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## Effects of Experimental Malaria Infection On Migration of Yellow-rumped Warblers (*Setophaga coronata*)

Rebecca J. Howe, *The University of Western Ontario*

Supervisor: MacDougall-Shackleton, Elizabeth, *The University of Western Ontario*

Joint Supervisor: Guglielmo, Christopher, *The University of Western Ontario*

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

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## Abstract

The potential of migratory animals to spread infectious diseases depends on how infection affects movement. If infection delays or slows the speed of travel, transmission to uninfected individuals may be reduced. Whether and how malaria (*Plasmodium* spp.) affects bird migration has received little experimental research. I captured 40 actively-migrating Yellow-rumped Warblers (*Setophaga coronata*) at a migration stopover site and held them in captivity. I inoculated 25 with *P. cathemerium* while 15 received sham inoculations. After 12 days the birds were released. Six *P. cathemerium*-inoculated birds (24%) developed *P. cathemerium* infections after inoculation. I radio-tagged all birds, and used radio signal strength variability as an index of activity before and after release. I radiotracked birds at the release site to measure stopover duration. Experimental groups (Infected, Exposed but uninfected, Sham) did not differ in activity levels before or after release, nor in stopover duration. This research suggests that birds do not alter the migratory stopover behavior in response to avian malaria.

## Keywords

migration, avian malaria, experimental infection, migratory stopover, *Plasmodium*

## Summary for Lay Audience

Migration is increasingly recognized as a potential factor that may influence the spread of infectious diseases. Most migratory birds do not migrate in one long flight, but instead take breaks of days to weeks to refuel at migratory stopovers, and diseases may be particularly likely to spread among individuals (or indirectly through insect vectors) at these stopover sites. If infected birds are delayed in their migration, e.g. taking longer to refuel at stopover, this may reduce their encounters with uninfected birds, thus limiting opportunities for disease to spread. Approximately two-thirds of bird species are affected by avian malaria, a common disease that causes symptoms such as reduced movement, reduced eating, and damage to blood cells. Little is known about how this disease affects bird migration, and experimental information is particularly lacking. I captured and kept captive 40 Yellow-rumped Warblers during fall migration, fitted them with radio-tags to track their movement and activity, and experimentally inoculated 25 of them with avian malaria. Six birds developed malaria (the Infected group) and 19 did not (the Exposed but uninfected group). The remaining fifteen birds were not inoculated with malaria (the Sham group). Twelve days after inoculation I released the birds and continued radio-tracking them until most individuals had left the release site. There was no difference in activity between groups either in captivity or after release, nor in duration of stay at the release site. This suggests that being exposed to and/or infected with avian malaria does not change how birds behave at a stopover. If so, infection-induced delays are not likely to restrict the spread of avian malaria, at least in this combination of bird and parasite species.

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## Co-Authorship Statement

The work presented here was completed under the supervision of Dr. Elizabeth MacDougall-Shackleton and Dr. Christopher Guglielmo at the University of Western Ontario. All work was done in collaboration with Dr. Elizabeth MacDougall-Shackleton, Dr. Christopher Guglielmo, and Dr. Leticia Soares, who helped develop study objectives and methodology. Bird collection and experimental inoculation were done in collaboration with Dr. Leticia Soares. Data collection and analysis was completed by Rebecca Howe. Dr. Yolanda Morbey helped with the analysis of radio telemetry data. This thesis was written by Rebecca Howe with editorial input from Dr. Elizabeth MacDougall-Shackleton and Dr. Christopher Guglielmo. A modified version will be published in an academic journal with Dr. Elizabeth MacDougall-Shackleton, Dr. Leticia Soares, Dr. Yolanda Morbey and Dr. Christopher Guglielmo as coauthors.

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## List of Abbreviations

- AFAR- Advanced Facility for Avian Research  
ANOVA- Analysis of Variance  
BPBO- Bruce Peninsula Bird Observatory  
CI- Confidence Interval  
df- Degrees of Freedom  
CV- Coefficient of Variance  
DNA- Deoxyribonucleic Acid  
dNTP- Deoxyribonucleotide Triphosphate  
EDTA- Ethylenediaminetetraacetic Acid  
GLMM- Generalized Linear Mixed Model  
GPS- Global Positioning System  
LPBO- Long Point Bird Observatory  
LPS- lipopolysaccharide (a bacterial endotoxin that causes an immune response)  
PCR- Polymerase Chain Reaction  
SD- Standard Deviation  
TBE- Tris/Borate/EDTA  
VHF- Very High Frequency

## Chapter 1

### 1 Introduction

#### 1.1 Disease and Migration

When we hear the word migration, we think of the dramatic, long-distance journeys of animals like monarch butterflies (*Danaus plexippus*) (Bradley and Altizer, 2005), caribou (*Rangifer tarandus*) (Fancy et al., 1990), and birds (DeLuca et al., 2019). Migratory behavior is so widespread that it likely evolved early in the history of life on Earth and its selective advantages maintain it in a wide variety of extant animals (Baker, 1978). The hypothesized origin of migratory movement was in response to seasonality of resources, and this remains the driving force behind most migrations today (Dingle, 2014). However, migration does not come without costs. While we enjoy watching geese fly south for the winter, we may think about the endurance marathon they have begun. Migration is a highly energy intensive period, and animals face unique challenges as they move across ecological barriers and through novel landscapes (Dingle, 2014). The stress and depletion of stored energy during migration can carry-over into other stages of an individual's life history and create longer term reductions in fitness (Drake et al., 2014; Harrison et al., 2011). Migration puts individuals at risk, and forces trade-offs between different physical attributes (Risely et al., 2018).

Migration may make animals more susceptible to parasites. Intense exercise, such as migratory flight, can cause a decrease in immune function immediately after exercise ceases (Nebel et al., 2012). In a study comparing migrant and resident Common Blackbirds (*Turdus merula*), the migrants exhibited reduced immune function (Eikenaar and Hegemann, 2016). If parasites are encountered when these birds are already under energetic stress, they could have an impact on subsequent migratory performance and life history (Pérez-Tris and Bensch, 2005).

There is abundant evidence that migrants can spread parasites, and that parasites associated with avian malaria (*Plasmodium* sp.) have evolved to take advantage of this. European migrant songbirds that overwinter in Africa acquire African haemoparasites,

but not all haemoparasites are also spread on the breeding grounds (Samuel et al., 2015; Waldenström et al., 2002). When avian malaria evolves to be infective year-round, migrating birds can spread it into larger ranges and potentially to new host species (Clark et al., 2015; Pérez-Tris and Bensch, 2005). Birds have also been responsible for outbreaks of West Nile virus and avian influenza virus, and outbreaks have been linked to specific migratory flyways and stopovers (Galsworthy et al., 2011; Rappole et al., 2000).

Migration can expose animals to a greater variety of parasites, or novel parasites to which they may have little resistance (Altizer et al., 2011; Yorinks and Atkinson, 2000). In migratory birds, such exposure may be particularly likely at stopover sites, defined as the time and space where migrating birds rest and refuel before their next migratory flight (Taylor et al., 2011; Woodworth et al., 2014). The time spent at a stopover is affected by the bird's condition at arrival (Dossman et al., 2016), the rate at which it can refuel (Moore, 2018), and weather or wind conditions which influence departure choice (Dossman et al., 2016; Drake et al., 2014; Haest et al., 2018). Birds may also make small relocations within a region to maximize refueling (Taylor et al., 2011). Stopover periods can last from days to weeks, (Moore, 2018; Smith and McWilliams, 2014).

Stopover populations of Mallard ducks (*Anas platyrhynchos*) experience pulses of local infections when uninfected birds arrive at a stopover where other birds are infected with avian influenza. Uninfected migrants quickly become infected with the local influenza, and have a higher prevalence of infection than resident Mallards (van Dijk et al., 2014). Migratory birds are also most vulnerable at stopovers, as the majority of migration is spent resting and refueling between bouts of migratory flight (Hedenström and Alerstam, 1997). These stopovers can have high densities of individuals in close proximity, and can provide a suitable environment for parasites and pathogens to spread among conspecifics and species (Altizer et al., 2011; McKay and Hoye, 2016; Samuel et al., 2015).

Despite considerable attention paid to migratory animals as potential “super-spreaders” of infectious disease (McKay and Hoye 2016), migration may instead dampen

the transmission of disease. The migratory escape hypothesis suggests that animals use migration to escape areas with high numbers of parasites (Altizer et al., 2011; Bradley and Altizer, 2005; Johns and Shaw, 2016). Parasites can also influence habitat use and have severe impacts under certain circumstances. Migrating shorebirds prefer to make their migratory stopovers, where they rest and refuel, in saltwater habitats. This preference may be a behavior to reduce avian malaria exposure, as shorebirds in freshwater habitats have higher infection prevalence than shorebirds in saltwater habitats (Mendes et al., 2005). When *Plasmodium relictum capistranoae* was introduced to Hawaii it devastated populations of local bird species and forced survivors into higher altitude habitats where mosquito vectors of the parasite could not survive (van Riper III et al., 1986). When parasitism is high in a specific location, shortening the period of the life cycle spent in that area, through migration, is a benefit to the host species. Both modeling and studies of the monarch (*Danaus plexippus*) and its parasite (*Ophryocystis elektroscirrha*) support the hypothesis that migratory escape will reduce the overall prevalence of parasitism in the species (Bradley and Altizer, 2005; Hall et al., 2014).

Other ways in which animal migration may dampen the spread of infectious disease involve effects of infection on mobility and survival. Such effects have been hypothesized to separate infected individuals from the uninfected population, through either death (migratory culling) or infection-induced delay (migratory separation; Emmenegger et al., 2018; Johns and Shaw, 2016; Risely et al., 2018). Migratory culling refers to a situation in which infected individuals are less likely to survive the demands of migration. Migratory separation refers to infection delaying and/or slowing migration and thereby temporally separating infected individuals from the uninfected population (Risely et al., 2018). Either mechanism could reduce population level-infection prevalence by separating uninfected from infected individuals.

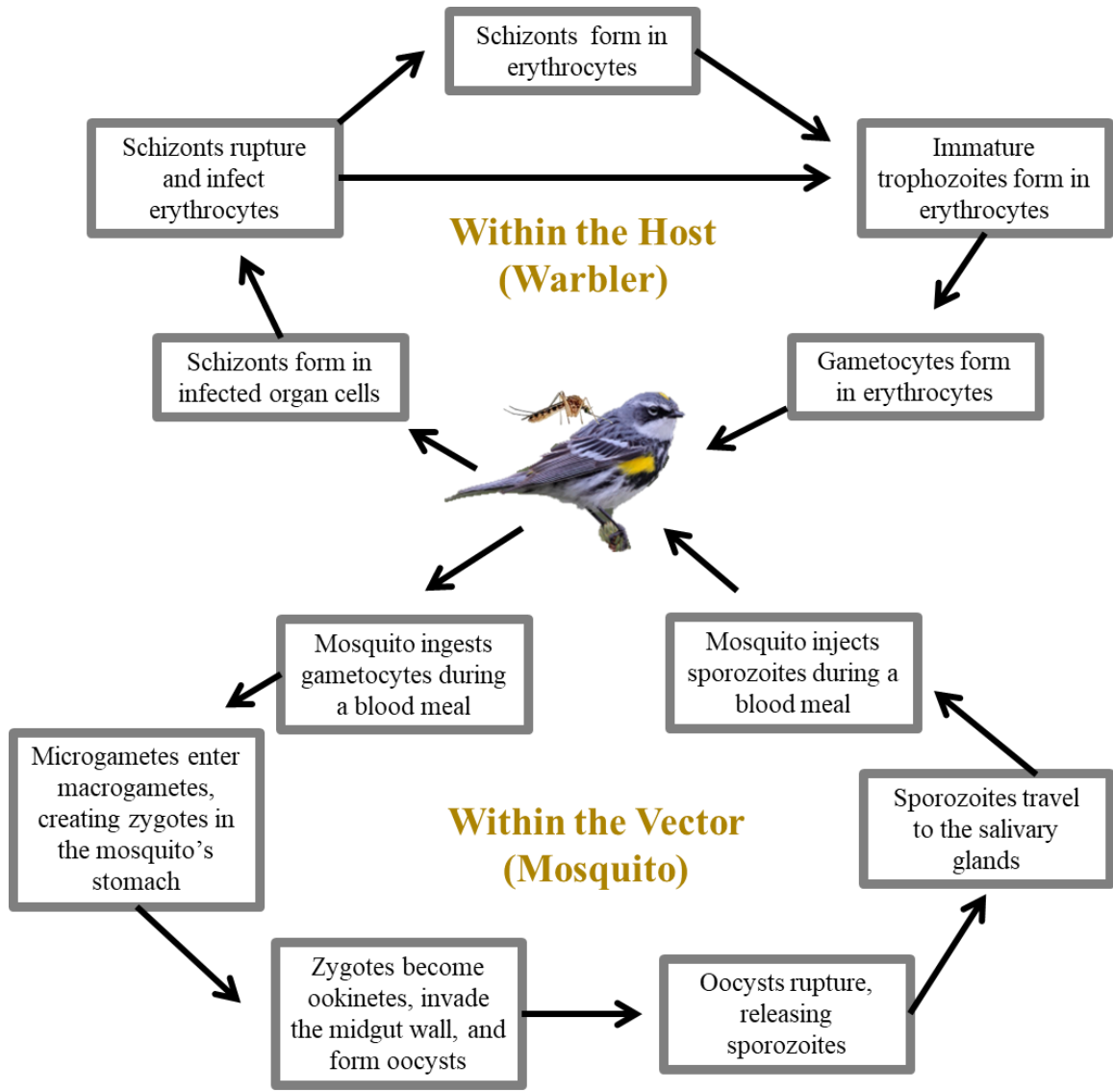
There is evidence for both migratory culling and migratory separation in birds. Long-distance migrant Lesser Black-Backed Gulls (*Larus fuscus*) have lower seroprevalence of avian influenza, and a higher constitutive immunity than their resident or short-distance migratory counterparts (Arriero et al., 2015). This implies that constitutive immunity and infection prevalence influence migratory strategies, and the stress of migration may cull



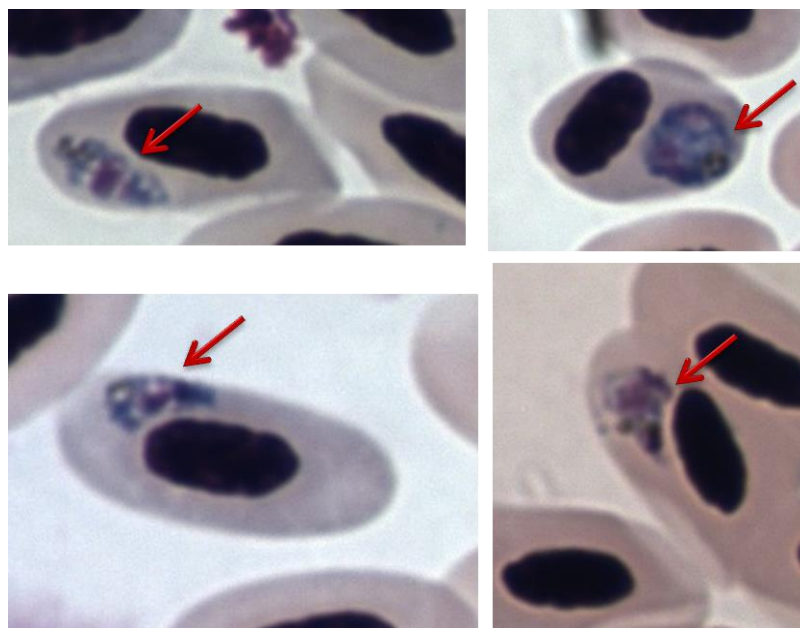
birds with poor immunity (Arriero et al., 2015). Modeling of avian influenza infection dynamics in Mallard populations shows that delayed migration due to infection results in migratory separation, and can reduce the total number of infections in a population (Galsworthy et al., 2011). General migration models and observational studies show that if migration of infected individuals is delayed, separating them from the healthy population, it can reduce influenza transmission (Bauer and Hahn, 2016; van Gils et al., 2007). Thus, there are numerous hypothesis (*e.g.* migratory escape, migratory culling, migratory separation) regarding how migration may effect and control the spread of disease.

