decrease reproductive success: an experimental study in a passerine bird. *Oecologia* 142, 541-545.

Merilä, J., Björklund, M. and Bennett, G.F. (1995). Geographic and individual variation in haematozoan infections in the greenfinch, *Carduelis chloris*. *Canadian Journal of Zoology* 73, 1798-1804.

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

Meyer, C.L. and Bennett, G.F. (1976). Observations on the sporogony of *Plasmodium circumflexum* and *Plasmodium polare* in New Brunswick, Canada. *Canadian Journal of Zoology* 54, 133-141.

Miller, B.R., Monath, T.P., Tabachnick, W.J. and Ezike, V.I. (1989). Epidemic yellow fever caused by an incompetent mosquito vector. *Annals of Tropical Medicine and Parasitology* 40, 396-399.

Mok, F.F. (1951). Transmission of *Plasmodium relictum grassi* and *feletti* by *Anopheles freeborni aitken*. *Science* 113, 485-485.

Molaei, G. and Andreadis, T.G. (2006). Identification of avian- and mammalian-derived bloodmeals in *Aedes vexans* and *Culiseta melanura* (Diptera : Culicidae) and its implication for West Nile virus transmission in Connecticut, USA. *Journal of Medical Entomology* 43, 1088-1093.

Molaei, G., Andreadis, T.G., Armstrong, P.M., Anderson, J.F. and Vossbrinck, C.R. (2006). Host feeding patterns of *Culex* mosquitoes and West Nile virus transmission, northeastern United States. *Emerging Infectious Diseases* 12, 468-474.

Molaei, G., Andreadis, T.G., Armstrong, P.M., Bueno, R.J., Dennett, J.A., Real, S.V., Sargent, C., Bala, A., Randle, Y., Guzman, H., Wuithiranyagool, T. and Tesh, R.B. (2007). Host feeding pattern of Culex quinquefasciatus (Diptera: Culicidae) and its role in transmission of West Nile Virus in Harris County, Texas. *American Journal of Tropical Medicine & Hygiene* 77, 73-81.

Molaei, G., Oliver, J., Andreadis, T.G., Armstrong, P.M. and Howard, J.J. (2006). Molecular identification of blood-meal sources in *Culiseta melanura* and *Culiseta morsitans* from an endemic focus of eastern equine encephalitis virus in New York. *American Journal of Tropical Medicine and Hygiene* 75, 1140-1147.

Nayar, J.K., Young, M.D. and Forrester, D.J. (1981). *Culex restuans*: an experimental vector for wild turkey malaria *Plasmodium hermani*. *Mosquito News* 41, 748-750.

Nayar, J.K., Knight, J.W. and Telford, S.R., Jr. (1998). Vector ability of mosquitoes for isolates of *Plasmodium elongatum* from raptors in Florida. *Journal of Parasitology* 84, 542-546.

Nayar, J.K., Young, M.D. and Forrester, D.J. (1981). *Plasmodium hermani*: experimental transmission by *Culex salinarius* and comparison with other susceptible Florida mosquitoes. *Experimental Parasitology* 51, 431-437.

———. (1982). Experimental transmission by mosquitoes of *Plasmodium hermani* between domestic turkeys and pen-reared bobwhites. *Journal of Parasitology* 68, 874-876.

Niare, O., Markianos, K., Volz, J., Oduol, F., Toure, A., Bagayoko, M., Sangare, D., Traore, S.F., Wang, R., Blass, C., Dolo, G., Bouare, M., Kafatos, F.C., Kruglyak, L., Toure, Y.T. and Vernick, K.D. (2002). Genetic loci affecting resistance to human malaria parasites in a West African mosquito vector population. *Science* 298, 213-216.

Patrican, L.A., Hackett, L.E., Briggs, J.E., McGowan, J.W., Unnasch, T.R. and Lee, J.H. (2007). Host-feeding patterns of *Culex* mosquitoes in relation to trap habitat. *Emerging Infectious Diseases* 13, 1921-1923.

Peirce, M.A. (1981). Distribution and host-parasite check-list of the haematozoa of birds in Western Europe. *Journal of Natural History* 15, 419-458.

Ponlawat, A. and Harrington, L.C. (2005). Blood feeding patterns of *Aedes aegypti* and *Aedes albopictus* in Thailand. *Journal of Medical Entomology* 42, 844-849.

Reeves, W.C., Herold, R.C., Rosen, L., Brookman, B. and Hammon, W. (1954). Studies on avian malaria in vectors and hosts of encephalitis in Kern county, California. 2. Infections in mosquito vectors. *American Journal of Tropical Medicine and Hygiene* 3, 696-703.

Richner, H., Christe, P. and Oppliger, A. (1995). Paternal investment affects prevalence of malaria. *Proceedings of the National Academy of Sciences USA* 92, 1192-1194.

Rohner, C., Krebs, C.J., Hunter, D.B. and Currie, D.C. (2000). Roost site selection of Great Horned Owls in relation to black fly activity: An anti-parasite behavior? *Condor* 4, 950-955.

Savage, H.M., Aggarwal, D., Apperson, C.S., Katholi, C.R., Gordon, E., Hassan, H.K., Anderson, M., Charnetzky, D., McMillen, L., Unnasch, E.A. and Unnasch, T.R. (2007). Host choice and West Nile virus infection rates in blood-fed mosquitoes, including members of the Culex pipiens complex, from Memphis and Shelby County, Tennessee, 2002-2003. *Vector Borne Zoonotic Diseases* 7, 365-386.

Schrenzel, M.D., Maalouf, G.A., Keener, L.L. and Gaffney, P.M. (2003). Molecular characterization of malarial parasites in captive passerine birds. *Journal of Parasitology* 89, 1025-1033.

Scott, T.W., Lorenz, L.H. and Edman, J.D. (1990). Effects of house sparrow age and arbovirus infection on attraction of mosquitoes. *Journal of Medical Entomology* 27, 856-863.

Sol, D., Jovani, R. and Torres, J. (2000). Geographical variation in blood parasites in feral pigeons: the role of vectors. *Ecography* 3, 307-314.

Spencer, K.A., Buchanan, K.L., Leitner, S., Goldsmith, A.R. and Catchpole, C.K. (2005). Parasites affect song complexity and neural development in a songbird. *Proceedings of the Royal Society Series B-Biological Sciences* 272, 2037-2043.

Stoops, C.A., Gionar, Y.R., Shinta, Sismadi, P., Elyazar, I.R., Bangs, M.J. and Sukowati, S. (2007). Environmental factors associated with spatial and temporal distribution of Anopheles (Diptera: Culicidae) larvae in Sukabumi, West Java, Indonesia. *Journal of Medical Entomology* 44, 543-553.

Stoskopf, M.K. and Beier, J. (1979). Avian malaria in African black-footed penguins. *J Am Vet Med Assoc* 175, 944-947.

Szymanski, M.M. and Lovette, I.J. (2005). High lineage diversity and host sharing of malarial parasites in a local avian assemblage. *Journal of Parasitology* 91, 768-774.

Takken, W. and Knols, B.G. (1999). Odor-mediated behavior of Afrotropical malaria mosquitoes. *Annual Reviews in Entomology* 44, 131-157.

Telford, S.R., Jr., Nayar, J.K., Foster, G.W. and Knight, J.W. (1997). *Plasmodium forresteri* n. sp., from raptors in Florida and southern Georgia: its distinction from *Plasmodium elongatum* morphologically within and among host species and by vector susceptibility. *Journal of Parasitology* 83, 932-937.

Tomás, G., Merino, S., Moreno, J., Morales, J. and Martinez-de la Puente, J. (2007). Impact of blood parasites on immunoglobulin level and parental effort: a medication field experiment on a wild passerine. *Functional Ecology* 21, 125-133.

Tripet, F., Aboagye-Antwi, F. and Hurd, H. (2008). Ecological immunology of mosquito-malaria interactions. *Trends in Parasitology* 24, 219-227.

Valkiunas, G., Zehtindjiev, P., Dimitrov, D., Krizanauskiene, A., Iezhova, T.A. and Bensch, S. (2008). Polymerase chain reaction-based identification of *Plasmodium (Huffia) elongatum*, with remarks on species identity of haemosporidian lineages deposited in GenBank. *Parasitology Research* 102(6), 1185-1193.

Valkiunas, G., Salaman, P. and Iezhova, T.A. (2003). Paucity of haematozoa in Colombian birds. *Journal of Wildlife Diseases* 39(2), 445-448.

Valkiunas, G. (2005) *Avian malaria parasites and other haemosporidia*. CRC press, Boca Raton.

Van Dyken, M., Bolling, B.G., Moore, C.G., Blair, C.D., Beaty, B.J., Black, W.C.t. and Foy, B.D. (2006). Molecular evidence for trypanosomatids in *Culex* mosquitoes collected during a West Nile virus survey. *International Journal for Parasitology* 36, 1015-1023.

Van Riper III, C., Van Riper, S.G., Goff, M.L. and Laird, M. (1986). The epizootiology and ecological significance of malaria in Hawaiian USA land birds. *Ecological Monographs* 56, 327-344.

Waldenström, J., Bensch, S., Kiboi, S., Hasselquist, D. and Ottosson, U. (2002). Cross-species infection of blood parasites between resident and migratory songbirds in Africa. *Molecular Ecology* 11, 1545-1554.

Warner, R.E. (1968). The role of introduced diseases in the extinction of the endemic Hawaiian avifauna. *Condor* 70, 101-120.

Warrell, D.A., and H.M. Gilles. (2002) *Essential Malariology*. Arnold, London ; New York.

White, E.M., Greiner, E.C., Bennett, G.F. and Herman, C.M. (1978). Distribution of the hematozoa of Neotropical birds. *Revista De Biología Tropical* 26, 43-102.

Wood, M.J., Cosgrove, C.L., Wilkin, T.A., Knowles, S.C., Day, K.P. and Sheldon, B.C. (2007). Within-population variation in prevalence and lineage distribution of avian malaria in blue tits, *Cyanistes caeruleus*. *Molecular Ecology* 16, 3263-3273.

Woodworth, B.L., Atkinson, C.T., LaPointe, D.A., Hart, P.J., Spiegel, C.S., Tweed, E.J., Henneman, C., Gregor, A., Smith, T. and Duffy, D. (2005). Host population persistence in the face of introduced vector-borne diseases: Hawaii amakihi and avian malaria. *Proceedings of the National Academy of Sciences* USA 5, 1531-1536.

Work, T.M., Washino, R.K. and Vanriper, C. (1990). Comparative susceptibility of *Culex tarsalis*, *Anopheles franciscanus*, and *Culiseta inornata* (Diptera, Culicidae) to *Plasmodium relictum* (Haemosporidia, Plasmodiiae). *J. Med. Entomology* 27, 68-71.

Yezerinac, S.M. and Weatherhead, P.J. (1995). Plumage coloration, differential attraction of vectors and haematozoa infections in birds. *Journal of Animal Ecology* 64, 528-537.

CHAPTER 2

PHYLOGEOGRAPHIC STRUCTURING OF *PLASMODIUM* LINEAGES ACROSS THE NORTH AMERICAN RANGE OF THE HOUSE FINCH (*CARPODACUS MEXICANUS*)

Abstract

The determinants of the geographic distribution of avian haematozoa are poorly understood. Sampling parasites from one avian host species across a wide geographic range is one approach to separate the potential influence of host species distribution from geographic effects not directly related to host species biology. We PCR-screened samples for haematozoan infection from 490 house finches (*Carpodacus mexicanus*) collected at 8 sites spanning continental North America. To explore geographic patterns of parasite lineage distributions, we sequenced a portion of the mitochondrial cytochrome *b* gene of *Plasmodium* infecting 77 house finches. We identified 5 distinct *Plasmodium* haplotypes representing 3 lineages that likely represent 3 species. One lineage was common at all sites where we detected *Plasmodium*. The second lineage contained 3 haplotypes that showed phylogeographic structuring on a continent-wide scale, with 1 haplotype common in eastern North America and 2 common in western North America. The third divergent lineage was recovered from 1 individual host. Considered together, the partial phylogeographic structuring of *Plasmodium* cytochrome *b* lineages over the range of the house finch suggests that parasite lineage distribution is not solely dependent on host species distribution, and other factors such as arthropod vector competence and distribution may be important.

Introduction

Avian malarial parasites in *Plasmodium* are widespread and cosmopolitan, occurring in virtually all bird species on every continent, except Antarctica (Valkiunas, 2005). Despite the ubiquity of avian *Plasmodium*, the geographic distribution of particular *Plasmodium* species remains poorly understood. For example, the extent to which *Plasmodium* species distribution is influenced by the distribution of 1, or more, of their avian host species is unknown. One reason for this lack of knowledge is the evolving state of haematozoan taxonomy. Because most molecular marker-based studies of parasites lack corresponding morphological data, there is currently no robust definition of what determines a *Plasmodium* species based on levels of genetic differentiation (Bensch et al., 2004). Recent comparisons of congruent mitochondrial and nuclear DNA variation in haematozoa infecting Old World warblers suggests that parasite species diversity is much higher than previously thought based on morphological descriptions alone, and that distinct mitochondrial lineages probably represent reproductively isolated entities (Bensch et al., 2004). Refining our understanding of the geographic distribution of parasitic organisms like *Plasmodium* is important to studies of host-parasite relationships and parasite community dynamics. Previous molecular marker-based studies of relationships between haematozoa and avian hosts have generally focused on either wide geographic and taxonomic sampling of the hosts (Ricklefs et al., 2004) or intensive sampling of multiple species in particular avian host communities (Beadell et al., 2004; Fallon et al., 2003; Fallon et al., 2005; Szymanski and Lovette, 2005).

In general, a parasite's geographic distribution may be influenced by the distribution of its host species and arthropod vector species, by ecological variables that show geographic variation, such as host or vector availability, or by interactions between these variables. A parasite may be limited geographically by a narrow host

range (the breadth of species it infects) (Lajeunesse and Forbes, 2002). For example, a parasite that specialized on a single rare or endemic host would clearly have a more restricted distribution than one that could infect a wide range of more broadly distributed host species. Alternatively, other environmental factors with a geographic component could influence a parasite's geographic distribution. For example, Fallon et al. (2005) found that even regionally common, host-generalist lineages of avian haematozoa were absent from some islands in the Lesser Antillean archipelago despite the availability of suitable avian host species on those islands and the presence of those parasite lineages at high frequency on neighboring islands.

