8-19-2015 12:00 AM

Natural Variation In Malarial Infection And Immune Investment In A Migratory Songbird, And The Effects Of Infection On Flight Performance

Laura Rooney, *The University of Western Ontario*

Supervisor: Dr. Chris Guglielmo, *The University of Western Ontario*

A thesis submitted in partial fulfillment of the requirements for the Master of Science degree in Biology

NATURAL VARIATION IN MALARIAL INFECTION AND IMMUNE INVESTMENT IN A MIGRATORY SONGBIRD, AND THE EFFECTS OF INFECTION ON FLIGHT PERFORMANCE

(Thesis format: Monograph)

by

Laura Rooney

Graduate Program in Biology

A thesis submitted in partial fulfillment
of the requirements for the degree of
Masters of Science in Biology

The School of Graduate and Postdoctoral Studies
The University of Western Ontario
London, Ontario, Canada

Abstract

Endurance flight and immune defense are energetically costly activities for birds, and there may be a trade-off between migration and immune investment. Avian blood parasites consume host resources, which may decrease a bird's resource pool so that endurance flight and immune defense may not be possible simultaneously. I developed a molecular method to detect and quantify blood parasite infection in migrating yellow-rumped warblers (*Setophaga coronata*), and measured immune activity. I assessed the impact of infection on endurance flight performance and refueling in captive birds. Infection status was not related to age or sex, or timing of migration, and haptoglobin decreased with capture date during autumn migration. Spring relapses in infection were seen in captive birds, and no effect of infection status on flight performance or refueling was evident. My results suggest that warblers are well adapted to malarial parasites, and may facilitate the spread of disease across large geographic distances.

Keywords

Wildlife disease, ecoimmunology, avian malaria, migration, host-parasite interactions, flight performance

Co-Authorship Statement

This thesis incorporates material that is a result of joint research with Christopher Guglielmo at The University of Western Ontario, and Susan Shriner at USDA APHIS. The blood smear analyses and the majority of the laboratory work were completed by myself. Lin Zhao assisted with qPCR method development, and Greg Mitchell and myself conducted the immune assays. Morag Dick was responsible for bird husbandry and conducted the majority wind tunnel flights and warbler sampling. Chris Guglielmo and Greg Mitchell assisted with data analysis. This thesis will be submitted to the Journal of Avian Biology for publication with Morag Dick, Greg Mitchell, Lin Zhao, Susan Shriner, and Christopher Guglielmo as co-authors.

Acknowledgments

I thank my supervisor, Christopher Guglielmo, and colleagues at The University of Western Ontario who provided much expertise and assisted the research, members of my advisory committee, Beth Macdougall Shackleton, The University of Western Ontario, and Phil Taylor, Acadia University, who provided insight into the project design, and data analysis.

I also thank Morag Dick for the use of her captive birds, Greg Mitchell for help during the field season and with data analysis, and Lin Zhao for help with the molecular method development.

In addition, I would like to thank the staff and volunteers at Bird Studies Canada and Long Point Bird Observatory for their help and support during my field season.

This research was partially supported by the USDA APHIS National Wildlife Research Centre Susan Shriner, and NSERC, Christopher Guglielmo The University of Western Ontario.

Table of Contents

List of Tables

List of Figures

List of Appendices

Chapter 1

1 Introduction

The discipline of disease ecology involves the study of host-pathogen interactions in the context of population wide spread and emergence of disease (Brock et al., 2014). The field aims to determine how pathogens spread over time and space, and how the spread of disease impacts host populations (Hudson et al., 2002). Pathogens (disease causing organisms) and parasites (organisms that may or may not be pathogenic but live in or on a host and sequester resources from their host) impact host populations by affecting survival and reproduction (Scott, 1988). For example, population size of feral Soay sheep (*Ovis aries L.*) infected with parasitic nematodes has been controlled by mortality caused by the pathogen (Gulland, 1992). Cyclic population crashes of Red grouse (*Lagopus lagopus scoticus*) in England are also thought to be controlled by parasitic nematode infection (Hudson et al., 1998). The degree to which a pathogen outbreak controls population size and possible extinction events depends on the density of available hosts, as well as the virulence of the specific pathogen (Vredenburg et al., 2010).

Virulence of pathogens and parasites is thought to be a result of a trade-off between pathogen reproduction within the host, and transmission to new hosts (de Roode et al., 2008). Damage to host tissues is a result of pathogen replication and reproduction within host tissues and cells, as well as consumption of host resources, and increased damage to host tissues can lead to increased mortality (Day et al., 2007). If a host dies before the pathogen can be transmitted into another host, the pathogen may also die (Day et al., 2007). Therefore, pathogens may have evolved an optimal level of virulence whereby pathogens can maximize their fitness through reproduction while minimizing potential costs of reduced transmission as a result of host mortality (Anderson and May, 1982). Hosts may also evolve tolerance (minimizing damage without clearing the infection) or resistance (decreased probability of contracting an infection or increased recovery) to pathogens that they are frequently exposed to (Boots et al., 2009). One of the most well known examples of evolved resistance to a pathogen is in the European rabbits (*Oryctolagus cuniculus*) of Australia and Myxoma virus (genus *Leporipoxvirus*) (Best and Kerr, 2000). Initially introduced to control the rabbit population in Australia, Myxoma virus wiped out almost 90 % of the rabbit population. However rapid selection for a less virulent strain occurred, followed by selection for enhanced

innate resistance to the virus in the remaining rabbit population (Best and Kerr, 2000). Not all populations or species will be resistant or tolerant to every pathogen, as there may be a trade-off between the type of immune defence aimed at different pathogens, and pathogens may co-evolve alongside their hosts in order to evade immune defences (Schmid-Hempel, 2005).

The spread of pathogens from reservoir hosts into highly susceptible, and naïve, populations can result in significant declines, and even extinction of some threatened or endangered species (Hudson et al., 2002). For example, severe population declines have been documented in the threatened Baikal seal (*Pusa sibirica*) as a result of canine distemper virus that spread from domestic dogs into the population of seals (Mamaev et al., 1995). An introduced fungal infection, chytridiomycosis, is responsible for mortality and population crashes in a variety of species of rainforest anurans in Australia and Central America (Berger et al., 1998). It is also possible for pathogens to spread from natural reservoir hosts into domestic animals and humans, causing substantial harm (Altizer et al., 2011). For example, Influenza pandemics in humans are a result of transmission of the virus from birds and pigs (Daszak et al., 2000). Ebola virus is also spread to humans via natural reservoir hosts, most likely species of bats (Daszak et al., 2000). Understanding how disease spreads between populations and species helps to mitigate possible epidemics in threatened and endangered species, as well as in humans (Altizer et al., 2011). Although the field of disease ecology has been relatively successful in explaining disease dynamics in terms of populations, the organismal level traits that mediate the spread of disease from one host to another have largely been ignored (Brock et al., 2014).

At the organismal level, whether a pathogen will cause an infection or not is dependent on the immune system of that particular animal (Murphy et al. 2008). The vertebrate immune system is generally split into two branches, the innate, and the adaptive branch (Murphy et al. 2008). The innate branch of the immune system involves a generic response to pathogens, and does not result in lasting protection (Buehler et al., 2010). The adaptive branch on the other hand, involves pathogen-specific responses that confer lasting protection against specific pathogens that the organism has encountered previously (Murphy et al. 2008). The immune system can be further divided into constitutive responses, and induced responses (Buehler et al., 2010). The first defences that animals have against invading pathogens are physical barriers, such as the skin and mucosal membranes (Murphy et al. 2008). If the pathogen gets past these defences, the always-present constitutive aspects of the immune system respond to the threat (Schmid-Hempel and Ebert 2003). The

constitutive branch of immune system includes soluble blood proteins, such as proteins involved in the complement cascade and natural antibodies, and cellular responses, such as phagocytes that engulf pathogens (Murphy et al. 2008). As phagocytes engulf pathogens, they release chemicals called cytokines that attract more phagocytes as well as dendritic (antigen presenting) cells to the infection site (Murphy et al. 2008). If the pathogen is not cleared at this point, the induced aspects of the immune system take over (Murphy et al. 2008). Macrophages continue to release pro-inflammatory cytokines, and the acute phase response is initiated (Schmid-Hempel and Ebert 2003). This involves the production of acute phase proteins that requires the use of amino acids that would otherwise be used for growth and reproduction (Buehler et al., 2010). Sickness behaviours, including lethargy and loss of appetite are promoted, and fever is induced (Buehler et al., 2010). Once dendritic cells have engulfed pathogens, they migrate to the lymph nodes in order to initiate the adaptive branch of the immune system, where T cells and B cells (antibody producing) proliferate and recognize the specific pathogen (Murphy et al. 2008).

Ecoimmunology is a relatively new field that seeks to explain variation in immunity, both at the individual level (Brock et al., 2014), and throughout evolution (Nunn, 2002). Immunity varies temporally and spatially within populations, as well as with life-history strategy (Brock et al., 2014). Levels of circulating immune cells tends to be elevated during winter months (Martin et al., 2008), and immune activity of anthropoid primates was positively correlated with rainfall, as primates tend to suffer higher parasite burdens in wetter habitats (Semple et al., 2002). Fast-living ecotypes of garter snakes (*Thamnophis elegans*) have higher levels of constitutive immune activity than slow-living ecotypes, indicating the life history strategy of each affects immune investment (Sparkman and Palacios, 2009). Immunity also interacts with other aspects of physiology, resulting in physiological trade-offs (Brock et al., 2014); this is due to the fact that nutrients and energy are required in order to produce components of the immune response such as acute phase proteins, and antibodies, and may be diverted away from other activities (Buehler et al., 2010). Stressful situations have been implicated in the suppression of immune activity; this may be due to the reallocation of resources to activities that may be more important for immediate survival and fitness (Martin, 2009). For example, during reproduction, territorial Galapagos marine iguanas (*Amblyrhynchus cristatus*) had higher levels of corticosterone and lower levels of immune activity (swelling) compared to bachelor males after being injected with phytohemaglutinin (PHA), a lectin that binds to T cells and causes swelling as a result of infiltration of immune cells (Berger et al., 2005). This indicates a

possible reallocation of resources towards reproduction and territory defense and away from immune activity. It is also possible that immune activity is reduced during stressful situations in order to minimize damage from autoimmune activity, as stress hormones induce the production of self-antigens as a result of tissue degradation, directing an immune response against the host (Råberg et al., 1998). Ecoimmunology has, however, mostly ignored how the individual traits that affect variation in immunity impact the dynamics of disease within individuals and populations (Brock et al., 2014). Merging ecoimmunology with disease ecology may help to determine how an individual responds to disease in the context of its life history stage and environment, and what traits may affect the likelihood of contracting an infection. During energetic stress, for example, infection may lead to energetic trade-offs due to the high energy costs of immune defence (Demas et al., 2012). Individuals have limited energy and nutrients to allocate to different physiological functions, potentially resulting in trade-offs between costly activities (Demas et al., 2012).

Trade-offs between immune defence and other physiological processes have been documented in a variety of animals. Lactating bighorn ewes (*Ovis canadensis*) have been found to have larger parasite burdens than non-lactating ewes (Festa-Bianchet, 1989), indicating a possible trade-off between reproductive activity and immune defence. Mosquitoes (*Anopheles gambiae*) mounting an immune response allocated less protein to egg yolk formation and egg production than control insects (Ahmed et al., 2002). Laboratory mice (C57BL/6J) stimulated to mount an immune response involving antibody production consumed more oxygen, and therefore increased metabolic rate compared to controls (Demas et al., 1997). In great tits (*Parus major),* mounting an immune response was found to increase basal metabolic rate (BMR) by 9 %, and was associated with body mass loss (Ots et al., 2001). Some moderate infections can increase gluconeogenesis rates by 150 – 200 %, which may result in lean tissue wasting as the infection persists (Lochmiller and Deerenberg, 2000). Challenge with lipopolysaccharide (LPS), a component of the outer membrane of Gram-negative bacteria commonly used to elicit a strong immune response in animals, resulted in increased body mass loss, as well as a decreased rate of food intake and reproductive effort in house sparrows (*Passer domesticus*) (Bonneaud et al., 2003). In addition, Pap and Márkus (2003) found that experimentally increasing brood size in barn swallows (*Hirundo rustica*), and therefore increasing workload to parents, led to a decrease in measures of cellular immunity. Collectively, this evidence suggests that mounting an immune response comes at a cost to the individual, and immune activity may be down regulated in favour of other activities.

1.1 Migration

Animal migration is a phenomenon that has attracted much attention throughout history. Many different species make incredible movements across the Earth, to and from different habitats (Dingle and Drake, 2007). For instance, Monarch butterflies (*Danaus plexippus*) carry out a complex multi-generational migration from Canada to Mexico in order to take advantage of favourable environmental conditions in different habitats along the way (Flockhart et al., 2013). Humpback whales (*Megaptera novaeangliae*) migrate to the warm waters of the Caribbean in order to give birth where it is warm enough for newborn calves to survive, even though there is no food for the mother to eat (Brown et al., 1995). They then migrate up to 8500 km back to the cold waters of the Arctic where food is plentiful once the calf is able to undertake the long journey (Brown et al., 1995). Species of Pacific salmon migrate first as juveniles out to the ocean from freshwater streams, then as adults undergo the perilous journey back upstream sometimes over 3000 km to their natal stream, where they will spawn, and die (Fleming and Gross, 1989). These astonishing movements require a substantial amount of energy, and animals need to prepare for their journey by storing fuel in advance, for example Monarch butterflies are able to store up to 125 % of their lean body mass as fat in preparation for migration (Brower and Fink, 2006).

Of all of the examples of migratory animals, migratory birds are the best studied. Migration is very demanding for birds in terms of energy expenditure (Buehler and Piersma, 2008). Migratory songbirds make non-stop endurance flights up to thousands of kilometers between breeding and wintering grounds (Bairlein et al., 2012). For example, blackpoll warblers (*Setophaga striata*) make nonstop migratory flights over the Atlantic Ocean from North America to South America (Nisbet, 1970), and these flights were recently found to last from 49 to 73 hours (up to 2770 km) (DeLuca et al., 2015). During migratory flights of this magnitude, energy expenditure increases 8 – 10 times above the basal metabolic rate (BMR) (Pennycuick and Battley, 2003). To fuel these incredible athletic movements, birds accumulate 30 – 50 % of their body mass in fat (Berthold, 1975). By the time a bird reaches a stopover location, most of the energy stores built up by the bird will have been used (Kerlinger, 1987), and portions of the digestive tract will have been catabolized to provide necessary key metabolites and water during the journey (Gerson and Guglielmo, 2011). In addition, during refuelling at stopover birds may reach the maximum rate of energy deposition, indicating that it may not be possible for birds to acquire enough resources to support more than one physiologically demanding process at a time (Lindström, 1991). Infection during migration may

negatively affect a birds ability to migrate due to the increase in energy expenditure required to mount an immune response, as well as through direct damage to host tissues as a result of the infection or subsequent immune response.

The high energy costs of migration and immune activity have led to the hypothesis that there is a trade-off between immune system functioning and migration (Altizer et al., 2011), and several studies have found evidence to support this proposed trade-off. Swainson's thrush (*Catharus ustulatus*) in a migratory state were found to have a reduced cell-mediated immune response compared to control birds (Owen and Moore, 2008). Bewick's swans (*Cygnus columbianus bewickii*) infected with avian influenza delayed migration for more than one month, and flew shorter distances than un-infected conspecifics (van Gils et al., 2007). In addition, measures of constitutive immunity were reduced in European starlings (*Sturnus vulgaris*) after prolonged flight in a wind tunnel (Nebel et al., 2012), and Monarch butterflies (*Danaus plexippus*) experimentally infected with protozoan parasites flew at slower flight speeds and covered less distance overall than uninfected butterflies (Bradley and Altizer, 2005). Finally, immune challenge with LPS during stopover elicits an acute phase response in yellow-rumped warblers (*Setophaga coronata*) and causes a delay in departure (G. Mitchell, unpublished data). This evidence suggests that infection may have a profound impact on the ability of a migratory bird to carry out an endurance flight as a result of energetic trade-offs between immune defense and flight.