## 1.2 Avian Malaria

Avian malaria is a common bloodborne disease vectored by mosquitoes (genera *Aedes*, *Anopheles*, *Culex*, *Culiseta*, and *Psorophora*) and caused by haemosporidian protozoan parasites in the genus *Plasmodium* (phylum Apicomplexa) that specialize on birds as the intermediate host (Figure 1, LaPointe et al., 2012; Marzal, 2012, Valkiunas, 2005). Avian malaria has a world-wide distribution, and areas where this disease is historically not present are either remote and/or lack mosquitoes to act as vectors (LaPointe et al., 2012; Palinauskas et al., 2011). Avian haemosporidians are very species-rich, with over forty species recognized in 2012, and tend to be generalists (LaPointe et al., 2012; Marzal, 2012; Valkiunas, 2005). Avian malaria infections affect two-thirds of all known bird species, and are particularly common in passerine birds (Marzal, 2012; Valkiūnas, 2005). Birds are also often co-infected (that is, simultaneously infected) with *Plasmodium* spp. and haemoparasites from the genera *Haemoproteus* and *Leucocytozoon*, which can compete with *Plasmodium* spp. infections and alter infection impacts (Clark et al., 2016; Figuerola et al., 1999; Martínez-de la Puente et al., 2010; Marzal et al., 2008; Palinauskas et al., 2011; Shurulinkov and Chakarov, 2006).



**Figure 1: A Summary of the *Plasmodium* spp. lifecycle within the avian and mosquito hosts (after Valkiunas, 2005).**



**Figure 2: *Plasmodium cathemerium* (indicated by the red arrows) infecting Yellow-rumped Warbler red blood cells. Images taken for this study using a 100x oil immersion microscope.**

When birds initially become infected with *Plasmodium* spp. they undergo an acute phase which can begin anywhere from six to twelve days after parasites first appear in the blood of infected birds (LaPointe et al., 2012). Birds express visible signs of illness such as lethargy and ruffled feathers, and may stop eating (Hayworth et al., 1987; LaPointe et al., 2012; Palinauskas et al., 2011). Internally, birds experience anemia as red blood cells lyse and parasites degrade haemoglobin to obtain protein for growth (Goldberg, 1993; LaPointe et al., 2012). There may be enlargement of the liver, spleen, and kidneys due to a build-up of materials from lysed cells, and some species may experience lowered body temperatures (Hayworth et al., 1987; Palinauskas et al., 2008; Yorinks and Atkinson, 2000).

If they survive the acute phase, birds enter a chronic phase around forty-five days after infection (Asghar et al., 2012). Parasites persist at low levels in the blood and organs, and prevalence in the blood stream changes throughout the year, peaking in breeding season (Cornelius et al., 2014; Townsend et al., 2018). This peak is due to both an increase in vector activity creating new infections and relapses in chronically infected

birds due to the allocation of host resources away from immune defense allowing parasitemia to increase (Applegate and Beaudoin, 1970; Cornelius et al., 2014; Rooney, 2015; Zehtindjiev et al., 2008). While low-level chronic infections appear to have no short-term costs, chronically infected birds have lower reproductive success, and chronic infections are associated with an increased rate of telomere loss (Asghar et al., 2015). Shorter telomeres are associated with aging and age-related diseases in humans, and increased telomere degradation may impact a bird's longevity (López-Otín et al, 2013).

Although *Plasmodium* parasites are normally transmitted to the avian host by the bite of an infected mosquito (Figure 1), experimental infection can also be accomplished by inoculating a bird with blood from an infected bird (either conspecific or heterospecific; Palinauskas et al. 2008). This approach is possible because *Plasmodium* spp. can replicate asexually in the blood cells, not merely in the organs, of its avian host (Valkiunas, 2005).

## 1.3 Past Research

### 1.3.1 Avian Malaria Across the Annual Cycle of Migratory Birds

Migratory birds all share a similar life history cycle; breeding, migration, time spent in non-breeding habitat, followed by migration back to the breeding grounds (Dingle, 2014). Avian malaria can be present within the population at any point in this cycle (Samuel et al., 2015). Observational and experimental studies during the breeding season consistently detect infections. For instance, infections in juveniles are frequently detected, and could only occur on the breeding grounds (Davidar and Morton, 1993; Shurulinkov and Chakarov, 2006). Transmission on the wintering grounds has similar evidence, with only adult birds in European populations being infected with parasites native to Africa. These infections could not occur anywhere other than their wintering grounds, and these infections in breeding adults prove that birds carry infections with them during migration (Shurulinkov and Chakarov, 2006; Waldenstrom et al., 2002).

However, infections during migration and their effects are the least studied part of this cycle, and the dynamics of infection at migratory stopovers is poorly understood. Existing research on migration and avian malaria is outlined below.

### 1.3.2 Migratory Timing, Condition, and Chronic Avian Malaria Infection

Migratory timing refers to the time of occurrence of a migratory behavior. Chronic malaria infection appears to have mixed effects on migratory timing. Arrival timing at breeding sites is not correlated with chronic infection in Purple Martins (*Progne subis*) (Davidar and Morton, 1993), but more heavily infected male Barn Swallows (*Hirundo rustica*) arrive later than uninfected counterparts (Møller et al., 2004). Migratory timing is not correlated with chronic infection in Garden Warblers (*Sylvia borin*) (Lopez et al., 2013), but is affected only in female Great Reed Warblers (*Acrocephalus arundinaceus*) (Asghar et al., 2011). Additionally, when Great Reed Warblers depart their wintering grounds there is no association between infection and departure date (Sorenson et al., 2016). Blackcaps (*Sylvia atricapilla*) arriving later in spring were more likely to have infections and higher parasitemia, but arrival date at the breeding ground was similar to uninfected counterparts (Santiago-Alarcon et al., 2013).

Physiological condition, usually measured as fuel stores, does not appear to be affected by chronic malaria infection during the migration season (Risely et al., 2018). Researchers surveying Neotropical migrants at a stopover found no effects of chronic infection on body condition measures (Cornelius et al., 2014). Similarly, Red Crossbills (*Loxia curvirostra*) and American Crows (*Corvus brachyrhynchos*) had no relationship between body condition or fat deposits and chronic infection status or intensity (Cornelius et al., 2014; Townsend et al., 2018), and chronically infected Blackcaps (*Sylvia atricapilla*) and Great Reed Warblers (*Acrocephalus arundinaceus*) had no differences in body condition from uninfected birds (Santiago-Alarcon et al., 2013; Sorenson et al., 2016). A meta-analysis of forty-one observational studies of migration and disease found that there was a relatively small association between infection status and physiological condition, movement, and migratory timing, but half of the studies in the meta-analysis reported no significant effects (Risely et al., 2018). It appears that if there are impacts from avian malaria, they are more likely to be present in the acute phase.

### 1.3.3 Chronic Infection and Correlational Avian Malaria Research

The studies reviewed above are subject to two important caveats. The first is that most avian malaria research has focused on correlational studies comparing migratory performance of uninfected individuals to those with chronic infections, partly because it is difficult to catch acutely infected birds in the wild (Asghar et al., 2012; Mukhin et al., 2016) and partly because of logistical difficulties in manipulating infection status experimentally. The acute period is brief and infected birds may be inactive, making them less likely to be captured in traps or nets (Lachish et al., 2011; Mukhin et al., 2016). Moreover, birds with very high acute parasitemia often die in the wild and would therefore not be sampled (Asghar et al., 2012; Palinauskas et al., 2011). However, if birds survive the acute phase, the resulting chronic infections often do not influence survival (Davidar and Morton, 1993; Risely et al., 2018). At any given time, most infected individuals in a host population are survivors with chronic infections. A population-level study of Blue Tits (*Cyanistes caeruleus*) found that uninfected birds were also more likely to be recaptured than infected individuals, so common sampling methods may not produce a true representation of infection with *Plasmodium* spp. (Lachish et al., 2011). The higher likelihood of catching healthy birds has likely led to an underestimation of the frequency and severity of infection in the wild (Mukhin et al., 2016).

### 1.3.4 Inoculation, Immune Challenges, and Medication Experiments

The second major caveat is that observational studies comparing the performance of naturally-infected and uninfected individuals are limited in the conclusions that can be drawn. Specifically, such studies cannot disentangle whether group differences in migratory performance (e.g., later arrival dates for infected individuals) reflect the effects of infection or effects of some other correlated but unmeasured variable such as variation in age, experience, or individual quality. For this reason, experimental studies are the most effective way to research how birds at a migratory stopover respond to infection: such studies can control for other variables that may be affecting condition and behavior. These can take the form of immune challenge experiments (where birds are exposed to a substance that mimics, but does not actually induce, parasitic infection), inoculation

experiments (where birds receive an inoculation with avian malaria to experimentally increase parasite load), and medication experiments (where birds receive antimalarial drugs to experimentally reduce parasite load).

When birds are given an immune challenge using the bacterial protein lipopolysaccharide (LPS), challenged birds stay longer at migratory stopovers and reduce their activity (Hegemann et al., 2018) relative to controls. Zebra Finches (*Taeniopygia guttata*) injected with LPS had an early reduction in activity, but this reduction was over within four days of inoculation (Sköld-Chiriac et al., 2014). These studies suggest that encountering parasites at a stopover impacts activity, but that the effect may be short-lived. It is unlikely that such a short response would be detectable without an experimental study (Lachish et al., 2011).

Great Reed Warblers (*Acrocephalus arundinaceus*) and Greenfinches (*Chloris chloris*) that were experimentally infected with *P. relictum* and *P. ashfordi* did not show effects on body mass, stored fat, or mass gain, but acute infection with *P. relictum* impacted activity in Siskins (*Spinus pinus*) (Mukhin et al., 2016; Palinauskas et al., 2009; Zehtindjiev et al., 2008). However, these studies only considered effects in infected and uninfected birds. Using experimental inoculation allows us to test birds that resist infection, which is not a treatment group we can identify in the wild. Resistant birds show unique responses to *Plasmodium* exposure. For instance, Song Sparrows (*Melospiza melodia*) inoculated with *P. relictum* had no difference in departure timing when compared to controls, but birds that resisted infection had a lower mass after inoculation than control or infected birds. This suggests that loss of fuel stores from resisting infection can be greater than the loss of fuel stores from tolerating it (Kelly et al., 2018).

Medication experiments that clear malaria infections are much less common, and generally medicate chronic infections. Blue Tits treated with Malarone to reduce chronic *Plasmodium* spp. infections showed no effect of medication on body mass, but did show positive effects on clutch size and fledging success (Knowles et al., 2010). Similarly, male Red-winged Blackbirds (*Agelaius phoeniceus*) that received antimalarial drugs showed no effect on their body condition, immune metrics, or corticosterone

concentrations (Schoenle et al., 2017). These studies are much more effective than observational chronic infection research because they can manipulate exposure to disease, control for variation in individual quality through random assignment to groups, and isolate the effect of the main variable of interest (infection).

## 1.4 Study System

### 1.4.1 Yellow-rumped Warbler

The Yellow-rumped Warbler (*Setophaga coronata*) is a common insectivorous and frugivorous warbler species abundant throughout North America (Hunt and Flaspohler, 1998; Terrill and Ohmart, 1984; Toews et al., 2014). They are habitat generalists that nest in coniferous woodlands and use almost any microhabitat during migration and winter (Hunt and Flaspohler, 1998; Parnell, 1969). Their ability to digest waxes in berries of bayberry (*Morella pensylvanica*) provides winter foraging and allows them to winter farther north than other warblers (Hunt and Flaspohler, 1998). There are four recognized subspecies with the main two being the Myrtle (*S.c. coronata*) and the Audubon's Warbler (*S.c. auduboni*). Audubon's Warblers breed in western North America while Myrtle Warblers breed in the eastern U.S.A and Canadian boreal forest, and hybrids are present where populations overlap (Toews et al., 2014). Myrtle Warblers, the species used in this experiment, overwinter in eastern North America, Central America, and the Caribbean (Ball, 1952; Hunt and Flaspohler, 1998; Toews et al., 2014; Woodworth et al., 2015; Yaukey, 2010).

Myrtle Warblers are primarily nocturnal migrants but are known to make diurnal migratory movements (Seewagen et al., 2019). Based on band recoveries they move an average of 312 km/day during spring migration and 88 km/day during fall migration, and are some of the first warblers to arrive in spring and last warblers to leave in fall (Hunt and Flaspohler, 1998). Males overwinter further north than females, with first year males overwintering the furthest north and first year females overwintering the furthest south (Hunt and Flaspohler, 1998). Myrtle Warblers also use stopovers during migration, and make small daytime flights to maximize refueling. When leaving a stopover, older, more experienced birds, and those with larger fat stores, are more likely to depart than younger



or leaner birds, but sex does not influence departure timing (Dossman et al., 2016; Seewagen et al., 2019).

Cozzarolo et al. (2018) reported that Myrtle Warblers are more likely to be chronically infected with *Plasmodium* species than Audubon's Warblers. Chronic infection measurements during spring migration in northwestern Ohio and southern Ontario found 58.3% of the sampled birds to be infected, with the number dropping to 41% during fall migration and spiking to 81% in the breeding season relapse (DeGroot and Rodewald, 2010; L. Soares (unpublished data)). Birds captured at stopovers later in the season are more likely to be chronically infected, with second year birds having more intense infections than older birds, but the prevalence of chronic infections is not related to age or sex (DeGroot and Rodewald, 2010; Rooney, 2015).

#### 1.4.2 *Plasmodium cathemerium*

*Plasmodium cathemerium* has a long history of use in avian malaria research due to its number of hosts (approximately 50 species), so its morphology, natural history and lifecycle are well understood. Its known vertebrate hosts are mainly birds in the order Passeriformes, and it is present in all zoogeographical regions except for Australia and the Antarctic. It is capable of completing sporogony in at least seventeen mosquito species, including species from genera *Aedes*, *Anopheles*, *Culex*, *Culiseta*, and *Psorophora*. The best vectors of *P. cathemerium* are *Culex* mosquitoes (Valkiunas, 2005), which have six species native to Ontario (Giordano et al. 2015).

When a mosquito takes a blood meal from a bird infected with *P. cathemerium*, it ingests gametocytes that begin the sexual phase of *P. cathemerium*'s life cycle within the mosquito (Figure 1). Microgametocytes and macrogametocytes in the mosquito's abdomen combine to form zygotes that mature into ookinetes. The ookinetes enter the gut wall and form oocytes, which grow until they rupture and release sporozoites. This phase, from ingestion to sporogony, usually takes around six days. Sporozoites move to the mosquito's salivary glands, and can be transmitted to a new avian host when the infected mosquito takes a blood meal (Valkiunas, 2005).

Once a bird is bitten by an infected mosquito the sporozoites move to the bird's liver, spleen and other organs where they create the first generation of primary exoerythrocytic meronts, called cryptozoites (Figure 1). These cryptozoites mature quickly and produce merozoites that then produce the second generation of primary exoerythrocytic meronts called metacryptozoites. The metacryptozoites produce both metacryptozoites and merozoites, and the merozoites enter the bloodstream. At this stage secondary exoerythrocytic meronts, called phanerozoites, can appear in the brain, lungs, and other organs (Valkiunas, 2005).