Teasing apart host effects from other geographic effects could be accomplished by documenting the geographic distribution of haematozoa found in a single host taxon that itself shows low genetic structuring across a broad geographic range. The house finch (*Carpodacus mexicanus*) is a common North American songbird whose recent history makes it an appropriate model species for this study. This host species is native to western North America, but small numbers were introduced to eastern North America approximately 60 yr ago (Elliott and Arbib, 1953) and subsequently expanded their range so that the introduced eastern population now abuts the western population (Fig. 2.1). Compared to broadly distributed host taxa with much longer tenures in their current distributions, house finches across their recent introduced range are presumably genetically similar to their native western conspecifics, except for modest differences in microsatellite (Hawley et al., 2006) and AFLP allele frequencies (Wang et al., 2003) between native and introduced populations, that probably stem from founder effects or local natural selection (Badyaev et al., 2002), respectively. Moreover, house finches are reported to be infected with species of *Plasmodium* at high rates relative to other passerines (Greiner et al., 1975; White et al., 1978).

If avian host species distribution alone influenced parasite distributions, then we would expect to see parasite haplotypes randomly distributed among house finch populations across their transcontinental range; if other factors drove parasite distributions, then we would see phylogeographic structure in parasite lineages. Here, we test these alternatives by describing the distribution of *Plasmodium* lineages in house finch populations across the North American continent.

Materials and methods

We obtained house finch blood and/or tissue samples from eight sampling sites covering most of the species' range across the United States (Table I). Most of the blood samples were placed into Queen's lysis buffer immediately after collection (Seutin et al., 1991) and subsequently stored at –80 C. The blood samples from some of the New York and Georgia birds were centrifuged, the plasma removed for other purposes, and the erythrocytes stored in their original collection hematocrits at –80 C or 4 C until extraction. Because house finches were sampled in Ithaca, New York during the same years as Szymanski and Lovette's study (2005), we combined our data with theirs.

Genomic DNA was extracted from blood and tissue using an Eppendorf® gDNA blood kit (Hamburg, Germany) following the manufacturer's instructions. We amplified and sequenced 550 bp of cytochrome *b* using primers L15175B (5' GTGCAACYGTTATTACTAATTTATTAT3' modified from Ricklefs and Fallon, 2002) and H15725 (Ricklefs and Fallon, 2002). An additional, overlapping fragment of cytochrome *b* (478 bp) was sequenced using primers HaemF and HaemR2 (Bensch et al., 2000) for one representative of each haplotype. Both primer sets amplify species of both *Plasmodium* and *Haemoproteus*, another common haematozoan genus. All sets of PCR reactions included three negative controls using PCR water in lieu of

template DNA and a positive control of a known infected avian blood sample. Reactions were set up using aerosol barrier pipet tips for all samples and reagents. Amplification volumes totalled 10 µl and included 1 µl purified DNA solution (concentration variable), 1.0 µl 10X Sigma PCR Buffer, 2.5 mM $MgCl_2$, 0.25 mM dNTPs (Sigma, St. Louis, Missouri), 0.15 µM each primer, and 0.625 U Sigma JumpStart Taq polymerase. Reactions were denatured for 3 min at 95 C, followed by 35 cycles of 95 C denaturing for 50 sec, 53 C annealing for 30 sec and a 72 C extension for 1 min, and terminated with a 5 min extension at 72 C. Sequencing reactions were conducted with ABI dye terminator chemistry (Applied Biosystems, Inc., Foster City, California) following the standard ABI cycle sequencing protocol and visualized on an ABI 3100 automated sequencer. The resulting sequences were aligned and assembled using Sequencher 4.2 (GeneCodes Corp., Ann Arbor, Michigan).

We assessed phylogeographic structure in *Plasmodium* spp. using cytochrome *b* sequence obtained from 77 individuals from 8 sites. Because we did not have morphological data for our DNA samples, we identified the parasites as species of *Plasmodium* or *Haemoproteus* by their placement in a phylogenetic topology of previously published sequences following Fallon et al. (2005). We generated a pairwise distance matrix in PAUP* (Swofford, 2003) using absolute number of differences. We used this matrix to draw a minimum spanning network of *Plasmodium* cytochrome *b* haplotypes. As the pairwise relationships among the few haplotypes recovered were straightforward, this network was readily constructed by eye.

We used Fisher's exact test to determine whether haplotype proportions at each site deviated significantly from a null expectation of random distribution across sites.

We also conducted a power analysis to determine the required sample sizes to obtain a power of 0.80 at the 0.05 significance level (Casagrande et al., 1978; Zar, 1984).

Results

Haematozoan prevalence

Of 490 house finches screened from 8 sites, parasite infection was detected in 232 individuals (Table I). Sequences diagnostic of *Plasmodium* spp. were recovered from 77 birds from 6 sites and sequences of *Haemoproteus* spp. from 28 birds at 2 sites. Despite the high prevalence overall, all of the individuals we examined appeared to be healthy. This result was not unexpected as indications of infection are sometimes caused by the high parasitemia that eventually renders birds immobile and thus less likely to be captured in the wild (Valkiunas, 2001). The highest prevalence was found at the northern California site, with 95% of birds infected, whereas the Arizona site had the lowest with 12.5% infected (Table I). Prevalence data reported here represent minimum prevalence because the repeatability of PCR for samples that yield very weak bands tends to be low, and there is consequently a high probability of mistakenly scoring weak infections as negatives. Contamination that would have resulted in false positives is less likely, however, because we employed rigorous negative controls.

Haematozoan cytochrome b sequences

A number of samples yielded weak PCR amplifications, which resulted in unreadable or poor-quality sequences. We did not obtain strong amplification for these samples following optimization of PCR conditions and use of alternative primer sets as previously described (Szymanski and Lovette, 2005). To avoid the high risk of contamination when re-amplifying using PCR product as templates for secondary amplifications, we sequenced only those samples that produced strong amplification in

the initial PCR. Of those samples that did yield sequence; we only included for analysis those that were free of double chromatogram peaks, following the methods of Fallon et al. (2003), as these samples are likely to represent multiple infections that cannot be resolved without cloning the individual amplicons prior to sequencing (Szymanski and Lovette, 2005). Our data set was therefore biased in favor of those lineages that amplified well. Other lineages could have been undersampled in this study because of low parasitemia or sequence differences at priming sites.

The neighbor-joining tree constructed with our cytochrome *b* sequences combined with published haematozoan sequences from Szymanski and Lovette (2005) consisted of the 2 reciprocally monophyletic clades previously reported for avian species of *Plasmodium* and *Haemoproteus* (Bensch et al., 2004; Fallon et al., 2005; Ricklefs et al., 2004). Therefore we assigned our haplotypes to 1 of these 2 genera according to whether they grouped with the *Plasmodium* or *Haemoproteus* clade (Fig. 2). Identification of *Plasmodium* species by visual examination of blood smears from 2 New York birds was consistent with their assignment by DNA sequence, but we did not have sufficient morphological information to identify these parasites below the genus level.

Five unique *Plasmodium* cytochrome *b* haplotypes were defined by 39 variable nucleotide sites. Transition differences occurred at 15 sites and transversions at 22 sites, and 2 sites contained both transitions and transversions. There were no insertions or deletions and thus the nucleotide alignment was unambiguous. Sequence divergence among haplotypes (uncorrected "p" distance) ranged from 0.18% to 6.4%.

Patterns of nucleotide substitutions among haplotypes

The 5 *Plasmodium* haplotypes we recovered and the 1 previously recovered by Szymanski and Lovette (2005) formed 3 clusters in the minimum spanning network (Fig. 2.1b). The common widespread haplotype E1 differed from F2 by 34 nucleotide

substitutions. Two haplotypes found in Idaho and California, F2 and F3, differed from each other by 1 nucleotide substitution (0.18%), while F1 differed from F2 by 4 substitutions (0.73%). Haplotype AZ differed from F1 and F2 by an intermediate level of divergence of 12 to 13 substitutions (2.2% and 2.4%), respectively.

Employing the divergence threshold of 1.2% used by Ricklefs and Fallon (2002), which defines strongly supported sister parasite lineages in a diverse avian host range on a global scale, we detected 3 lineages of *Plasmodium*: E1, F1-F2-F3, and AZ. House finches from the western region of North America (California, Idaho, and Arizona) harbored 4 haplotypes representing 3 lineages; birds from the eastern region (New York and Georgia) harbored 2 haplotypes representing 2 lineages.

Distribution of Plasmodium haplotypes among house finch populations

Haplotype E1 was found in high proportions in all house finch populations. Haplotype F1 was detected only in New York and Georgia at lower frequencies than E1. Haplotypes F2 and F3 were detected only in California and Idaho (Fig. 2.1a).

Proportions of E1 and non-E1 haplotypes did not differ significantly between New York and Georgia (Fisher's exact test, 2-tailed P=0.13,) or between California and Idaho (P=0.35). We therefore pooled New York with Georgia (n=45) and California with Idaho (n=28) and compared proportions of E1 between pooled sites. Haplotype E1 occurred at a significantly higher frequency in New York and Georgia than in California and Idaho (P=0.003). Power analysis indicated that a sample size of 24 was required to detect differences in the proportions of E1 versus non-E1 haplotypes in each pooled population with a power of 0.80 at the 0.05 significance level.

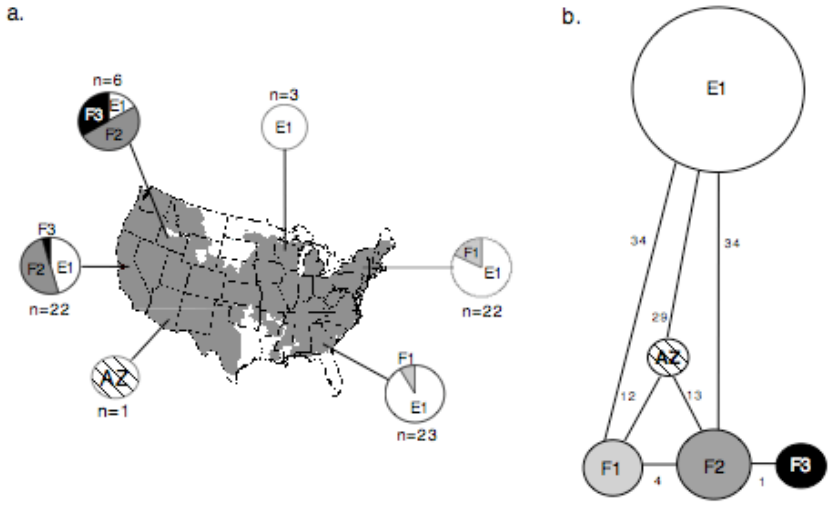

Figure 2.1. (a) Distribution of *Plasmodium* cytochrome *b* haplotypes among house finch populations. Haplotypes E1 and F1 were previously reported in house finches by Szymanski and Lovette (2005). (b) Minimum spanning network of *Plasmodium* cytochrome b haplotypes. Each circle represents a unique haplotype. The size of each circle is proportional to its representation in the total sample from all sites combined. Numbers on the connecting lines indicate the inferred number of base pair changes among haplotypes.

Discussion

Plasmodium cytochrome *b* haplotypes were distributed in a non-random pattern across the North American range of the house finch, consistent with the idea that geographic factors contribute to parasite phylogeographic structuring. This pattern of haplotype distribution suggests that host-parasite dynamics differ between the eastern and western portions of the house finch's range. The genetic differences in parasite haplotypes found in different house finch populations are unlikely to be due to genetic variation among the birds, because eastern house finches are derived from a relatively small number of recently introduced native western individuals.

An intensive survey of passerines in Ithaca, New York found 3 haplotypes of *Plasmodium* infecting house finches (Szymanski and Lovette, 2005), of which we

recovered two (E1 and F1). Haplotype E1 was identical to PA of Fallon et al. (2004), which they found in 2 avian host species on 5 islands in the Lesser Antilles (*Coereba flaveola*, Bananaquit [Coerebidae] and *Loxigilla noctis*, Lesser Antillean bullfinch [Emberizidae]), and to haplotype 56 of Ricklefs and Fallon (2002) which was identified in Missouri, USA in *Passerina cyanea* (Indigo bunting [Cardinalidae]). Moreover, a more recent, broader survey by Fallon et al. (2005) recovered E1 (PA) from 52 individuals representing 27 host species from 9 locations in the Lesser Antilles, North America and South America. E1 was also previously found in three other host species in New York besides house finches: *Tachycineta bicolor* (Tree swallow [Hirundinidae]) from New York and California, *Dendroica petechia* (Yellow warbler [Parulidae]) and *Melospiza melodia* (Song sparrow [Emberizidae]); F1 was found in *T. bicolor* and *M. georgiana* (Swamp sparrow [Emberizidae]), also in New York, both distantly related to house finches (Szymanski and Lovette, 2005). Haplotype E1 may, therefore, represent a *Plasmodium* species with a particularly broad geographic and host distribution.

A study of haematozoan prevalence in house finches using blood smear microscopy found 6% infected with *P. relictum* and 8% with *H. fringillae* in Georgia, and 2% with *P. relictum* and 3% with *H. fringillae* in New York (B. Hartup, pers. comm.). Since *P. relictum* was the only species identified using morphological criteria in house finches, and haplotype E1 occurs in such high frequency at the 2 sites in Hartup's study, we tentatively hypothesize that E1 represents *P. relictum*. Blood samples were not available from the individuals examined in Hartup's study, however, so we cannot definitively link this morphological species to mtDNA data. At least 5 morphologically defined *Plasmodium* species are documented to occur in the house finch: *P. relictum (= P. praecox*), *P. cathemerium*, *P. elongatum*, *P. vaughani* (= *P. hexamerium*) and *P. nucleophilum* (Bennett et al., 1982), but experimental infections

under controlled conditions would be required to determine the degree of concordance between morphological and molecular data in these parasite species.