Migratory birds are often thought to play a significant role in the spread of disease between geographic regions (Altizer et al., 2011). There is evidence that many migratory bird species are infected with a number of pathogens that may have the potential to cause harm to their hosts, as well as to other populations and species. These pathogens include avian influenza A (Altizer et al., 2011), West Nile virus (Rappole and Hubálek, 2003), and avian malaria (Valkiunas, 2005). In order to understand how migratory birds may facilitate the transmission of disease across large geographic distances, it is important to study host-pathogen interactions. Recent research has begun to focus on blood parasitic infection in migratory birds, and the effect that these infections have on individuals and populations. In an experiment using house martins (*Delichon urbica*) infected with *Heomoproteus prognei*, researchers found that clutch size was reduced in birds with heavier infections (Marzal et al., 2005). Experimental infection of Hawaii Amakihi (*Hemignathus virens*) with *Plasmodium relictum* significantly decreased food consumption and body mass, and mortality rate was 65 % (Atkinson et al., 2000).

1.2 Haemosporidians

Haemosporidian parasites are a group of apicomplexan protozoa that are widespread across the Earth (Valkiunas, 2005). Haemosporidia have an indirect life cycle in which they infect both invertebrate and vertebrate hosts, and are known to be pathogenic. The most well known example is malaria. Avian malaria and pseudo-malarial type diseases are caused by *Plasmodium* spp. and *Haemoproteus* spp., respectively (Valkiunas, 2005). These two groups of parasites are transmitted from host to host by blood sucking insect vectors, such as mosquitoes, biting midges, and louse flies (Santiago‐Alarcon et al., 2012). When an infected insect takes a blood meal from a bird, sporozoites are injected into the avian host's blood stream, and migrate into the bird's tissues (liver, spleen) (Asghar et al., 2012). In the cells within the bird's tissues, the parasite undergoes schizogony, and produces merozoites, which re-enter the blood stream and penetrate red blood cells (Valkiunas, 2005). Within the red blood cells, the malarial parasites mature into gametocytes, consume haemoglobin and eventually cause the erythrocyte to burst; this is the infective stage where a mosquito may take up gametocytes when they take a blood meal (Valkiunas, 2005). During the erythrocytic phases, infection intensity (parasitemia) rises as the parasite invades an increasing number of red blood cells; the infection is considered in acute phase (Valkiunas, 2005).

During the acute phase of malarial infection, a crisis peak of parasitemia is reached and birds may die due to severe anaemia, emaciation, myopathy as parasite megalomeronts develop in musculature, and increased risk of predation as a result of an inability or impaired ability to fly (Valkiunas, 2005). The majority of the pathology of malaria infections is caused by the cytokine cascade triggered as a result of the parasite bursting out of red blood cells (Zambrano-Villa et al., 2002). Destruction of erythrocytes by the malaria parasite induces chemical changes in the blood, increasing protein concentration and decreasing pH (Valkiunas, 2005). This change leads to a decrease in the oxygen binding capacity of haemoglobin (Valkiunas, 2005). In addition, glycogen, protein, and RNA levels in the liver, spleen and brain may decrease in infected birds (Reddy et al., 1980), indicating that malaria may impose considerable changes to the metabolism of birds (Valkiunas, 2005). During spring migration, young yellow-rumped warblers, magnolia warblers (*D. magnolia*), and yellow warblers (*D. petechial*) with higher parasitemia had reduced energetic condition compared to birds with less intense infections (DeGroote and Rodewald, 2010). It has been suggested that younger birds may be particularly susceptible to the stress of their first migration and as such may lower their ability to mitigate the possible energetic costs of infection.

Additionally, summer and scarlet tanagers (*Piranga rubra* and *P. olivacea*) infected with *Haemoproteus* have been found to have lower fat scores and lower body mass compared to uninfected individuals (Garvin et al., 2006). If a bird survives the acute phase, parasitemia is reduced to a very low level, and the infection is then considered to be chronic (Valkiunas, 2005). Chronic malarial infections have been found to persist with the individual for the remainder of the individual's life if the initial infection is not cleared (Snounou et al., 2000).

Once an individual is infected and in the chronic stage, it is common for the infection to relapse during periods of stress (Valkiūnas et al., 2004). This is due to activation of exoerythrocytic merogony, where the malaria parasites emerge from tissue cells and enter circulation (Valkiunas, 2005). Spring relapse in infection seems to be a common occurrence in migratory birds, with increasing parasitemia tending to coincide with reproductive activity (Applegate and Beaudoin, 1970). Increased parasitemia has been documented in house sparrows (*Passer domesticus*) given corticosterone as a treatment to mimic stress compared to control birds (Applegate and Beaudoin, 1970). Another potential mechanism of spring relapse is that the parasite may time its emergence from tissues and reproduction to coincide with the increasing abundance of vectors and hosts in the spring, and over the course of the breeding season (Atkinson and van Riper, 1991).

1.3 Sources of variation in Haemosporidian infection status in birds

Both Haemosporidian infection prevalence and parasitemia have been found to vary in nature with the age and sex of the avian host. Adult birds are generally more likely to be infected with malarial parasites than younger birds (Allander and Bennett, 1994; Hasselquist et al., 2007). This may be due to the fact that adult birds have had an increased time of exposure to infected vectors, increasing the probability of infection. Although van Oers et al. (2010) found that adult Seychelles warbler (*Acrocephalus sechellensis*) were less likely to be infected with blood parasites than younger birds, they attributed this finding to selective mortality of some heavily infected birds, and acquired immunity by others. Most studies have found that infected juveniles tend to have higher parasitemia compared to adults (Allander and Bennett, 1994; Sol et al., 2003). Two possible explanations have been suggested for this pattern; the immune system of juveniles has not developed sufficiently to fight the infection, or younger birds are more prone to the stress of migration, which may suppress immunity and allow infection to proliferate (Hasselquist et al., 2007). It is also possible that juveniles may be in the acute phase of infection, resulting in higher recorded parasitemia.

Confounding the effect of age on malarial infection prevalence and parasitemia is the sex of the host. Testosterone is thought to have suppressive effects on the immune system (O'Neal and Ketterson, 2012), which may result in males being more susceptible to infection. In song sparrows (*Melospiza melodia)*, testosterone has been found to decrease both cell-mediated and humoral immunity in non-breeding males (Owen‐Ashley et al., 2004). However, testosterone may act indirectly on immune functioning, as male song sparrows implanted with 5∝-dihydrotestosterone (DHT), an androgen similar to testosterone in function that cannot be aromatized into estradiol, did not supress cell-mediated or humoral immunity (Owen‐Ashley et al., 2004). This suggests that testosterone may be converted to estradiol through aromatization in order to act on steroid receptors of immune cells resulting in immune suppression (Owen‐Ashley et al., 2004). Although this effect of estradiol has been proposed, estrogen has also been linked to the enhancement of certain immune components (Olsen and Kovacs, 1996). Humoral immunity is thought to be enhanced by increased concentrations of circulating estrogen, although cell-mediated immunity may be suppressed at certain concentrations (Olsen and Kovacs, 1996; Owen‐Ashley et al., 2004). In general, females (including birds and mammals) are thought to have stronger immune systems when compared to male conspecifics (Sheldon and Verhulst, 1996). Higher levels of circulating IgM antibodies have been demonstrated in female humans compared to males (Butterworth et al., 1967), indicating a stronger humoral immune response. However, a female sex-bias in parasite prevalence has been documented during the breeding season in birds in a comparison of polygynous and monogamous species (McCurdy et al., 1998). In addition to hormonal influences, the mobility of birds may affect the probability of infection. For example, birds that spend more time incubating the brood (typically females) over the course of the breeding season will be less mobile than those that spend more time foraging, and this decrease in activity increases the possibility of being bitten by an infected vector, thereby increasing the chances of infection (Valkiunas, 2005).

Both infection prevalence and parasitemia appear to vary over the annual cycle, as well as among years. In dark-eyed juncos (*Junco hyemalis*), prevalence of infection tends to be high at the arrival to the breeding grounds, and variable during the breeding season, increasing for most parasites (Deviche et al., 2001). Parasitemia of *Leucocytozoon* was also found to increase from spring to summer, and then decrease during the onset of autumn migration (Deviche et al., 2001). Typically, parasitemia tends to be much lower during autumn migration than during the spring migratory period (Valkiunas, 2005). *Plasmodium relictum* infection in house sparrows was found to

vary substantially with season, no infections were found in the autumn, while in the spring infection prevalence rose to 36.8 % within the same population (Applegate, 1971). This is thought to be due to the malarial parasites timing emergence from host tissues and their reproduction to coincide with the increasing temperature and vector abundance over the course of the spring months, facilitating transmission to new susceptible hosts over the course of the spring and summer breeding period (Lalubin et al., 2013). Due to the somewhat contradictory evidence supporting age and sex differences in infection status, and limited support for temporal effects within the migratory period, determining how each of these variables affects the likelihood a bird will become infected, and the resulting infection intensity will be important in order to predict how avian malaria will spread throughout the population of migratory birds, and potentially affect other naïve, and highly susceptible wild and domestic species. For example, *Plasmodium relictum* was introduced to Hawaii and infection has threatened the variety of Hawaiian honeycreepers (Drepanididae) native to the islands, where most species exhibit 50-100% mortality (Ricklefs et al. 2005).

1.4 Diagnostic Methods to Detect and Quantify Haemosporidian Infections

In the past, avian malaria has been primarily diagnosed via blood smear analysis. This involves visually scanning a blood smear under the microscope for cells infected with *Plasmodium* spp. or *Haemoproteus* spp. (Valkiunas, 2005). Typically, parasitemia is measured as the number of infected erythrocytes per 10,000 red blood cells and low intensity infections are commonly missed due to human error (Bentz et al., 2006). Recently, molecular techniques such as polymerase chain reaction (PCR), and quantitative polymerase chain reaction (qPCR) that amplify portions of the cytochrome b gene of the parasite have been used in order to diagnose and quantify infections more accurately and efficiently than traditional microscopy diagnostics (Bentz et al., 2006). Although these methods can detect infections of very low intensity, efficacy of newly developed assays may need validation by traditional blood smear analysis (Valkiūnas et al., 2006), and there is a pressing need to determine the reliability of molecular methods.

1.5 Yellow-rumped Warblers as a Model Study System

The yellow rumped warbler is an intermediate distance migratory songbird that breeds throughout the boreal forest of Canada and Alaska and winters throughout the southern United

States and into Central America (Hunt and Flaspohler, 1998). It is split into two subspecies, the Audubon warbler that occupies the western portion of the species range, and the Myrtle warbler, which occupies the eastern portion of the species range. Full grown, both the Myrtle and Audubon subtype typically weigh between 12 and 13 g (Hunt and Flaspohler, 1998). Breeding season occurs over the course of the summer months in the boreal forest where females are primarily responsible for nest construction, and breeding pairs will have one to two broods per season of up to six eggs in each (Hunt and Flaspohler, 1998). Nestlings remain in the nest for 10 – 14 days, after which they depart as fledglings, and are cared for by parents for up to 14 days (Hunt and Flaspohler, 1998). Autumn migration begins in early August for birds at the most northern limit of the range, and continues until mid October, and Spring migration occurs from late March and early April up until the end of April into early May (Hunt and Flaspohler, 1998). Yellow-rumped warblers are commonly caught during the migratory periods in Southern Ontario, they are known to be infected with Haemosporidian parasites (DeGroote and Rodewald. 2010), and tend to be easy to keep in captivity, making them a good candidate for a model species (an organism that may be used to study a particular biological phenomenon and that may provide insight into how other organisms may function). It is important to use a model species in order to test host-parasite interactions and infection dynamics in order to determine how migratory birds may respond to and spread disease over large geographic distances.

1.6 Objectives

My thesis research had three major objectives: 1) to develop a molecular method to accurately determine infection prevalence and parasitemia in yellow-rumped warblers; 2) to determine the effects of age and sex on infection status and immune functioning, and the effect of infection status and immune activity on the timing of passage through stopover during autumn migration; and 3) to investigate seasonal changes in malarial infection, and the effect of infection on refuelling and flight performance of captive warblers.

I hypothesized that infection prevalence and intensity would vary with age and sex of the avian host, and that infection status would affect timing of passage through stopover. Individuals passing through stopover later would have higher infection prevalence and intensity. Late migrants may have been forced to delay migration in order to fight infection, or may be required to take shorter flights due to a decreased aerobic and endurance capacity. Regarding measures of immune system function in wild birds I hypothesized that immune defense would vary with infection status

and intensity. I predicted that as infection intensity increases, measures of immune defense would increase in order to control infection, or alternatively, as infection intensity increases, levels of immune defense may be decreased as a result of the underlying quality or condition of the bird.

For captive warblers I hypothesized that parasitemia should vary with season and that fuelling rate and endurance flight performance would be negatively affected by blood parasite presence and infection intensity. I predicted: 1) that parasitemia would increase from the winter to spring (indicating spring relapse in infection); 2) that infected birds would have lower weight gain than uninfected birds as a result of parasites sequestering resources from the host, and 3) that birds with higher parasitemia would have poorer flight performance in a wind tunnel than uninfected birds and those birds with lower parasitemia. Decreased levels of haemoglobin will diminish the ability of blood to efficiently carry oxygen to working tissues, and long distance flight may not be possible.

2 Methods

2.1 Sample Collection and husbandry

Yellow-rumped warblers were caught using mist-nets at the Old Cut field station at Long Point Ontario Bird Observatory, Port Rowan, Ontario, from September 26 to October 23, 2013. A total of 96 birds for use in the wild portion of the study were sexed by plumage and aged by skull ossification (Pyle, 1997). Twenty-two of the birds caught were after hatch year (AHY) birds and 74 were hatch year (HY) birds, 35 of which were female, 45 male, and 16 of unknown sex based on plumage (Table 1). Birds of unknown sex were molecularly sexed by PCR (see below) (Griffiths et al., 1998). Body mass, wing chord and fat score (0-8 scale) were recorded (Table 1) and a blood sample (approximately 100 μL per bird) was taken from the brachial vein. The birds were then released. A drop of blood was added to lysis buffer (50 mL 1M Tris pH 8.0, 100 mL 0.5M EDTA pH 8.0, 1 mL 5M NaCl, 25 mL 10 % Sodium Dodecyl Sulphate, 324 mL water), two blood smears per bird were prepared using 3 μL of blood and air-dried, and excess blood was centrifuged at 2000 x g for 10 minutes; red cells and plasma were separated and stored at -80 °C. Dried blood smears were fixed while in the field by soaking in 100 % methanol for 5 minutes within 1 hour of blood smear preparation, and stored in a slide box for later staining and investigation of parasite prevalence and infection intensity using microscopy techniques. Staining was performed using a Fisher HealthCare™ PROTOCOL™ Hema 3™ Manual Staining System, following the instructions included in the kit. (Fisher Scientific, Pittsburgh).

During the autumn field season of 2013, 60 additional yellow-rumped warblers were caught at the Old Cut field station at Long Point Bird Observatory, Port Rowan, Ontario, and transported back to the Advanced Facility for Avian Research (AFAR) at the University of Western Ontario, London, Ontario for a concurrent diet manipulation study examining flight performance (M. Dick and C.G. Guglielmo, unpublished data). Birds were kept in groups of 30 in two large indoor aviaries (3.7 x 2.4 x 3.1 m) at a natural photoperiod of 12h light: 12h dark and fed a carbohydrate mash diet that included canola oil as the main fat source (16.29 % Glucose, 3.62 % caesin, 1.63 % Agar, 1.59 % Brigg's Salt, 0.54 % Vitamin Mix, 72.39 % Water, 3.08 % Oil, 0.87 % Cellufil), as well as meal worms. Birds were allowed to acclimate for 2 weeks, after which each bird's flight propensity was assessed in the wind tunnel (see below). Photoperiod was then changed to a short day cycle (9h light: 15h dark), simulating winter. Prior to the beginning of the experimental wind tunnel flights, birds were assigned to 10 experimental blocks of 6 birds each (1 control bird and 1 flown bird for each of the 3 experimental diets) and weighed weekly using an electronic balance to the nearest 0.001 g. At the end of February 2014, one at a time, blocks were switched to a long day photoperiod (18h light: 6h dark) stimulating birds to become migratory. Switching the photoperiod of each block one at a time allowed each bird to be sampled at roughly the same migratory state. Overall body mass gain was recorded over the course of the experiment, and average rate of body mass gain of each bird after the switch to long day photoperiod was calculated using repeated measures of body mass. The diet manipulation began at the time of separation into blocks. Diets were composed of the carbohydrate mash, and altered to differ in fatty acid composition. The three experimental diets included a high monounsaturated fatty acid diet, a high omega-3 polyunsaturated fatty acid diet, and a high omega-6 polyunsaturated fatty acid diet. Effects of diet on flight performance are presented elsewhere (M. Dick and C.G. Guglielmo, unpublished data), and diet is taken into account as a factor within all statistical analyses. All animal procedures were approved by the University of Western Ontario Animal Use Sub-Committee (protocols 2010-020, 2010-216, appendix B) and birds were sampled under scientific collection permits from the Canadian Wildlife Service (CA-0255, CA-0256, appendix C).