Once merozoites enter the blood they begin to form sexual and asexual stages. These stages are visible within the bloodstream within seventy-two hours of initial infection, and at peak parasitemia up to 50% of erythrocytes can be parasitized. High parasitemia lasts for around a week before declining to chronic infection levels (Valkiunas, 2005). Long term studies of avian populations suggest that birds generally remain chronically infected and do not clear the parasite (Podmokła et al., 2014; Santiago-Alarcon et al., 2013).

## 1.5 Study Objective and Predictions

The purpose of this study was to test the hypothesis that exposure to avian malaria at a migratory stopover affects condition, activity, and stopover duration. To test this hypothesis, I simulated infection at a stopover by capturing Yellow-rumped warblers during their fall migration, inoculating them with *P. cathemerium* in captivity, determining which individuals became infected following the inoculation and which did not, and releasing them to continue their migration. I used radio-telemetry during captivity and after release to measure activity levels and stopover departure timing.

If experimental inoculation with avian malaria causes birds to invest energy into resisting or tolerating infection, I expected to see reduced body condition, reduced activity, and a delayed departure date in all inoculated birds relative to controls. Moreover, I expected birds that were inoculated but resisted infection to have a larger reduction in condition than those that were inoculated and became infected, based on previous work suggesting that resistance is more energetically taxing than becoming

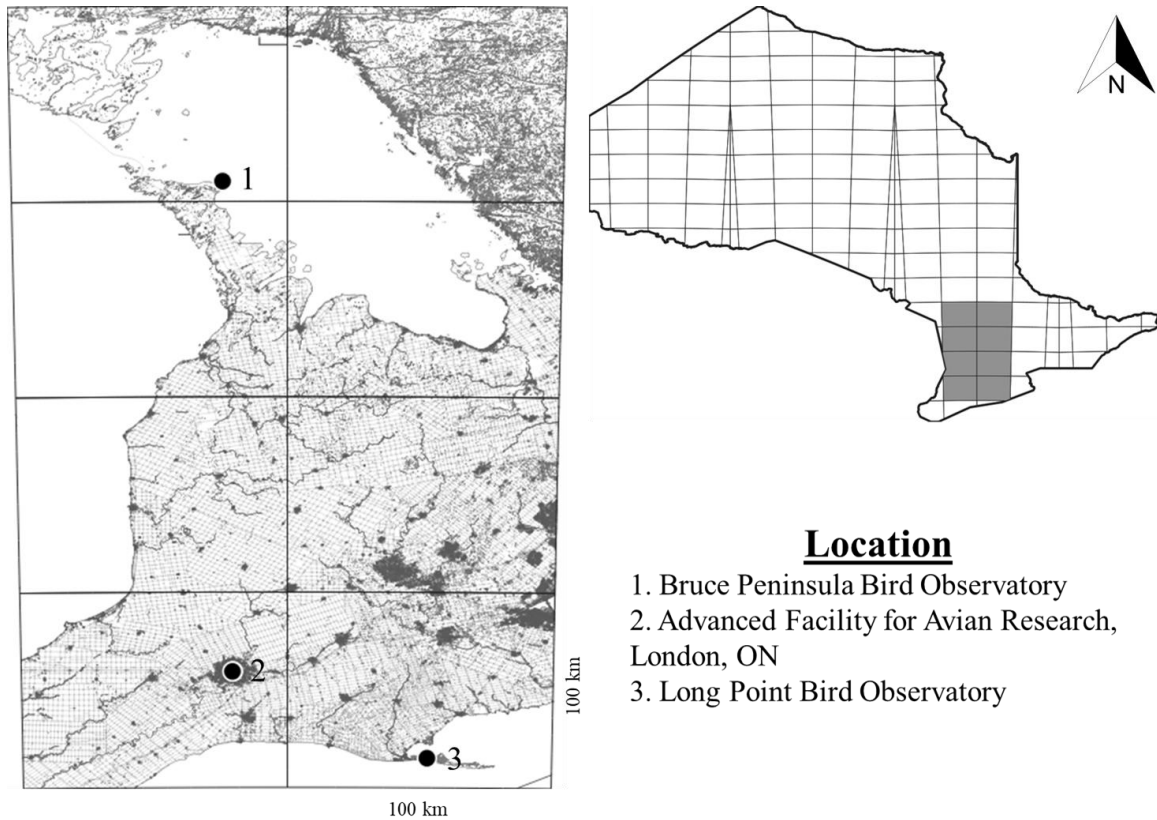
infected during migration (Kelly et al., 2018). I predicted that departure date would be delayed in all inoculated birds, as birds that have reduced energy stores would need to regain them before departure. I also expected that inoculated birds would decrease activity immediately after inoculation if they become acutely infected. The energetic savings of reduced feeding activity can be seven times higher than the cost of fighting off an infection (Hasselquist and Nilsson, 2012). If birds are minimizing impacts on migratory fuel stores, they should therefore respond by reducing their activity to minimize energy loss. While other studies have found no impacts on condition when birds are experimentally inoculated, they have not considered birds that resist the inoculation. Research by Kelly et al. (2018) suggests that effects are observed in these birds, and this may be where the effects of exposure to *Plasmodium* and other pathogens are detectable.

## Chapter 2

### 2 Methods

#### 2.1 Capture and Husbandry

I captured thirty-six Yellow-rumped warblers at the Bruce Peninsula Bird Observatory (BPBO), at Dyers Bay, Ontario (45°14'47.1''N 81°17'57.7''W), and received an additional four birds captured at the Long Point Bird Observatory (LPBO), Port Rowan, Ontario (42°34'58.5''N 80°23'54.5''W) (Figure 3). All birds were captured during early fall migration between September 13<sup>th</sup> and 26<sup>th</sup>, 2019 as they flew south from their boreal breeding grounds. I used mist nets, according to banding station protocols (North American Banding Council, 2001), between a half-hour before sunrise and six hours after sunrise. At capture, I banded birds using standard aluminum Canadian Wildlife Service bands, scored the fat in their furcular hollow on a standard 0-8 scale used by banding stations, and weighed them. Birds were aged at capture using feather quality (hatch year  $n = 33$ , after hatch year  $n = 7$ ), and were sexed later using genetic analysis (see below).



**Figure 3: Map of study sites throughout Southern Ontario showing the capture site (1), captivity site (2), and release site (3).**

I transported birds in groups to the Advanced Facility for Avian Research (AFAR) at the University of Western Ontario, London, ON (43°00'37.5''N 81°16'47.2''W) using small pet carriers modified with two small branches for perching, a water dish, and live mealworms.



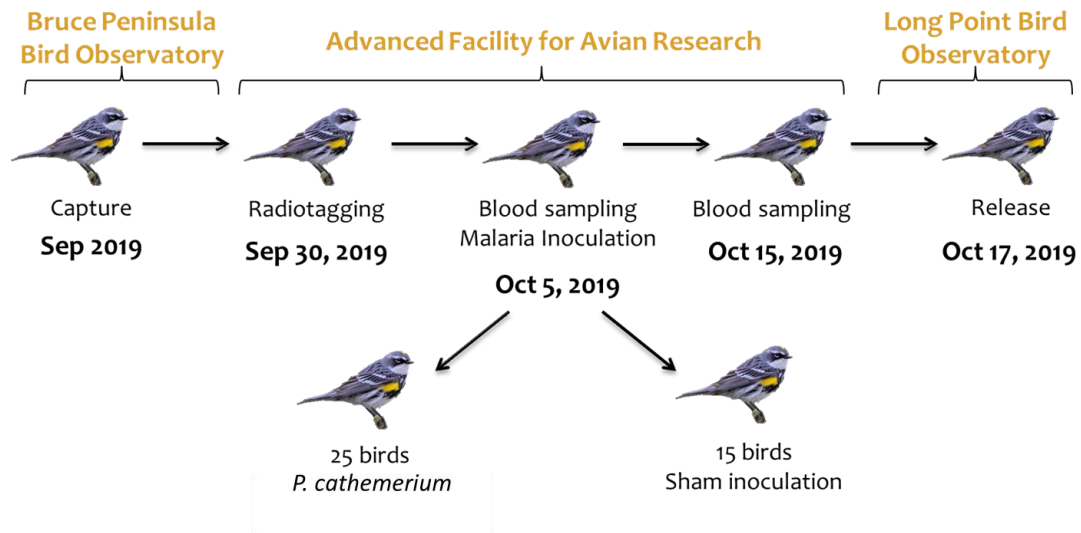
**Figure 4: An example of one of the four free-flight aviaries at the Advanced Facility for Avian Research where birds were housed in captivity.**

I kept birds indoors in groups of ten, in rooms free of mosquitos or other insect vectors, at the AFAR. The aviaries measured 2.4 m by 3.6 m by 2.7 m and included a minimum of six hanging tree branch perches and one floor mounted perch to provide ample perching space at different heights. I included three water dishes for drinking and bathing, two on the floor and one elevated on a potted plant stand (Figure 4).

Birds received *ad libitum* synthetic warbler diet (16.29% glucose, 3.62% casein, 1.63% agar, 1.59% Brigg's Salt, 0.54% vitamin mix, 72.93% water, 3.08% oil, 0.87% cellulose), blueberries, and live mealworms (*Tenebrio molitor*) using three floor dishes and four hanging feed cups (Guglielmo et al. 2017). I calculated meal weights to provide each bird with 20 g of fresh diet and 5 g of live mealworms per day. I removed old food and emptied and refilled water bowls daily while doing daily health checks. I cleaned the aviary floors every seven days.

I scheduled lights on from 7:00 to 19:35 from September 13<sup>th</sup> to October 10<sup>th</sup> (10.5L:13.5D), and from 7:10 to 19:20 from October 10<sup>th</sup> to October 17<sup>th</sup> (10L:14D).

This light schedule approximated the natural timing of sunset and sunrise in London, ON for this time of year and allowed the birds to maintain migratory condition. I housed birds in the aviaries from the date of capture (earliest capture date September 13<sup>th</sup>) to October 17<sup>th</sup> (Figure 5). The longest time a bird spent in captivity was 38 days, with an average of 27.3 (SE  $\pm$  1 day) days.



**Figure 5: Summarized timeline of the experimental phase showing date, location, and treatment group size.**

## 2.2 Experimental Infection

### 2.2.1 Blood Sampling and Inoculation

I conducted blood sampling and inoculation on October 5<sup>th</sup>, 2019 (Figure 5). I collected blood samples (used in genetic sexing and microscopic analysis of initial parasite load) using brachial venipuncture. I swabbed the puncture site with 70 % ethanol to mat feathers away from the site and remove dander, and allowed the site to air dry. I then used a 26 gauge needle to puncture the vein and collected the blood into capillary tubes (Millet et al., 2007). I drew 100  $\mu$ L of blood from each bird. A portion of this blood was used to create two to three thin-film blood smears, by placing a drop of blood at the top of a glass microscope slide and drawing the drop across the slide using the edge of a

second microscope slide. Smears were allowed to air dry, immersed in methanol for 60 seconds, and air dried again to fix the samples. Slides were stained using Giemsa stain (Sigma-Aldrich) after each round of sampling. I stored 80  $\mu$ L of the remaining blood sample in Longmire's lysis buffer for genetic analysis (Longmire et al. 1988). Whole blood in lysis buffer was stored at  $-20^{\circ}$  C until genetic analysis (see below). I collected a second blood sample on October 15<sup>th</sup>, 2019 (ten days after inoculation, see below) using the same protocol to collect blood smears in order to assess infection outcome. Data on mass and fat were also collected on this date (see below).

On October 5<sup>th</sup>, 2019 I inoculated twenty-five birds with 0.3 ml of inoculant containing *P. cathemerium* and fifteen birds with a 0.3 ml sham inoculation known to be *P. cathemerium* free. Blood samples for the inoculant came from wild Yellow-rumped Warblers captured the previous spring at LPBO, whose infections were verified using microscopy, PCR, and sequencing. The blood for treatment inoculations was collected from captive infected donor birds twice a month from May to August, and blood for sham inoculations was collected from euthanized uninfected birds. One capillary tube of blood (approximately 80  $\mu$ l) was collected from infected donors at each sampling point and blood was drawn from euthanized sham donors until no more blood could be collected. Treatment blood was pooled into a common sample upon collection, and mixed at a 1:2 ratio with a glycerol-based solution (57.1 g Glycerol, 1.23 ml DL-Lactic Acid, 0.17 g Sodium Phosphate dehydrate ( $\text{Na}_2\text{HPO}_4$ ), 0.086 g of Sodium Phosphate anhydrous ( $\text{Na}_2\text{HPO}_4$ ), 0.03 g Potassium Chloride). Sham blood was mixed with the same solution, and blood samples were stored in liquid nitrogen until inoculation day.

The morning of October 5<sup>th</sup>, 2019, inoculant was made by thawing out stored blood samples and removing glycerol using a NaCl wash. Blood was mixed in a 3:1 ratio with 0.9% sodium chloride (30  $\mu$ L of blood, 10  $\mu$ l of 0.9% sodium chloride) and sham and treatment syringes were prepared and kept on ice immediately before injection into the pectoral muscle (Kelly et al., 2018). I assigned treatment using an online random number generator (<http://www.randomnumbergenerator.com/>), with an equal ratio of treatment and Sham birds in each aviary. Because I did not know the sex of the birds at this point, I could not randomize sex in treatment groups. Sex was not balanced across



treatment groups, with eleven males and fourteen females in the treatment group and one male and fourteen females in the Sham group (see below).

### 2.2.2 Parasitology

I viewed blood smears at 100x magnification using an oil-immersion microscope, reading the feathered and monolayer sections. I viewed 10,000 cells per slide, and recorded total number of haemoparasite infected cells following Kelly et al. (2016). I used oil immersion light microscopy to identify infections with *P. cathemerium* and other haemoparasites according to characteristics outlined in Valkiunas (2005). All parasites were photographed at 100x magnification and confirmed visually by Leticia deSouza Soares, a professional parasitologist. Trophozoite stage infections could not be identified to species and were not used to determine response groups (Infected versus Exposed but Uninfected).

In reviewing post-inoculation blood smears in the Treatment group, I categorized birds with one or more confirmed *P. cathemerium* occurrences in 10,000 red blood cells as the “Infected” group. Birds in the Treatment group with no *P. cathemerium* observed in a scan of 10,000 red blood cells were categorized as the “Exposed but Uninfected” group. While one bird was observed to be infected with *Plasmodium* spp. before inoculation (Sham group, 5 infected cells), no birds were found to be (naturally) infected with *P. cathemerium* before inoculation.

## 2.3 DNA Sexing

To determine the sex of my study birds I used whole blood in lysis buffer samples collected during captivity to extract and image DNA. These samples were stored at -20° C immediately after collection and retrieved at a later date for DNA extraction. I placed 100 µL of packed red blood cells into 200 µL of Longmire’s lysis buffer and 10 µL of 20 mg/ml Proteinase K, and incubated samples at 60° C for twenty-four hours (Longmire et al. 1988). I then added 125 µL of 97% 6.7-7.3 pH ammonium acetate, shook the tubes, and incubated them for thirty minutes at 7° C. I centrifuged samples for fifteen minutes at 14,000 rpm and poured the supernatant into new tubes. Samples were mixed with 400 µL of ice cold 99.99% isopropanol and let sit for two minutes before gentle mixing and

fifteen minutes of centrifuging at 14,000 rpm. Liquid waste was discarded and 300  $\mu\text{L}$  of ice cold 70% ethanol was added. I centrifuged samples, discarded the liquid waste, and then repeated the ethanol and centrifuging step. I discarded the liquid waste and placed samples in a 45° C dry bath until the ethanol evaporated from the DNA pellet. I added 200  $\mu\text{L}$  of ddH<sub>2</sub>O to each tube and stored them at 7° C for four days.