Plasmodium relictum is 1 of 3 *Plasmodium* species that infects Passeriformes on every continent (Valkiunas, 2005). This cosmopolitan distribution would be consistent with its broad representation in our study and others. A recent study of the distribution of haematozoa across the range of a long-distance migratory bird, *Luscinia svecica* (Bluethroat), found a similar pattern in *Leucocytozoon*, with 1 widespread common cytochrome *b* haplotype and rarer haplotypes exhibiting frequency differences among sites (Hellgren, 2005). The similar breadth of host range of haplotypes E1 and F1, considered with their different distributions and congruent patterns with other haematozoan genera, further suggests that non-host geographic effects could be important drivers of parasite geographic distribution. Both spatial and temporal variability in *Haemoproteus* cytochrome *b* haplotype distribution occur across Scandinavian populations of the willow warbler, a long-distance migratory bird (Bensch and Åkesson, 2003). Because house finches are short-distance partial migrants, with only eastern populations undertaking predictable seasonal movements (Belthoff and Gauthreaux, 1991), they may harbor fewer haplotypes over time than long-distance migratory bird species, which are known to acquire haematozoan infections in both their breeding and over-wintering regions (Waldenström et al., 2002).

If avian host species distribution is not the sole determinant of phylogeographic structuring in *Plasmodium* spp., it is possible that vector interactions are important. The role of vectors in avian host-parasite associations has been examined in a few pioneering studies (Yezerinac and Weatherhead, 1995; Fonseca et al., 2000; Sol et al., 2000; Garvin and Greiner, 2003), but virtually nothing is known about geographic variation in vector competence for avian haematozoan parasites or

the level of vector specificity. In most cases we do not even know which vector species are competent for particular species of avian haematozoa. Future studies would benefit from a consideration of vector interactions with birds and parasites.

The high prevalence of *Plasmodium* species in house finches contrasts with other studies that have found it to be uncommon in many avian taxa relative to other haematozoa, especially *Haemoproteus* (Greiner et al., 1975; Valkiunas et al., 2003). Because they are more sensitive and better able to detect chronic infections, PCR assays consistently reveal higher prevalences than previously estimated using the traditional method of microscopic examination of stained blood films (Perkins et al., 1998; Richard et al., 2002; Jarvi et al., 2003; Fallon et al., 2004; Ribeiro et al., 2005). PCR screening revealed prevalence in New York house finches of 39% compared to 7% found by B. Hartup using blood smears; in Georgia, 45% were found to be infected using PCR and 17% using smears (Table I). Different individuals were sampled in these 2 studies, but the same locations were sampled over the same time period. Our prevalence may thus be elevated by the greater efficiency of detection by PCR over microscopy, rather than reflecting a unique interaction between parasite and host in this study. *Plasmodium* spp. prevalence is often underestimated using blood smear microscopy (Valkiunas and Iezhova, 2001). This bias would be particularly strong in the house finches in our study that were mostly sampled during the non-breeding season, when parasitemia tends to be low. One hypothesis is that malarial parasitemia is highest during the breeding season because the physiological stress of reproduction facilitates relapse (Richner et al., 1995), possibly due to hormonal changes (Pearson, 2002).

Our finding of regional differences in disease dynamics among host populations is not surprising given the wide range of environmental variation across North America and the variable community composition of both alternate hosts and

potential vectors. Other house finch diseases have a geographic component. For example, avian pox is a viral disease whose prevalence is unusually high among house finch populations in western North America, but which is virtually nonexistent in the east (although it has been recently reported in low prevalence in one eastern population (Hartup et al., 2004)). Another well-studied example is the different susceptibility of eastern and western house finch populations to *Mycoplasma gallisepticum*, a pathogenic bacterium that causes conjunctivitis that frequently leads to host mortality. Eastern populations are currently suffering an intense epidemic that has not caused the same high levels of mortality in western populations (Dhondt et al., 2005). These continent-wide differences in pox prevalence, *Mycoplasma* sp. susceptibility, and *Plasmodium* haplotype frequencies could be due to geographic variation in ecological variables or differences in coevolutionary associations between native and introduced populations of the house finch, or an interaction between these. Ecological differences between regions, such as host or vector availability, are more likely to explain differences in pox prevalence than native or introduced status, however, as exemplified by introduced house finches on the Hawaiian islands that harbor a high prevalence of pox infection similar to birds on their native range (Zahn & Rothstein, 1999).

By demonstrating phylogeographic structuring of haematozoan lineages across a single avian host species, we have shown that parasite distribution may be influenced by geographic variables independent of host species distribution. Further study is needed to explore the relative importance of such ecological factors as variation in the distribution, abundance, and competence of arthropod vectors, different avian host communities, or evolutionary influences such as coadaptation between parasite, vector and host in affecting haematozoan distribution among their avian hosts.

REFERENCES

Badyaev, A.V., Hill, G.E., Beck, M., Dervan, A.A., Duckworth, R., Mcgraw, K.J., Nolan, P. and Whittingham, L. (2002). Sex-biased hatching order and adaptive population divergence in a passerine bird. *Science* 295, 316-318.

Beadell, J.S., Gering, E., Austin, J., Dumbacher, J.P., Peirce, M.A., Pratt, T.K., Atkinson, C.T. and Fleischer, R.C. (2004). Prevalence and differential host-specificity of two avian blood parasite genera in the Australo-Papuan region. *Molecular Ecology* 12, 3829-3844.

Belthoff, J.R. and Gauthreaux, S.A. (1991). Partial migration and differential winter distribution of House Finches in the eastern United-States. *Condor* 93, 374-382.

Bensch, S. and Åkesson, S. (2003). Temporal and spatial variation of hematozoans in Scandinavian willow warblers. *Journal of Parasitology* 89, 388-391.

Bensch, S., Pérez-Tris, J., Waldenström, J. and Hellgren, O. (2004). Linkage between nuclear and mitochondrial DNA sequences in avian malaria parasites: Multiple cases of cryptic speciation? *Evolution* 58, 1617-1621.

Bensch, S., Stjernman, M., Hasselquist, D., Östman, Ö., Hansson, B., Westerdahl, H. and Torres, P.R. (2000). Host specificity in avian blood parasites: A study of *Plasmodium* and *Haemoproteus* mitochondrial DNA amplified from birds. *Proceedings of the Royal Society Series B-Biological Sciences* 267, 1583-1589.

Casagrande, J.T., Pike, M.C. and Smith, P.G. (1978). Improved approximate formula for calculating sample sizes for comparing 2 binomial distributions. *Biometrics* 34, 483-486.

Dhondt, A.A., Altizer, S., Cooch, E.G., Davis, A.K., Dobson, A.P., Driscoll, M.J.L., Hartup, B.K., Hawley, D.M., Hochachka, W.M., Hosseini, P.R., Jennelle, C.S., Kollias, G.V., Ley, D.H., Swarthout, E.C.H. and Sydenstricker, K.V. (2005). Dynamics of a novel pathogen in an avian host: Mycoplasmal conjunctivitis in house finches. *Acta Tropica* 94, 77-93.

Elliott, J.J. and Arbib, R.S. (1953). Origin and status of the house finch in the eastern United States. *Auk* 70, 31-37.

Fallon, S.M., Bermingham, E. and Ricklefs, R.E. (2003). Island and taxon effects in parasitism revisited: Avian malaria in the Lesser Antilles. *Evolution* 57, 606-615.

Fallon, S.M., Bermingham, E. and Ricklefs, R.E. (2005). Host specialization and geographic localization of avian malaria parasites: A regional analysis in the Lesser Antilles. *American Naturalist* 165, 466-480.

Fallon, S.M., Ricklefs, R.E., Swanson, B.L. and Bermingham, E. (2004). Detecting avian malaria: an improved polymerase chain reaction diagnostic. *Journal of Parasitology* 89(5), 1044-1047.

Fonseca, D.M., Lapointe, D.A. and Fleischer, R.C. (2000). Bottlenecks and multiple introductions: population genetics of the vector of avian malaria in Hawaii. *Molecular Ecology* 11, 1803-1814.

Garvin, M.C. and Greiner, E.C. (2003). Ecology of *Culicoides* (Diptera: Ceratopogonidae) in southcentral Florida and experimental *Culicoides* vectors of the avian hematozoan *Haemoproteus danilewskyi kruse*. *Journal of Wildlife Diseases* 39, 170-178.

Greiner, E.C., Bennett, G.F., White, E.M. and Coombs, R.F. (1975). Distribution of avian hematozoa of North America. *Canadian Journal of Zoology* 53, 1762-1787.

Hartup, B.K., Stott-messick, B., Guzy, M. and Ley, D.H. (2004). Health survey of house finches (*Carpodacus mexicanus*) from Wisconsin. *Avian Diseases* 48, 84-90.

Hawley, D.M., Hanley, D., Dhondt, A.A. and Lovette, I.J. (2006). Molecular evidence for a founder effect in invasive house finch (*Carpodacus mexicanus*) populations experiencing an emergent disease epidemic. *Molecular Ecology* 15, 263-275.

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

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

Lajeunesse, M.J. and Forbes, M.R. (2002). Host range and local parasite adaptation. *Proceedings of the Royal Society Series B-Biological Sciences* 269, 703-710.

Pearson, R.D. (2002). Is prolactin responsible for avian, saurian, and mammalian relapse and periodicity of fever in malarial infections? *Canadian Journal of Zoology* 7, 1313-1315.

Perkins, S.L., Osgood, S.M. and Schall, J.J. (1998). Use of PCR for detection of subpatent infections of lizard malaria: Implications for epizootiology. *Molecular Ecology* 7, 1587-1590.

Ribeiro, S.F., Sebaio, F., Branquinho, F.C.S., Marini, M.A., Vago, A.R. and Braga, E.M. (2005). Avian malaria in Brazilian passerine birds: parasitism detected by nested PCR using DNA from stained blood smears. *Parasitology* 130, 261-267.

Richard, F.A., Sehgal, R.N.M., Jones, H.I. and Smith, T.B. (2002). A comparative analysis of PCR-based detection methods for avian malaria. *Journal of Parasitology* 4, 819-822.

Richner, H., Christe, P. and Oppliger, A. (1995). Paternal investment affects prevalence of malaria. *Proceedings of the National Academy of Sciences USA* 92, 1192-1194.

Ricklefs, R.E. and Fallon, S.M. (2002). Diversification and host switching in avian malaria parasites. *Proceedings of the Royal Society Series B-Biological Sciences* 269, 885-892.

Ricklefs, R.E., Fallon, S.M. and Bermingham, E. (2004). Evolutionary relationships, cospeciation, and host switching in avian malaria parasites. *Systematic Biology* 53, 111-119.

Seutin, G., White, B.N. and Boag, P.T. (1991). Preservation of avian blood and tissue samples for DNA analysis. *Canadian Journal of Zoology* 69, 82-90.

Sol, D., Jovani, R. and Torres, J. (2000). Geographical variation in blood parasites in feral pigeons: the role of vectors. *Ecography* 3, 307-314.

Swofford, David L. (2003). PAUP* Phylogenetic Analysis Using Parsimony (*and Other Methods).

Szymanski, M.M. and Lovette, I.J. (2005). High lineage diversity and host sharing of malarial parasites in a local avian assemblage. *Journal of Parasitology* 91, 768-774.

Valkiunas, G. (2001). Blood parasites of birds: some obstacles in their use in ecological and evolutionary biology studies. *Avian Ecology & Behavior* 7, 87-100.

———. (2005) *Avian malaria parasites and other haemosporidia*. CRC press, Boca Raton.

Valkiunas, G. and Iezhova, T.A. (2001). A comparison of the blood parasites in three subspecies of the yellow wagtail *Motacilla flava*. *Journal of Parasitology* 87, 930-934.

Valkiunas, G., Iezhova, T.A. and Shapoval, A.P. (2003). High prevalence of blood parasites in hawfinch *Coccothraustes coccothraustes*. *Journal of Natural History* 37, 2647-2652.

Waldenström, J., Bensch, S., Kiboi, S., Hasselquist, D. and Ottosson, U. (2002). Cross-species infection of blood parasites between resident and migratory songbirds in Africa. *Molecular Ecology* 11, 1545-1554.

Wang, Z., Baker, A.J., Hill, G.E. and Edwards, S.V. (2003). Reconciling actual and inferred population histories in the house finch (*Carpodacus mexicanus*) by AFLP analysis. *Evolution* 57, 2852-2864.

White, E.M., Greiner, E.C., Bennett, G.F. and Herman, C.M. (1978). Distribution of the hematozoa of Neotropical birds. *Revista De Biología Tropical* 26, 43-102.

Yezerinac, S.M. and Weatherhead, P.J. (1995). Plumage coloration, differential attraction of vectors and haematozoa infections in birds. *Journal of Animal Ecology* 64, 528-537.

Zahn, S.N. and Rothstein, S.I. (1999). Recent increase in male house finch plumage variation and its possible relationship to avian pox disease. *Auk* 116, 35-44.

Zar, J.H. (1984) *Biostatistical analysis*. Prentice Hall, Englewood Cliffs.

CHAPTER 3

AVIAN MALARIA PARASITES SHARE CONGENERIC MOSQUITO VECTORS

Abstract

Assessing a parasite's specificity to vector species is crucial to understanding the emergence of vector-borne diseases into new hosts and the temporal and spatial dynamics of parasite infections. Avian malaria parasites in the genus *Plasmodium* have a cosmopolitan distribution and broad avian host ranges, which together suggest that they are likely vector-generalists. We tested this prediction by asking if and to what extent different mosquito species were naturally infected in the field with identical avian *Plasmodium* lineages. We sequenced a standard *Plasmodium* cytochrome *b* marker from parasites infecting individuals of three ornithophilic mosquito species abundant in the northeastern United States: *Culex pipiens pipiens*, *Cx. restuans*, and *Ochlerotatus canadensis*. We found five clades of *Plasmodium* haplotypes in these mosquitoes. Four of these clades were found in both *Cx. pipiens* and *Cx. restuans*, and one of these also infected *Oc. canadensis*. The fifth clade was found only in *Cx. restuans*. One of the clades contained many unique haplotypes that did not match any previously known avian *Plasmodium* sequences, suggesting that the diversity of *Plasmodium* lineages is higher than has been found via limited sampling avian communities. One common *Plasmodium* haplotype found previously in birds was also present in all three mosquito species. However, another common *Plasmodium* haplotype previously found in numerous bird species, occurred in low frequency, and only in *Cx. restuans*. We therefore found limited support for our prediction that avian *Plasmodium* vector breadth accompanies host breadth. The association of both *Culex* species with four of five *Plasmodium* clades we found in our

study, and the presence of a single parasite haplotype in three mosquito species representing two genera, suggests that avian *Plasmodium* parasites have not tightly coevolved with their vectors at lower taxonomic levels.