2.2 Flight Group Assignment and Wind Tunnel Flights

Prior to the beginning of experimental wind tunnel flights of up to 6 h, captive birds were flown in the hypobaric wind tunnel at the AFAR for 20 min and assigned a flight score ranging from 1 (unable to maintain flight, continuous landing, needed consistent prodding) to 5 (strong flight,

flew at consistent speed without any prompting required). Birds were then separated into Flown (*N* = 30) and Control (*N* = 30) birds based on the top 30 and bottom 30 flight scores, respectively. Control birds were not flown, but were included in the data analysis to determine if flight score assignment was affected by infection status.

Starting at the end of April 2014, the remaining 26 birds (4 birds died during the course of the experiment) from the flown group were flown in the wind tunnel at the AFAR. All birds were fasted for 2 hours before the normal lights-off time. Control birds were sampled within 30 minutes of lights out. Birds were weighed using an electronic balance to the nearest 0.001 g, and scanned by quantitative magnetic resonance (QMR) to determine body composition (Guglielmo et al., 2011). Flown birds were then allowed to rest for 5 minutes before the flights took place. Flights occurred at night and were controlled under low light at 8 m/s, 70 % relative humidity, 15 °C, and voluntary flight duration was recorded. Flights were ended either when a bird stopped flying voluntarily, or reached 6 hours of voluntary flight.

2.3 Blood Sampling of Captive Birds

Initial short day blood samples were taken from all captive birds (N = 60) 2 weeks prior to the switch to long day photoperiod. Additional blood samples were taken from all flown birds immediately post flight, and from control birds prior to the scheduled flight each night. From each bird, 100 µL of blood was taken using 70 µL heparinized capillary tubes and transferred to 0.6 mL heparinized microcentrifuge tubes. Two blood smears per bird were made from the blood samples taken during short day photoperiod (Valkiunas, 2005), 10 µL of blood was stored in lysis buffer within 0.6 mL heparinized microcentrifuge tubes, and the remainder was centrifuged at 2000 x g for 10 min, separated into individual microcentrifuge tubes containing plasma and red cells, and stored at -80 °C. Blood taken from flown birds post flight and from control birds during the sampling period just prior to the fights each night was not stored in lysis buffer in order to save time during sampling and dissection. Birds were euthanized under isoflurane anaesthesia for analysis of tissues (muscle, adipose, liver, heart) for the concurrent diet study and sex was confirmed post mortem.

2.4 Plasma Indicators of Immunocompetence

Immunocompetence of birds was determined by measuring plasma concentrations of haptoglobin and immunoglobulin Y (IgY). Haptoglobin is an acute phase protein involved in the acute phase response and increases during non-specific inflammation (Matson et al., 2012), whereas

IgY is the major antibody produced by birds. These two assays were chosen to provide a general measure of both the innate and adaptive branches of the immune system, respectively.

Plasma haptoglobin concentration was measured for all birds using a Tridelta Phase™ haptoglobin colorimetric assay (TP801; Tri- Delta Diagnostics, NJ, USA) kit according to Matson et al. (2012). Immunoglobulin Y (IgY) concentration was measured with an Enzyme-linked immunosorbant assay (ELISA) using anti-chicken antibodies as described by Bourgeon et al. (2006) yielding an absorbance value as a unit-less measure of the antibody concentration. Due to a limited amount of plasma, IgY concentration was not measured for birds kept in captivity.

2.5 Blood Smear Analysis

Each blood smear was examined visually by oil immersion under high magnification (1000 x) for *Plasmodium* and *Haemoproteus* infection per 10,000 erythrocytes using identification methods described by Valkiunas (2005). For each slide, 25 pictures of random fields of view were taken using SPOT Imaging Solutions™ (SPOT Basic Image Capture V. 5.2); these pictures were used to determine parasitemia by counting the number of infected cells per red blood cells. For each bird, approximately 2000 red blood cells were counted. This method of determining parasitemia has been shown to be correlated to counting the number of infected cells per 10,000 red blood cells under the microscope (Lewicki, 2013). Identification of infection and subsequent parasitemia was used to calibrate and assess the accuracy of results from PCR and quantitative PCR assays (qPCR).

2.6 PCR and Quantitative Real-time PCR for Sex Identification, Infection Prevalence and Intensity

Genomic DNA was extracted from blood samples using a QIAGEN DNeasy Blood and tissue kit (Cat. 69504) following the directions included in the kit, but increasing incubation with proteinase k to 15 minutes and repeating the wash with Buffer AW2 for blood samples stored in lysis buffer. DNA concentration was measured using a Thermo-Scientific Nano-drop 2000. Wild birds of unknown or ambiguous sex based on plumage were molecularly sexed following methods described by Griffiths et al. (1998). The chromo-helicase-DNA-binding (CHD) genes located on the avian sex chromosomes (Z and W) were targeted. A single set of primers, P2 and P8, was used which amplify the CHD genes on each chromosome during PCR (Griffiths et al. 1998). Products were separated by electrophoresis for 60 – 90 minutes at 100 V in a 1.5 % agarose gel stained with

RedSafe™ Nucleic Acid Staining Solution (iNtRON Biotechnology, Inc.). Results were visualized under UV light and compared to a known male and a known female control. Since female birds possess both a Z and a W chromosome, two bands present on the gel represent a female, whereas male birds have two copies of the Z chromosome, one band present on the gel represent a male (Griffiths et al., 1998). Sizes of the PCR products were also compared, as a polymorphism in the Z chromosome has led to the appearance of two bands (smaller in size compared to a typical female banding pattern) in some males (Dawson et al. 2001).

Samples from wild birds were screened for *Plasmodium* and *Haemoproteus* DNA using a nested PCR design targeting the cytochrome b gene of the parasite mitochondrial DNA following the protocol outlined by Hellgren et al. (2004). Primers HaemNF1 and Haem NR3 were first used for amplification of the Haemosporidian cytochrome b gene, then primers HaemF and HaemR2 were used for amplification of *Plasmodium* and *Haemoproteus* lineages (Hellgren et al. 2004). A random subset of positive PCR products (N = 26) were cleaned up using Geneaid Gel/PCR DNA Fragment Extraction Kit (Cat. # DF100) and sent for sequencing using the ABI377 DNA sequencer at the DNA Sequence Facility at The John P. Robarts Research Institute, the University of Western Ontario, London, ON. Sequences were aligned using Sequencer 5.3 (Gene Codes) and results were BLAST searched in the MalAvi database to determine parasite lineage and partial or whole DNA sequence. Parasite DNA sequences were used to determine suitability of both newly designed and published primers.

The published primers L4050Plasmo (5’-GCTTTATGTATTGTATTTATAC-3’), H4121Plasmo (5’-GACTTAAAAGATTTGGATAG-3’) (Christe et al., 2012) amplified both *Plasmodium* spp. and *Haemoproteus* spp. identified within our sample. L4050Plasmo and H4121Plasmo primers target a region of the cytochrome b gene of *Plasmodium* and *Haemoproteus* mitochondrial DNA that is conserved across these genera (Christe et al., 2012). The newly designed primers were created using IDT PrimerQuest, and were found to be too specific to different lineages within our sample to be used as a general diagnostic test of avian malarial infection (Table 1 in appendix A). Primers targeting the SFSR gene were chosen to amplify host DNA, SFSR/3Fb (5’-ACTAGCCCTTTCAGCGTCATGT-3’) and SFSR/3Rb (5’ -CATGCTCGGGAACCAAAGG-3’) (Podmokła et al., 2014). The SFSR gene is a single copy of nuclear DNA that is ultra conserved across vertebrates (Bejerano et al., 2004), and comparison of the quantity of this gene to the quantity

of parasite DNA allowed us to determine the approximate amount of parasite DNA per cell for each sample.

A SYBR-green chemistry qPCR assay was developed using a similar protocol to Christe et al. (2012) and Podmokła et al. (2014), and was used to measure prevalence and parasitemia in all birds. Final reaction volume for each assay was 7 µL; including 5 µL SYBR-Green master mix (3.5 µL SensiFAST™ SYBR® No-Rox, 0.014 µL of each primer at 100 nM, and 1.5 µL Ambion® RT-PCR Grade Water), and 2 µL of 2 ng/µL genomic DNA. qPCR was performed using the Rotor-gene 6000 (Corbett Science) with the following thermal profile: hold at 95 °C for 5 min, followed by 42 cycles of 95 °C for 15 sec, 47 °C for 10 sec (60 °C for SFSR3b), 72 °C for 5 sec. Samples were run in duplicate, and Ct (cycle threshold) was scored as an average across duplicates. If Ct values between duplicates were greater than 1 Ct apart, analysis was repeated. Efficiency of qPCR assays was evaluated based on fluorescence curve slope and determined to be equal in order to assess parasitemia by relative quantification. Parasitemia was then calculated as $P=2^{\Delta\Delta Ct}$.

2.7 Data Analysis

2.7.1 Wild population (Autumn migration)

All data analysis was performed using R version 3.1.2. Parasitemia of all wild birds and 6 captive birds as determined from microscopy and from qPCR was correlated using a Pearson correlation. Following this correlation, qPCR parasitemia data were used for all further analysis. Parasitemia data were not normally distributed, and was log transformed to in order to normalize the data.

I tested whether infection prevalence and parasitemia of wild birds varied with age, sex, body condition, and capture date of the host, and whether haptoglobin and IgY varied with infection status of individual wild birds, as well as age, sex, body condition, and capture date with generalized linear models and linear models, respectively. To determine the most parsimonious model, I evaluated all possible model sub-sets and ranked models based on Akaike Information Criterion (AICc) using the glmulti package (which uses maximum likelihood estimation). If the top model was within two AICc units of the null model, model selection was complete and the null model was considered most parsimonious. When the top three models had AICc values less than two AICc units relative to the null model, I fit interaction terms between the remaining variables to determine if the interactions improved the fit of the models. I evaluated collinearity between predictor variables using

correlation coefficients and in no case were predictors strongly correlated ($r < 0.7$). For the three top models from each analysis, I visually assessed homogeneity of variance, normality of error, and curvilinear relationships between response and predictor variables.

2.7.2 Captive population (Spring migration)

I tested whether the likelihood of infection prevalence varied with the initial flight or control group assignment of captive birds using a Chi-Squared test. Mean infection intensity of the flight and control groups were compared using a two-tailed t-test.

To assess the effect of season on parasitemia, the change in parasitemia of captive birds from winter to spring was analyzed with a linear mixed effect model with individual fitted as a random effect (taking into account repeated measures on the same individual). Birds that tested negative for infection in both the winter and spring were excluded from analysis. I tested whether sex, haptoglobin levels, and body condition of birds affected the change in parasitemia from winter to spring by using all sub-set model selection, following the same criteria previously outlined.

To assess the effect of infection prevalence on flight performance of captive birds, flight duration was modeled linearly as a function of infection prevalence, sex, body mass, diet, and haptoglobin. All subset model selection was carried out using the same procedure previously outlined. I then analyzed whether parasitemia affected flight duration in infected birds only, and included sex, body mass, diet, and haptoglobin as additional predictor variables in the linear and generalized linear models. All subset model selection (using linear models) was then carried out using the same criteria previously outlined.

To determine the effect of infection on refuelling of birds, I carried out the same all-subset model selection procedure for both overall body mass change of captive birds and for the average rate of body mass change for captive birds from the switch to long day light cycle to the end of the experiment. Sex, diet, and haptoglobin were included as predictors in the initial linear models. Finally, I tested whether haptoglobin was affected by flight duration and parasitemia, and included sex, body mass and diet as additional predictor variables in the initial linear model. All subset model selection was carried out using the same procedure used for each set of model selection.

3 Results

For birds used in the wild portion of the study, mean body mass of adult male and female birds at time of capture was 12.80 g (SD = 0.87) and 11.86 g (SD = 0.67), respectively, and mean body

mass of juvenile male and female birds was 12.97 g (SD = 1.20) and 12.02 g (SD = 0.97), respectively (Table 1). Males were significantly heavier than females (GLM; F = 6.85; p = 0.03). Mean fat score of male and female juvenile birds and male and female adult birds was 2.24 (SD = 1.40) and 1.65 (SD = 1.35), respectively, and 1.92 (SD = 0.79) and 2.5 (SD = 1.43) respectively (Table 1). Fat score did not differ significantly between males and females (GLM; F = 1.44; p = 0.23) or between adult and juvenile birds (GLM; F= 0.53; p = 0.46). However, juvenile males tended to have higher fat scores, this trend was approaching significance (GLM; F = 3.31; p = 0.07). Mean wing length of adult male and female birds was 73.50 mm (SD = 1.83) and 69.90 mm (SD = 1.19), respectively, and for juvenile males and females was 72.30 mm (SD = 1.92) and 69.02 mm (SD = 2.33), respectively (Table 1). Females had significantly shorter wing length than males (GLM; F = 23.49; p < 0.001).

Table 1. Mean body mass, wing length, and fat score of male and female juvenile (HY) and adult (AHY) yellow-rumped warblers (96 total) caught at Long Point Ontario during the autumn migration of 2013

		Body mass (g)		**Wing length (mm)**		**Fat score**	
	N	Mean	SD	Mean	SD	Mean	SD
HY Males	37	12.97	1.20	72.30	1.94	2.24	1.40
HY Females	37	12.02	0.97	69.02	2.33	1.65	1.35
AHY Males	12	12.80	0.87	73.5	1.83	1.92	0.79
AHY Females	10	11.86	0.67	69.90	1.19	2.5	1.43

3.1 Efficacy of PCR and qPCR based techniques at diagnosing of avian blood parasite infection

Overall, 46.5 % of the blood smears analysed (including all blood smears from wild birds, and blood smears from 6 captive birds) were diagnosed as infected with *Plasmodium* and/or *Haemoproteus* spp. Results obtained from qPCR indicated that 62.3 % of birds (including all wild birds sampled and 6 captive birds) were infected with *Plasmodium* and/or *Haemoproteus* spp.

Mean parasitemia of wild birds as well as 6 captive birds as identified by microscopy was determined to be 0.01 (SD = 0.01), or 1 % of erythrocytes were found to contain *Plasmodium* and/or *Haemoproteus* spp. The mean parasitemia score of wild birds and 6 captive birds as determined by

qPCR was 0.06 (SD = 0.10), or infected erythrocytes made up approximately 6 % of the blood sample analyzed. Although both prevalence and parasitemia tend to be slightly under-estimated by microscopy, results of a Pearson correlation revealed that parasitemia of birds as determined by microscopy and by qPCR were significantly correlated ($r = 0.91$; $n = 102$; $p < 0.001$; Figure 1). Following this result, parasitemia as determined by qPCR only was used in all further analysis.