I used a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific) to measure absorbance of the sample at wavelengths between 190 and 840nm and calculate the DNA concentration according to the Beer-Lambert equation. Samples were diluted to 30 ng/ $\mu\text{L}$  using ddH<sub>2</sub>O.

The reaction mixture contained a final concentration of 1x PCR Buffer (Invitrogen, Burlington, Canada),  $3.0 \times 10^{-6}$   $\mu\text{M}$  of each dNTP,  $4.0 \times 10^{-6}$   $\mu\text{M}$  of each primer (SexFwd 5'-GTATCGTCAATTTCCATTTTCAGGT-3', SexRev 5'-CCATCAAGTCTCTAAAGAGATTGA-3'; Morbey et al., 2018), and 0.5U of DreamTaq Green DNA Polymerase (Thermo Scientific), and approximately 35ng of DNA template. 0.4  $\mu\text{L}$  of MgCl<sub>2</sub> (25 mM) was also added, and combined with the MgCl<sub>2</sub> in the DreamTaq created a final concentration of 3 mM MgCl<sub>2</sub>. I placed samples in a Biometra TGradient thermocycler to amplify the target gene using the thermal cycle: initial step of 95 °C (5 minutes); 40 cycles of 95 °C (30 seconds), 59.5 °C (20 seconds), 72 °C (20 seconds); and a final step of 72 °C (2 minutes) (Morbey et al., 2018).

I imaged reaction products using gel electrophoresis. I made a 1% agarose gel (1 g of agarose, 100 ml of 1X TBE buffer) and added 5  $\mu\text{L}$  of RedSafe dye (Intron Biotech) immediately before the gel set. I mixed 10  $\mu\text{L}$  of PCR product with 1  $\mu\text{L}$  of loading dye (Fischer Scientific) and placed the mix into premade wells. I added 3  $\mu\text{L}$  of Invitrogen Low Mass DNA ladder to the far left well to provide a measurement for DNA fragment lengths. I ran each gel at 96 V for 50 minutes before imaging it with a Bio-Rad Gel Doc 2000 using ultra-violet light. CHD-Z shows a band at 520 base pairs, and CHD-W shows a band at 319 base pairs for this species (Morbey et al., 2018). Male birds (ZZ) are identifiable by only having a CHD-Z band, while female birds (ZW) have both (Griffiths et al. 1998). Three individuals only had a single CHD-W band detectable. Because only

females have CHD-W, I assumed that mutations in the CHD-Z primer binding site could have occurred, and categorized these birds as female.

## 2.4 Body Condition

In order to assess the effects of exposure to and/or infection with *P. cathemerium* on body condition, I measured fat score and mass of all subjects both before and after inoculation. On October 5<sup>th</sup> and 15<sup>th</sup>, 2019, I scored fat in the furcular hollow on a standard 0-8 scale used to generate a pre- and post-inoculation fat score (North American Banding Council, 2001). I weighed birds to the nearest 0.01 g using a digital table-top scale to generate pre- and post-inoculation mass measurements. I then separated fat score and mass data into pre- and post-inoculation periods for statistical comparison.

I ran paired t-tests to determine if mass or fat score were significantly different between time periods (pre- vs. post-inoculation). I ran two-way ANOVAs (rstatix; Kassambara, 2021) to model mass and fat score as functions of day since radio tagging and response group (Infected, Exposed but Uninfected, Sham).

## 2.5 Movement

### 2.5.1 Radio tagging

To monitor activity levels before and after release, and to locate birds after release, I radio-tagged birds on September 30<sup>th</sup> and October 1<sup>st</sup>, 2019 with Lotek NTQB2-1 radio-tags (Deakin et al., 2021). I affixed tags using an elasticized thread in a figure-eight leg-loop harness. These tags were VHF radio-tags set to 12.5 s bursts at 166.380 MHz, with an expected life span of 63 days (model NTQB2-1, Lotek Wireless, Oakville, ON, <https://www.lotek.com/products/nanotags/>). Tag ID# 038 had identical specifications but a 12.7 s burst. With elastic harnesses included, tags weighed between 0.29 g and 0.35 g (average and median = 0.32 g). I determined harness size based on the weight of the bird immediately before tagging to ensure a comfortable fit, and secured the harness elastic to the tag with superglue. I also gave each bird a unique combination of colored plastic leg bands to allow individual identification in the aviaries.

## 2.5.2 Radiotracking

### 2.5.2.1 Aviary Radiotracking

I began radiotracking September 30<sup>th</sup>, 2019 using a radio receiver (Lotek Wireless SRX600) with a single 12 inch whip antenna located in the hallway outside the four aviary rooms. The receiver ran continuously except for periodic data downloads, and recorded tag ID, signal strength, and the timing of the pulse. When multiple pulses occurred at once the receiver documented an interference signal in place of a tag ID.

### 2.5.2.2 Manual Radiotracking

I released birds at LPBO on October 17<sup>th</sup>, 2019 (Figure 3). Following release, I radiotracked the birds manually using a handheld five-element yagi antenna and the SRX600 receiver daily from October 17<sup>th</sup>, 2019 to November 4<sup>th</sup>, 2019. I manually surveyed areas accessible by road within an approximately 6 km radius of the release site, with the first detection of a strong signal being recorded for each bird. I considered a strong signal strength  $\geq 170$  dB (max) and gain  $\leq 40$  dbi. Signal strength is internally converted by the receiver onto a scale ranging from 0 to 255 as a ratio of signal decibels relative to maximum decibels. Higher SRX600 receiver values indicate a stronger signal. Gain refers to the sensitivity of the antenna, and as gain decreases the reception range decreases. Therefore, a signal at low gain means the observer is closer to the bird than a signal at high gain. By repeatedly finding the strongest signal, moving towards it, and reducing gain I could move towards the bird. Only days with persistent heavy rain were not sampled. I recorded time and global positioning system (GPS) location ( $\pm 5$  m) using a Samsung Galaxy A5 (2017), and attempted visual confirmation of each bird with the GPS location being the bird's location if the bird was sighted. Otherwise the GPS location was where signal strength was strongest. By November 4<sup>th</sup>, 2019, only a single bird was detected at the release site and I discontinued further radiotracking.

### 2.5.2.3 Automated Radiotracking

The radio-tags used on birds were also registered with and automatically detected by the Motus Wildlife Tracking System, an automated radiotelemetry system with stations throughout Southern Ontario and the U.S.A. (Figure 6). These automated towers

have a maximum detection radius of approximately 20 km, and allow measurement of activity at the release site (Taylor et al., 2017). The Old Cut Tower, a tower equipped with five nine-element yagi antennas, is at the release site. The tower collects detections including the time, antenna number, tag number, and signal strength (Morbey et al., 2018).



**Figure 6: Motus towers (dots) and estimated antenna ranges (loops) in the Motus Wildlife Tracking System surrounding the release site (Motus.org, 2021)**

### 2.5.3 Data Management

All data were analyzed in R version 3.6.3 for 64-bit (R Core Team, 2018). Captivity data, collected during captivity using an SRX600 receiver, were analyzed for activity. Release data, collected after release using the Motus Wildlife Tracking System, were analyzed for activity and departure timing. Motus database access, downloading, and initial data management were done using the packages *remotes* (Hester et al., 2021), *motus*, *motusData* (Crewe et al., 2020), and *RSQLite* (Müller et al., 2021). Modeling, model selection, analysis of variance, and other calculations were done using the packages *tidyverse* (Wickham, 2021), *rstatix* (Kassambara, 2021), *ggpubr* (Kassambara, 2021), *lme4* (Bates et al., 2021), *arm* (Gelman et al., 2021), *multcomp* (Hothorn et al., 2021), *nlme* (Pinheiro et al., 2021), and *AICcmodavg* (Mazerolle, 2020). Visualizations

were made using the packages *lattice* (Sarkar et al., 2021), *hrbrthemes* (Rudis et al., 2020), *gridExtra* (Auguie and Antonov, 2017), *gmap* (Kahle et al., 2019), *mapproj* (McIlroy, 2020), and *ggplot2* (Wickham, 2016).

I downloaded release data from the Motus database according to instructions in the Motus R Book (Crewe et al., 2020) and excluded all runs with a length  $\leq 4$ . A run is a set of continuous detections by a receiver, and runs with a length of four or less tend to be false positives and should be excluded from analysis. When an hour included over 50 runs with at least 75% of those runs being longer than 5, I excluded runs with a length less than 10 to increase the likelihood of removing false positives without losing true detection data. I saved a file including all of these excluded runs (false positives) in case they needed further analysis, and continued working with the main file (Crewe et al., 2020). I then excluded all data from the dataset occurring before the release date, excluded all data from other species, and excluded all data for tags that were not part of my study. I excluded data from all Motus towers other than the Old Cut tower at the release site to ensure I was analyzing data only at the simulated stopover. Once all extraneous Motus data was removed I added data for aviary room, sex, treatment group (Exposed, Sham), and response group (Infected, Exposed but Uninfected, Sham) (Morbey et al., 2018).

## 2.5.4 Signal Coefficient of Variation to Measure Activity

### 2.5.4.1 Activity in Captivity

Data were downloaded from the SRX600 Receiver periodically to ensure the internal memory was not full, and these subsets of the data were merged into a single file containing all data for the captivity period. I removed interference and false positives by selecting only data from my tags, and added data for aviary room, sex (male, female), treatment group (Exposed, Sham), and response group (Infected, Exposed but Uninfected, Sham). Because there may be a lag between the aviary light timer clock and the SRX600's internal clock I removed a buffer of 5 minutes before and 5 minutes after the light changes from the dataset, so I could be confident that data labeled as night (lights off) was truly night. I also recalculated timestamp data so that midnight became 0,

for ease of future calculation, and created a value for lighting (day, night) (Morbey et al., 2018).

Because of the high number of tags transmitting in a small area at the AFAR I had a large amount of signal interference. I needed to determine how well each individual, time period, and group was detected and if those data were strong enough to calculate activity. I also wanted to determine if certain individuals or rooms had poor detection. To do this I calculated signal coverage of each tag as the number of signals detected in a time period divided by the maximum possible number of signals in that time period (the time period in seconds divided by the pulse rate of the tag in seconds). I removed all data with poor coverage, defined as coverage less than 0.05 (5% of expected detections), from the dataset, and removed days when birds were handled (October 2<sup>nd</sup>, 5<sup>th</sup>, 15<sup>th</sup>, and 17<sup>th</sup>) as this alters behavior. I then visually determined whether there was a difference in coverage between sex, aviary, treatment, or response group using boxplots (Morbey et al., 2018). There were no differences, so no further work needed to be done based on coverage.

Both Motus and SRX600 receivers calculate signal strength as a ratio of signal decibels relative to maximum decibels, and periods of inactivity are identifiable as periods of low variability in signal strength. This allows calculation of an activity value using the coefficient of variation of the signal strength, by dividing the standard deviation of signal power, a value recorded by the receivers, by the mean of signal power (Deakin et al., 2021). Because I anticipated activity to differ between day (lights on) and night (lights off), I separated data into day and night datasets. I also separated data based on pre- and post-inoculation time periods, leaving me with four datasets (pre-inoculation day, pre-inoculation night, post-inoculation day, and post-inoculation night). I created linear mixed models using the R package lme4 (Bates et al., 2021) to determine whether the coefficient of variation was affected by response group (Infected, Exposed but Uninfected, Sham), day since radio tagging, an interaction between response group and day, room, sex, or individual. Initial models included all variables and I used Akaike Information Criterion (AIC) (*tidyverse*; Wickham, 2021) to compare models and remove terms until I was left with a model that explained the greatest amount of variation using

the fewest possible independent variables. I created and fitted one model per time period, and checked model assumptions by graphing residuals and leverage (Morbey et al., 2018). I then ran Analysis of Variance (ANOVA)s (rstatix; Kassambara, 2021) for each final model to determine if there was a statistically significant difference in mean CV.

**Table 1: The variables, descriptions, and types included in linear models of the coefficient of variation of activity.**

<b>Variable</b>	<b>Description</b>	<b>Effect</b>	<b>Type</b>
<b>Response</b>	Inoculation outcome or Sham	Fixed	Categorical
<b>Day</b>	Day of captivity	Fixed	Continuous
<b>Response:day</b>		Interaction	Interaction
<b>Room</b>	Aviary ID	Random	Categorical
<b>Tag.ID</b>	Individual radiotag ID	Random	Categorical
<b>Sex</b>	Sex of bird	Random	Categorical

#### 2.5.4.2 Activity after Release

I calculated the coefficient of variation (my measure of activity level) for the Motus data collected after release, using the same method as the captivity data. I removed all runs with a length less than twenty to remove false positives, and included only the first 24 hours of data to ensure the maximum numbers of birds were present and sample size was as high as possible (Morbey et al., 2018).

I used linear modeling (lme4; Bates et al., 2021) to compare the coefficient of variation between response groups (Infected, Exposed but Uninfected, Sham), sex, hour, an interaction between response group and hour, and individual. The initial model included all variables, and I used Akaike Information Criterion (c) from the package AICcmodavg (Mazerolle, 2020) to correct for small sample sizes and select the model that explained the greatest amount of variation using the lowest number of independent



variables. Once I had determined the model with the best fit (lowest AICc score; AICcmodavg; Mazerolle, 2020) I ran an ANOVA (rstatix; Kassambara, 2021) to determine which if any variables remaining in the best model were statistically significant predictors of the coefficient of variation.

### 2.5.5 Departure from Stopover

I calculated the number of signals per hour per bird, and considered birds present at the release site if they had twenty-five or more detections per hour to avoid false positives (Morbey et al., 2018; Seewagen et al., 2019). I removed data for periods when the birds had fewer than twenty-five detections per hour, calculated the last date a bird was detected, and used this value as their departure date. For each individual bird I also graphed the signal strength over time on their departure date to visually assess signals, as true departure flights make a distinctive parabolic shape (Morbey et al., 2018). I then calculated the frequency (number of detections) during the first hour after release on October 17<sup>th</sup>, 2019, the first detection date, and the first detection time (Morbey et al., 2018).

I created a both a General Linear Model (GLM) and a General Linear Mixed Model (GLMM) with binomial error distribution using nlme (Pinheiro et al., 2021) to determine if the frequency of detection correlated with time, sex, individual, response group (Infected, Exposed but Uninfected, Sham), or room. I used AIC (rstatix; Kassambara, 2021) to select the models that explained the greatest amount of variation using the fewest independent variables, and calculated the ANOVA (rstatix; Kassambara, 2021) of those models to determine if frequency of detection was significantly related to any of those variables. To assess model fit of the GLM I calculated confidence intervals and created a Binned Residual plot (nlme; Pinheiro et al., 2021). To assess model fit of the GLMM I plotted the Pearson residuals (nlme; Pinheiro et al., 2021).

To analyze the probability of departure I created Kaplan-Meier survival curves for the response groups (Infected, Exposed but Uninfected, Sham). Kaplan-Meier estimates are typically used for survivorship studies and calculate the fraction of individuals still living at time points after treatment. By graphing survival at these time points we can

create a visual curve that represents survival (Hess and Hess, 2020). If we view presence at the release site as “survival” we can use Kaplan-Meier curves to calculate the probability of a bird still being at the release site. The Kaplan-Meier statistics (survival; Therneau and Lumley, 2017; *survminer*; Kassambara et al., 2021) included in the model provide a chi-square and p-value that I used to determine if there was a significant difference between the survival (presence at the release site) of response groups (Dossman et al., 2016; Morbey et al., 2018). A group with higher “survival” would be a group that stayed longer at the release site and delayed departure.