Introduction

Vector-borne parasitic diseases present a major challenge to understanding host-parasite dynamics, because vector-parasite as well as vector-host interactions must be considered. Many vertebrate parasites rely on blood-feeding insect vectors for transmission to new hosts. A parasite ingested in a blood meal must evade the insect's immune system and complete development while the insect remains alive long enough to feed on another host. In the case of malaria (Haemosporida: Plasmodiidae), vector-parasite associations are particularly important because the sexual reproduction of the parasite occurs in the mosquito vector (Diptera: Culicidae), making the vector the definitive host.

Malaria is a particularly well-studied vector-borne disease in humans, but also has enormous impacts on the health of humans and wildlife. Despite many years of research and eradication efforts, however, human malaria has experienced a major resurgence (Lindsay and Martens, 1998). In many cases, the interactions between malaria parasites and their mosquito vectors are complex: they can be affected by both genetic and environmental factors as well as interactions among them (Tripet et al., 2008). Crucial to understanding the potential role of vectors in influencing the dynamics of vector-borne diseases is assessing the parasite's specificity to the vector as well as to the host. Parasites able to exploit a wider range of vector species gain access to a higher diversity of potential hosts, and thus could be expected to emerge into new host populations or species more readily than would vector specialists.

Malaria epidemics have had profound impacts on birds as well as humans; for example, the inadvertent introduction in the early 1800s of the avian malaria vector, *Culex quinquefasciatus*, and subsequent emergence of *Plasmodium relictum* into the native avifauna on the Hawaiian islands has been implicated in the extinction of many bird species in that archipelago (Atkinson et al., 1995). In zoos, naïve birds become vulnerable to avian malaria when they are brought into captivity in close proximity to infected birds and local mosquito vectors (Fix et al., 1988; Schrenzel et al., 2003). In nature, avian malaria parasites in the genus *Plasmodium* have a cosmopolitan distribution and occur naturally in nearly all avian taxa (Valkiunas, 2005). The relative scarcity of data on the ecology of vectors of avian malaria and other related blood parasites seems particularly acute given the recent attention to the role of these parasites in the ecology and evolution of birds (Hamilton and Zuk, 1982; Ricklefs, 1992; Martin et al., 2001; MacDougall-Shackleton et al., 2002; Waldenström et al., 2002; Marzal et al., 2005; Møller and Nielsen, 2007; Marzal et al., 2008).

Avian *Plasmodium* lineages form geographic patterns in single avian host species, at both large and small geographic scales (Beadell et al., 2006; Kimura et al., 2006; Wood et al., 2007), suggesting that these patterns may be driven by variation in vector ecology across geographic regions. Two recently published long-term studies of avian host populations have revealed temporally fluctuating patterns of prevalence in different *Plasmodium* lineages that may be tied to variation in vector phenology (Bensch et al., 2007; Cosgrove et al., 2008), but data on vectors are needed to evaluate their importance in these systems. Vector ecology could be a particularly important factor in the dynamics of avian blood parasites in their hosts if parasite species exhibited vector-specificity. Spatial variation in vector community composition could be caused by variation among mosquito species in the specificity of their oviposition sites (Eldridge and Edman, 2000), resting sites, canopy height (Lothrop and Reisen,

2001), passive aggregation (e.g. via wind) or spatial patterns in adult mortality (Ellis, 2008). These habitat differences could result in highly localized differences in the transmission of different *Plasmodium* lineages. Furthermore, mosquito species exhibit variation in phenology, with some species maintaining high population densities throughout the season and others showing a peak and decline (Barker et al., 2003). Temporal patterns of the prevalence of vector-specific *Plasmodium* lineages could likewise be driven by these differences in vector phenology.

Because many *Plasmodium* lineages show little specificity to avian species, genus, family or sometimes order (Ricklefs and Fallon, 2002; Beadell et al., 2004; Fallon et al., 2005; Beadell et al., 2006), even if avian host specificity had originally facilitated the diversification of these parasites via cospeciation, subsequent host switching and sharing have obscured most coevolutionary associations (Ricklefs et al., 2004). Evidence exists, however, for the coevolution of avian blood parasites with their vectors. A recent multi-gene phylogeny of many diverse avian and saurian haemosporidian taxa showed that major deeper nodes in the tree correspond to switches of parasites into families of Dipteran vectors (Martinsen et al., 2008). Moreover, Gager et al. (2008) found unique avian *Plasmodium* lineages isolated from two tropical mosquito genera, but the absence of infected congeneric mosquito species in that study did not allow for a test of within-genus vector-specificity. Human malaria parasite species are known to be specific to mosquito species in the genus *Anopheles* alone and these mosquito species are known to differ in their ability to evade malaria infection (Ponnudurai et al., 1988). To date, no studies have asked whether avian *Plasmodium* lineages are shared among congeneric ornithophilic mosquito species.

The broad avian host breadth and widespread geographic distribution of many *Plasmodium* lineages could be mediated by a broad vector range, where parasites

could exploit multiple vector species and ultimately gain access to diverse avian hosts in different habitats. The high lineage diversity found in avian *Plasmodium* coupled with the absence of avian host specificity (Beadell et al., 2004), however, leads to the opposite prediction: that parasite-vector specificity reflects a close coevolutionary history between parasite and vector. In this study, we tested these opposing predictions by asking if and to what extent different mosquito species are naturally infected with identical avian *Plasmodium* lineages. This study represents a step towards understanding the evolution of avian malaria parasites across an entire natural transmission system by explicitly asking whether avian malaria parasites in the genus *Plasmodium*, some of which are known to be avian host-generalists, are shared among vector species.

Materials and methods

Mosquito collection, species, and identification

Mosquitoes were collected in traps and resting on vegetation in aspirator collections in 2003 and 2004 in Ithaca, New York, USA. Two mosquito species, *Culex pipiens pipiens* and *Culex restuans*, were caught in bird-baited traps from June to September 2004, using *Gallus domesticus* (domestic chicken) or *Passer domesticus* (house sparrow) as described by Darbro and Harrington (2006). Only mated adult *Cx. p. pipiens* females will seek a blood meal; consequently, we increased the probability of sampling *Plasmodium*-infected individuals by utilizing bird-baited traps. Standard mosquito resting collection techniques, which involve aspirating mosquitoes from their resting habitat, produce high numbers of mosquitoes including males (that do not feed on blood) and virgin teneral females (that would not yet be seeking a blood meal). Females that have taken at least one previous blood meal represent the proportion capable of transmission in subsequent blood meals.

Individual mosquitoes were identified to species using standard taxonomic keys and confirmed with molecular methods (for details, see Darbro & Harrington, 2006; Harrington & Poulson, 2008). The two focal mosquito species were chosen because they represented over 90% of the individuals captured in the bird-baited traps throughout the sampling season. We also included three other abundant mosquito species, *Ochlerotatus aurifer*, *Ochlerotatus canadensis* and *Culiseta melanura*, which were collected by aspirating resting individuals on vegetation. These additional species were readily identified morphologically using standard taxonomic keys (Darsie and Ward, 2004). We also included a smaller number of aspirated resting *Cx. pipiens* and *Cx. restuans* individuals in our sample. All five mosquito species occupy similar forested habitat and utilize woodland pools or artificial containers for breeding, so we expected that they would encounter a similar suite of potential avian host species.

Culex pipiens is one of the best-studied candidate vector for avian *Plasmodium*, in part because of its continuing medical importance in West Nile virus transmission to humans (Turell et al., 2000), and because of its amenability to rearing in the laboratory. Most early laboratory avian malaria transmission experiments used *Cx. pipiens* as a vector for *P. relictum* (Huff, 1929; Tate and Vincent, 1934) and *P. cathemerium* (Huff, 1931). Both *Cx. pipiens* and *Cx. restuans* were previously found to be competent in the laboratory for *P. elongatum* in captive penguins (Beier and Trpis, 2001), and *Cx. restuans* was found to be competent for *P. forresteri*, which infects raptors (Telford et al., 1997). Blood meal analyses have shown *Cs. melanura* to be a strongly ornithophilic species, only occasionally feeding on mammals (Magnarelli, 1977; Molaei and Andreadis, 2006; Molaei et al., 2006). Little is known about the host feeding preferences of *Oc. aurifer* or *Oc. canadensis*, although the latter is known to feed on both birds and mammals (LeDuc et al., 1972).

In mosquitoes, *Plasmodium* life stages develop after infected red blood cells are ingested from an avian host. The parasite gametes fuse to form a zygote within the mosquito gut lumen. Zygotes then form oocysts on the mosquito that penetrate the mosquito midgut wall, eventually giving rise to sporozoites. Once sporozoites infect the salivary glands, they can be transmitted to a new host during mosquito blood feeding (Garnham, 1966). Because we wanted to include in this study only those parasites that had successfully reached the oocyst or sporozoite stage, rather than those parasites ingested in a blood meal from a chicken or sparrow inside the traps, for bird-baited mosquitoes we only sampled individual mosquitoes that 1) were physically excluded from feeding on birds in the trap by a mesh barrier or 2) did not contain blood in the abdomen upon visual inspection. For individuals collected via aspiration from resting sites, we only included individuals where 1) no blood was seen in the abdomen or 2) the abdomen had been removed.

Molecular identification of Plasmodium from mosquitoes and birds

Because malaria taxonomy is based only on morphology in the vertebrate blood stages, molecular methods are the only way to distinguish parasite taxa in their mosquito stages. Genomic DNA extracted from individual mosquitoes was screened for *Plasmodium* infection using primers R2 (Beadell et al., 2004) and HaemF2 (GGATGGTGTTTTAGATATATGCATG, modified from Bensch et al. (2000), which amplify 359 bp of *Plasmodium* cytochrome *b*, and where possible we sequenced an overlapping fragment using primers F2.Plas.2 (modified from Beadell et al., 2004) and H15725 (Ricklefs and Fallon, 2002), to obtain a total of 726 bp. Our primers were designed in regions of *Plasmodium* cytochrome *b* that are highly conserved among parasites that infect birds and those that infect rodents (*P. chabaudi*, Perkins et al., 2007). The fact that the same primers we used in our study readily amplify *Plasmodium* from infected bird blood samples suggests that these primers are robust

enough to detect infection in a broad range of background host DNA, and a consistent inability to amplify a particular parasite lineage from any given mosquito species most likely represents the true absence of that lineage, not simple PCR failure. We employed a conservative standard for scoring individual mosquitoes as infected; we only scored positive infections if we were able to obtain readable sequence. Although some samples showed faint amplification of a similar-sized fragment, we scored faint amplifications as not infected.

Sequences were aligned using Sequencher 4.2 (GeneCodes Corp., Ann Arbor, MI). Because there are no taxonomic characters for *Plasmodium* taxa while they are in the mosquito stages, lineages isolated from mosquitoes could not be assigned to morphological species. Where possible, we inferred taxonomic identity by assessing the phylogenetic affinities of mosquito-isolated *Plasmodium* lineages with published sequences from GenBank that were reliably identified to morphological species. To explore phylogenetic relationships among *Plasmodium* lineages isolated from mosquitoes, we used MrModeltest v.2 (Nylander, 2004) to determine the appropriate model of molecular evolution using hierarchical likelihood ratio tests. We then conducted a Bayesian phylogenetic analysis using MrBayes v.3.1.2 (Huelsenbeck and Ronquist, 2001), with 2 simultaneous runs of 10 million generations, with four incrementally heated chains, sampling trees every 2000 generations, and discarding the first 5 million generations as burn-in. We used 5 outgroup taxa: one avian haematozoan parasite species, *Haemoproteus balmorali*, and 4 *Plasmodium* species infecting mammals: *P. reichenowi*, *P. falciparum*, *P. malariae* and *P. chabaudi*. We performed another Bayesian analysis including all previously published avian *Plasmodium* cytochrome *b* sequences that aligned with the fragment we used in this study (tree not shown) to link these sequences to morphologically-defined

Plasmodium species identified by microscopy, their avian hosts and their mosquito vectors.

Results

Plasmodium prevalence in mosquitoes

A slightly greater proportion of *Cx. pipiens* (16.6%) than *Cx. restuans* (14.8%) yielded readable *Plasmodium* sequence. None of the vegetation-aspirated *Cx. restuans* individuals were infected with *Plasmodium*, but aspirated *Cx. pipiens* were infected in a similar proportion (13.3%) to the bird-baited *Cx. pipiens*. *Plasmodium* infection was detected in only 1 of the 181 *Oc. canadensis* screened. We did not detect *Plasmodium* infection in any of the *Oc. aurifer* or *Cs. melanura* individuals screened (Table 3.1).

Table 3.1. Mosquito species included in this study and their respective prevalences of *Plasmodium* infection.

Mosquito species	n screened	n infected and sequenced (%)
Culex p. pipiens	276	46 (16.6)
Culex restuans	150	22 (14.8)
Culiseta melanura	177	0
Ochlerotatus canadensis	161	1 (0.6)
Ochlerotatus aurifer	110	0
Total	813	68 (8)

Phylogenetic analysis and vector sharing across clades

The MrModelTest analysis indicated that GTR + I + G (general time-reversible + gamma + proportion invariant) was the most appropriate model of evolution. The phylogenetic analysis revealed five well-supported clades (99-100% posterior probability, Figure 3.1). Clade A contained many unique *Plasmodium* cytochrome *b* haplotypes not previously isolated from birds or assigned to morphospecies. This

clade included a single haplotype, Cx-1, found in high frequency in both *Cx. pipiens* (n=22) and *Cx. restuans* (n=10). *Plasmodium* isolated from both *Cx. pipiens* and *Cx. restuans* were represented in clades A-D, while only *Cx. restuans* was represented in clade E.