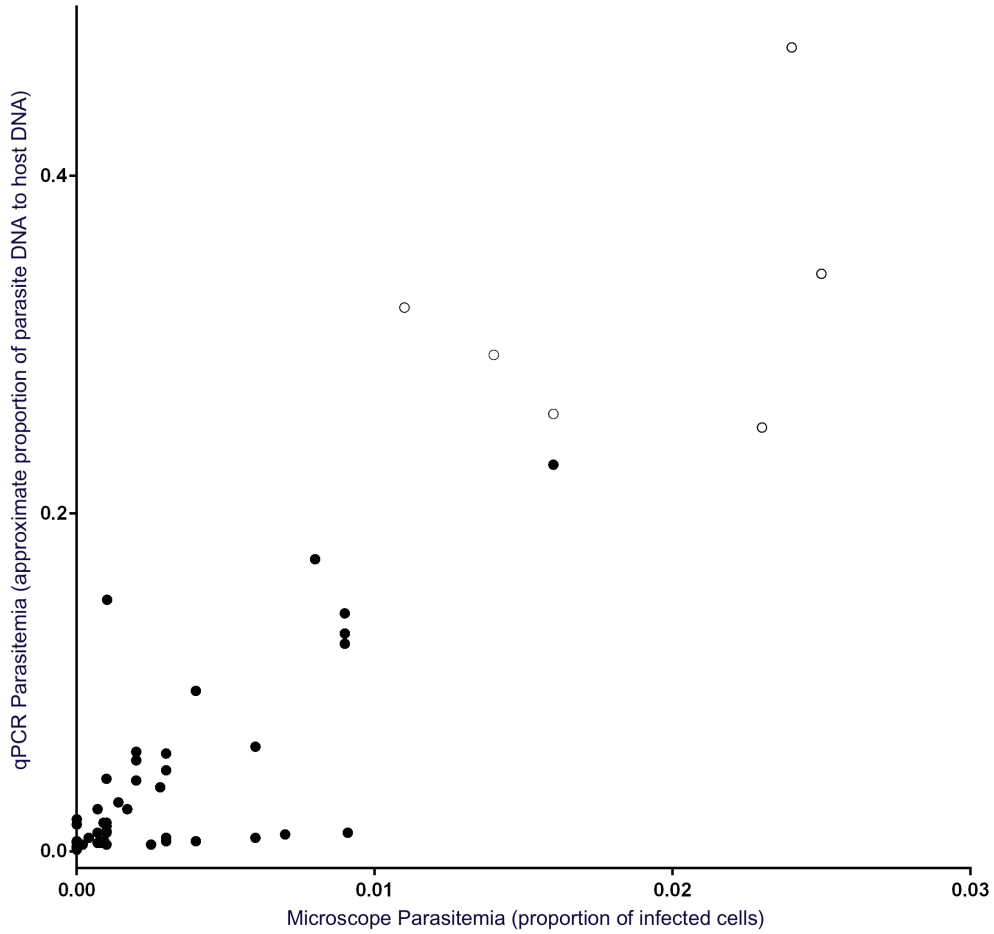

Figure 1. The relationship between parasitemia as measured by microscopy (proportion of infected cells) and parasitemia as measured by qPCR (proportion of parasite DNA to host DNA) of all wild caught yellow-rumped warblers sampled during the autumn migration period (n=96) and a subset of captive birds sampled during the winter period (n=6). The black circles represent wild birds and the white circles represent captive birds. Pearson correlation $r = 0.91$, $p < 0.001$.

3.2 Infection Prevalence in Wild Warblers

59.4 % of wild warblers were infected with *Haemoproteus* and/or *Plasmodium* based on qPCR results. 54.5 % of adult birds and 60.8 % of juvenile birds were infected, and 53.2 % of females and 65.3 % of males were infected with *Haemoproteus* and/or *Plasmodium* (Table 2). Overall, model selection suggested that the null model was the most parsimonious model, i.e., infection prevalence was not explained by any of the factors considered (difference in AICc between the top model and the null model = 0.56; Table 2 in appendix A). Interaction terms did not improve the fit of the models.

3.3 Infection Intensity in Wild Warblers

Parasitemia of *Haemoproteus* and/or *Plasmodium* ranged from 0 to 0.32 (approximate proportion of parasite DNA to host DNA). Parasitemia of adult birds ranged from 0 to 0.13, and parasitemia of juvenile birds ranged from 0 to 0.32 (approximate proportion of parasite DNA to host DNA) (Table 2). Males had parasitemia ranging from 0 to 0.32, and females had parasitemia ranging from 0 to 0.13 (approximate proportion of parasite DNA to host DNA) (Table 2). Again, none of the predictor variables considered significantly explained the variation in parasitemia data (difference in AICc between the top model and the null model = 1.67 ; Table 3 in appendix A). Interaction terms did not improve the fit of the models.

3.4 Immune Activity

Mean plasma haptoglobin concentration of males and females were 0.26 mg/mL (SD = 0.10) and 0.29 mg/mL (SD = 0.10), respectively, and 0.28 mg/mL (SD = 0.09) and 0.27 mg/mL (SD = 0.10) for adults and juveniles, respectively (Table 2). The top haptoglobin model included capture date, body mass, and fat score as predictor variables (difference in AICc between the top model and the null model = 24.72; Table 4 in appendix A). Plasma haptoglobin concentration decreased with date and this decrease was stronger earlier in the season (estimate = - 2.33e-4 + 8.43e-14; $\beta + \beta^2$ = 7.95e-5 + 2.88e-14; Table 3; Figure 2), and decreased linearly with body mass (estimate = -0.02; β = 0.01; Figure 3). Overall R^2 for the model was 0.36. Adding interaction terms between the variables did not improve the fit of the model.

Mean IgY concentration of adult and juvenile birds was 0.37 (SD = 0.11), and 0.36 (SD = 0.11), respectively, and was 0.35 (SD = 0.11) and 0.34 (SD = 0.11) in males and females, respectively

(Table 2.). None of the variables examined explained the variation in IgY (difference in AICc between the top model and the null model = 0.50 ; Table 5 in appendix A).

Table 2. Infection prevalence (percentage of total wild birds sampled), parasitemia, and plasma Haptoglobin and IgY concentration of wild yellow-rumped warblers sampled (96 total) during the autumn migration at Long Point Ontario, 2013.

		Infection prevalence (%)	**Parasitemia (approximate proportion of infected cells)**		**Haptoglobin (mg/mL)**		**IgY (absorbance)**	
	N		Median	Range	Mean	SD	Mean	SD
HY Males	74	67.5	0.005	0-0.32	0.25	0.10	0.36	0.11
HY Females	22	57	0.0032	0-0.03	0.29	0.11	0.37	0.10
AHY Males	49	50	0.002	0-0.13	0.30	0.09	0.35	0.11
AHY Females	47	50	0.0002	0-0.06	0.28	0.09	0.41	0.09

Table 3. Top 3 models and the null model for plasma haptoglobin concentration data of wild caught birds during autumn migration.

Model	**AICc**	**ΔAICc**	**Relative likelihood**	**Akaike weight (w)**	**F**	**df**	**p value**	**R^2**
Haptoglobin ~ 1	-132.12	24.72	0.00	0.00				
Haptoglobin ~ 1 + Capture Date + Capture date^2 + Body mass + Fat Score	-156.48	0.00	1.00	0.47	9.84	4,71	0.0000022	0.36
Haptoglobin ~ 1 + Capture Date + Body mass	-155.64	1.21	0.55	0.26	11.63	3,72	0.0000027	0.33
Haptoglobin ~ 1 + Capture Date	-155.74	1.10	0.57	0.27	16.19	2,73	0.0000015	0.31

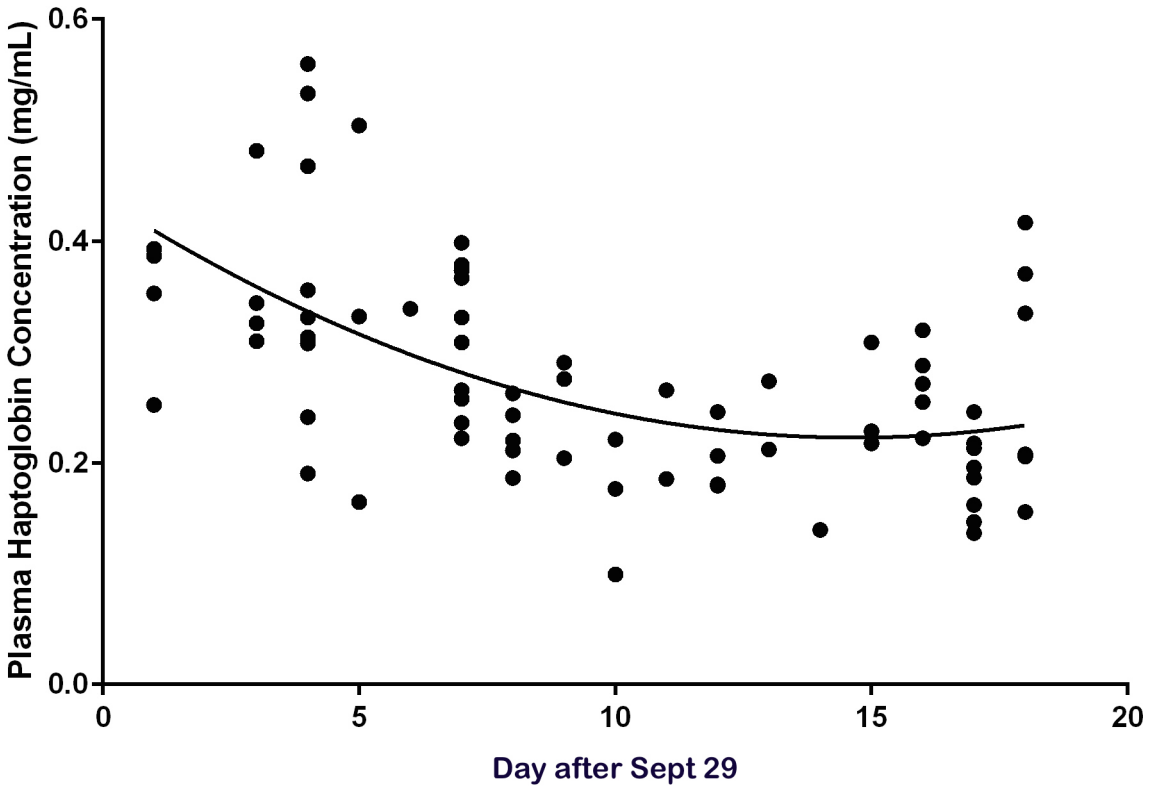

Figure 2. Change in plasma haptoglobin concentration (mg/mL) of wild yellow-rumped warblers sampled over the course of autumn migration at Long Point Ontario, 2013.

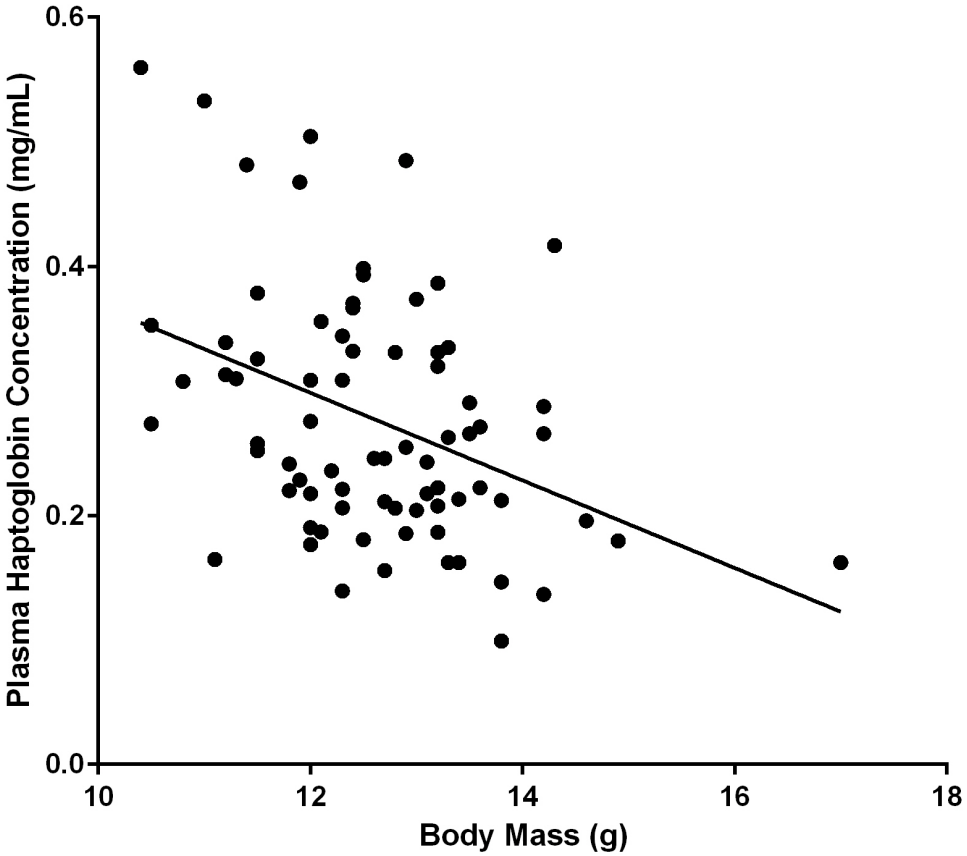

Figure 3. Plasma haptoglobin concentration (mg/mL) versus body mass of wild yellow-rumped warblers sampled during autumn migration at Long Point Ontario, 2013.

3.5 Seasonal Effects

Mean parasitemia of all captive birds was 0.12 (SD = 0.11) in the winter, and 0.22 (SD = 0.14) in the spring. In captive birds, parasitemia increased significantly with the switch from short days (winter) to long days (spring), this included some birds that tested negative for infection during the winter but positive during the spring (difference in AICc between the top model and the null model = 21.69; Table 6 in appendix A), indicating a spring relapse of infection ($F = 14.91$; $df = 1, 51$; $p = 0.00$; Figure 4). Average change in parasitemia was 0.10 (SD = 0.16) for all captive birds. None of the other predictor variables considered explained changes in parasitemia from winter to spring (difference in AICc between the top model and the null model = 1.81; Table 7 in appendix A).

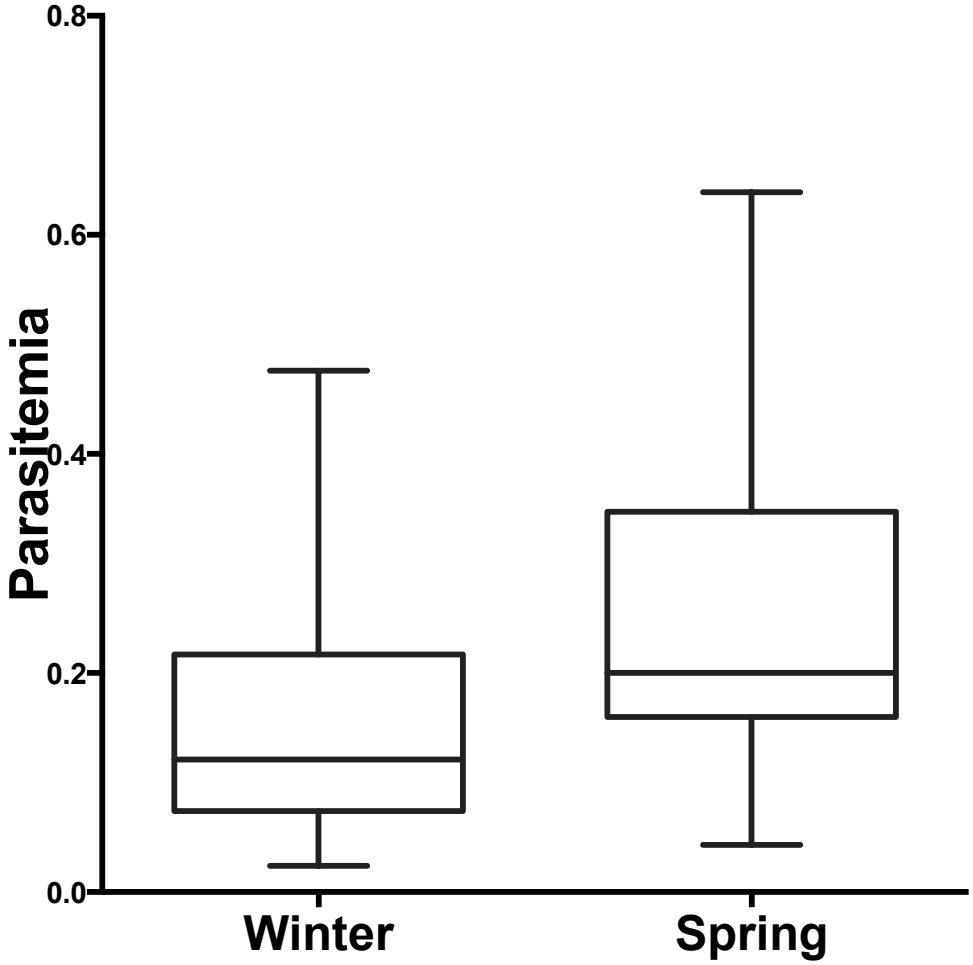

Figure 4. Change in parasitemia (proportion of infected cells) from winter to spring in captive birds. Median and range are shown.