## Chapter 3

### 3 Results

#### 3.1 Microscopy and Infection Outcome

I viewed between 10,000 and 10,855 cells per bird per sample period (n=40 pre-inoculation samples, n=40 post-inoculation samples), with a mean of 10,062 cells, and considered a bird infected with haemoparasites if it had at least one parasitized cell. One bird (Sham) was infected with *Plasmodium* spp. before inoculation (infected cells = 5), although not with *P. cathemerium*. Six birds of the 25 (24%) in the Treatment group, were found to be infected with *P. cathemerium* after inoculation (average infected cells = 1.5, range = 1 to 3) and were categorized as “Infected” and the remaining 19 were categorized as “Exposed but Uninfected”.

In addition to *P. cathemerium* infections, I detected unidentifiable haemoparasite stages in four birds before inoculation (Sham = 3, Treatment = 1, average infected cells = 2.25, range = 1 to 6), and six birds after inoculation (Sham = 3, Treatment = 3, average infected cells = 3, range = 1 to 8). Four (Sham = 3, Treatment = 1) of the pre-inoculation infections were only unidentifiable stages, and three (Sham = 2, Treatment = 1) of the post-inoculation infections were only unidentifiable stages. Early life stages of haemosporidian parasites cannot be visually differentiated, so while these birds were infected I cannot confidently say that they were infected with *P. cathemerium*. To be conservative, I excluded birds with only unidentifiable infections from the response groups (Infected, Exposed but Uninfected, Sham). Among the Sham-inoculated birds (n = 15) none developed *P. cathemerium* infections, although one had an existing (naturally-acquired) infection with another *Plasmodium* species before inoculation, two had unidentifiable stages before inoculation, and two different birds had unidentifiable stages after infection. Among the treatment birds (n = 25) one had an unidentifiable infection before inoculation, and three individuals had unidentifiable infections (Infected = 2, Exposed but Uninfected = 1) after inoculation.

Two birds (Sham = 1, Treatment = 1) were infected with other haemoparasites before the first blood sampling (*Parahaemoproteus* = 1, *Leucocytozoon* = 1) but did not have detectable infections during the second blood sampling. Three birds (Sham = 2, Treatment = 1) developed detectable infections with other haemoparasites after the first blood sampling (*Haemoproteus* = 1, *Leucocytozoon* = 2). One bird in the Infected response group was co-infected by *P. cathemerium* and *Leucocytozoon* at the second sampling.

## 3.2 DNA Sexing

I sexed all forty birds according to the methods developed by Morbey et al. (2018), identifying 28 females, and 12 males. The treatment group contained 11 males and 14 females, and the Sham group contained one male and fourteen females. Six males and thirteen females resisted infection and became the “Exposed but Uninfected” group, and five males and one female were classified in the “Infected” group.

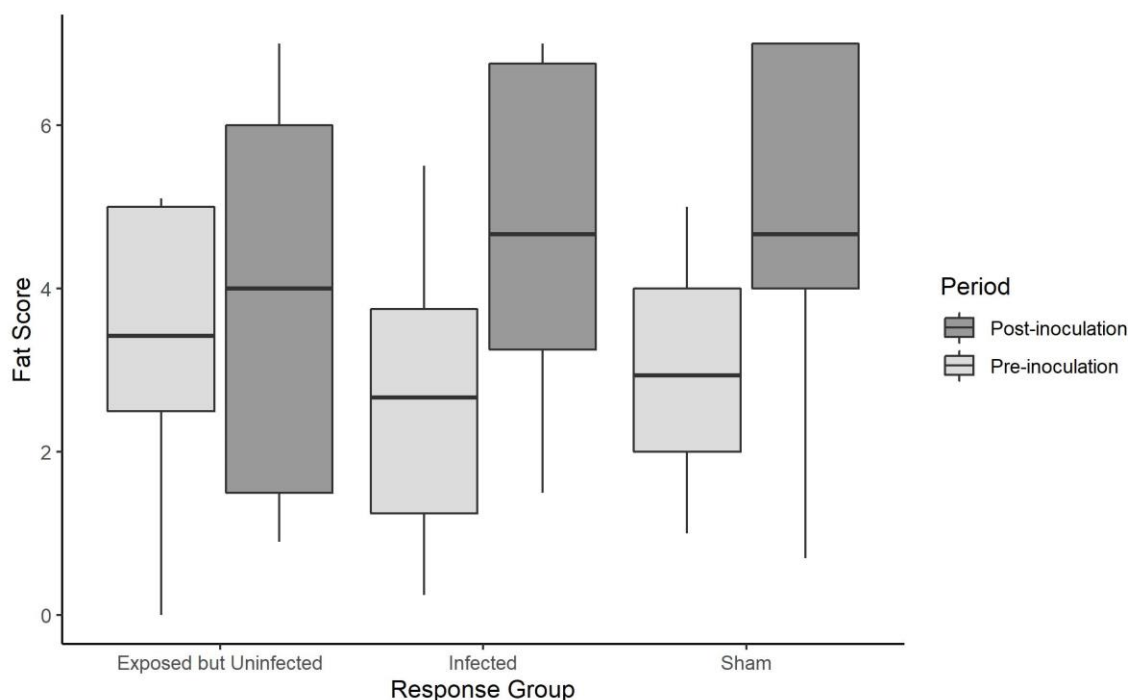
Because birds were assigned randomly to Treatment before DNA sexing, sex could not be balanced across Treatments. Thus, sex was not independent of Treatment ( $\chi^2_1 = 6.2$ ,  $P < 0.0001$ ).

## 3.3 Captivity

### 3.3.1 Condition

#### 3.3.1.1 Fat Score

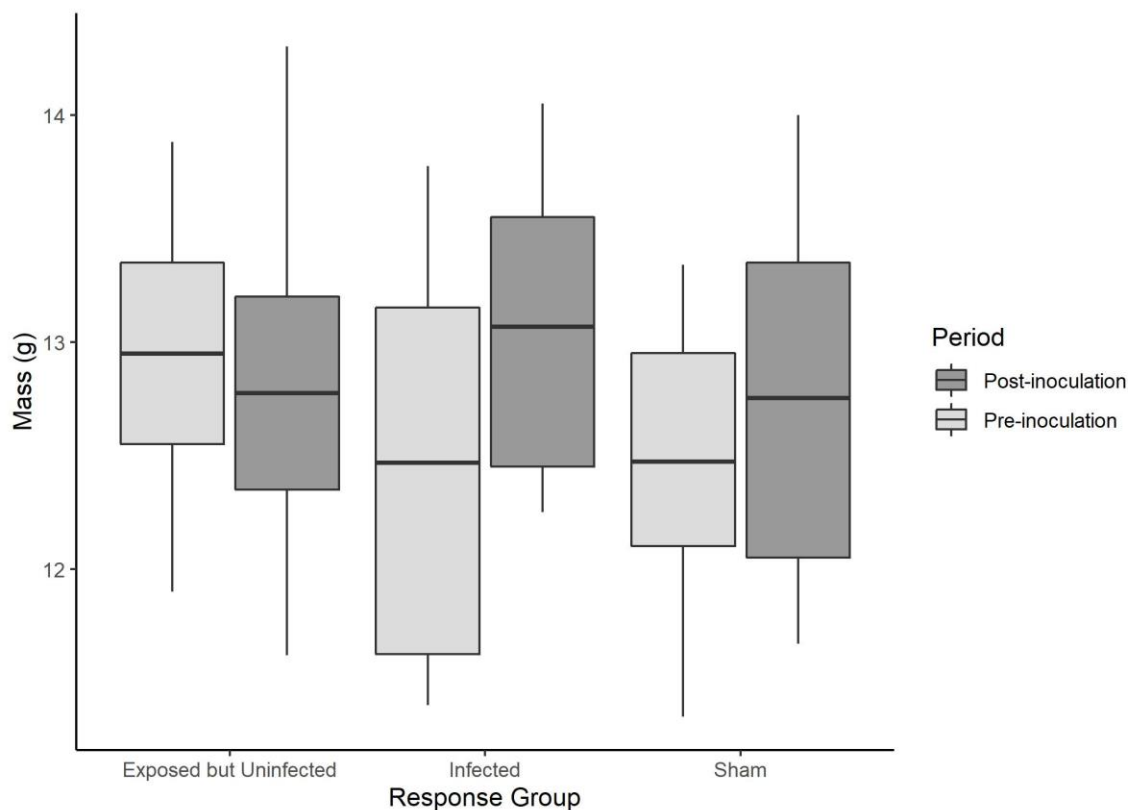
If inoculation with *P. cathemerium* impacts condition there should have been a detectable difference in the increase of the fat score of the response groups (Infected, Exposed but Uninfected, Sham). Mean ( $\pm$  SD) fat scores across all groups were  $0 \pm 1$  at capture (Sep 9 to Sep 26, 2019);  $3 \pm 2$  at inoculation (Oct 5, 2019), and  $4 \pm 2$  at their second blood sampling (Oct 15, 2019; all  $n = 40$ ). There was a significant increase in fat score over time ( $F_{1,78}=6.68$ ,  $P = 0.012$ ), but no effect of response group (Infected, Exposed but Uninfected, Sham) ( $F_{2,77} = 0.02$ ,  $P = 0.977$ ).



**Figure 7: The fat score of captive Yellow-rumped Warblers (*Setophaga coronata*) (n = 40) inoculated with *Plasmodium cathemerium* at inoculation day and 10 days after inoculation day. The top of the boxplot represents the 75% confidence interval and the bottom represents the 25% confidence interval. The whiskers represent the 5% and 95% confidence intervals. The horizontal line represents the mean fat score. Fat score increased significantly after inoculation, but was not significantly different between groups.**

### 3.3.1.2 Mass

If inoculation with *P. cathemerium* impacts condition there should have been a detectable difference in the mass of the response groups (Infected, Exposed but Uninfected, Sham). Mean ( $\pm$ SD) mass across all groups were 11.74 g  $\pm$  0.7 g (Sep 9 to Sep 26, 2019); of 12.7 g  $\pm$  0.8 g at inoculation (Oct 5, 2019), 12.8 g  $\pm$  0.8 g at their second blood sampling (Oct 15, 2019; all n = 40) (Figure 8). There was no significant difference between response groups in the change in mass ( $F_{1,38} = -0.86$ ,  $P = 0.4$ ) or in mass at either time point ( $F_{2,77} = 0.79$ ,  $P = 0.46$ ).

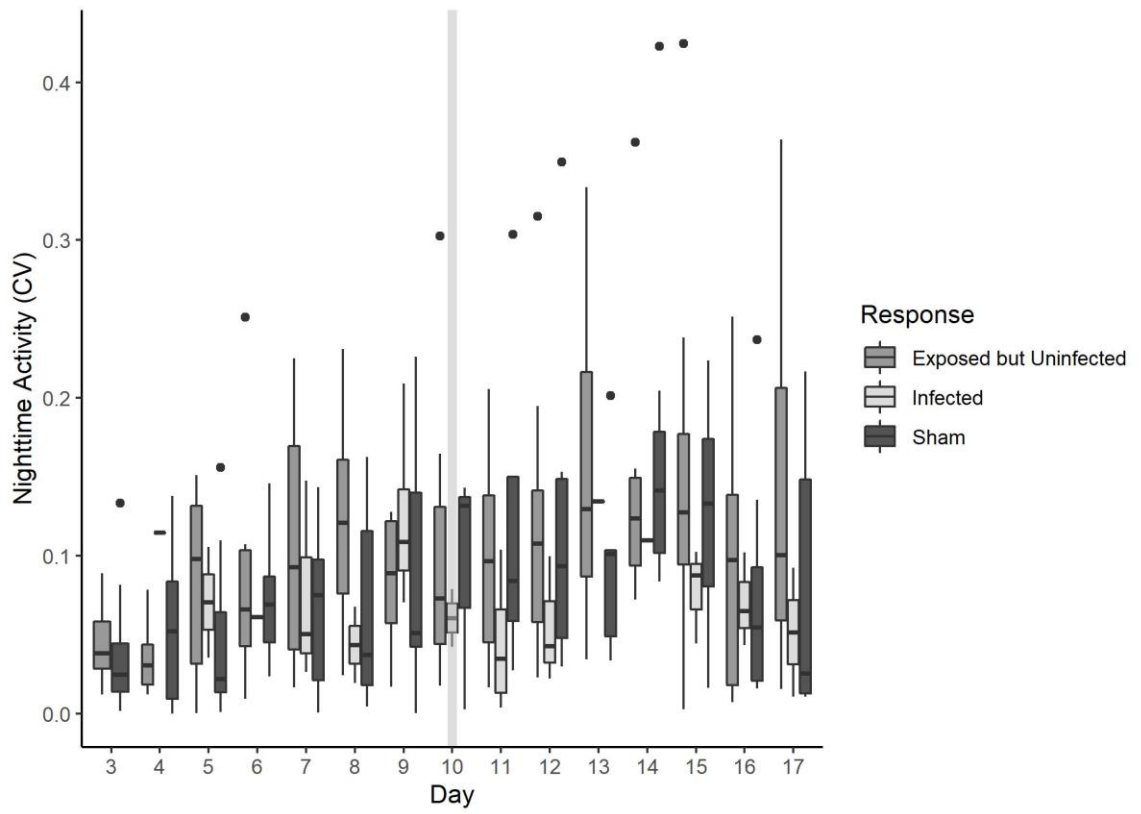
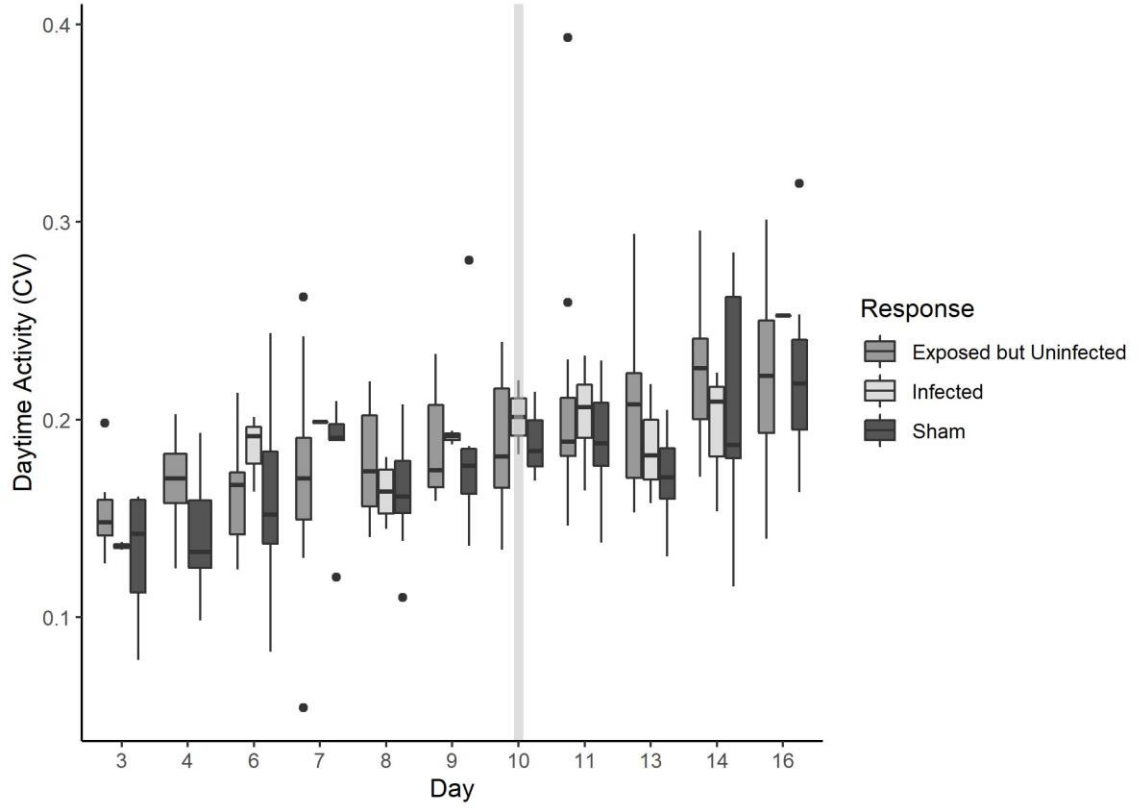


**Figure 8: The mass in grams of captive Yellow-rumped Warblers (*Setophaga coronata*) (n = 40) before and 10 days after inoculation with *Plasmodium cathemerium*. The top of the boxplot represents the 75% confidence interval and the bottom represents the 25% confidence interval. The whiskers represent the 5% and 95% confidence intervals. The horizontal line represents the mean mass. There was not a significant difference in mass between response groups.**

### 3.3.2 Activity

If inoculation with *P. cathemerium* impacts activity, measured as CV of signal strength, there should have been a detectable difference in the post-inoculation activity of the response groups (Infected, Exposed but Uninfected, Sham). Before inoculation, neither daytime nor nighttime CV were significantly related to date ( $F_{1,38} = 2.7$ ,  $P = 0.1$ ;  $F_{1,38} = 0.06$ ,  $P = 0.81$ , respectively). Linear models of post-inoculation activity show that CV significantly increased over time in both day and night ( $F_{8,31} = 79.67$ ,  $P < 0.001$ ;

$F_{12,27} = 19.32$ ,  $P < 0.0001$ , respectively). An increase in activity over time, during both periods, is natural and expected for this species (Woodworth et al., 2015). Final models for CV did not include response group and response groups did not differ significantly in CV for any period (Appendix E, table E1).