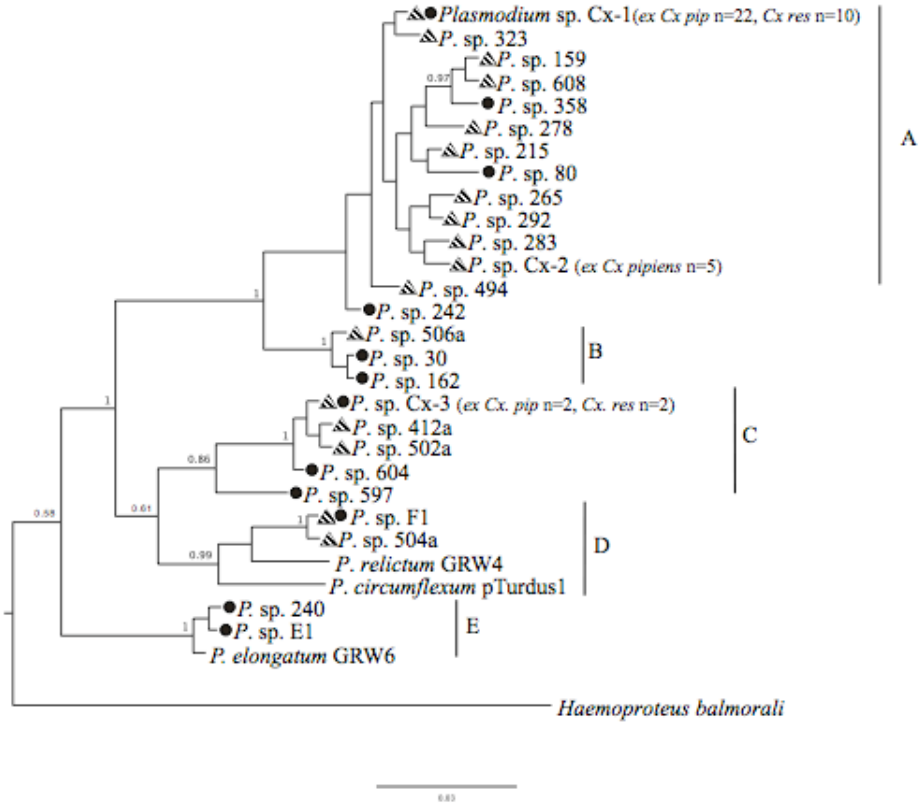

Figure 3.1. Bayesian phylogenetic analysis of mosquito-isolated *Plasmodium* cytochrome *b* (726 bp). All haplotypes were found in a single mosquito unless otherwise noted, with hatched triangles representing *Plasmodium* haplotypes isolated from *Cx. pipiens* and filled circles *Cx. restuans*. Major clades are indicated by letters. Numbers at nodes indicate mean posterior probabilities, with values under 0.5 omitted.

Parasite associations with mosquito vectors and avian hosts

Two distinct mosquito-isolated haplotypes were identical to those previously isolated from infected wild birds. Haplotype E1, isolated in this study from *Cx. restuans* only, was previously found in the same study area in bird species

representing several avian families (Szymanski and Lovette 2005; also reported in several bird species throughout N. America and the Lesser Antilles as haplotype PA by Fallon et al. 2005). Haplotype F1 was likewise isolated from bird species representing different families (Szymanski and Lovette, 2005) and was the second most common *Plasmodium* haplotype found in *Carpodacus mexicanus* (house finch) populations in the eastern United States (Kimura et al., 2006).

Three mosquito-isolated *Plasmodium* haplotypes matched bird-isolated haplotypes from this study (Table 3.2). Haplotype Cx-3 was found in both *Cx. pipiens* and *Cx. restuans* and a common local bird species, *Turdus migratorius* (American robin). Haplotype F1 was found in three mosquito species: *Cx. pipiens*, *Cx. restuans* and *Oc. canadensis*, and in three bird species, *C. mexicanus*, *Melospiza georgiana* (swamp sparrow) and *Tachycineta bicolor* (tree swallow), from previous studies conducted in the same study area (Kimura et al. 2006; Szymanski & Lovette, 2005). Haplotype F1 grouped closely with haplotype GRW4, previously identified as *P. relictum* and found to infect unrelated avian taxa across the globe (Waldenström & Bensch, 2002; Beadell et al. 2006). The most common haplotype isolated from both *Cx. pipiens* and *Cx. restuans*, Cx-1, was not previously isolated from any birds in this study or other studies.

Table 3.2. Avian hosts and *Plasmodium* morphospecies associated with clades A-E from Figure 3.1. Avian host associations with clades A-E are based on an additional Bayesian phylogenetic analysis (tree not shown). References are noted for previously published GenBank sequences.

	Clade A	Clade B	Clade C	Clade D	Clade E
Culicine hosts	*Cx. pipiens* *Cx. restuans*	*Cx. pipiens* *Cx. restuans*	*Cx. pipiens* *Cx. restuans*	*Cx. pipiens* *Cx. restuans* *Oc. canadensis*	*Cx. restuans*
Avian hosts		*T. migratorius*[1]		*C. mexicanus*[2,8] *T. bicolor*[2] *M. georgiana*[2]	*C. mexicanus*[2,8] *A. herodias*[7] *T. bicolor*[2] *M. melodia*[2] *D. petechia*[2] *P. cyanea*[1] *C. flaveola*[3] *L. noctis*[3]
Plasmodium morphospecies				*P. relictum* (GRW4)[4] *P. circumflexum* (pTURDUS1)[5]	*P. elongatum* (GRW6)[6]

[1](Ricklefs et al., 2005); [2](Szymanski and Lovette, 2005); [3](Fallon et al. 2005); [4](Valkiunas et al., 2007); [5](Palinauskas et al., 2007); [6](Valkiunas et al., 2008); [7](Beadell et al., 2006); [8](Kimura et al., 2006)

In an ongoing effort to integrate molecular and taxonomic data on avian blood parasites, recent studies have linked morphospecies to mtDNA cytochrome *b* haplotype by subinoculating naïve birds with parasites of known morphospecies (Palinauskas et al., 2007; Valkiunas et al., 2007). We used the same species to identify our mosquito-isolated *Plasmodium* lineages to species. Our phylogenetic analysis suggests that haplotype E1 corresponds to *P. elongatum* because both cluster in clade E, differing by a single nucleotide change. Both *P. relictum*, the most common morphospecies infecting passerines, and *P. circumflexum* group together in clade D. None of the three morphospecies for which DNA sequences are available falls into clade A.

Discussion

Plasmodium lineages share vector species

We found moderate support for our prediction that host breadth accompanies vector breadth, with one of the most common *Plasmodium* haplotypes infecting birds (F1, possibly *P. relictum*) also infecting three mosquito species, but another *Plasmodium* haplotype (E1, probably *P. elongatum*, widespread among bird taxa and geographic areas) infecting just one mosquito species. A previous study (Kimura et al. 2006) found E1 to be the most common *Plasmodium* haplotype occurring in *C. mexicanus* in Ithaca, NY and at several other sampling sites across N. America; Szymanski and Lovette (2005) isolated it from four avian host species (*Tachycineta bicolor*, *Melospiza melodia*, *Dendroica petechia* and *C. mexicanus*) in the same study area, and Fallon et al. (2005) found the same haplotype ("PA") in both resident and migratory birds on several islands across the Lesser Antilles. The high *Plasmodium* prevalence in *Cx. pipiens* and *Cx. restuans* relative to other abundant mosquitoes in our study area suggests that they are important vectors. A similar proportion of adult female *Cx. quinquefasciatus* (12%) was found to be infected with *P. relictum* in Hawaii, where they are known to be the dominant vector species (Reiter and LaPointe, 2007). It is therefore curious that we isolated E1 just once from *Cx. restuans* and not at all from *Cx. pipiens*.

The high level of sharing of *Plasmodium* lineages between *Cx. pipiens* and *Cx. restuans* might be due to their similar ecology; they are known ecological competitors in their larval stages (Reiskind and Wilson, 2008) and both feed primarily on birds (Magnarelli, 1977). Their high rate of capture in the same traps indicate that they have similar foraging habits (Darbro and Harrington, 2006). The finding of haplotype F1 in two distantly-related mosquito genera (*Ochlerotatus* and *Culex*) further suggests that

vector phylogenetic relatedness may not be a reliable index of their ability to become infected with the same *Plasmodium* lineage.

The evolution of major clades of haemosporidian parasites correlates with vector shifts into different Dipteran families, presumably by giving parasites access to new hosts (Martinsen et al., 2008). This has been shown on a broad phylogenetic scale, and evidence exists for partitioning of lineages among divergent mosquito genera (Gager et al. 2008). Our data demonstrate that at least some *Plasmodium* lineages are found across these congeneric and locally sympatric mosquito species. The same *Plasmodium* haplotype, F1, was shared by two mosquito species (*Cx. pipiens* and *Oc. canadensis*) representing two genera, showing that *Plasmodium* vector sharing also occurs at the mosquito genus level. More sampling of divergent mosquito taxa needs to be added to the growing data set of vector-isolated *Plasmodium* to understand patterns of Dipteran host sharing. Although robust molecular phylogenies for the *Culex* and *Ochlerotatus* genera are presently not available, one phylogenetic hypothesis based on morphological characters suggests that *Cx. pipiens* and *Cx. restuans* are not sister taxa (Miller et al., 1996). The position of *Ochlerotatus* relative to *Culex* is not known.

Differential Plasmodium prevalence in mosquitoes and evidence for local transmission

Just 7 of 78 described species in the mosquito genus *Culex* have documented competence for avian *Plasmodium* species, most commonly *Cx. quinquefasciatus*, closely related to *Cx. pipiens* (LaPointe et al., 2005). We were therefore not expecting to find equal *Plasmodium* prevalences in all the mosquito species we tested. Our results confirm a general finding from other studies (LaPointe et al., 2005; Gager et al., 2008) that vector competence, and by extension the probability of avian *Plasmodium* infection, varies among mosquito species. Our inability to detect

Plasmodium infection in *Oc. aurifer* and *Cs. melanura* suggest that these species are not as important as *Cx. pipiens* and *Cx. restuans* as competent vectors at our study site. Given the high prevalence of *Plasmodium* (15-17%) in these two latter species, it is unlikely that the absence of *Plasmodium* infection in the other species can be explained entirely by the difference in sampling effort.

The occurrence of so many *Plasmodium* cytochrome *b* haplotypes in the two most abundant ornithophagic mosquito species, and the co-occurrence of some of those haplotypes in diverse avian host species at our study site, provide evidence that transmission occurs at our study site. This finding contrasts with the conclusion of Bensch et al. (2000) that transmission of avian haemosporidia to migratory birds occurs largely on the wintering grounds. Other studies have inferred low rates of transmission on the wintering grounds due to the low prevalence of infected migrants sampled there (Bennett et al., 1980; Garvin et al., 2004). Consistent with the results from this study, Szymanski et al. (2005) concluded that transmission occurs on the breeding grounds because they detected infections of both *Haemoproteus* and *Plasmodium* species in recently fledged birds still in their natal area. More sampling of *Plasmodium* vectors in both breeding and wintering areas of migratory birds would help to elucidate transmission dynamics over the annual cycle of migratory birds.

Patterns of Plasmodium lineage diversity

The high representation among mosquitoes of *Plasmodium* haplotypes forming a clade not previously recovered from vertebrate hosts suggests that we did not sample the relevant avian hosts in our system. Testing this hypotheses would require future studies to sample a greater diversity of hosts and other vertebrates in our study area, and to conduct blood meal analyses to identify the range of hosts fed upon by local mosquitoes. Most studies on avian malaria parasite genetic diversity are heavily

biased towards small passerine birds that are easily captured in mist nets, and our results suggest that these may not be the most important hosts in our study area.

The high frequency of *Plasmodium* haplotype Cx-1 and closely related haplotypes in our sample of mosquito-isolated *Plasmodium* and their absence in birds to date suggests that avian *Plasmodium* is even more diverse, at least at the cytochrome *b* locus, than previously realized. The elevated diversity in our sample relative to that found by Gager et al. (2008) could simply reflect differences found in the two study areas (temperate versus tropical). It could also indicate the possibility that pooling multiple mosquitoes during DNA extraction, as employed by Gager et al., reduces the probability of isolating unique parasite haplotypes by increasing the ratio of mosquito background DNA in the sample.

Conclusions

The dynamics of vector-borne diseases depend upon a complex interaction among host, vector and parasite. Because many birds regularly migrate vast geographic distances, the ability to infect different vector species could further facilitate a parasite's colonization of new hosts inhabiting different regions. For example, a broad vector range has been hypothesized to facilitate the radiation of primate malarias in southeast Asia which may have led to the emergence of *Plasmodium* in humans (Escalante et al., 1998). Our results provide mixed support for the hypothesis that avian *Plasmodium* vector breadth accompanies host breadth: one of the most common avian malaria parasite haplotypes was isolated from three mosquito species in our study area, but the other was found in just one mosquito species. We have presented definitive evidence that avian malaria parasites infect multiple vector species, that major parasite clades are shared between congeneric mosquito species, and that mosquito vectors harbor a high diversity of avian malaria parasites.

REFERENCES

Atkinson, C.T., Woods, K.L., Dusek, R.J., Sileo, L.S. and Iko, W.M. (1995). Wildlife disease and conservation in Hawaii: Pathogenicity of avian malaria (*Plasmodium relictum*) in experimentally infected Iiwi (*Vestiaria coccinea*). *Parasitology* 111, S59-S69.

Barker, C.M., Paulson, S.L., Cantrell, S. and Davis, B.S. (2003). Habitat preferences and phenology of *Ochlerotatus triseriatus* and *Aedes albopictus* (Diptera: Culicidae) in southwestern Virginia. *Journal of Medical Entomology* 40, 403-410.

Beadell, J.S., Gering, E., Austin, J., Dumbacher, J.P., Peirce, M.A., Pratt, T.K., Atkinson, C.T. and Fleischer, R.C. (2004). Prevalence and differential host-specificity of two avian blood parasite genera in the Australo-Papuan region. *Molecular Ecology* 12, 3829-3844.