3.6 Flight group assignment

Infection prevalence did not vary with initial flight group assignment, i.e., 70.9 % of the control birds were infected, and 73.1 % of the flight group birds were infected ($\chi^2 = 0$; df = 1; p = 1.00). Mean parasitemia of control birds was 0.12 (SD = 0.15), and of flown birds was 0.10 (SD = 0.13). Parasitemia also did not differ between birds in the control and flight groups (t = 0.46; df = 54; p = 0.65).

3.7 Flight Performance and Infection Status

Uninfected birds flew for an average of 355 minutes (SD = 9.24), and infected birds flew for an average of 248 minutes (SD = 135.80). None of the predictor variables explained the variation in

flight duration between infected and uninfected birds (difference in AICc between the top model and the null model = 0.48 ; Table 8 in appendix A). Flight duration of captive birds ranged from 54 minutes up to 360 minutes; mean = 260 minutes (SD = 132.1). None of the variables measured explained the variation in the flight duration (difference in AICc between the top model and the null model = 1.45; Table 9 in appendix A).

3.8 Refueling and Parasitemia

Mean total body mass change (change in body mass over the course of the experiment) of all captive birds was 1.17 g (SD = 1.52). Mean rate of body mass gain (change in body mass per day during the course of the experiment) for all birds was 0.05 g/day (SD = 0.05). Females had a mean body mass change of 0.74 g (SD = 1.52), and a mean rate of body mass change of 0.04 g/day (SD = 0.04), males had a mean body mass change of 1.54 g (SD = 1.44), and a mean rate of body mass change of 0.05 g/day (SD = 0.06). Uninfected birds on average gained 1.33 g (SD = 1.10) at an average rate of 0.03 g/day (SD = 0.22), while infected birds gained an average of 1.15 g (SD = 1.55) at an average rate of 0.05 g/day (SD = 0.05). The overall body mass change of birds from winter to spring was not affected by any of the variables considered (difference in AICc between the top model and the null model = 1.55; Table 10 in appendix A). None of the parameters measured explained the variation in rate of body mass gain of captive birds (difference in AICc between the top model and the null model = 0.37; Table 11 in appendix A).

3.9 Immune Activity

Overall, mean haptoglobin concentration (post flight or at the end of the study) was 0.56 mg/mL (SD = 0.22) for all captive birds. This was consistent with the mean haptoglobin concentration for both male and female captive birds (Table 2). None of the factors considered explained the variance in haptoglobin data in captive birds. Interaction terms did not significantly improve the fit of the models (difference in AICc between the top model and the null model = 1.07; Table 12 in appendix A).

4 Discussion

The qPCR technique was successful at accurately determining infection prevalence and parasitemia, and was correlated with results from blood smear analysis. I did not find that infection

prevalence or parasitemia varied with the age or sex of the bird, or throughout the autumn migratory period. This may indicate that there is selective mortality among younger birds, or that warblers are well adapted to the particular parasite strains that they encounter. Alternatively, measuring pooled infection prevalence and parasitemia may affect results. Haptoglobin decreased throughout the autumn migratory period, indicating that warblers may either be from different geographic origins and subjected to different pathogen pressures, or may be at different points in the refuelling process. In captive birds, infection intensity increased from winter to spring, indicating a relapse in infection. This could be a result of stress or seasonal susceptibility to disease and immune enhancement over the winter months. Finally, I found no effect of infection status on flight performance or refuelling in captive birds, indicating that the particular parasite lineages in this study may have evolved to be less virulent in order to maximize transmission over larger geographic distance, or that warblers are well adapted to their parasites.

4.1 Efficacy of qPCR based techniques at diagnosing avian blood parasite infection

I found that the qPCR technique was successful at both diagnosing malaria, and quantifying infection. Both infection prevalence and parasitemia were underestimated when using traditional blood smear analysis when compared to qPCR results. Blood smear analysis missed approximately 15 % of infections compared to qPCR. This is likely due to human error, and some infections may be of too low an intensity to visualize on blood smears (Jarvi et al. 2010). Bentz et al. (2006) conducted a similar comparison of microscopy and PCR and qPCR based techniques for identifying parasitic infection, and found that blood smear examination drastically underestimated parasite prevalence compared to qPCR. All blood smears with positive infection identification also tested positive with the qPCR technique, but not all positive infections diagnosed via qPCR were found to be positive on blood smears. Using blood smear analysis alone therefore may bias results, as some infections would be excluded from analysis.

One limitation of the qPCR assay that I developed is that it is a general assay that cannot determine which specific lineage of parasite a bird is infected by without sequencing the products. It is also possible that the primers used may bind to parasite DNA other than *Plasmodium* and *Haemoproteus* species. In the PCR protocol developed by Hellgren et al. (2004), DNA derived from *Leucocytozoon* has been found to bind to the primers specifically designed to amplify portions of the

cytochrome b gene of *Plasmodium* and *Haemoproteus* (Cosgrove et al., 2006). There is a possibility that this may have occurred in my sample of birds, as a small sample of the birds with higher recorded parasitemia from qPCR were found to have *Leucocytozoon* parasites on their blood smears. This could contribute to the higher parasitemia of some qPCR results compared to microscopy results, as *Leucocytozoon* was not included in parasitemia during blood smear analysis.

For the purpose of my study, a general diagnostic test that determines the overall parasitemia (including *Plasmodium, Haemoproteus* and potentially *Leucocytozoon* species) was adequate to test my predictions about infection status and parasitemia. Future research could further develop the assay that I have developed to include more specific primers for particular parasite lineages of interest. However, these primers would be very specific, and would potentially miss infections of some lineages if the sequence of target parasite DNA were more than a few base pairs off at either end of the sequence. Particular lineages of interest (based on prevalence) should be identified before designing new primers.

4.2 Infection Prevalence

Infection prevalence in wild yellow-rumped warblers did not vary with age or sex within the population studied. Although malarial infection prevalence has been demonstrated to vary with both age and sex in some studies there is also evidence that age and sex have no effect on infection prevalence (Davidar and Morton, 1993; Zuk and McKean, 1996). In a survey of Brazilian passerine species, Ribeiro and coworkers found that *Plasmodium* infection prevalence did not vary with the age and sex of the avian host (Ribeiro et al., 2005). It is also possible that I did not find an effect of age or sex due to the fact that my measure of infection prevalence included both *Plasmodium* and *Haemoproteus.* In blue tits (*Cyanistes caeruleus*), infection prevalence did not vary depending on the age of the host when analyzing overall prevalence, but when each parasite lineage was analyzed separately, infection prevalence was found to be higher in older birds for certain parasite lineages, an effect that was found to be stronger in males than females (Wood et al., 2007). Similarly, in a study comparing blood parasite prevalence between monogamous and polygynous species, McCurdy et al. (1998) found that overall infection prevalence did not vary depending on the sex of the host, but when broken down and analyzed by taxon, female birds were found to have higher *Haemoproteus* infection prevalence than males. It is surprising that infection prevalence in juveniles was approximately equal to that of adult birds rather than lower. This could be a result of nestlings being particularly susceptible to infection while in the nest as a result of low mobility. However, it

would then be expected that infection prevalence would increase with age and adult prevalence would be high. Selective mortality of infected juveniles over the winter could result in approximately equal infection prevalence between birds of different ages. Prevalence of infection in returning juvenile birds in the spring may be lower than the prevalence of infection in the juvenile birds during autumn migration, and as adults birds may have a decreased probability of becoming infected, which eventually could return infection prevalence to approximately 50 % of the population. This is supported by the findings of Davidar and Morton (1993) who found that infection prevalence in returning second year juvenile birds was lower than expected, and attributed this to high mortality among naïve first year juvenile birds. However, this would depend on whether transmission of infection occurs only over the breeding season, or year-round.

Capture date did not affect the likelihood of a bird being infected – late migrants were no more likely to be infected with *Plasmodium* and/or *Haemoproteus* than early migrants. This is contradictory to results presented by DeGroote and Rodewald (2010), where yellow-rumped warblers captured later in the spring migratory period were more likely to be infected than birds caught earlier, however since my study of wild caught birds occurred during autumn migration, the trend may be different. Similar to my results, Santiago-Alarcon et al. (2013) found no relationship between infection status and arrival date to breeding grounds in blackcaps (*Sylvia atricapilla*) infected with Haemosporidian parasites, although again, the study was conducted during spring migration. One more recent study reported that infection prevalence of *Plasmodium* and *Haemoproteus* in blackcaps tended to decrease throughout both the autumn and spring migratory period, although this was not significant (Arizaga et al., 2010). My results could indicate that transmission of the malarial parasites in this study occurs over the course of the breeding season, not during autumn migration, which could correspond to vector abundance as a result of both geographic location and temperature sensitivity of vectors (Santiago-Alarcon et al., 2012).

4.3 Infection Intensity

I found no sex or age effect on parasitemia, male and female and adult and juvenile birds had similar levels of infection intensity. This is contradictory to many previous studies that have found sex or age effects on recorded parasitemia. Sol et al. (2003) found that juvenile feral pigeons (*Columba livia*) infected with *Haemoproteus columbae* had higher parasitemia than adult birds. Younger yellow-rumped warblers were found to have infections of higher intensity than older birds

(DeGroote and Rodewald, 2010). Female great reed warblers (*Acrocephalus arundinaceus*) were found to have higher parasitemia than males in one study, although in the same study, no age effect was evident (Asghar et al., 2011). In blue tits (*Cyanistes caeruleus*), no sex bias in infection intensity of *Plasmosium* and *Haemoprotues* infection was found, supporting my findings (Podmokła et al., 2014). Analyzing overall parasitemia may eliminate the age or sex effect found by studies using lineage specific parasitemia because each different parasite lineage may have different dynamics with the host depending on age and sex. However, Podmokła et al (2014) found that analyzing parasitemia of separate lineages did elucidate any effect of sex on infection status. Parasitemia of some lineages may be affected by the age and sex of the host, whereas others may not be affected at all. Warblers may be well adapted to the parasite lineages frequently encountered and have sufficient immune activity to maintain infections at low intensities, juveniles with higher infection intensities may have died before the autumn migration, or may have not been caught at the stopover location (Valkiunas, 2005). Additionally, the immunosuppressive effect of testosterone on humoral immunity could be negligible, or cell-mediated immunity may be more important for parasite defence than humoral immunity, which is suppressed by both testosterone and oestrogen (Grossman, 1985). B-cell deficient mice chronically infected with *Plasmodium* spp. have been found to be resistant to re-infection by similar parasite species, suggesting that the immune response involved in resisting secondary infection is controlled by cell-mediated immune processes (Grun and Weidanz, 1983).

Parasitemia did not vary with capture date. These results are consistent with that of Santiago-Alarcon et al. (2013) who found that parasitemia of Haemosporidian parasites in blackcaps did not vary with arrival date to breeding grounds. Other studies, however, have found that infection intensity varies with capture date. Asghar et al. (2011) found that infection intensity increased with capture date in female great reed warblers. Infection intensity was also found to increase with later capture date during a study conducted throughout the spring migratory period (DeGroote and Rodewald, 2010). These results could indicate that parasitemia does not vary with date during autumn migration in the population used in my study, infection may be going into a period of latency at the time of autumn migration, which suggests that infection may not affect migration in the autumn, or could be a result of pooled measures of parasitemia. Alternatively, those birds that are affected by high parasitemia may not have been captured as a result of decreased mobility during stopover (Valkiunas, 2005), or may have not migrated at all.

4.4 Immunocompetence

Circulating levels of IgY did not vary within the population studied during autumn migration. My results are consistent with results of several studies. In Swainson's Thrush (*Catharus ustulatus*), Veery (*C. fuscescens*), and Wood Thrush (*Hylocichla mustelina*), IgG titres did not vary throughout the study period (Owen and Moore, 2006). Antibody response to immune challenge in blue peafowl (*Pavo cristatus*) has also been found to be independent of individual condition (Møller and Petrie, 2002). It is possible that during migration, IgY levels may be suppressed in order to reallocate resources to other processes more important for flight, or that IgY production is too important to down regulate, and will be maintained at a threshold level in order to mitigate potential infections during times of stress.

I found that plasma haptoglobin concentration decreased with capture date, more strongly at the beginning of the migratory period than towards the end. Early migrants could have had higher plasma haptoglobin concentrations than late migrants as a result of the underlying quality of the bird. Early migrants during the autumn migration may have been failed or non-breeders (Reed et al., 2003), or late breeders (Mitchell et al., 2012) leaving the breeding grounds early, indicating possibly that earlier migrants during the autumn may be of poorer quality compared to birds migrating later. Since circulating haptoglobin levels were also found to be lower in heavier birds, it is likely that elevated haptoglobin levels in lighter birds, and earlier migrants may indicate that these birds are of lower quality than heavier birds and those migrating later in the autumn, and could be fighting infection (other than malaria). It is also possible that birds migrating at different times were from different geographic locations at varying distances from the stopover study site, and subsequently have been exposed to different pathogen pressures resulting in varied immune activity. Haptoglobin concentration was not related to infection with *Plasmodium* and/or *Haemoproteus* in wild birds during autumn migration. Although haptoglobin was not affected by the specific parasites considered in this study, other parasite or pathogen pressures could affect circulating levels. Plasma haptoglobin concentration has been found to vary over the annual cycle within a population of skylarks (*Alauda arvensis*), with peaks during spring migration and during breeding, and lower levels documented during moult and autumn migration (Tieleman et al., 2012). It is also possible that birds caught earlier in the season were at a different point in refuelling than birds caught later in the season, affecting the measured haptoglobin concentration. Buehler et al. (2010) found that haptoglobin was lower in Red Knots (*Calidris canutus rufa*) building up protein stores while at

stopover than birds storing fat to fuel their subsequent flights. They proposed that this may be due to compromised immune function after migratory flight in recent arrivals, or that birds that are in the processes of refuelling fat stores may have increased rates of infection as a result of crowded stopover conditions (Buehler et al., 2010). It is therefore possible that birds caught later during the study period with lower haptoglobin levels were recent arrivals to stopover and were recovering protein, and birds that were sampled earlier in the study period with higher haptoglobin levels had recovered enough protein prior to the initiation of the study or being caught, and were storing fat.

4.5 Seasonal Changes in Parasitemia

Parasitemia of captive birds increased from simulated winter (short day photoperiod) to spring (long day photoperiod). This included positive infection identification in the spring in some birds that were diagnosed as uninfected during the winter. Seasonal relapses are common in *Plasmodium* and *Haemoproteus* (Valkiunas, 2005). *Plasmodium* has been found to show a bimodal distribution in prevalence thought the year (Cosgrove et al., 2008), and parasitemia of *P. relictum* in house sparrows was found to increase from April to June (Applegate, 1971). The magnitude of change in parasitemia seen here was not related to haptoglobin, sex, or body condition of the host. Although the potential mechanisms responsible for relapses in infection were not clear in my study, several hypotheses have been proposed in the literature. Since relapses in infection are most common in the spring, many researchers have proposed that they result from hormonal changes, particularly increased gonadotropin hormones and corticosterone (Applegate and Beaudoin, 1970; Atkinson and van Riper, 1991). Corticosterone was found to induce relapse of infection in house sparrows, however no effect of gonadotropin hormones were established (Applegate and Beaudoin, 1970).