**Figure 9: The activity of captive Yellow-rumped Warblers (*Setophaga coronata*) (n = 40), measured as the coefficient of variation of signal strength, over time. The y axis is the coefficient of variation of the signal strength of the radio tags. The x axis is the day since activation of the radio tags. The vertical line indicates the day Yellow rumped Warblers were inoculated with *Plasmodium cathemerium*. The lower and upper edges of the box are the 25<sup>th</sup> and 75<sup>th</sup> percentiles, the whiskers extend to the largest and smallest values at 1.5\*IQR. The middle line is the median value. Points outside the whiskers are outliers. While activity increased over time during both the day and night there was no significant difference between groups (Exposed but Uninfected, Infected, Sham).**

## 3.4 Release

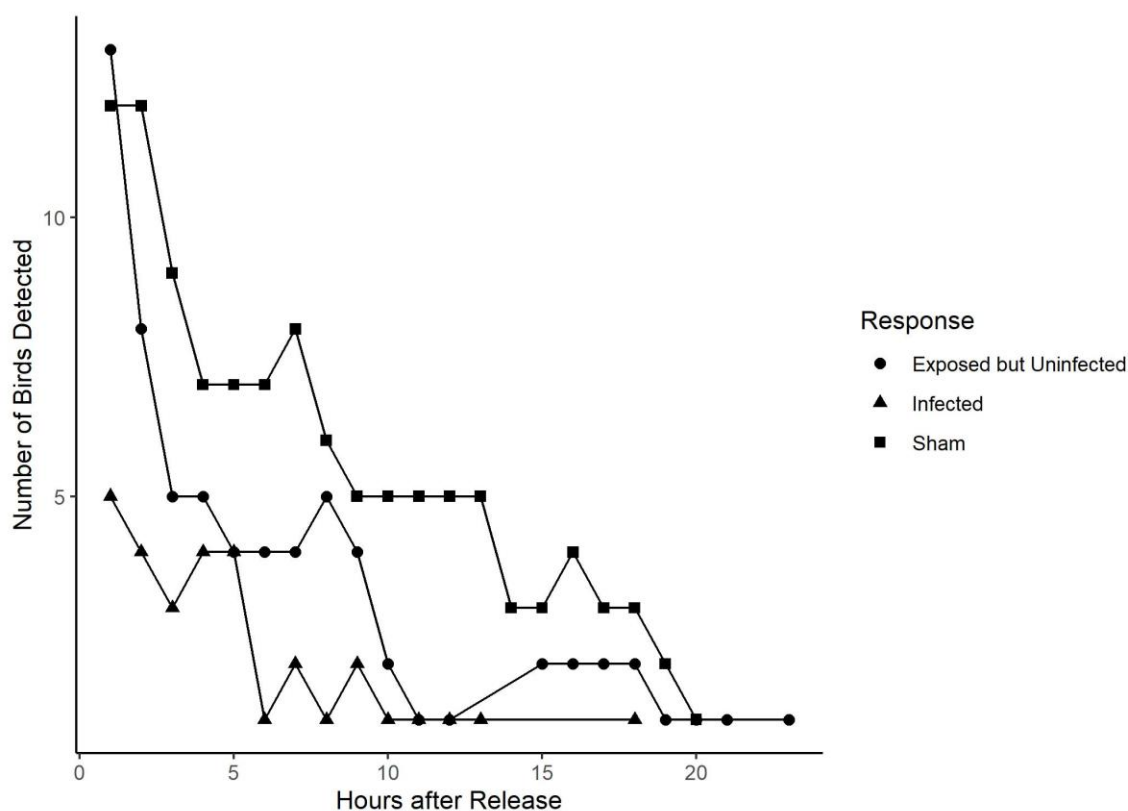
### 3.4.1 Activity

If inoculation with *P. cathemerium* affected activity, measured as CV of signal strength, there should have been a detectable difference in the activity of the response groups (Infected, Exposed but Uninfected, Sham) after release. The initial linear model compared CV to response group, sex, hour, an interaction between response group and hour, and individual. After using AIC to select the model that explained the greatest amount of variance with the fewest independent variables, the final model (CV ~ hour + individual) showed a significant increase in CV per hour after release but no relationship between CV and response group ( $F_{1,182} = 49.32$ ,  $P < 0.0001$ ; Appendix E, table E2).

### 3.4.2 Departure Timing

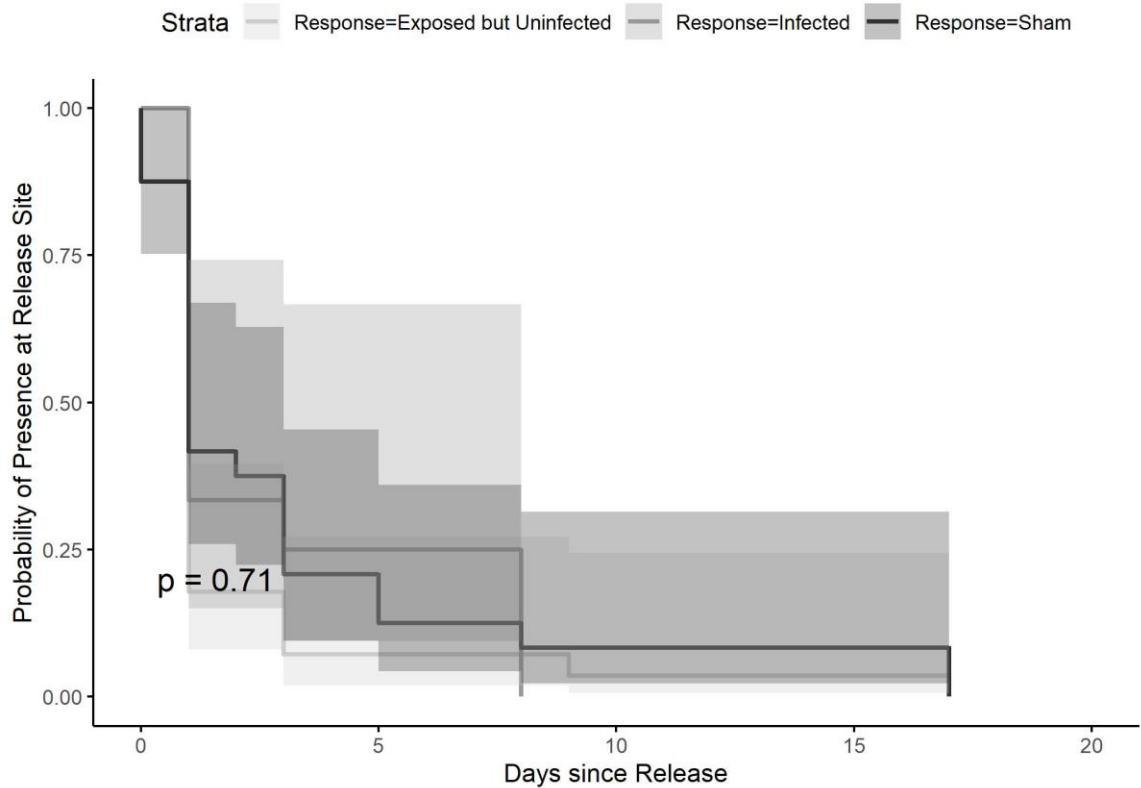
If inoculation with *P. cathemerium* affected departure timing, there should have been a detectable difference in the departures of the response groups (Infected, Exposed but Uninfected, Sham). Detections per hour show that by 20 hours after release, most birds were no longer at the release site (Figure 10). The initial general linear model of detection per hour included the time since release, sex, response group (Infected, Exposed but Uninfected, Sham), treatment group (Exposed or Sham), and room. The final model (Frequency ~ time since release + response + room) showed that detections

per hour significantly decreased with time since release ( $F_{1,73} = 28.54$ ,  $P < 0.0001$ ; Appendix E, E3).



**Figure 10: The number of individual Yellow-rumped Warblers (*Setophaga coronata*) (n = 40) detected at the Old Cut Motus Tower release site per hour after release on Oct 17, 2019. The y axis is the number of individual birds detected. Most birds departed by 20 hours after release, and there was no significant difference in departure timing between groups.**

A chi-square test of the Kaplan-Meier statistics modeling the probability of presence at the release site showed that the numbers of days birds remained did not differ among response groups (Infected, Exposed but Uninfected, Sham) ( $\chi^2_2 = 0.7$ ,  $P = 0.7$ ; Figure 11).



**Figure 11: The daily probability of an individual Yellow-rumped Warbler (*Setophaga coronata*) (n = 40) remaining at the Old Cut Motus Tower release site after release on Oct 17, 2019. The x axis is the number of days since release day. Line color indicates the response group and shading around the lines indicates the 95% confidence interval. There was no significant difference in departure probability between response groups.**

## Chapter 4

### 4 Discussion

Birds have been implicated in the spread of zoonoses, and parasite phylogeny suggests that migration plays an important role in the population dynamics of *Plasmodium* spp. (Clark et al., 2015; Galsworthy et al., 2011; Rappole et al., 2000). If *Plasmodium* spp. infection affects avian migration, it could control the spread of avian malaria through either migratory culling or migratory separation (Emmenegger et al., 2018; Johns and Shaw, 2016; Risely et al., 2018). Since birds spend the majority of the migratory period resting and refueling at stopovers, stopover duration and ecology is a logical focus of disease ecology research (Taylor et al., 2011; Woodworth et al., 2014). Yellow-rumped Warblers are a migratory species known to both be chronically infected with *Plasmodium* spp. and to use stopovers, making them an ideal candidate for researching the impacts of infection at migratory stopovers. (DeGroot and Rodewald, 2010; L. Soares (unpublished data)). From past inoculation experiments it is clear that the acute phase of infection, which would occur if a bird was infected at a migratory stopover, can cause physical and behavioral changes that could impact migration (Hayworth et al., 1987; Kelly et al., 2018; LaPointe et al., 2012; Palinauskas et al., 2011). I hypothesized that experimental infection of Yellow-rumped Warblers with *P. cathemerium* at a simulated migratory stopover would affect their condition, activity, and departure timing. I also expected some birds would resist infection, based on resistance to *P. relictum* inoculation in Song Sparrows, and that these birds would see larger impacts than infected individuals (Kelly et al., 2018). Through captivity measurements and radio-tracking, I found that the opposite was true. Condition, activity, and departure date were not significantly different between Infected, Exposed but Uninfected, and Sham birds.

#### 4.1 Resistance to Infection

Only six of the twenty-five birds inoculated (24%) developed detectable *P. cathemerium* infections. This may be because migratory species are known for their increased immune investment, making Yellow-rumped Warblers resistant to parasitism in

general (Møller and Erritzøe, 1998), or because of previous exposure to *P. cathemerium*. Migration may also drive increased investment in adaptation to reliably encountered parasites, based on modeling by Gandon and Michalakis (2002). Yellow-rumped Warblers are exposed to *P. cathemerium* during their entire migratory cycle, so they are likely adapted to this common parasite. Because birds did not die due to infection, it is unlikely that migratory culling occurs in Yellow-rumped Warblers. Infected birds showed no effects that would suggest future death during migration, but it is possible that mortality occurred elsewhere during the migratory cycle.

There is abundant evidence that previous exposure to *Plasmodium* species reduces the severity of new infections (Atkinson et al., 2001). Repeated exposure to *P. relictum* in domestic Canaries (*Serinus canaria*) showed that primary infections have the greatest impact on hematocrit, with subsequent infections having lesser effects and clearing faster (Cellier-Holzem et al., 2010). More intense infections in younger birds also support this hypothesis (DeGroot and Rodewald, 2010). By using wild birds in my experiment, I was unable to control for previous exposure, but the majority of my birds were young of the year and thus likely had similar levels of exposure to this and other strains of *Plasmodium*. Most birds are exposed before their first migration, and the prevalence of infection does not generally differ between age classes, so it is likely that these birds had some acquired immunity (Ágh et al., 2019; Ricklefs et al., 2005; Rintamaki et al., 1998; van Riper III et al., 1986).

It is well known that the stress of avian migration can negatively impact immune function (Gylfe et al., 2000). Migrating birds divert resources to migratory flight, and exercise experiments show immediate negative effects of long-distance flight on constitutive immunity (Eikenaar and Hegemann, 2016; Nebel et al., 2012). This reduction in immune quality can trigger relapses where chronic illnesses reenter the acute phase, and may make birds more susceptible to infection (Gylfe et al., 2000). However, because Yellow-rumped Warblers in this experiment were given time to adjust to captivity and radio-tags prior to inoculation, they may have avoided flight-induced reductions in their constitutive immunity at the time of inoculation.

Breeding season relapses have been observed in Yellow-rumped Warblers, but sex differences in immune investment are not present in the fall migration (Asghar et al., 2011; Soares, unpublished data). Sex also has no impact on infection prevalence or peak parasitemia in a wide variety of species (European Robins (*Erithacus rubecula*), White-throated Sparrows (*Zonotrichia albicollis*), Red Crossbills, Great Tits (*Parus major*), Red-bellied Woodpeckers (*Melanerpes carolinus*), Blue Tits) (Ágh et al., 2019; Asghar et al., 2012; Boyd et al., 2018; Cornelius et al., 2014; Dunn et al., 2011; Podmokła et al., 2014; Rintamäki et al., 1998; Schrader et al., 2003). While sex was not evenly distributed between my treatment groups, I doubt that sex differences in immunity affected my findings because the experiment took place in the fall where these differences are less pronounced (Asghar et al., 2011).

Co-infection with multiple haemoparasites is common in Yellow-rumped Warblers (L. Soares, unpublished data) and occurred during this experiment, but was not included in the analysis of results. Two birds were co-infected at the first blood sampling, and three birds were co-infected after inoculation, but no birds were co-infected during both samplings. While co-infection would be an interesting direction for future research, DNA sequencing would be necessary to determine all the haemoparasites present and accurately include co-infection as a statistical variable. Substantial increases in sample size would also be needed to ensure adequate statistical power.