Beadell, J.S., Ishtiaq, F., Covas, R., Melo, M., Warren, B.H., Atkinson, C.T., Bensch, S., Graves, G.R., Jhala, Y.V., Peirce, M.A., Rahmani, A.R., Fonseca, D.M. and Fleischer, R.C. (2006). Global phylogeographic limits of Hawaii's avian malaria. *Proceedings of the Royal Society Series B-Biological Sciences* 273, 2935-2944.

Beier, J.C. and Trpis, M. (2001). Incrimination of natural culicine vectors which transmit *Plasmodium elongatum* to penguins at the Baltimore Zoo. *Canadian Journal of Zoology* 59, 470-475.

Bennett, G.F., Witt, H. and White, E.M. (1980). Blood parasites of some Jamaican birds. *Journal of Wildlife Diseases* 16, 29-38.

Bensch, S., Waldenstrom, J., Jonzen, N., Westerdahl, H., Hansson, B., Sejberg, D. and Hasselquist, D. (2007). Temporal dynamics and diversity of avian malaria parasites in a single host species. *Journal of Animal Ecology* 76, 112-122.

Bensch, S., Stjernman, M., Hasselquist, D., Östman, Ö., Hansson, B., Westerdahl, H. and Torres, P.R. (2000). Host specificity in avian blood parasites: A study of *Plasmodium* and *Haemoproteus* mitochondrial DNA amplified from birds. *Proceedings of the Royal Society Series B-Biological Sciences* 267, 1583-1589.

Cosgrove, C.L., Wood, M.J., Day, K.P. and Sheldon, B.C. (2008). Seasonal variation in *Plasmodium* prevalence in a population of blue tits *Cyanistes caeruleus*. *Journal of Animal Ecology* 77, 540-548.

Darbro, J.M. and Harrington, L.C. (2006). Bird-baited traps for surveillance of West Nile mosquito vectors: Effect of bird species, trap height, and mosquito escape rates. *Journal of Medical Entomology* 43, 83-92.

Darsie, R.F., and R.A. Ward. (2004) *Identification and geographical distribution of the mosquitoes of North America, north of Mexico*. University Press of Florida, Gainesville.

Eldridge, B.F., and J.D. Edman. (2000) *Medical Entomology: a textbook on public health and veterinary problems caused by arthropods*. Kluwer Academic Publishers, Dordrecht.

Ellis, A.M. (2008). Linking movement and oviposition behaviour to spatial population distribution in the tree hole mosquito *Ochlerotatus triseriatus*. *Journal of Animal Ecology* 77, 156-166.

Escalante, A.A., Freeland, D.E., Collins, W.E. and Lal, A.A. (1998). The evolution of primate malaria parasites based on the gene encoding cytochrome *b* from the linear mitochondrial genome. *Proceedings of the National Academy of Sciences* 95, 8124-8129.

Fallon, S.M., Bermingham, E. and Ricklefs, R.E. (2005). Host specialization and geographic localization of avian malaria parasites: A regional analysis in the Lesser Antilles. *American Naturalist* 165, 466-480.

Fix, A.S., Waterhouse, C., Greiner, E.C. and Stoskopf, M.K. (1988). *Plasmodium relictum* as a cause of avian malaria in wild-caught magellanic penguins (*Spheniscus magellanicus*). *Journal of Wildlife Diseases* 24, 610-619.

Gager, A.B., Del Rosario Loaiza, J., Dearborn, D.C. and Bermingham, E. (2008). Do mosquitoes filter the access of *Plasmodium* cytochrome *b* lineages to an avian host? *Molecular Ecology* 17, 2552-2561.

Garnham, P.C.C. (1966) *Malaria parasites and other haemosporidia*. Blackwell Scientific Publications, Oxford.

Garvin, M.C., Marra, P.P. and Crain, S.K. (2004). Prevalence of hematazoa in overwintering American Redstarts (*Setophaga ruticilla*): no evidence for local transmission. *Journal of Wildlife Diseases* 40, 115-118.

Hamilton, W.D. and Zuk, M. (1982). Heritable true fitness and bright birds: a role for parasites? *Science* 218, 384-387.

Harrington, L.C. and Poulson, R.L. (2008). Considerations for accurate identification of adult *Culex restuans* (Diptera: Culicidae) in field studies. *Journal of Medical Entomology* 45, 1-8.

Huelsenbeck, J.P. and Ronquist, F. (2001). MRBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics* 17, 754-755.

Huff, C.G. (1931). The inheritance of natural immunity to *Plasmodium cathemerium* in two species of *Culex*. *Journal of Preventive Medicine* 5, 249-259.

———. (1929). The effects of selection upon susceptibility to bird malaria in *Culex pipiens*. *Linn Ann Trop Med Parasitol.* 23, 427–439.

Kimura, M., Dhondt, A.A. and Lovette, I.J. (2006). Phylogeographic structuring of *Plasmodium* lineages across the North American range of the House finch (*Carpodacus mexicanus*). *Journal of Parasitology* 92, 1043-1049.

LaPointe, D.A., Goff, M.L. and Atkinson, C.T. (2005). Comparative susceptibility of introduced forest dwelling mosquitoes in Hawai'i to avian malaria, *Plasmodium relictum*. *Journal of Parasitology* 91, 843-849.

LeDuc, J.W., Suyemoto, W., Eldridge, B.F. and Saugstad, E.S. (1972). Ecology of arboviruses in a Maryland freshwater swamp. II. Blood feeding patterns of potential mosquito vectors. *American Journal of Epidemiology* 96, 123-128.

Lindsay, S.W. and Martens, W.J. (1998). Malaria in the African highlands: past, present and future. *Bull World Health Organ* 76, 33-45.

Lothrop, H.D. and Reisen, W.K. (2001). Landscape affects the host-seeking patterns of *Culex tarsalis* (Diptera: Culicidae) in the Coachella Valley of California. *Journal of Medical Entomology* 38, 325-332.

MacDougall-Shackleton, E.A., Derryberry, E.P. and Hahn, T.P. (2002). Nonlocal male mountain white-crowned sparrows have lower paternity and higher parasite loads than males singing local dialect. *Behavioral Ecology* 13, 682-689.

Magnarelli, L.A. (1977). Host feeding patterns of Connecticut mosquitoes (Diptera: Culicidae). *American Journal of Tropical Medicine & Hygiene* 26, 547-552.

Martin, T.E., Møller, A.P., Merino, S. and Clobert, J. (2001). Does clutch size evolve in response to parasites and immunocompetence? *Proceedings of the National Academy of Sciences of the United States of America* 98, 2071-2076.

Martinsen, E.S., Perkins, S.L. and Schall, J.J. (2008). A three-genome phylogeny of malaria parasites (*Plasmodium* and closely related genera): Evolution of life-history traits and host switches. *Molecular Phylogenetics and Evolution* 47, 261-273.

Marzal, A., Bensch, S., Reviriego, M., Balbontin, J. and De Lope, F. (2008). Effects of malaria double infection in birds: one plus one is not two. *Journal of Evolutionary Biology*

Marzal, A., de Lope, F., Navarro, C. and Møller, A.P. (2005). Malarial parasites decrease reproductive success: an experimental study in a passerine bird. *Oecologia* 142, 541-545.

Miller, B.R., Crabtree, M.B. and Savage, H.M. (1996). Phylogeny of fourteen *Culex* mosquito species, including the *Culex pipiens* complex, inferred from the internal transcribed spacers of ribosomal DNA. *Insect Mol Biol* 5, 93-107.

Molaei, G. and Andreadis, T.G. (2006). Identification of avian- and mammalian-derived bloodmeals in *Aedes vexans* and *Culiseta melanura* (Diptera : Culicidae) and its implication for West Nile virus transmission in Connecticut, USA. *Journal of Medical Entomology* 43, 1088-1093.

Molaei, G., Oliver, J., Andreadis, T.G., Armstrong, P.M. and Howard, J.J. (2006). Molecular identification of blood-meal sources in *Culiseta melanura* and *Culiseta morsitans* from an endemic focus of eastern equine encephalitis virus in New York. *American Journal of Tropical Medicine and Hygiene* 75, 1140-1147.

Møller, A.P. and Nielsen, J.T. (2007). Malaria and risk of predation: a comparative study of birds. *Ecology* 88, 871-881.

Nylander, J.A.A. (2004). MrModeltest v2. *Evolutionary Biology Centre, Uppsala University*

Palinauskas, V., Kosarev, V., Shaploval, A., Bensch, S. and Valki_nas, G. (2007). Comparison of mitochondrial cytochrome *b* lineages and morphospecies of two avian malaria parasites of the subgenera *Haemamoeba* and *Giovannolaia* (Haemosporida: Plasmodiidae). *Parasitology Research* 1626, 39–50.

Perkins, S.L., Sarkar, I.N. and Carter, R. (2007). The phylogeny of rodent malaria parasites: simultaneous analysis across three genomes. *Infection, Genetics and Evolution* 7, 74-83.

Ponnudurai, T., Billingsley, P.F. and Rudin, W. (1988). Differential infectivity of *Plasmodium* for mosquitoes. *Parasitology Today* 4, 319-321.

Reiskind, M.H. and Wilson, M.L. (2008). Interspecific competition between larval *Culex restuans* Theobald and *Culex pipiens* L. (Diptera: Culicidae) in Michigan. *Journal of Medical Entomology* 45, 20-27.

Reiter, M.E. and LaPointe, D.A. (2007). Landscape factors influencing the spatial distribution and abundance of mosquito vector *Culex quinquefasciatus* (Diptera: Culicidae) in a mixed residential-agricultural community in Hawai'i. *J Med Entomol* 44, 861-868.

Ricklefs, R.E. (1992). Embryonic development period and the prevalence of avian blood parasites. *Proceedings of the National Academy of Sciences of the United States of America* 89, 4722-4725.

Ricklefs, R.E. and Fallon, S.M. (2002). Diversification and host switching in avian malaria parasites. *Proceedings of the Royal Society Series B-Biological Sciences* 269, 885-892.

Ricklefs, R.E., Fallon, S.M. and Bermingham, E. (2004). Evolutionary relationships, cospeciation, and host switching in avian malaria parasites. *Systematic Biology* 53, 111-119.

Schrenzel, M.D., Maalouf, G.A., Keener, L.L. and Gaffney, P.M. (2003). Molecular characterization of malarial parasites in captive passerine birds. *Journal of Parasitology* 89, 1025-1033.

Szymanski, M.M. and Lovette, I.J. (2005). High lineage diversity and host sharing of malarial parasites in a local avian assemblage. *Journal of Parasitology* 91, 768-774.

Tate, P. and Vincent, M. (1934). The susceptibility of autogenous and anautogenous races of *Culex pipiens* to infection with avian malaria. *Parasitology* 512-522.

Telford, S.R., Jr., Nayar, J.K., Foster, G.W. and Knight, J.W. (1997). *Plasmodium forresteri* n. sp., from raptors in Florida and southern Georgia: its distinction from *Plasmodium elongatum* morphologically within and among host species and by vector susceptibility. *Journal of Parasitology* 83, 932-937.

Tripet, F., Aboagye-Antwi, F. and Hurd, H. (2008). Ecological immunology of mosquito-malaria interactions. *Trends in Parasitology* 24, 219-227.

Turell, M.J., O'Guinn, M. and Oliver, J. (2000). Potential for New York mosquitoes to transmit West Nile virus. *Am J Trop Med Hyg* 62, 413-414.

Valkiunas, G., Zehtindjiev, P., Hellgren, O., Ilieva, M., Iezhova, T.A. and Bensch, S. (2007). Linkage between mitochondrial cytochrome b lineages and morphospecies of two avian malaria parasites, with a description of *Plasmodium (Novyella) ashfordi* sp. nov. *Parasitology Research* 100, 1311-1322 .

Valkiunas, G. (2005) *Avian malaria parasites and other haemosporidia*. CRC press, Boca Raton.

Waldenström, J., Bensch, S., Kiboi, S., Hasselquist, D. and Ottosson, U. (2002). Cross-species infection of blood parasites between resident and migratory songbirds in Africa. *Molecular Ecology* 11, 1545-1554.

Wood, M.J., Cosgrove, C.L., Wilkin, T.A., Knowles, S.C., Day, K.P. and Sheldon, B.C. (2007). Within-population variation in prevalence and lineage distribution of avian malaria in blue tits, *Cyanistes caeruleus*. *Molecular Ecology* 16, 3263-3273.

CHAPTER 4

AMPLIFICATION OF AVIAN *PLASMODIUM* BY THE *CULEX P. PIPIENS* MOSQUITO

Abstract

Most ecological studies of avian blood parasites have focused on bird-parasite interactions to the exclusion of their Dipteran vectors. For parasites in the genus *Plasmodium* (Plasmodiidae), the mosquito is crucial not just to the transmission of parasites among hosts but also for sexual reproduction and completion of the parasite life cycle. It is therefore important to consider whether parasite-vector interactions influence which parasites ultimately infect birds. We tested the hypothesis that vectors limit *Plasmodium* infection in an avian community by experimentally testing whether a common *Plasmodium* lineage found in wild birds in our study area exhibits variation in its ability to infect a model avian malaria vector, *Culex pipiens pipiens,* using adult mosquitoes reared from field-collected eggs. A second aim of our study was to assess the ability of local *Cx. pipiens* to amplify *Plasmodium* parasites in wild birds with naturally occurring parasitemias. Lab-reared adult female mosquitoes were used in replicated feeding trials upon 4 wild-caught Red-winged blackbirds (*Agelaius phoeniceus*) naturally infected with a locally common *Plasmodium* lineage. The polymerase chain reaction (PCR) was used to screen both blood meals and mosquito bodies following incubation for the presence of *Plasmodium* infection. The low (0-17%) *Plasmodium* prevalence in female mosquitoes following incubation probably reflects natural variation in avian gametocytemia and mosquito immune responses to infection.

Introduction

Malaria parasites (Plasmodiidae) impose fitness costs to both vertebrate host and mosquito vector. Parasite associations with both host and vector must therefore be considered together as potential drivers of parasite evolution. Most studies of avian blood parasites have focused on assessing fitness costs to birds (Ots and Horak, 1998; Merino et al., 2000; Marzal et al., 2005; Spencer et al., 2005; Tomás et al., 2007). Our limited knowledge of fitness costs to arthropod vectors comes from studies on mosquitoes that are medically important to humans (Hurd, 2003). For example, malaria parasite infection can reduce *Anopheles* mosquito flight distance (Schiefer et al., 1977), fecundity (Hopwood et al., 2001) and speed of response to vertebrate host defensive behavior, which may in turn lead to increased mosquito mortality (Anderson et al., 2000).