Another possible explanation for spring relapses in infection could be the host's seasonal susceptibility to disease. Changes, due to for example, a change in the daily melatonin pulse controlled by photoperiod (Dowell, 2001; Valkiūnas et al., 2004). In mammals, melatonin has been linked to enhanced immune function (Nelson and Drazen, 2000). An experimental study using Japanese Quail (*Coturnix coturnix japonica*) found that birds exposed to light dark cycles had enhanced immune responses compared to birds held at 24h of light, and that melatonin supplementation to birds held at 24h of light had immune-enhancing effects (Moore and Siopes, 2000). During short-day photoperiod, longer nights may increase the amount of melatonin released, which may enhance immunity leading to a decrease in infection intensity during the winter months. With the onset of long-day photoperiod, increased day light decreases the amount of melatonin

released, which may decrease immune functioning and allow infection intensity to rise. This hypothesis would be also consistent with the seasonal pattern of infection prevalence increasing over the spring, and decreasing during the autumn. However, increased melatonin has also been found during the migratory period (Fusani and Gwinner, 2005). In blackcaps (*Sylvia atricapilla*), melatonin has been found to increase during in both autumn and spring, presumably to facilitate nocturnal migratory flights (Fusani and Gwinner, 2005). Nevertheless, melatonin levels were much lower in the spring compared to the autumn (Fusani and Gwinner, 2005), and spring relapses in infection due to immunosuppression may be affected by melatonin.

4.6 Flight and Immune Investment

Plasma haptoglobin concentration was not affected by flight duration in captive warblers, suggesting there was no trade-off between immune investment and endurance flight. Similar results have been found in western sandpipers (*Calidris mauri*), where flight performance of birds flown while mounting an acute phase immune response to LPS injections was not diminished (Nebel et al., 2013). However, in the same study, bacterial killing ability of birds flown while mounting an acute phase response was decreased after flight compared to control birds (Nebel et al., 2013). It is possible that other aspects of immune function in the birds used in my study were diminished after endurance flight. Since there was only enough plasma available to measure haptoglobin, additional experiments are required to determine whether endurance flight and infection affect immune functioning.

4.7 Flight Performance

Neither infection prevalence nor parasitemia affected the voluntary flight duration of captive birds in the wind tunnel flights. This finding could suggest that, at least within chronic infections as seen here, malaria infection could be relatively benign in the short term to the host. Birds were able to allocate sufficient resources and energy to migration while allowing the parasite to survive and reproduce. This could be a result of evolved tolerance. In the classic example of the highly pathogenic introduced *Plasmodium relictum* in Hawaii, some species of birds living at lower elevation have now evolved tolerance for the parasite (Atkinson et al., 2013). In a population of Hawaii Amakihi (*Hemignathus virens*) residing at low elevation, experimental infection with *Plasmodium relictum* resulted in similar parasitemia, no change in feeding rate, lower body mass

loss, and lower mortality than experimentally infected birds residing at higher elevation (Atkinson et al., 2013). It is also possible that the parasite lineages within our sample may have a reduced virulence compared to other lineages, allowing the host to survive and carry out its migration without negative consequences in order to facilitate transmission to new hosts on either the wintering grounds or breeding grounds where density of potential new hosts is high. There may be an optimal level of virulence for parasites where they may increase their own fitness through within-host replication or reproduction to a threshold level, beyond which they pay a cost of excessive host mortality and reduced or potentially failed transmission (de Roode et al., 2008). The variation in voluntary flight duration of infected birds was quite high, making it difficult to detect an effect of infection. Thus, future studies should use repeated measures designs to more sensitively test effects on flight performance. Birds should be flown uninfected, and then experimentally infected and flown in a wind tunnel multiple times as the infection progresses in order to determine if infection does indeed impair flight performance.

Body mass gain and average rate of body mass gain of captive birds was not affected by either infection prevalence or parasitemia. This could have been because infection within the birds sampled was chronic and of lower intensity on average, even when accounting for relapse in infection in the spring, or warblers could be well adapted to deal with infection with parasites that they commonly encounter. Some research suggests that malaria infection is benign to the avian host during chronic infection. Cornlius et al. (2014) found that common yellow- throats (*Geothlypis trichas)*, and western palm warblers (*Dendroica palmarum palmarum)* infected with *Plasmodium* or *Haemoproteus* did not differ in stress levels, body condition, or fat score from uninfected birds, suggesting that chronic malaria infection may have minimal impacts on the host. Similarly, blackcaps (*Sylvia atricapilla*) infected with either *Plasmodium* or *Haemoproteus* did not differ from uninfected birds in body mass or fat score (Arizaga et al., 2009). A recent study found that the costs of chronic *Plasmodium* and *Haemoproteus* infection might be long-term rather than short-term (Asghar et al., 2015). Activity levels of great reed warblers did not differ between uninfected and infected individuals, indicating little short-term cost of chronic infection, although uninfected individuals were found to have longer lifespans and produce more fledglings over the course of their lifespan than infected individuals (Asghar et al., 2015).

Additional research is needed to further assess whether infection prevalence and parasitemia have an effect on the ability of migratory birds to carry out long endurance flights. A possible

experiment could involve experimentally infecting naïve captive birds with *Plasmodium* or *Haemoproteus*, then flying both uninfected and infected birds in a wind tunnel. Parasitemia should be measured throughout the course of the infection to determine whether uninfected birds are able to fly longer, and therefore further than infected birds, and whether there is a threshold level of infection intensity above which birds cannot carry out endurance exercise.

5 Conclusions

Relatively few definitive conclusions can be made regarding the variation in blood parasite infection prevalence and parasitemia from my results. This may be due to analyzing pooled parasite prevalence and parasitemia. Overall, there may be no age or sex effect on prevalence and parasitemia in the study population and there may be no effect of infection status on migration timing, or there may be selective mortality of infected birds, particularly in the younger birds, or differences may be species or lineage specific. All of the contradictory evidence in the literature supports that there are species or lineage specific differences in how age, sex, and time affect the likelihood of contracting an infection as well as the intensity of infection that may result. Future research should aim to determine if these types of effects exist in my study population by measuring infection prevalence and parasitemia of specific lineages or species of the more common parasites found within the population.

Innate immune response varied with date during autumn migration. This may be due to birds of different underlying quality migrating at different times during the season, and this hypothesis is supported by the fact that heavier birds had lower levels of haptoglobin than lighter birds, indicating a potential acute phase response. It is also possible that the birds used in this study came from different geographic locations, and therefore have been exposed to different pathogen pressures resulting in varied immune activity. Natural markers such as stable isotope signatures should be used in future studies to determine the geographic origin of each bird. Additionally, the type of fuel during refuelling (protein vs. fat) may affect innate immune responses. Future studies should aim to determine whether the quality of bird, geographic origin, or refuelling state is responsible for the variation in haptoglobin concentration over the course of autumn migration.

Relapses in infection from winter to spring occurred in captive birds. The duration of melatonin pulse as a result of photoperiod changes, or corticosterone may be responsible for the increase in parasitemia. Future research should aim to determine the definitive cause of *Plasmodium* and *Haemoproteus* infection relapse. There was little effect of parasite infection (prevalence and

parasitemia) on flight performance and refuelling of captive birds. Yellow-rumped warblers could have evolved tolerance to the *Plasmodium* and *Haemoproteus* parasites that are commonly encountered, or the parasites may have evolved a less virulent strategy to maximize transmission over larger geographic distances. Furthermore, since pooled parasitemia was used in data analysis, it is possible that flight performance and refuelling are adversely affected by more virulent strains of *Plasmodium* and *Haemoproteus*, and future work should aim to determine the effects of infection by specific lineages and strains.

Literature Cited

Ahmed, A. M., S. L. Baggott, R. Maingon, and H. Hurd. 2002. The costs of mounting an immune response are reflected in the reproductive fitness of the mosquito *Anopheles gambiae*. Oikos 97:371-377.

Allander, K., and G. F. Bennett. 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.

Altizer, S., R. Bartel, and B. A. Han. 2011. Animal migration and infectious disease risk. Science 331:296-302.

Anderson, R. M., and R. M. May. 1982. Coevolution of hosts and parasites. Parasitology 85:411-426.

Applegate, J., and R. Beaudoin. 1970. Mechanism of spring relapse in avian malaria: effect of gonadotropin and corticosterone. Journal of Wildlife Diseases 6:443-447.

Applegate, J. E. 1971. Spring relapse of plasmodium relictum infections in an experimental field population of English sparrows (*Passer domesticus*). Journal of wildlife diseases 7:37-42.

Arizaga, J., E. Barba, and A. Hernández. 2009. Do Haemosporidians affect fuel deposition rate and fuel load in migratory blackcaps *Sylvia atricapilla*. Ardeola 56.1:41-47.

Arizaga, J., X. Esparza, and E. Barba. 2010. Haemosporidians in migratory blackcaps (*Sylvia atricapilla*), a comparison between autumn and spring periods of passage. Anales de Biología 32:87-93.

Asghar, M., H. Westerdahl, P. Zehtindjiev, M. Ilieva, D. Hasselquist, and S. Bensch. 2012. Primary peak and chronic malaria infection levels are correlated in experimentally infected great reed warblers. Parasitology 139:1246-1252.

Asghar, M., D. Hasselquist, B. Hansson, P. Zehtindjiev, H. Westerdahl, and S. Bensch. 2015. Chronic infection. Hidden costs of infection: chronic malaria accelerates telomere degradation and senescence in wild birds. Science 347:436.

Asghar, M., D. Hasselquist, S. Bensch. 2011. Are chronic avian haemosporidian infections costly in wild birds? Journal of Avian Biology 42:530-537.

Atkinson, C. T., and C. van Riper III. 1991. Pathogenicity and epizootiology of avian haematozoa: Plasmodium, Leucocytozoon & Haemoproteus. Oxford Ornithology Series:19-48.

Atkinson, C. T., K. S. Saili, R. B. Utzurrum, and S. I. Jarvi. 2013. Experimental evidence for evolved tolerance to avian malaria in a wild population of low elevation Hawai‘i ‘amakihi (*Hemignathus virens*). EcoHealth 10:366-375.

Atkinson, C., R. Dusek, K. Woods, and W. Iko. 2000. Pathogenicity of avian malaria in experimentally-infected Hawaii Amakihi. Journal of Wildlife Diseases 36:197-204.

Bairlein, F., D. R. Norris, R. Nagel, M. Bulte, C. C. Voigt, J. W. Fox, D. J. T. Hussell, and H. Schmaljohann. 2012. Cross-hemisphere migration of a 25 g songbird. Biology Letters 8:505-507.

Bejerano, G., M. Pheasant, I. Makunin, S. Stephen, W. J. Kent, J. S. Mattick, and D. Haussler. 2004. Ultraconserved elements in the human genome. Science 304:1321-1325.

Bentz, S., T. Rigaud, M. Barroca, F. Martin-Laurent, D. Bru, J. Moreau, and B. Faivre. 2006. Sensitive measure of prevalence and parasitaemia of haemosporidia from European blackbird (*Turdus merula*) populations: value of PCR-RFLP and quantitative PCR. Parasitology 133:685-692.

Berger, L., R. Speare, P. Daszak, D. E. Green, A. A. Cunningham, C. L. Goggin, R. Slocombe, M. A. Ragan, A. D. Hyati, K. R. McDonald, H. B. Hines, K. R. Lips, G. Marantelli, and H. Parkes. 1998. Chytridiomycosis causes amphibian mortality associated with population declines in the rain forests of Australia and Central America. Proceedings of the National Academy of Sciences of the United States of America 95:9031-9036.

Berger, S., L. B. Martin, M. Wikelski, L. M. Romero, E. K. V. Kalko, M. N. Vitousek, and T. Rödl. 2005. Corticosterone suppresses immune activity in territorial Galápagos marine iguanas during reproduction. Hormones and Behavior 47:419-429.

Berthold, P. 1975. Migration: Control and Metabolic Physiology . Academic Press, New York.

Best, S. M., and P. J. Kerr. 2000. Coevolution of host and virus: The pathogenesis of virulent and attenuated strains of Myxoma virus in resistant and susceptible European rabbits. Virology 267:36-48.

Bonneaud, C., J. Mazuc, G. Gonzalez, C. Haussy, O. Chastel, B. Faivre, and G. Sorci. 2003. Assessing the cost of mounting an immune response. The American Naturalist 161:367-379.

Boots, M., A. Best, M. R. Miller, and A. White. 2009. The role of ecological feedbacks in the evolution of host defence: what does theory tell us? Philosophical Transactions of the Royal Society B: Biological Sciences 364:27-36.

Bourgeon, S., J. Martinez, F. Criscuolo, Y. Le Maho, and R. Raclot. 2006. Fast-induced changes of immunological and stress indicators in breeding female eiders. General and Comparative Endocrinology 147:336-342.

Bradley, C. A., and S. Altizer. 2005. Parasites hinder Monarch butterfly flight: implications for disease spread in migratory hosts. Ecology Letters 8:290.

Brock, P., C. Murdock, and L. Martin. 2014. The history of ecoimmunology and its integration with disease ecology. Integrative and Comparative Biology 54:353-362.

Brower, L. P., and L. S. Fink. 2006. Fueling the fall migration of the Monarch Butterfly. Integrative and Comparative Biology 46:1123-1142.

Brown, M. R., P. J. Corkeron, P. T. Hale, K. W. Schultz, and M. M. Bryden. 1995. Evidence for a sex-segregated migration in the Humpback Whale (*Megaptera novaeangliae*). Proceedings of the Royal Society of London Series B: Biological Sciences 259:229-234.

Buehler,D.M., and Piersma, T. 2008. Travelling on a budget: predictions and ecological evidence for bottlenecks in the annual cycle of long-distance migrants. Philosophical Transactions of the Royal Society B. 363: 247-266.

Buehler, D. M., B. I. Tieleman, and T. Piersma. 2010a. How do migratory species stay healthy over the annual cycle? A conceptual model for immune function and for resistance to disease. Integrative and Comparative Biology 50:346-357.

Buehler, D. M., B. I. Tieleman, and T. Piersma. 2010b. Indices of immune function are lower in red knots (Calidris canutus) recovering protein than in those storing fat during stopover in Delaware Bay. The Auk 127:394-401.

Butterworth, M., B. Mcclellan, and M. Klansmith. 1967. Influence of sex on immunoglobulin levels. Nature 214:1224-1225.

Christe, P., O. Glaizot, N. Strepparava, G. Devevey, and L. Fumagalli. 2012. Twofold cost of reproduction: an increase in parental effort leads to higher malarial parasitaemia and to a decrease in resistance to oxidative stress. Proceedings of the Royal Society Biological sciences / The 279:1142-1149.

Cornelius, E. A., A. K. Davis, and S. A. Altizer. 2014. How important are Hemoparasites to migratory songbirds? Evaluating physiological measures and infection status in three Neotropical migrants during stopover. Physiological and Biochemical Zoology 87:719-728.

Cosgrove, C. L., K. P. Day, and B. C. Sheldon. 2006. Coamplification of Leucocytozoon by PCR diagnostic tests for avian malaria: A cautionary note. Journal of Parasitology 92:1362-1365.

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

Daszak, P., A. Cunningham, and A. Hyatt. 2000. Wildlife ecology - Emerging infectious diseases of wildlife - Threats to biodiversity and human health. Science 287:443-449.

Davidar, P., and E. S. Morton. 1993. Living with parasites - prevalence of a blood parasite and its effect on survivorship in the purple Martin. Auk 110:109-116.

Dawson D.A., Darby S., Hunter F.M., Krupa A.P., Jones I.L., Burke T. 2001. A critique of avian CHD-based molecular sexing protocols illustrated by a Z-chromosome polymorphism detected in auklets. Molecular Ecology Notes. 1:201–204.

Day, T., A. L. Graham, and A. F. Read. 2007. Evolution of parasite virulence when host responses cause disease. Proceedings of the Royal Society B: Biological Sciences 274:2685-2692.

de Roode, J. C., A. J. Yates, and S. Altizer. 2008. Virulence-transmission trade-offs and population divergence in virulence in a naturally occurring butterfly parasite. Proceedings of the National Academy of Sciences of the United States of America 105:7489-7494.

DeGroote, L. W., and P. G. Rodewald. 2010. Blood parasites in migrating wood-warblers (Parulidae): Effects on refueling, energetic condition, and migration timing. Journal of Avian Biology 41:147-153.

DeLuca, W. V., B. K. Woodworth, C. C. Rimmer, P. P. Marra, P. D. Taylor, K. P. McFarland, S. A. Mackenzie, and D. R. Norris. 2015. Transoceanic migration by a 12 g songbird. Biology letters 11:20141045.

Demas, G.E., Grieves, T., Chester, E., and French, S. 2012. Energetics of immunity: Mechanisms mediating trade-offs in ecoimmunology. In Demas, G. E., and R. J. Nelson (Eds.). Ecoimmunology (259-296). Oxford New York, NY: University Press.