## 4.2 Migratory Condition

I predicted that inoculated birds, or at least Exposed but Uninfected birds, would have reduced migratory condition, measured as mass and fat score, because migratory resources would be diverted to resisting infection. Inoculated Song Sparrows (*Melospiza melodia*) had measurable condition changes in resistant birds (Kelly et al, 2018), and half the studies in a meta-analysis of observational studies observed a relationship between chronic infection and migratory performance (Risely et al., 2018). I had expected an inoculation study would be more likely to detect effects than an observational study, and that I would observe effects, at the least, in my Exposed but Uninfected birds. When comparing pre and post-inoculation measurements, however, this was not the case. The lack of response suggests that neither tolerating nor resisting *P. cathemerium* infection is

a significant burden for Yellow-rumped Warblers. This is supported by other experimental research finding no difference in body mass when Great Reed Warblers (*Acrocephalus arundinaceus*) were inoculated with a native pathogen (Asghar et al., 2012). A similar lack of relationship has been observed in European Robins, Chipping Sparrows (*Spizella passerine*), Black-chinned Sparrows (*Spizella atrogularis*), and migratory waders (Ágh et al., 2019; Carbó-Ramirez and Zuria, 2015; Clark et al., 2015). The lack of difference in fat scores has similar supporting evidence (Ágh et al., 2019; Cornelius et al., 2014).

While it is possible that I did not observe changes because of the variables I measured, it is unlikely that those changes would not have been reflected by changes in mass measurements. Lean mass was affected by experimental exposure to *Plasmodium* in Song Sparrows (Kelly, 2018), but was only detectable using Quantitative Magnetic Resonance analysis, which cannot be done on radio-tagged birds. If substantial mass changes occurred, they likely would have been reflected in total mass and therefore detected. In general, studies of migrating birds have failed to find an impact of infection status on mass, fat score, or feather length, regardless of sex or age (Emmenegger et al., 2018). The fact that I observed no physical costs (in terms of mass and fat score) in birds that were inoculated, relative to those that were not, suggests that activity and departure date might also be robust to exposure to *P. cathemerium*.

### 4.3 Activity at the Stopover

During both captivity and release birds became more active as time increased. This is normal behavior, as birds experience Zugunruhe (nocturnal migratory restlessness triggered by the migratory state) and become increasingly restless to depart the stopover (Seewagen et al., 2019). While Kelly (2018) found that *P. relictum*-exposed Song sparrows reduced Zugunruhe, this may be because Song Sparrows are exclusively nocturnal migrants and also experienced a reduction in condition. Yellow-rumped Warblers migrate during both the day and night so their activity is spread out during both periods, and their condition was not impacted by inoculation. Chronically infected Yellow-rumped Warblers also have no difference in activity measurements from uninfected birds (Asghar et al., 2015; Seewagen et al., 2019). The lack of condition

differences and different migratory style likely explain why reduced activity was not present in acutely infected or resistant birds, and all birds displayed normal migratory activity.

#### 4.4 Departure Timing

Perhaps not surprisingly, given *P. cathemerium*'s lack of effect on condition, I observed no significant difference between response groups in departure timing. Migratory departure decisions are mainly dependent on weather, and birds will delay departure if conditions are not favorable (Dossman et al., 2016; Robinson et al., 2010; Woodworth et al., 2015). High wind speeds and precipitation reduce departures, and clear skies and helpful tail-winds increase departures (Dossman et al., 2016; Drake et al., 2014; Haest et al., 2018; Packmor et al., 2020; Woodworth et al., 2015). The availability of celestial navigation cues is also important, and Yellow-rumped Warblers prefer to migrate in clear skies when the sun and stars are visible (Dossman et al., 2016; Liu and Swanson, 2015; Woodworth et al., 2015). When crossing ecological barriers like Lake Erie, Yellow-rumped Warblers will wait for favorable conditions, spending a minimum of  $6.96 \pm 2.98$  days at the Lake Erie shoreline before risking the flight (Dossman et al. 2016).

By releasing my birds at the same time and location I controlled for variation in weather effects. Experience and fat score can also influence departure decisions, but because fat score was not impacted by inoculation there were no differences to impact departure. Older birds are more likely to depart than inexperienced birds when crossing Lake Erie, but the majority of my birds were young of the year and lacked experience migrating, controlling for age as much as possible (Dossman et al., 2016).

While birds in the Infected group departed the release site sooner than Exposed but Uninfected or Sham group birds it was not a statistically significant difference. Birds that become infected at a stopover may be motivated to leave sooner to avoid further infections, but it is not possible to draw conclusions with such a small sample size. Studies of chronically infected birds have found differences in migratory arrival, but cannot discern if this is due to differences in departure timing or flight speed (Ágh et al.,



2019; Møller et al., 2004). My results suggest that these differences are not due to departure timing, so if migratory separation is occurring it must be due to differences elsewhere in migration.

## 4.5 Implications for Future Research

### 4.5.1 Sample Size

The lack of detectable changes in my study may be due to small sample sizes. Animal husbandry resources are a limiting factor in any captivity experiment; and forty to fifty birds were the most that could be accommodated for this research. The number of birds of this species captured at banding stations also became a limiting factor, and I needed supplementary birds from LPBO to reach 40 individuals.

I also lacked control over how many birds became infected after inoculation. While I expected some birds to resist and form the Exposed but Uninfected group, I did not expect the proportion to be as large as I observed. DNA sequencing of parasites, in addition to or instead of microscopy, might have helped to confirm infections and increase sample size in the Infected group. However, despite some infections being in stages that could not be identified through microscopy, only one treatment bird had such an infection after inoculation. This suggests that DNA sequencing would not have confirmed many additional *P. cathemerium* infections. The only way to accommodate for this in future studies would be to have a larger treatment sample size, and larger number of birds. Conversely, future research could use a more infective species of *Plasmodium* to increase the number of infected individuals. By using a *Plasmodium* naturally occurring in Myrtle Warblers there was a large amount of natural resistance that could be avoided if a novel *Plasmodium* was used, but this method would lose ecological relevance.

Radio-tagging so many birds in a small space led to unexpected signal interference issues. This could be avoided by having tags with longer burst intervals in future studies. Reducing the total number of tags would reduce available data, and may make it harder to detect small activity changes. It is also difficult to detect wild birds in a landscape because tree canopies and other landscape features can block signals. There is nothing that can be done about the landscape, but larger sample sizes increase the chance

of tags being detected by Motus. Increasing the number of birds, and including tags with other burst rates, would have led to larger datasets. In general, having more data and more than 6 birds in the Infected group, or a more impactful *Plasmodium*, would also have increased statistical power.

#### 4.5.2 Species Specificity

Each parasite species and lineage has unique characteristics (Asghar et al., 2011; Zehtindjiev et al., 2008). Parasitemia and the effects of chronic infection frequently depend on the parasite in question, even when the effects of past exposure are discounted (Asghar et al., 2011; Lachish et al., 2011; Zehtindjiev et al., 2008). The species of bird used in experimental malaria research is also important, as patterns of infection are different even among closely related species (Dimitrov et al., 2015; Palinauskas et al., 2009). Inoculation experiments exposing multiple avian species to a single malaria strain have shown that resistance differs even when all other variables are kept the same (Dimitrov et al., 2015; Palinauskas et al., 2008). Chronic infection research surveying multiple species crossing the Gulf of Mexico found species-specific effects on mass and fat score (Garvin et al., 2006). Choosing appropriate host and pathogen species are an integral part of experimental malaria research, ensuring that natural infections are simulated, but research cannot be generalized among species groups (Dimitrov et al., 2015). Because of this I am hesitant to generalize my findings to other *Plasmodium* or warbler species.

However, there are some important lessons learned from this research. Avian and haemosporidian species that commonly interact in nature have likely adapted to minimize the severity of infection, benefiting both the host and parasite (Frank, 1996). Also, this proves that resistance is both present and common in Yellow-rumped Warblers, and should be taken into account when researching disease ecology. Inoculation experiments are the best way to research these relationships, as observational studies are unable to tell if birds have resisted infection. Inoculation experiments can also control for the effects of previous exposure by using novel *Plasmodium* species, at the cost of simulating natural host-parasite relationships. There is no way to control for previous exposure in observational studies.

### 4.5.3 Future Research

Condition effects, leading to activity and departure effects, may not have been present because captive birds were provided *ad libitum* food in a climate controlled environment. Future studies could avoid this either by diet restriction, or avoiding the captivity segment of this experiment. If birds were captured at the stopover, radio tagged, and inoculated immediately they would be exposed without the cushion of captivity, and this may make activity and departure changes visible. The acute phase would take several days to develop, but using the Motus Wildlife Tracking System we would be able to detect delays in movement towards Lake Erie. This study design would lose the ability to measure condition changes and determine infection success, but may be a better measure of migratory behavior changes. Blood samples could also be collected to determine co-infections and sex. Finally, an extreme way to ensure that research subjects are immunologically naive is to hand-raise subjects from hatch under controlled (vector-free) conditions. Wild-caught birds have unknown exposure histories and co-infections, so controlling exposure history is the only way to determine if resistance is innate or acquired. While using *Plasmodium* spp. that birds could not have been exposed to is also a good measure of resistance, it does not simulate natural species interactions and is difficult to compare to natural behavior. Because of the uniqueness of host-parasite interactions, avian malaria researchers must choose their species wisely.

## 4.6 Conclusion

None of the variables measured here (condition, activity, timing) were significantly affected by exposure to, or infection with, avian malaria. This suggests that neither migratory culling nor migratory separation is likely to occur in this host-parasite system, and migration will not reduce the spread of *P. cathemerium*. Infected and uninfected birds intermingle during migration and infected birds successfully complete migration, allowing transmission to occur throughout the yearly cycle. These conditions allow *P. cathemerium* to spread, and help explain why it is an extremely widespread lineage (Valkiunas, 2005).

It is likely that evolution with and previous exposure to *P. cathemerium* by Yellow-rumped Warblers has conferred resistance and created tolerance. *Plasmodium* species would not have such global coverage if they severely affected major life history events like migration, and selection on parasites would favor not killing the host (Frank, 1996). That being said, *Plasmodium* species vary in the degree to which they affect their host, avian species likely differ in their response to exposure and infection, and in all likelihood the unique combination of host and parasite species also influences the effects of infection. We need to keep this in mind when estimating malaria's impact and designing future studies.

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# Appendices

## Appendix A: Animal Use Protocol

**AUP Number:** 2019-020

**PI Name:** MacDougall-Shackleton, Elizabeth

**AUP Title:** Flying while diseased: Effects of malaria parasites on songbird migratory performance.

**Approval Date:** 04/01/2019

**Official Notice of Animal Use Subcommittee (AUS) Approval:** Your new Animal Use Protocol (AUP) 2019-020:1: entitled " Flying while diseased: Effects of malaria parasites on songbird migratory performance" has been APPROVED by the Animal Care Committee of the University Council on Animal Care. This approval, although valid for up to four years, is subject to annual Protocol Renewal.

Prior to commencing animal work, please review your AUP with your research team to ensure full understanding by everyone listed within this AUP.

As per your declaration within this approved AUP, you are obligated to ensure that:

- 1) Animals used in this research project will be cared for in alignment with:
  - a) Western's Senate MAPPs 7.12, 7.10, and 7.15  
[http://www.uwo.ca/univsec/policies\\_procedures/research.html](http://www.uwo.ca/univsec/policies_procedures/research.html)
  - b) University Council on Animal Care Policies and related Animal Care Committee procedures  
[http://uwo.ca/research/services/animalethics/animal\\_care\\_and\\_use\\_policies.htm](http://uwo.ca/research/services/animalethics/animal_care_and_use_policies.htm)
- 2) As per UCAC's Animal Use Protocols Policy,
  - a) this AUP accurately represents intended animal use;
  - b) external approvals associated with this AUP, including permits and scientific/departmental peer approvals, are complete and accurate;
  - c) any divergence from this AUP will not be undertaken until the related Protocol Modification is approved by the ACC; and
  - d) AUP form submissions - Annual Protocol Renewals and Full AUP Renewals - will be submitted and attended to within timeframes outlined by the ACC.
  - e) [http://uwo.ca/research/services/animalethics/animal\\_use\\_protocols.html](http://uwo.ca/research/services/animalethics/animal_use_protocols.html)

3) As per MAPP 7.10 all individuals listed within this AUP as having any hands-on animal contact will

- a) be made familiar with and have direct access to this AUP;
- b) complete all required CCAC mandatory training ([training@uwo.ca](mailto:training@uwo.ca)); and
- c) be overseen by me to ensure appropriate care and use of animals.

4) As per MAPP 7.15,

- a) Practice will align with approved AUP elements;
- b) Unrestricted access to all animal areas will be given to ACVS Veterinarians
- c) UCAC policies and related ACC procedures will be followed, including but not limited to:
  - i) Research Animal Procurement
  - ii) Animal Care and Use Records
  - iii) Sick Animal Response
  - iv) Continuing Care Visits

5) As per institutional OH&S policies, all individuals listed within this AUP who will be using or potentially exposed to hazardous materials will have completed in advance the appropriate institutional OH&S training, facility-level training, and reviewed related (M)SDS Sheets, <http://www.uwo.ca/hr/learning/required/index.html>

**Submitted by:** Copeman, Laura on behalf of the Animal Care Committee University Council on Animal Care



## Appendix B: Scientific Permit to Capture and Band Migratory Birds

 Environment and Climate Change Canada / Environnement et changement climatique Canada		<b>SCIENTIFIC PERMIT TO CAPTURE AND BAND MIGRATORY BIRDS</b> <b>PERMIS DE CAPTURE ET DE BAGUAGE D'OISEAUX MIGRATEURS</b>	
In the Province(s) / Territories - Dans la (les) provinces(s) / territoires <b>Ontario</b>		Permit No. / N° de permis <b>10169 BU</b>	
<b>Issued under the Migratory Birds Regulations Sections 4 and 19.</b> <i>If the authorizations include any species that are not protected under the Migratory Bird Convention Act, 1994, this document represents an agreement between Environment Canada and the holder for the use of federal bird bands on those species. All conditions listed on the back of this document apply.</i> <b>Émis en vertu des articles 4 et 19 des règlements concernant les oiseaux migrateurs.</b> <i>Si les autorisations visent des espèces qui ne sont pas protégées en vertu de la Loi de 1994 sur la convention concernant les oiseaux migrateurs, le présent document tient lieu d'entente entre Environnement Canada et le titulaire aux fins de l'utilisation de bagues fédérales sur ces espèces d'oiseaux. Toutes les conditions figurent au verso du présent document s'appliquent.</i>			
Name and Address - Nom et adresse <b>CHRISTOPHER GUGLIELMO</b> <b>PROFESSOR, DEPT OF BIOLOGY</b> <b>UNIVERSITY OF WESTERN ONTARIO</b> <b>1151 RICHMOND STREET N.</b> <b>LONDON, ON N6A 5B7</b>		Issue Date / Date d'émission <b>2019/08/23</b> Expiration Date / Date d'expiration <b>2020/12/31</b>	
		For the Minister - Pour le Ministre Name (Print) - Nom (Lettres) <b>L. HOWES</b>	
		Signature 	
<b>AUTHORIZATIONS -- AUTORISATIONS</b>			