Laboratory experiments on captive birds have shown that *Culex pipiens pipiens* is a competent vector species for several *Plasmodium* species that infect birds (Huff, 1929; Valkiunas, 2005). These experiments typically involved infecting a bird by direct inoculation from an infected bird into a previously unexposed bird, waiting for the parasite to amplify in the new host's blood, then placing the bird into a cage for mosquitoes to feed upon. Experimental conditions necessarily departed from natural conditions by utilizing 1) unnatural host-vector combinations, 2) manipulated parasitemias, or 3) mosquitoes reared in small colonies for many generations, potentially resulting in high levels of inbreeding.

In many cases parasitemias were manipulated (Work et al., 1990) and feeding trials timed to occur when parasitemias are elevated (LaPointe et al., 2005) to maximize the probability that experimental mosquitoes ingested large numbers of parasites with the blood meal. Such studies laid the foundation for understanding variation among mosquito and parasite species interactions, and were most valuable

for eliminating possible vector candidates (*i.e.,* those mosquito species that did not become infected under ideal conditions were unlikely to be important in the wild).

It is rare that competence trials are conducted with the parasitemic levels found in wild birds (0.1-13% erythrocytes infected, (Kirkpatrick and Sluthers, 1988), which are often lower than those achieved by inoculating captive birds in the laboratory (up to 63% erythrocytes infected, (Valkiunas, 2001). Parasitemia is an important consideration because it directly affects the probability that a female mosquito feeding on a host will ingest both male and female gametocytes in its blood meal which are required for the parasite to complete its life cycle in the mosquito. Failure to ingest at least a single male and single female gametocyte would eliminate any possibility of transmission to a new host regardless of the mosquito's physiological capacity to do so.

The mosquitoes used for feeding trials often originate from captive breeding colonies, which are inbred for many generations. Recent molecular genetic and genomics research on human malaria vectors has suggested that wild type *Anopheles gambiae* is immune to *Plasmodium* infection and that susceptibility to infection, and by extension vector competence, may be a result of loss of immune function (Riehle et al., 2006). Inbreeding may increase the frequency of such a loss of function in a population. Inbreeding has also been shown empirically to increase the mosquito's susceptibility to parasite infection (Niare et al., 2002). Using inbred mosquitoes alone for vector competence trials might therefore result in overestimating vector competence.

Experiments demonstrating a mosquito species' *potential* ability to be a vector does not mean that it is an important vector in the wild. Whether a mosquito species is an important vector in an avian community depends on complex interacting factors including vector and host density, host availability, feeding preference for particular

hosts and a threshold reservoir of infected hosts. Moreover, a single vector species can exhibit intraspecific geographic variation in competence. This variation may represent genetic variation in competence among populations, or it might reflect variation in vectorial capacity, which includes competence and other extrinsic factors such as host and vector density (Frost, 1976). It is therefore not always possible to predict a vector species' competence based on its performance under laboratory conditions.

A previous study explored patterns of lineage distribution among avian malaria vectors and revealed that a unique clade of *Plasmodium* cytochrome *b* haplotypes sampled from mosquitoes largely excluded published *Plasmodium* haplotypes sampled from birds, and that one mosquito species, *Cx. restuans*, harbored more parasite haplotypes than *Cx. pipiens* (Kimura in prep.). It is not clear whether this difference in parasite-vector association is due to an intrinsic evolutionary interaction between vector and parasites, or a reflection of differences between mosquito species in their avian host feeding preference. Analyses of mosquito blood meal contents show that mosquitoes often prefer to feed on particular bird species, independent of the relative availability of those birds (Hassan et al., 2003). If *Plasmodium* parasites were specific to bird species, and mosquito species were specific in their avian host feeding preferences, parasites would be vector-specific, irrespective of any host preferences among vectors. Variation in vectorial competence (ability to transmit parasites to a new host) could therefore contribute to observed patterns in avian malaria lineage diversity and distribution across avian hosts and mosquito vectors.

Here, we test experimentally whether an abundant vector species, *Cx. pipiens*, is able to amplify a *Plasmodium* lineage naturally found in local wild birds, using adult mosquitoes reared from local field-collected eggs.

Methods

Molecular and morphological identification of Plasmodium in avian hosts.

Passerine birds were captured using mist nets in Tompkins County, NY in July 2007. All bird handling procedures were reviewed and approved under Cornell University Institutional Animal Care and Use Committee protocol #2006-0076. Approximately 50 μL of blood per bird was taken by brachial venipuncture. Genomic DNA was extracted from the erythrocytes and using Perfect gDNA Blood Mini Kits (Eppendorf), screened for *Plasmodium* infection via PCR, and the *Plasmodium* sequenced following methods of Kimura et al. (2006).

In addition, thin blood smears were made (4 smears per bird), air dried, fixed in 100% methanol for 1 minute and stained in 7% Giemsa for 1 hour. At least 150 fields of each slide were viewed at high magnification (1000X) with approximately 250 erythrocytes per field. Intensity of infection was estimated by counting the number of parasites per 1,000 erythrocytes. Parasites were identified to species or subgenus level, where infection levels allowed, following Valkiunas (2005), so that the morphology of the blood stages of the parasites could be related to their DNA sequence.

Mosquito collection and rearing.

Egg rafts of *Cx. pipiens* were collected from peridomestic aquatic habitats in Tompkins County, New York. Each egg raft was placed individually in a container with 1L deionized water containing approximately 20 ml diet slurry (1:2:1 fish food: rabbit pellets: bovine liver powder) and held at 25°C until hatching. After hatching, sub-samples of 2-4th instar larvae from each egg raft were removed and identified to species using standard taxonomic characters (Means, 1987). Pupae were removed from larval rearing trays on each day of pupation and placed into emergence bowls inside mesh-covered 5-liter buckets. To control for potential variation in feeding and

competence among progeny of different females, pupae from multiple egg rafts were combined into emergence bowls. Emerging adults were provided with *ad libitum* 20% sucrose. When adult females were between 4 and 14 days old, the sucrose solution was removed for 24 hours before each feeding trial.

Mosquito feeding trials and detection of infection.

Four thin blood smears were taken from each bird within 5 minutes of the feeding trials to assess parasitemia at the time of feeding. An individual restrained live bird was placed into a mesh-covered 5-liter bucket cage in a dark room with 40-50 adult female mosquitoes for 2-3 hours, or until most females were engorged or had stopped feeding. All unfed females were removed and discarded. Engorged and partially engorged females were placed into an environmental chamber under 70% relative humidity and a 14L:10D photoregime at 25-28°C and provided with 20% sucrose provided *ad libitum* on cotton wicks. To confirm that females had ingested blood stages of *Plasmodium* from the birds, engorged females from each bucket were removed and frozen. Genomic DNA was extracted from the whole mosquito body using DNAzol reagent (Invitrogen, Carlsbad, CA) followed by ethanol precipitation. The presence and identity of *Plasmodium* parasites in the blood meals were confirmed via PCR and sequencing using primers R2 and HaemF2. After 14 days of incubation, the remaining gravid mosquitoes were frozen whole at -20C. The right wing was removed from each female and measured to estimate body size. Genomic DNA was extracted from ground whole mosquito bodies, and *Plasmodium* was detected via PCR using the same method described for the blood meals.

Data analysis.

We used the exact logistic regression procedure in Stata version 10 because the data were sparse. Effects of individual bird, feeding trial replicate, and proportion of mosquitoes that ingested *Plasmodium* in blood meals on day 0 were used as

independent variables to predict the response variable of proportion of gravid mosquitoes infected with *Plasmodium* on day 14.

Results

Infected birds.

Using PCR-based detection of *Plasmodium* DNA in avian blood, four red-winged blackbirds (*Agelaius phoeniceus*) harbored infections of a common *Plasmodium* cytochrome *b* haplotype and one was infected with *Haemoproteus*. The *Plasmodium*-infected birds were subsequently held in captivity for feeding trials. None of the blood smears showed any cells infected with *Plasmodium* gametocytes, the life stage of the parasite that would eventually lead to sexual reproduction in the mosquito midgut, or schizonts, which would have facilitated assignment of the parasites to morphological subgenus or species. A previous study found *P. cathemerium* and *P. circumflexum* naturally occurring in a population of red-winged blackbirds in the northeastern United States (Herman, 1938). Five additional *Plasmodium* species are known to occur in red-winged blackbirds from around North America: *P. elongatum, P. hexamerium, P. polare, P. relictum* and *P. vaughani* (Bennett et al., 1982). The low parasitemias in our study birds did not allow identification to morphospecies, however.

Mosquito feeding trials.

Three replicated feeding trials were conducted with *Cx. pipiens* mosquitoes (n=368) on the four captive birds. Overall, a lower proportion of mosquitoes was found with disseminated *Plasmodium* infections (4%) than with blood meals containing *Plasmodium* (38%) (Figure 4.1). The proportion of mosquitoes infected following incubation was lower than the proportion of blood meals infected at time of feeding within each trial, with the exception of bird 41035 in replicate 2, where 10%

of engorged mosquitoes contained *Plasmodium* at the time of feeding and 17.6% disseminated *Plasmodium* following incubation. Considerable variation existed between individual birds in both the proportion of mosquitoes ingesting *Plasmodium* in the blood meal (10-100%). The logistic regression indicated a significant effect of individual bird in predicting the proportion of mosquitoes with disseminated *Plasmodium* following incubation (p= 0.01) as well as a significant effect of individual bird (p=0.05). The effect of feeding trial approached significance (p=0.06). To ensure that low levels of infection following incubation were not due to insufficient incubation time, in trial 3 we sampled female mosquitoes four more times, at days 19, 24, 29 and 33. Increased incubation time did not increase the occurrence of infection, as no mosquitoes fed on birds 41016 or 41026 sampled after day 14 were infected. A single mosquito that fed on bird 41033 showed infection on day 19.

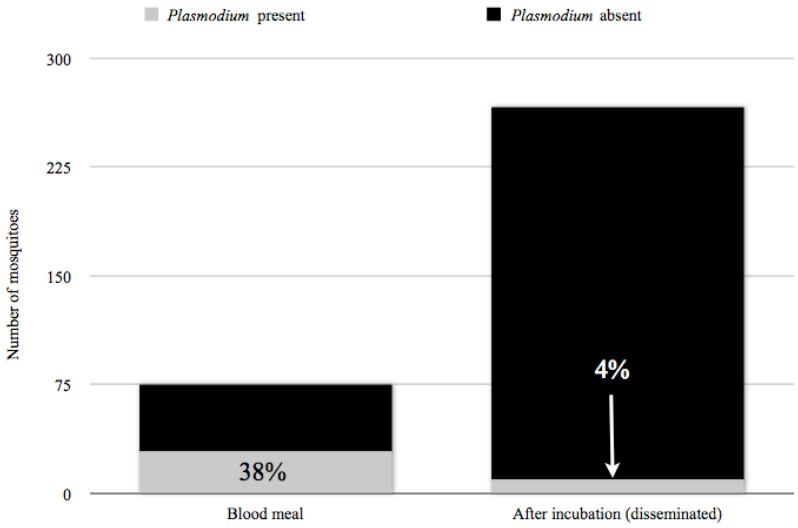

Figure 4.1. Summary of proportion of 1) blood meals and 2) mosquitoes following incubation in which *Plasmodium* was detected by PCR after feeding on red-winged blackbirds (n=368 mosquitoes).

Discussion

In our study, the probability that a mosquito ingested *Plasmodium* during feeding varied from 10-100%. This variation was likely an artifact of low power due to small sample sizes (n=2) of mosquito blood meals taken in trial 1. Like other studies, our study detected *Plasmodium* in bird blood using PCR even when infected erythrocytes were not seen in microscopic examination of blood smears (Jarvi et al., 2003).

The variation observed in *Plasmodium* presence in the blood meals and in the mosquitoes post-incubation likely reflects variation in parasitemia either among individual birds, or over time, or both. Because a mosquito needs to ingest both a male and female gametocyte to harbor the parasite's development through the sporozoite stage, and a single blood meal (about 3 μl) consists of only a minute sample of the total blood volume of a single bird, gametocytemia would need to be relatively high for transmission to be successful.

This reduction could result from mosquitoes ingesting only non-sexual stages of parasites in the blood, detected by PCR in the blood meal, which would prevent the parasite from starting its life cycle inside the mosquito. Gametocytes typically occur at a much lower level than non-sexual stages. If gametocytes had been completely absent, then we would not have observed any *Plasmodium* amplified in mosquitoes after the incubation period. However, *Plasmodium* did appear in of the14 feeding events for which we were able to compare infection at time of feeding to infection following incubation.

The proportion of *Cx. pipiens* individuals amplifying the various *Plasmodium* lineages found in local birds at our study site in a single feeding trial (0-17%) is not as high as reported for the same mosquito species fed on an unnatural host (canary) when feedings were conducted at a time where gametocytes could be seen and counted in

blood films (Rosen and Reeves, 1954). Our finding of relatively low rates of amplification is consistent with other studies examining rates of amplification of *Plasmodium* found at unmanipulated levels in human hosts (0-15%, Shin et al. 2002). This relatively low amplification rate could represent a more realistic approximation of the natural amplification rate. An earlier experiment found that *Cx. pipiens* could become infected even when no gametocytes were seen by microscopic examination (Tate and Vincent, 1934). A similar ability to amplify submicroscopic infections is seen in human malaria parasites (*P. falciparum*) in *Anopheles* mosquitoes (Schneider et al., 2007; Shekalaghe et al., 2007).