Demas, G. E., V. Chefer, M. I. Talan, and R. J. Nelson. 1997. Metabolic costs of mounting an antigen-stimulated immune response in adult and aged C57BL/6J mice. American Journal of Physiology - Regulatory, Integrative and Comparative Physiology 273:1631-1637.

Deviche, P., E. C. Greiner, and X. Manteca. 2001. Seasonal and age-related changes in blood parasite prevalence in Dark-eyed Juncos *(Junco hyemalis,* Aves, Passeriformes). Journal of Experimental Zoology 289:456-466.

Dingle, H., and V. A. Drake. 2007. What is migration? Bioscience 57:113-121.

Dowell, S. F. 2001. Seasonal variation in host susceptibility and cycles of certain infectious diseases. Emerging Infectious Diseases 7:369-374.

Festa-Bianchet, M. 1989. Individual differences, parasites, and the costs of reproduction for Bighorn Ewes (Ovis canadensis). Journal of Animal Ecology 58:785-795.

Fleming, I. A., and M. R. Gross. 1989. Evolution of adult female life history and morphology in a Pacific Salmon (Coho: *Oncorhynchus kisutch*). Evolution 43:141-157.

Flockhart, D. T., L. I. Wassenaar, T. G. Martin, K. A. Hobson, M. B. Wunder, and D. R. Norris. 2013. Tracking multi-generational colonization of the breeding grounds by Monarch butterflies in eastern North America. Proceedings of the Royal Society B: Biological Sciences 280:20131087.

Fusani, L., and E. Gwinner. 2005. Melatonin and nocturnal migration. Annals of the New York Academy of Sciences 1046:264-270.

Garvin, M. C., C. C. Szell, and F. R. Moore. 2006. Blood parasites of Nearctic–Neotropical migrant passerine birds during spring trans-gulf migration: impact on host body condition. Journal of Parasitology 92:990-996.

Gerson, A. R., and C. G. Guglielmo. 2011. Flight at low ambient humidity increases protein catabolism in migratory birds. Science 333:1434-1436.

Griffiths, R., M. C. Double, K. Orr, and R. J. Dawson. 1998. A DNA test to sex most birds. Molecular Ecology 7:1071-1075.

Grossman, C. J. 1985. Interactions between the gonadal steroids and the immune system. Science 227:257-261.

Grun, J. L., and W. P. Weidanz. 1983. Antibody-independent immunity to reinfection malaria in B-cell-deficient mice. Infection and Immunity 41:1197-1204.

Guglielmo, C. G., L. P. McGuire, A. R. Gerson, and C. L. Seewagen. 2011. Rapid, Non-invasive measurement of fat, lean, and total body water masses of live birds using quantitative magnetic resonance. Journal of Ornithology 152:S75-S85.

Gulland, F. M. D. 1992. The role of nematode parasites in Soay sheep (*Ovis aries L.*) mortality during a population crash. Parasitology 105:493-503.

Hasselquist, D., O. Ostman, J. Waldenstrom, and S. Bensch. 2007. Temporal patterns of occurrence and transmission of the blood parasite *Haemoproteus payevskyi* in the great reed warbler *Acrocephalus arundinaceus*. Journal of Ornithology 148:401-409.

Hellgren, O., J. Waldenström, and S. Bensch. 2004. A new PCR assay for simultaneous studies of Leucocytozoon, Plasmodium, and Haemoproteus from avian blood. Journal of Parasitology 90:797-802.

Hudson, P. J., A. P. Dobson, and D. Newborn. 1998. Prevention of population cycles by parasite removal. Science 282:2256-2258.

Hudson, P. J., A. Rizzoli, B. T. Grenfall, H. Heesterbeek, and A. P. Dobson. 2002. The Ecology of Wildlife Diseases. Oxford University Press, Oxford.

Hunt, P. D., and D. J. Flaspohler. 1998. Yellow-rumped Warbler (*Setophaga coronata*) . The Birds of North America Online (A. Poole, Ed.) Ithaca: Cornell Lab of Ornithology; Retrieved from the Birds of North America Online.

Jarvi, S. I., Schultz, J. J., and Atkinson, C. T. 2002. PCR diagnostics underestimate the prevalence of avian malaria (Plasmodium relictum) in experimentally-infected passerines. Journal of Parasitology, 88:153-158.

Kerlinger, P. 1987. Stopover and fat deposition by North American Wood-Warblers (Parulinae) following spring migration over the Gulf of Mexico. Oecologia 74:47-54.

Lalubin, F., A. Deledevant, O. Glaizot, and P. Christe. 2013. Temporal changes in mosquito abundance (*Culex pipiens*), avian malaria prevalence and lineage composition. Parasites and Vectors 6:307-307.

Lewicki, K. E. 2013. Haemosporidian parasites of Barred Owls (Strix varia) and Northern Spotted Owls (*S. Occidentalis Caurina*): Investigating the effects of an invasive species on parasite transmission and community dynamics. ProQuest, UMI Dissertations Publishing, Colorado State University.

Lindström, Å. 1991. Maximum fat deposition rates in migrating birds. Ornis Scandinavica:12-19.

Lochmiller, R. L., and C. Deerenberg. 2000. Trade-offs in evolutionary immunology: Just what is the cost of immunity? Oikos 88:87-98.

Mamaev, L. V., N. N. Denikina, S. I. Belikov, V. E. Volichikov, I. K. G. Visser, M. Fleming, C. Kai, T. C. Harder, B. Liess, A. D. M. E. Osterhaus, and T. Barrett. 1995. Characteristics of morbillviruses isolated from Lake Baikal seals (*Phoca sibrica*). Veterinary Microbiology 40:251-259.

Martin, L. B. 2009. Stress and immunity in wild vertebrates: Timing is everything. General and Comparative Endocrinology 163:70-76.

Martin, L. B., Z. M. Weil, and R. J. Nelson. 2008. Seasonal changes in vertebrate immune activity: mediation by physiological trade-offs. Philosophical Transactions of the Royal Society B: Biological Sciences 363:321-339.

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

Matson, K. D., N. P. Horrocks, M. A. Versteegh, and B. I. Tieleman. 2012. Baseline haptoglobin concentrations are repeatable and predictive of certain aspects of a subsequent experimentally-induced inflammatory response. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology 162:7-15.

McCurdy, D. G., D. Shutler, A. Mullie, and M. R. Forbes. 1998. Sex-biased parasitism of avian hosts: Relations to blood parasite taxon and mating system. Oikos 82:303-312.

Mitchell, G. W., A. E. M. Newman, M. Wikelski, and D. Ryan Norris. 2012. Timing of breeding carries over to influence migratory departure in a songbird: an automated radiotracking study. Journal of Animal Ecology 81:1024-1033.

Møller, A. P., and M. Petrie. 2002. Condition dependence, multiple sexual signals, and immunocompetence in peacocks. Behavioral Ecology 13:248-253.

Moore, C. B., and T. D. Siopes. 2000. Effects of lighting conditions and melatonin supplementation on the cellular and humoral immune responses in Japanese Quail *Coturnix coturnix japonica*. General and comparative endocrinology 119:95-104.

Murphy K, Travers P, Walport M. 2008. Janeway's immuno- biology. 7th Edition. New York: Garland Science.

Nebel, S., U. Bauchinger, D. M. Buehler, L. A. Langlois, M. Boyles, A. R. Gerson, E. R. Price, S. R. McWilliams, and C. G. Guglielmo. 2012. Constitutive immune function in European starlings, *Sturnus vulgaris*, is decreased immediately after an endurance flight in a wind tunnel. Journal of Experimental Biology 215:272-278.

Nebel, S., D. M. Buehler, A. MacMillan, and C. G. Guglielmo. 2013. Flight performance of western sandpipers, *Calidris mauri*, remains uncompromised when mounting an acute phase immune response. Journal of Experimental Biology 216:2752-2759.

Nelson, R. J., and D. L. Drazen. 2000. Melatonin mediates seasonal changes in immune function. Annals of the New York Academy of Sciences 917:404-415.

Nisbet, I. C. 1970. Autumn migration of the Blackpoll Warbler: evidence for long flight provided by regional survey. Bird-Banding:207-240.

Nunn, C. L. 2002. A comparative study of leukocyte counts and disease risk in primates. Evolution 56:177-190.

Olsen, N., and W. Kovacs. 1996. Gonadal steroids and immunity. Endocrine Reviews 17:369-384.

O'Neal, D.M., and Ketterson, E.D. 2012 Life history evolution, hormones and avian immune function. In Demas, G. E., and R. J. Nelson (Eds.). Ecoimmunology (7-44). Oxford New York, NY: University Press.

Ots, I., A. B. Kerimov, E. V. Ivankina, T. A. Ilyina, and P. Horak. 2001. Immune challenge affects basal metabolic activity in wintering great tits. Proceedings of the Royal Society: Biological sciences 268:1175-1181.

Owen-Ashley, N. T., D. Hasselquist, and J. C. Wingfield. 2004. Androgens and the immunocompetence handicap hypothesis: unraveling direct and indirect pathways of immunosuppression in song sparrows. The American Naturalist 164:490-505.

Owen, J. C., and F. R. Moore. 2006. Seasonal differences in immunological condition of three species of thrushes. The Condor 108:389-398.

Owen, J. C., and F. R. Moore. 2008. Swainson's thrushes in migratory disposition exhibit reduced immune function. Journal of Ethology 26:383-388.

Pap, P. L., and R. Márkus. 2003. Cost of reproduction, T-lymphocyte mediated immunocompetence and health status in female and nestling Barn Swallows *Hirundo rustica*. Journal of Avian Biology 34:428-434.

Pennycuick, C. J., and P. F. Battley. 2003. Burning the engine: a time-marching computation of fat and protein consumption in a 5420-km non-stop flight by great knots, *Calidris tenuirostris*. Oikos 103:323-332.

Podmokła, E., A. Dubiec, S. M. Drobniak, A. Arct, L. Gustafsson, and M. Cichoń. 2014. Determinants of prevalence and intensity of infection with malaria parasites in the Blue Tit. Journal of Ornithology 155:721-727.

Pyle, P. 1997. Identification Guide to North American Birds. Part I. Columbidae to Ploceidae. Slate Creek Press, Bolinas.

Råberg, L., M. Grahn, D. Hasselquist, and E. Svensson. 1998. On the adaptive significance of stress-induced immunosuppression. Proceedings of the Royal Society of London.Series B: Biological Sciences 265:1637-1641.

Rappole, J. H., and Z. Hubálek. 2003. Migratory birds and West Nile virus. Journal of Applied Microbiology 94:47-58.

Reddy, C., S. Damayanthi, and B. Rao. 1980. Host responce to *Haemoproteus columbae*. Rivista di Parassitologia 41:191-193.

Reed, E. T., J. Bêty, J. Mainguy, G. Gauthier, and J. Giroux. 2003. Molt migration in relation to breeding success in Greater Snow Geese. Arctic 56:76-81.

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

Ricklefs, R.E., Fallon, S.M., Latta, S.C., Swanson, B.L., and Bermingham, E. 2005. Migrants and their parasites. In Greenberg, R., and Marra, P.P (Eds.). Birds of two worlds (210-221). Baltimore, MD: Johns Hopkins University Press.

Santiago-Alarcon, D., V. Palinauskas, and H. M. Schaefer. 2012. Diptera vectors of avian Haemosporidian parasites: untangling parasite life cycles and their taxonomy. Biological Reviews 87:928-964.

Santiago-Alarcon, D., R. Mettler, G. Segelbacher, and H. M. Schaefer. 2013. Haemosporidian parasitism in the blackcap *Sylvia atricapilla* in relation to spring arrival and body condition. Journal of Avian Biology 44:521-530.

Schmid-Hempel, P. 2005. Evolutionary ecology of insect immune defenses. Annual Review of Entomology 50:529-551.

Schmid-Hempel, P. and Ebert, D. 2003. On the evolutionary ecology of specific immune defence. Trends in Ecology and Evolution 18:27-32

Scott, M. E. 1988. The impact of infection and disease on animal populations: Implications for conservation biology. Conservation Biology 2:40-56.

Semple, S., G. Cowlishaw, and P. M. Bennett. 2002. Immune system evolution among anthropoid primates: parasites, injuries and predators. Proceedings of the Royal Society of London.Series B: Biological Sciences 269:1031-1037.

Sheldon, B. C., and S. Verhulst. 1996. Ecological immunology: Costly parasite defences and trade-offs in evolutionary ecology. Trends in Ecology and Evolution 11:317-321.

Snounou, G., W. Jarra, and P. R. Preiser. 2000. Malaria multigene families: The price of chronicity. Parasitology Today 16:28-30.

Sol, D., R. Jovani, and J. Torres. 2003. Parasite mediated mortality and host immune response explain age-related differences in blood parasitism in birds. Oecologia 135:542-547.

Sparkman, A. M., and M. G. Palacios. 2009. A test of life-history theories of immune defence in two ecotypes of the Garter Snake, *Thamnophis Elegans*. Journal of Animal Ecology 78:1242-1248.

Tieleman, B. I., K. D. Matson, C. Both, and A. Hegemann. 2012. Immune function in a free-living bird varies over the annual cycle, but seasonal patterns differ between years. Oecologia 170:605-618.

Valkiunas, G. 2005. Avian Malaria Parasites and Other Haemosporidia. CRC, Boca Raton.

Valkiūnas, G., F. Bairlein, T. A. Iezhova, and O. V. Dolnik. 2004. Factors affecting the relapse of *Haemoproteus belopolskyi* infections and the parasitaemia of Trypanosoma spp. in a naturally infected European songbird, the blackcap, *Sylvia atricapilla*. Parasitology Research 93:218-222.

Valkiūnas, G., S. Bensch, T. A. Iezhova, A. Križanauskienė, O. Hellgren, C. V. Bolshakov. 2006. Nested cytochrome B polymerase chain reaction diagnostics underestimate mixed infections of avian blood Haemosporidian parasites: Microscopy is still essential. Journal of Parasitology 92:418-422.

van Gils, J. A., V. J. Munster, R. Radersma, D. Liefhebber, R. A. M. Fouchier, and M. Klaassen. 2007. Hampered foraging and migratory performance in swans infected with low-pathogenic avian influenza A virus. Plos One 2:e184.

van Oers, K., D. S. Richardson, S. A. Sæther, and J. Komdeur. 2010. Reduced blood parasite prevalence with age in the Seychelles Warbler: selective mortality or suppression of infection? Journal of Ornithology 151.1:69-77.

Vredenburg, V. T., R. A. Knapp, T. S. Tunstall, C. J. Briggs, and D. B. Wake. 2010. Dynamics of an emerging disease drive large-scale amphibian population extinctions. Proceedings of the National Academy of Sciences of the United States of America 107:9689-9694.

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

Zambrano-Villa, S., D. Rosales-Borjas, J. C. Carrero, and L. Ortiz-Ortiz. 2002. How Protozoan Parasites Evade the Immune Response. TRENDS in Parasitology 18:272-278.

Zuk, M., and K. A. McKean. 1996. Sex differences in parasite infections: Patterns and processes. International Journal for Parasitology 26:1009-1024.

6 *Appendix A:* Primer Design and Results of Model Selection

Table 1. Newly designed primers and suitability for amplifying *Plasmodium* and *Haemoproteus* spp. within wild birds caught during autumn migration.

Primer	Lineages amplified (MalAvi)	Trial completed	Suitability
Plasmo01F: (5' – CATGGATTTGTGGTGGATATCTTG – 3') Plasmo01R: (5' – CCTAAAGGATTAGTGCTACCTTGT –3')	AFR013 AFR068 BT7 PSB1 BT7 GRW06	Yes	Too lineage specific
Plasmo 02F: (5' – GATATCTTGTAAGTGACCCAACCT – 3') Plasmo 02R: (5' – CCCTAAAGGATTTGTGCTACCT – 3')	SEIAUR01 CATUST05	Yes	Too lineage specific
Haemo01F: (5' – CCTGGACTTGTTTCATGGATTG – 3') Haemo01R: (5' – GAGCTACCTTGTAAGTGTAAGAAGA – 3')	DENPEN02 DUNNO01	Yes	Too lineage specific
Haemo02F: (5' – GCTACCGGTGCTACATTTGTT – 3') Haemo02R: (5' – TCCATGAAACAAGTCCAGGTATAA – 3')	DENPEN02 DUNNO02	Yes	Too lineage specific
Haemo03F: (5' – TGTTTCATGGATTTGTGGAGGA –3') Haemo03R: (5' – AAGGATTAGAGCTACCTTGTAAGTG – 3')	DENPEN02 DUNNO01	Yes	Too lineage specific

Table 2. Top 3 models for infection prevalence data in wild caught birds during autumn migration.

Model	AICc	ΔAICc	Relative likelihood	Akaike weight (w)
Prevalence ~ 1 + Fat_Lean	131.17	0.00	1.00	0.38
Prevalence ~ 1 + Sex + Fat_Lean	131.37	0.20	0.90	0.34
Prevalence ~ 1	131.73	0.56	0.75	0.28

Table 3. Top 3 models and the null model for parasitemia data in wild caught birds during autumn migration.

Model	AICc	ΔAICc	Relative likelihood	Akaike weight (w)	F	df	p value	R^2
Parasitemia ~ 1	210.16	1.67	0.43	0.15				
Parasitemia ~ 1 + Capture Date	208.48	0.00	1.00	0.35	0.85	2,39	0.43	0.04
Parasitemia ~ 1 + Capture Date + Wing	208.9302	0.45	0.79	0.28	0.77	3,38	0.51	0.05
Parasitemia ~ 1 + Age + Capture Date	209.5012	1.02	0.60	0.21	0.73	3,38	0.53	0.05

Table 4. Top 3 models for IgY data in wild caught birds during autumn migration.

Model	AICc	ΔAICc	Relative likelihood	Akaike weight (w)	F	df	p value	R^2
IgY ~ 1	-136.29	0.00	1.00	0.44				
IgY ~ 1 + Sex	-135.78	0.50	0.77	0.34	1.57	1,81	0.21	0.02
IgY ~ 1 + Wing	-134.85	1.44	0.48	0.21	0.64	1,81	0.42	0.008

Table 5. Top 3 models and the null model for plasma haptoglobin concentration data of wild caught birds during autumn migration.

Model	AICc	ΔAICc	Relative likelihood	Akaike weight (w)	F	df	p value	R^2
Haptoglobin ~ 1	-132.12	24.72	0.00	0.00				
Haptoglobin ~ 1 + Capture Date + Capture date^2 + Body mass + Fat Score	-156.48	0.00	1.00	0.47	9.84	4,71	0.0000022	0.36
Haptoglobin ~ 1 + Capture Date + Body mass	-155.64	1.21	0.55	0.26	11.63	3,72	0.0000027	0.33
Haptoglobin ~ 1 + Capture Date	-155.74	1.10	0.57	0.27	16.19	2,73	0.0000015	0.31

Table 6. Top 3 models and the null model for parasitemia data in the winter and spring in captive birds.

Model	AICc	ΔAICc	Relative likelihood	Akaike weight (w)	R^2
Parasitemia~ 1 + (1|ID)	-104.81	21.69			0.05
Parasitemia ~ Time + (1|ID)	-126.5	0.00	1.00	0.57	0.31
Parasitemia ~ Sex Time + (1|ID)	-125.3	1.29	0.40	0.30	0.32
Parasitemia ~ Diet + Time + (1|ID)	-122.9	3.66	0.39	0.09	0.32

Table 7. Top 3 models of the change in parasitemia data from winter to spring in captive birds.

Model	AICc	ΔAICc	Relative likelihood	Akaike weight (w)	F	df	p value	R^2
Change in parasitemia ~ 1 + Body mass	-39.95	0.00	1.00	0.55	3.25	1, 50	0.05	0.07
Change in parasitemia ~ 1	-38.14	1.81	0.40	0.22				
Change in parasitemia ~ 1 + Sex + Body mass	-38.10	1.84	0.39	0.22	2.15	2, 49	0.13	0.08

Table 8. Top 3 models of flight duration data with infection prevalence of captive birds.

Model	AICc	ΔAICc	Relative likelihood	Akaike weight (w)	F	df	p value	R^2
Duration ~ 1	330.86	0.00	1.00	0.46				
Duration ~ 1 + long day prevalence	331.35	0.48	0.78	0.36	1.79	1,24	0.19	0.06
Duration ~ 1 + long day prevalence	332.82	1.96	0.37	0.17	1.39	2,23	0.27	0.12

Table 9. Top 3 models of flight duration data with parasitemia of captive birds.

Model	AICc	ΔAICc	Relative likelihood	Akaike weight (w)	F	df	p value	R^2
Duration ~ 1	330.86	0.00	1.00	0.53				
Duration ~ 1 + Body mass	332.31	1.45	0.48	0.25	0.85	1,24	0.36	0.03
Duration ~ 1 + Sex	332.73	1.86	0.39	0.20	0.45	1,24	0.50	0.02

Table 10. Top 3 models of the overall body mass gain data of captive birds throughout the experimental period.

Model	AICc	ΔAICc	Relative likelihood	Akaike weight (w)	F	df	p value	R^2
Overall Body mass Change ~ 1	190.27	1.55	0.50	0.28				
Overall Body mass Change ~ 1 + Sex	188.72	0.00	1.00	0.61	3.71	1,49	0.059	0.07
Overall Body mass Change ~ 1 + long day parasitemia	192.06	3.34	0.19	0.11	0.37	1,49	0.55	0.01

Table 11. Top 3 models of the average rate of body mass gain data of captive birds.

Model	AICc	ΔAICc	Relative likelihood	Akaike weight (w)	F	df	p value	R^2
Average rate of body mass gain ~ 1 + long day parasitemia	- 153.12	0.00	1.00	0.43	2.50	1,49	0.12	0.05
Average rate of body mass gain ~ 1	- 152.75	0.37	0.83	0.36				
Average rate of body mass gain ~ 1 + Sex + long day parasitemia	- 151.68	1.44	0.49	0.21	1.63	2,48	0.21	0.06

Table 12. Top 3 models of haptoglobin concentration post-flight data in captive birds.

Model	AICc	ΔAICc	Relative likelihood	Akaike weight (w)	F	df	p value	R^2
Haptoglobin ~ 1	-7.17	0.00	1.00	0.49				
Haptoglobin ~ 1 + Body mass	-6.09	1.07	0.58	0.29	1.06	1,50	0.31	0.02
Haptoglobin ~ 1 + Long day parasitemia	-5.44	1.73	0.42	0.20	0.42	1,50	0.52	0.01

7 *Appendix B:* Animal Care Protocols

March 25, 2010

This is the Original Approval for this protocol
A Full Protocol submission will be required in 2014

Dear Dr. Guglielmo:

Your Animal Use Protocol form entitled:
Physiological Ecology of Migratory Birds During Stopover
Funding Agency NSERC - Grant - 311901-05

has been approved by the University Council on Animal Care. This approval is valid from **March 25, 2010 to March 31, 2011.** The protocol number for this project is **2010-020 which replaces 2006-014 which has expired.**.

1. This number must be indicated when ordering animals for this project.
2. Animals for other projects may not be ordered under this number.
3. If no number appears please contact this office when grant approval
   If the application for funding is not successful and you wish to proce ternal scientific peer review be performed by the Animal Use Subcommittee o
4. Purchases of animals other than through this system must be cleare certificates will be required.

ANIMALS APPROVED FOR 4 Years

Species	4 Year Total Numbers Estimated as Required	List All Strain(s)	Age / Weight
Songbird	22220	All native bird species as permitted by CWS and USFWS	all

REQUIREMENTS/COMMENTS
Please ensure that individual(s) performing procedures on live animals, as described in this protocol, are familiar with the contents of this document.
The holder of this Animal Use Protocol is responsible to ensure that all associated safety components (biosafety, radiation safety, general laboratory safety) comply with institutional safety standards and have received all necessary approvals. Please consult directly with your institutional safety officers.

c.c. Approval - C. Guglielmo, S. Waring, W. Lagerwerf

***The* University *of* Western Ontario**
Animal Use Subcommittee / University Council on Animal Care
Health Sciences Centre, ● London, Ontario ● CANADA – N6A 5C1
PH: 519-661-2111 ext. 86770 ● FL 519-661-2028 ● www.uwo.ca / animal

Western

April 8, 2010

This is the Original Approval for this protocol

Dear Dr. Guiglielmo:

Your Animal Use Protocol form entitled:
Energetics, fuel use, water balance and immunocompetence during exercise in migrating birds
Funding Agency NSERC - 311901-05 and up for renewal at present

has been approved by the University Council on Animal Care. This approval is valid from **April 8, 2010 to April 30, 2011.** The protocol number for this project is **2010-216 which replaces 2006-011-04 which has expired.**.

1. This number must be indicated when ordering animals for this project.
2. Animals for other projects may not be ordered under this number.
3. If no number appears please contact this office when grant approval is received.
   If the application for funding is not successful and you wish to proceed with the project, request that an internal scientific peer review be performed by the Animal Use Subcommittee office.
4. Purchases of animals other than through this system must be cleared through the ACVS office. Health certificates will be required.

ANIMALS APPROVED

Species	4 Year Total Numbers Estimated as Required	List All Strain(s)	Age / Weight
Songbird	660	All bird species as permitted by CWS and USFWS	all

REQUIREMENTS/COMMENTS
Please ensure that individual(s) performing procedures on live animals, as described in this protocol, are familiar with the contents of this document.
The holder of this Animal Use Protocol is responsible to ensure that all associated safety components (biosafety, radiation safety, general laboratory safety) comply with institutional safety standards and have received all necessary approvals. Please consult directly with your institutional safety officers.

c.c. Approval - C. Guglielmo, S. Waring

***The* University *of* Western Ontario**
Animal Use Subcommittee / University Council on Animal Care
Health Sciences Centre, ● London, Ontario ● CANADA – N6A 5C1
PH: 519-661-2111 ext. 86770 ● FL 519-661-2028 ● www.uwo.ca / animal

8 *Appendix C:* CWS Permits

Environment Canada / Environnement Canada

CANADIAN WILDLIFE SERVICE - PERMIT
PERMIS - SERVICE CANADIEN DE LA FAUNE

		Permit to/for Permis de/pour **SCIENTIFIC**	Permit no. No de permis **CA 0255**
Organization Organization **University of Western Ontario**	Issued under section Délivré en vertu de l'article 19	of de MIGRATORY BIRD REGULATIONS	
Surname Nom de famille **Guglielmo**	Name Prénom **Christopher**	Department Département **Department of Biology**	

1151 Richmond Street North
London On
N6A 5B7

Date of issue Date d'émission **June 13, 2012**	Date of expiry Date d'éxpiration **March 31, 2015**
Signature of holder Signature du détenteur	For the minister Pour le ministre

Special Conditions - Conditions spéciales

1. Prior to any use of this permit the permittee will notify the Ontario Ministry of Natural Resources relative to collecting procedures, times and localities of collection.
2. Landowner's permission must be obtained prior to collecting on private property.
3. Permit or a copy of the permit to be carried in the field by all collectors.
4. The permit holder is authorized to collect and to possess for scientific research purposes, samples of blood, feather, claw and urine, from migratory birds – to wit: White-throated Sparrow (Zonotrichia albicollis), Yellow-rumped Warbler (Setophaga coronata), Magnolia Warbler (Setophaga magnolia), Hermit Thrush (Catharus guttatus), Swainson's Thrush (Catharus ustulatus) and American Redstart (Setophaga ruticilla) – from locations as situated within Middlesex, Norfolk, Haldimand, Oxford, Perth, Elgin, Essex, Kent, Bruce & Grey Counties within the province of Ontario.
5. Take is limited to a maximum of 100 specimens per species per year.
6. All specimens are to be taken to the Department of Biology, University of Western Ontario, London, Ontario, for further analysis.
7. Capture, handling and sampling procedures are to be performed according to the Animal Care Committee protocols of the University of Western Ontario.
8. All other birds are to be released into the wild by the conclusion of the study or otherwise be humanely euthanized. No birds are to be donated or loaned to another individual or institution without the prior consent of the Canadian Wildlife Service.
10. Samples not to be retained are to be disposed of by the approved laboratory waste management system of the University of Western Ontario.
11. Permit holder shall submit a written report, by January 31, of each year following, indicating the results of the study to the Canadian Wildlife Service, 867 Lakeshore Road, Burlington, ON., L7R 4A6.
12. Nominees to this permit are: Department of Biology faculty/staff as acting under the direction of the permittee.

Amended: 13 June 2013 PK

Canada

Environment Canada / Environnement Canada

CANADIAN WILDLIFE SERVICE - PERMIT
PERMIS - SERVICE CANADIEN DE LA FAUNE

		Permit to/for Permis de/pour **SCIENTIFIC**	Permit no. No de permis **CA 0256**
Organization Organization **University of Western Ontario**	Issued under section Délivré en vertu de l'article 19	of de MIGRATORY BIRD REGULATIONS	
Surname Nom de famille **Guglielmo**	Name Prénom **Christopher**	Department Département **Department of Biology**	

1151 Richmond Street North
London On
N6A 5B7

Date of issue Date d'émission **June 13, 2012**	Date of expiry Date d'expiration **June 30, 2015**
Signature of holder Signature du détenteur	For the minister Pour le ministre

Special Conditions - Conditions spéciales

1. Prior to any use of this permit the permittee will notify the Ontario Ministry of Natural Resources relative to collecting procedures, times and localities of collection.
2. Landowner's permission must be obtained prior to collecting on private property.
3. Permit or a copy of the permit to be carried in the field by all collectors.
4. The permit holder is authorized to collect and to possess for scientific research purposes, migratory birds - to wit: Cedar Waxwing (Bombycilla cedrorum), Tree Swallow (Tachycinetra bicolor), Purple Martin (Progne subis), White-throated Sparrow (Zonotrichia albicollis), Yellow-rumped Warbler (Setophaga coronate), Hermit Thrush (Catharus guttatus), Swainson's Thrush (Catharus ustulatus), American Robin (Turdus migratorius) and Ruby-throated Hummingbird (Archilocus colubris) - from locations as situated within Oxford, Perth, Elgin, Middlesex, Norfolk and Haldimand Counties within Ontario. Take is limited to a maximum of 60 birds per species per year.
5. Additional permission is granted to receive from Simon Fraser University, Vancouver, BC, migratory birds - to wit: Western Sandpiper (Calidris mauri) - as currently held in a captive breeding facility. Take is limited to a maximum 100 birds per year. Upon completion of this study a number of the Western Sandpiper (Calidris mauril) will be returned to the captive colony at Simon Fraser University for further study or release.
6. All specimens are to be taken to the Department of Biology, University of Western Ontario, London, Ontario, for further analysis.
7. Capture, handling and housing procedures are to be performed according to the Animal Care Committee protocols of the University of Western Ontario.
8. All other birds are to be released into the wild by the conclusion of the study or otherwise be humanely euthanized. No birds are to be donated or loaned to another individual or institution without the prior consent of the Canadian Wildlife Service.
9. All specimens are to be retained at the University of Western Ontario for scientific study purposes.
10. Samples not to be retained are to be disposed of by the approved laboratory waste management system of the University of Western Ontario.
11. Permit holder shall submit a written report, by January 31, of each year following, indicating the results of the study to the Canadian Wildlife Service, 867 Lakeshore Road, Burlington, ON., L7R 4A6.
12. Nominees to this permit are: Department of Biology faculty/staff as acting under the direction of the permittee.

Canada

9 *Appendix D:* Curriculum Vitae

Laura Rooney

Education

Masters of Science – Biology — September / 2013 – present
The University of Western Ontario
London, Ontario

Bachelor of Sciences - Specialization in Biology — 2013
The University of Western Ontario
London, Ontario

Awards/Achievements

Deans Honor List — 2010-2013
The University of Western Ontario

Entrance Scholarship — 2008
The University of Western Ontario

Academic/Teaching Experience

Teaching Assistant – Physiology of Migration — 2014-2015
The University of Western Ontario

Teaching Assistant – Parasitology — 2014
The University of Western Ontario

Presentations

Rooney, L.A; Guglielmo, C.G. Shriner, S.A. **Sources of variation in blood parasite infection and its impact on immune function and flight performance in yellow–rumped warblers (Setophaga coronata).** *Annual Meeting of the Society of Integrative and Comparative Biology, West Palm Beach, Fl, USA. 2015*