One potential limitation of this study was our small sample size of mosquitoes. Sample sizes of blood-fed mosquitoes were low in some groups because not all mosquitoes offered a live bird actually took a blood meal. We used a single avian host species in this study so that that possible variation among individual birds could not be attributed to variation among bird species. Instead, the variation we found between feeding trials could represent within-host community dynamics, such as competition among lineages, that have been reported in human malaria parasites inoculated into laboratory mice (de Roode et al., 2005; Bell et al., 2006; Wargo et al., 2007), in human malaria in the field (Bruce et al., 2000a; Bruce et al., 2000b; Bruce and Day, 2003) and hypothesized to occur in avian hosts (Fallon et al., 2003; Fallon et al., 2005).

Our study represents a first step towards understanding how mosquito vectors may mediate host-parasite dynamics. The typically high rate of *Plasmodium* infection in birds at our study site (10-40%, Kimura et al. (2006), Szymanski and Lovette (2005)) might predict high competence in one of the dominant ornithophilic mosquito species at our site, *Cx. pipiens*, which was a likely candidate to be a highly competent vector based on high levels of transmission with this species in previous laboratory experiments. This study adds to existing evidence from studies of other vertebrate

hosts of distantly-related *Plasmodium* species that amplification rates by mosquito vectors of *Plasmodium* species is lower than expected from feeding experiments under unnatural conditions in the laboratory. Future work should explore the relative vector competence of other ornithophilic mosquito species.

REFERENCES

Anderson, R.A., Knols, B.G.J. and Koella, J.C. (2000). *Plasmodium falciparum* sporozoites increase feeding-associated mortality of their mosquito hosts *Anopheles gambiae s.L*. *Parasitology* 120, 329-333.

Bell, A., S., De Roode, J., C., Sim, D. and Read, A., F. (2006). Within-host competition in genetically diverse malaria infections: Parasite virulence and competitive success. *Evolution* 60, 1358-1371.

Bennett, G.F., M. Whiteway, and C.B. Woodworth-Lynas. (1982) *A host-parasite catalogue of the avian haematozoa*. Memorial University of Newfoundland.

Bruce, M.C. and Day, K.P. (2003). Cross-species regulation of *Plasmodium* parasitemia in semi-immune children from Papua New Guinea. *Trends in Parasitology* 19, 271-277.

Bruce, M.C., Donnelly, C.A., Alpers, M.P., Galinski, M.R., Barnwell, J.W., Walliker, D. and Day, K.P. (2000a). Cross-species interactions between malaria parasites in humans. *Science* 287, 845-848.

Bruce, M.C., Galinski, M.R., Barnwell, J.W., Donnelly, C.A., Walmsley, M., Alpers, M.P., Walliker, D. and Day, K.P. (2000b). Genetic diversity and dynamics of plasmodium falciparum and P. vivax populations in multiply infected children with asymptomatic malaria infections in Papua New Guinea. *Parasitology* 121, 257-272.

de Roode, J.C., Helinski, M.E.H., Anwar, M.A. and Read, A.F. (2005). Dynamics of multiple infection and within-host competition in genetically diverse malaria infections. *American Naturalist* 166, 531-542.

Fallon, S.M., Bermingham, E. and Ricklefs, R.E. (2003). Island and taxon effects in parasitism revisited: Avian malaria in the Lesser Antilles. *Evolution* 57, 606-615.

Fallon, S.M., Bermingham, E. and Ricklefs, R.E. (2005). Host specialization and geographic localization of avian malaria parasites: A regional analysis in the Lesser Antilles. *American Naturalist* 165, 466-480.

Frost, W.H. (1976). Some conceptions of epidemics in general. *American Journal of Epidemiology* 103, 141-151.

Hassan, H.K., Cupp, E.W., Hill, G.E., Katholi, C.R., Klingler, K. and Unnasch, T.R. (2003). Avian host preference by vectors of eastern equine encephalomyelitis virus. *American Journal of Tropical Medicine and Hygiene* 69, 641-647.

Herman, C.M. (1938). Epidemiology of malaria in eastern red-wings (*Agelaius p. phoeniceus*). *American Journal of Hygiene* 28, 232-243.

Hopwood, J.A., Ahmed, A.M., Polwart, A., Williams, G.T. and Hurd, H. (2001). Malaria-induced apoptosis in mosquito ovaries: a mechanism to control vector egg production. *Journal of Experimental Biology* 204, 2773-2780.

Huff, C.G. (1929). The effects of selection upon susceptibility to bird malaria in *Culex pipiens*. *Linn Ann Trop Med Parasitol.* 23, 427–439.

Hurd, H. (2003). Manipulation of medically important insect vectors by their parasites. *Annual Review of Entomology* 48, 141-161.

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

Kimura, M., Dhondt, A.A. and Lovette, I.J. (2006). Phylogeographic structuring of *Plasmodium* lineages across the North American range of the House finch (*Carpodacus mexicanus*). *Journal of Parasitology* 92, 1043-1049.

Kirkpatrick, C.E. and Sluthers, H.B. (1988). Epizootiology of blood parasite infections in passerine birds from central New Jersey USA. *Canadian Journal of Zoology* 66, 2374-2382.

LaPointe, D.A., Goff, M.L. and Atkinson, C.T. (2005). Comparative susceptibility of introduced forest dwelling mosquitoes in Hawai'i to avian malaria, *Plasmodium relictum*. *Journal of Parasitology* 91, 843-849.

Marzal, A., de Lope, F., Navarro, C. and Møller, A.P. (2005). Malarial parasites decrease reproductive success: an experimental study in a passerine bird. *Oecologia* 142, 541-545.

Means, R.G. (1987) *Mosquitoes of New York. Part II. Genera of Culicidae other than Aedes occurring in New York*. Bulletin No. 430b.

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

Niare, O., Markianos, K., Volz, J., Oduol, F., Toure, A., Bagayoko, M., Sangare, D., Traore, S.F., Wang, R., Blass, C., Dolo, G., Bouare, M., Kafatos, F.C., Kruglyak, L., Toure, Y.T. and Vernick, K.D. (2002). Genetic loci affecting resistance to human malaria parasites in a West African mosquito vector population. *Science* 298, 213-216.

Ots, I. and Horak, P. (1998). Health impact of blood parasites in breeding great tits. *Oecologia* 116, 441-448.

Riehle, M.M., Markianos, K., Niare, O., Xu, J., Li, J., Toure, A.M., Podiougou, B., Oduol, F., Diawara, S., Diallo, M., Coulibaly, B., Ouatara, A., Kruglyak, L., Traore, S.F. and Vernick, K.D. (2006). Natural malaria infection in *Anopheles gambiae* is regulated by a single genomic control region. *Science* 312, 577-579.

Rosen, L. and Reeves, W.C. (1954). Studies on avian malaria in vectors and hosts of encephalitis in Kern county, California. 3. The comparative vector ability of some of the local culicine mosquitoes. *American Journal of Tropical Medicine and Hygiene* 3, 704-708.

Schiefer, B.A., Ward, R.A. and Eldridge, B.F. (1977). *Plasmodium cynomolgi* - effects of malaria infection on laboratory flight performance of *Anopheles stephensi* mosquitos. *Experimental Parasitology* 41, 397-404.

Schneider, P., Bousema, J.T., Gouagna, L.C., Otieno, S., van de Vegte-Bolmer, M., Omar, S.A. and Sauerwein, R.W. (2007). Submicroscopic *Plasmodium falciparum* gametocyte densities frequently result in mosquito infection. *American Journal of Tropical Medicine and Hygiene* 76, 470-474.

Shekalaghe, S.A., Bousema, J.T., Kunei, K.K., Lushino, P., Masokoto, A., Wolters, L.R., Mwakalinga, S., Mosha, F.W., Sauerwein, R.W. and Drakeley, C.J. (2007). Submicroscopic *Plasmodium falciparum* gametocyte carriage is common in an area of low and seasonal transmission in Tanzania. *Tropical Medicine and International Health* 12, 547-553.

Shin, E.H., Kim, T.S., Lee, H.W., Lee, J.S. and Lee, W.J. (2002). Vector competence of *Anopheles lesteri* Baisas and Hu (Diptera: Culicidae) to *Plasmodium vivax* in Korea. *Korean Journal of Parasitology* 40, 41-44.

Spencer, K.A., Buchanan, K.L., Leitner, S., Goldsmith, A.R. and Catchpole, C.K. (2005). Parasites affect song complexity and neural development in a songbird. *Proceedings of the Royal Society Series B-Biological Sciences* 272, 2037-2043.

StataCorp. 2007. Stata Statistical Software: Release 10. College Station, TX: StataCorp LP.

Szymanski, M.M. and Lovette, I.J. (2005). High lineage diversity and host sharing of malarial parasites in a local avian assemblage. *Journal of Parasitology* 91, 768-774.

Tate, P. and Vincent, M. (1934). The susceptibility of autogenous and anautogenous races of *Culex pipiens* to infection with avian malaria. *Parasitology* 512-522.

Tomás, G., Merino, S., Moreno, J., Morales, J. and Martinez-de la Puente, J. (2007). Impact of blood parasites on immunoglobulin level and parental effort: a medication field experiment on a wild passerine. *Functional Ecology* 21, 125-133.

Valkiunas, G. (2001). Blood parasites of birds: some obstacles in their use in ecological and evolutionary biology studies. *Avian Ecology & Behavior* 7, 87-100.

———. (2005) *Avian malaria parasites and other haemosporidia*. CRC press, Boca Raton.

Wargo, A.R., de Roode, J.C., Huijben, S., Drew, D.R. and Read, A.F. (2007). Transmission stage investment of malaria parasites in response to in-host competition. *Proceedings of the Royal Society Series B-Biological Sciences* 274, 2759-2768.

Work, T.M., Washino, R.K. and Vanriper, C. (1990). Comparative susceptibility of *Culex tarsalis*, *Anopheles franciscanus*, and *Culiseta inornata* (Diptera, Culicidae) to *Plasmodium relictum* (Haemosporidia, Plasmodiiae). *Journal of Medical Entomology* 27, 68-71.

APPENDIX

PILOT EXPERIMENTS FOR MOSQUITO FEEDING TRIALS

Experiment 1. The goal of the first pilot experiment was to compare the ability of two putative avian *Plasmodium* vector species, *Culex p. pipiens* and *Culex p. quinquefasciatus*, to amplify *Plasmodium* after feeding on naturally infected house finches. All animal handling was reviewed and approved under IACUC protocol #2006-0076.

Methods. Feeding trials were conducted using protocols described in Chapter 4. Five house finches were found to be infected with *Plasmodium* using PCR protocols described in Chapter 2. All mosquitoes were reared in the laboratory under 70% RH and a 14L:10D photoregime. *Cx. quinquefasciatus* came from the SLAB, an insecticide-resistant colony originally derived from California populations and maintained in colony for more than 20 years. *Cx. pipiens* were derived from wild material collected in central New York State and augmented annually. Female mosquitoes were offered blood meals from a restrained chicken and provided with an oviposition substrate 4 days later. First instar larvae hatching from eggs were 3 days old when they were placed in groups of 200 in 1L of distilled water and a dry food mixture as described in Chapter 4. Larvae were maintained in incubators under the above conditions. Following pupation, pupae were placed in containers in mesh-covered bucket cages until eclosion commenced on day 14. Because males emerge before females, we discarded the adults that emerged from approximately the first 30% of the pupae. The remaining pupae were moved into a new bucket provided with 20% sucrose *ad libitum*. Adults in the second bucket were mostly females but contained a sufficient number of males to ensure that all females in the cohort mated.

When adult females were 3 days old, on day 18, sucrose solution was replaced with distilled water.

To enhance the probability that these nocturnal mosquito species would become infected, we exposed restrained birds to mosquitoes at 10 p.m., EST. On day 20, five *Plasmodium*-infected house finches and one uninfected bird were placed sequentially in two separate bucket cages with *Cx. quinquefasciatus* and *Cx. pipiens* individuals for a 25-minute feeding interval. Following the feeding period, birds were removed from the buckets. After 12 hours, unfed females and males were removed by aspiration. Engorged females were returned to the incubator, held between 24 and 28°C, and provided 20% sucrose on cotton pads ad libitum. On day 11, females were frozen for DNA analysis as described in Chapter 4. Mature sporozoites have been found in culicine vectors under laboratory conditions between 7 to 20 days following a blood meal, so we assumed that 11 days was sufficient time to reach infectivity under incubator conditions.

Results. None of the incubated mosquitoes showed evidence of *Plasmodium* infection by PCR. Feeding rates varied between *Cx. quinquefasciatus*, mean=18.5%, and *Cx. pipiens*, mean=48.3% (Table 1).

Table A1. Percentages of female mosquitoes that fed upon restrained house finches in pilot feeding trial.

Cx. quinquefasciatus	148	158	260	281	534	582
unfed females	n/a	24	8	15	30	12
fed females	0	6	1	6	5	7
TOTAL females	0	30	9	21	35	19
percentage fed	0%	20.0%	11.1%	28.6%	14.3%	36.8%
Cx. pipiens						
unfed females	15	23	14	65	26	26
fed females	25	46	5	30	22	34
TOTAL females	40	69	19	95	48	60
percentage fed	62.5%	66.7%	26.3%	31.6%	45.8%	56.7%

Experiment 2. The goal of this experiment was to induce high parasitemia in captive naïve canaries by inoculating them with blood from a *Plasmodium*-infected bird. All animal handling was reviewed and approved under IACUC protocol #2006-0076.

Methods. Captive house finches were screened for *Plasmodium* infection following methods described in Chapter 2. Once infected house finches were identified, approximately 20 µl of blood were drawn from each of two infected birds. The blood was immediately diluted with 1 part of 3.8% sodium citrate to 4 parts of whole blood (G. Valkiunas, pers. comm.). Within 10 minutes, approximately 50 µl of citrated blood were injected into the pectoral muscle of 2 recipient canaries (each canary was inoculated with blood from a different donor house finch). Canaries were bled and screened for *Plasmodium* infection 7 and 10 days following inoculation with infected blood. Individual *Cx. p. pipiens* were reared in the laboratory from field-collected eggs. After *Plasmodium* infection was detected in both canaries, mosquitoes were fed on the restrained birds as described in Chapter 4. On day 11 after incubation at 25-28°, mosquitoes were frozen for DNA analysis as described in Chapter 4.

Results. Thirty female *Cx. pipiens* were engorged from feeding on both canaries. None showed evidence of *Plasmodium* infection by PCR following the 11-day incubation period.