### Authorized to:

- Band incidentally captured migratory birds listed under Schedule 1 of the Species at Risk Act as Threatened or Endangered, pursuant to Section 74 of the Species at Risk Act (SARA). No SARA listed species are to be specifically targeted.
- Band passerines and other landbirds
- Take, possess, and transport blood samples (brachial vein) up to 70 uL from passerines; up to 100 samples per species in collab w. 10691 (not to exceed 1% body mass; if in possession of a valid Animal Care Committee Approval).
- Take, possess, and transport feather samples (1 rectrix and 2-3 head feathers) from passerines up to 100 samples per species in collab w. 10691 (if in possession of a valid Animal Care Committee Approval).
- Take, possess, and transport swabs (mouth swabs) from passerines, on up to 200 samples per species, in collab w. 10691 (includes oral, tracheal, cloacal, etc.; if in possession of a valid Animal Care Committee Approval).
- Use mist nets
- Use traps
- Use (81H) radio transmitter (incl. nanotags) on White-throated Sparrow (WTSP) (5580)  
Lotek NTQB-2 nanotag on up to 60 birds (leg-loop harness; marker and attachment materials not to exceed 3% total body weight; if in possession of a valid Animal Care Committee Approval) in Ontario
- Mark Black-throated Blue Warbler (BTBW) (6540) with (01A) plastic colour leg band  
Single color band; RED WHI GRN BLUE BLACK in Ontario
- Use (81H) radio transmitter (incl. nanotags) on Black-throated Blue Warbler (BTBW) (6540)  
Lotek NTQB-2 Nanotag (leg-loop harness; marker and attachment materials not to exceed 3% total body weight; if in possession of a valid Animal Care Committee Approval) in Ontario
- Mark Myrtle Warbler (MYWA) (6550) with (01A) plastic colour leg band  
Single color band; RED WHI GRN BLUE BLACK in Ontario
- Use (81H) radio transmitter (incl. nanotags) on Myrtle Warbler (MYWA) (6550)  
Lotek nanotag NTQB-2-1 (0.29g) in collab w. 10691 (leg-loop harness; marker and attachment materials not to exceed 3% total body weight; if in possession of a valid Animal Care Committee Approval) in Ontario

See Permit Conditions on the following page -- Voir les conditions du permis sur la page suivante



**SCIENTIFIC PERMIT TO CAPTURE AND BAND MIGRATORY BIRDS  
PERMIS DE CAPTURE ET DE BAGUAGE D'OISEAUX MIGRATEURS**

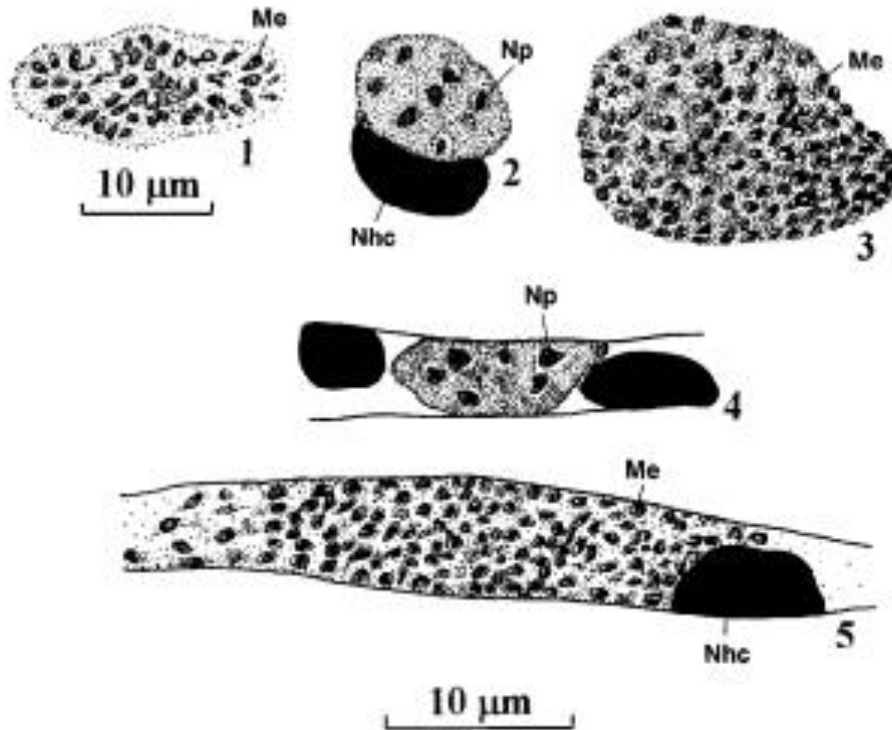
Permit No.  
N° de permis

**10169 BU**

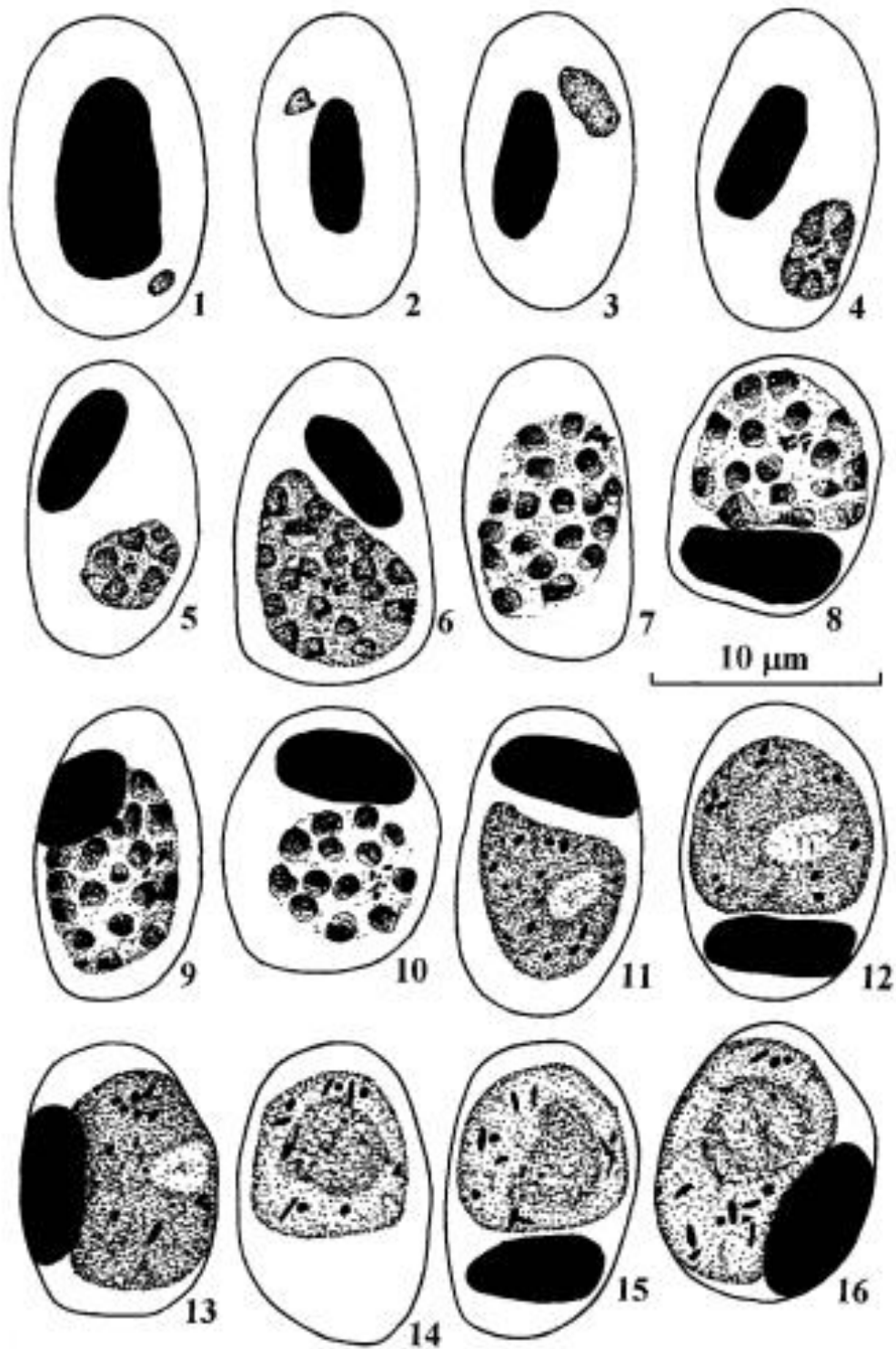
**PERMIT CONDITIONS - CONDITIONS DU PERMIS**

1. This permit is not transferable and is not valid unless it is signed by the person to whom it is issued. By signing this permit, the permittee agrees to abide by all permit terms and conditions. Permit holders must have their signed permit on their person at all times while undertaking permitted activities. Permit holders are responsible for the actions of the individuals under their supervision that conduct activities authorized under this permit.
  2. Permit holders must follow best practices to ensure the safety and welfare of the birds they capture and band.
  3. Unless otherwise stated, this permit does not authorize the collection of blood, feathers and other avian biological materials.
  4. Unless otherwise stated, this permit does not authorize the targeted capture, banding or disturbing of species listed as threatened or endangered or extirpated under the Species at Risk Act.
  5. If a bird is injured during the permitted activities and cannot be rehabilitated or released, the permit holder may euthanize the bird to prevent further suffering.
  6. The permit holder may salvage birds found dead, or killed as a result of normal banding operations, for the purpose of donating them to an individual or institution authorized by permit to acquire and possess migratory bird specimens for scientific or educational purposes.
  7. The issuance of this permit does not exempt the permit holder from compliance with all relevant Canadian, provincial and territorial laws and regulations or jurisdictional bylaws.
  8. This permit does not authorize the capture of any birds on any public, private or indigenous lands without the consent of all relevant land owners or custodians.
  9. Unless otherwise stated on this permit, the permit holder will use only the official numbered leg bands issued by the Canadian Wildlife Service.
  10. The permit holder shall not sell, exchange, or transfer any unused bands to any person without written authorization from the Bird Banding Office. Any unused bands remaining when this permit is voluntarily returned, revoked, or expired must be returned to the Bird Banding Office.
  11. The permit holder will submit all required data to the Bird Banding Office as outlined in the instructions to banding permit holders or within 30 days of receiving a written request from a representative of the Minister.
  12. The permit holder agrees that all banding and encounter data may be released to other users or the public.
  13. The permit holder agrees that the name, phone number, address and e-mail address of the permit holder will be stored in the shared North American Bird Banding and Encounter database, and may be shared with people reporting an encounter of a band used under this permit.
  14. The permit holder shall allow any authorized representative of the Minister to enter and inspect the premises where operations authorized by this permit are being conducted and to inspect the records related to these operations.
  15. This permit may be suspended or cancelled if the person to whom it was issued has failed to comply with any condition set out in the permit.
1. Le présent permis n'est pas transférable et n'est valide que s'il est signé par la personne à laquelle il est délivré. En signant ce permis, le titulaire accepte de respecter toutes ses modalités applicables. Les titulaires de permis doivent avoir leur permis signé en leur possession en tout temps lorsqu'ils réalisent des activités permises. Les titulaires de permis sont responsables des actions des personnes sous leur supervision qui réalisent des activités autorisées en vertu du permis.
  2. Les titulaires de permis doivent respecter les pratiques exemplaires pour assurer la sécurité et le bien-être des oiseaux qu'ils capturent et baguent.
  3. Sauf indication contraire sur le permis, ce permis n'autorise pas les prélèvements de sang, des plumes ou de tout autre matériel biologique aviaire.
  4. Sauf indication contraire sur le permis, il est interdit de capturer, de baguer ou de déranger intentionnellement toute espèce en péril inscrite comme étant menacée, en voie de disparition ou disparue sous la Loi sur les espèces en péril.
  5. Si un oiseau est blessé pendant les activités permises et que son rétablissement pour le relâcher est impossible, le détenteur du permis peut euthanasier l'oiseau afin d'éviter d'autres souffrances.
  6. Le titulaire de permis peut récupérer des oiseaux trouvés morts, ou encore tués par suite d'opérations normales de baguage, dans le but de les donner à une personne ou à un établissement autorisé par un permis à acquérir et à posséder des spécimens d'oiseaux migrateurs à des fins scientifiques ou éducatives.
  7. La délivrance du permis n'exempte pas son titulaire de l'obligation de respecter les lois et règlements fédéraux, provinciaux et territoriaux par ailleurs applicables.
  8. Ce permis n'autorise pas la capture d'oiseaux sur des terres publiques, privées ou autochtones sans le consentement de tous les propriétaires ou gardiens concernés.
  9. Sauf indication contraire sur le permis, le détenteur ne doit utiliser que les bagues officielles numérotées délivrées par le Service canadien de la faune.
  10. Le titulaire du permis ne peut vendre, échanger ou transférer des bagues inutilisées à quiconque sans l'autorisation écrite du Bureau de baguage des oiseaux. Toutes bagues inutilisées restantes, lorsque ce permis est révoqué, expiré ou volontairement retourné, doivent être retournées au Bureau de baguage des oiseaux.
  11. Le titulaire de permis doit présenter toutes les données requises au Bureau de baguage des oiseaux de la façon décrite dans les instructions de présentation de données relatives au baguage ou dans les 30 jours suivant la réception d'une demande écrite d'un représentant du ministre.
  12. Le titulaire du permis convient que toutes les données de baguage et les données demandées par suite d'un signalement peuvent être communiquées à d'autres utilisateurs ou au public.
  13. Le titulaire de permis doit convenir que son nom, numéro de téléphone, adresse postale et adresse de courriel seront conservés dans la base de données commune du programme nord-américain de baguage des oiseaux et pourront être communiqués aux personnes qui signalent une bague utilisée au titre du présent permis.
  14. Le titulaire du permis doit permettre à tout représentant autorisé du ministre d'entrer et d'inspecter les lieux où se déroulent les opérations autorisées par le présent permis et d'inspecter les dossiers liés à ces opérations.
  15. Le présent permis peut être suspendu ou révoqué si la personne à laquelle il a été délivré omet de se conformer à toute condition énoncée dans le permis.

Appendix C: *Plasmodium cathemerium* in organs and cells (Valkiunas, 2005).



**Figure 225** Exoerythrocytic meronts of *Plasmodium cathemerium*:  
 1 – mature cryptozoite; 2–5 – phanerozoites from *Fringilla coelebs*: immature (2) and mature (3) parasites in lungs, immature (4) and mature (5) parasites in brain; Me – merozoite; Nhc – nucleus of host cell; Np – nucleus of parasite (1 is modified from Garnham, 1966).



**Figure 226** *Plasmodium cathemerium* from the blood of *Serinus canaria*:  
 1-3 – trophozoites; 4-10 – erythrocytic meronts; 11-13 – macrogametocytes; 14-16 – microgametocytes.

## Appendix D: Warbler Sexing Protocol

### Warbler sexing PCR protocol Adapted from Morbey et al 2016 by Leticia Soares (Feb. 2020)

Primers:

SexFwd 5'-GTATCGTCAATTTCCATTTTCAGGT-3'

SexRev 5'-CCATCAAGTCTCTAAAGAGATTGA-3'

Reagent	Concentration	Volume $\mu$ L
ddH <sub>2</sub> O	-	6.4
Buffer [10x] containing MgCl <sub>2</sub> *	1x	1.0
dNTP [10 $\mu$ L each]	0.3 mM	0.3
MgCl <sub>2</sub> [25 mM]	3 mM [final]	0.4
SexFwd [10 $\mu$ M]	0.4 $\mu$ M	0.4
SexRev [10 $\mu$ M]	0.4 $\mu$ M	0.4
Golden Taq	0.5 U	0.1
Master Mix	-	9
DNA template	-	1
Final volume	-	10

The thermal cycle: 95 °C (5 minutes); 40 cycles of 95 °C (30 seconds), 59.5 °C (20 seconds), 72 °C (20 seconds); and 72 °C (2 minutes).

6–10  $\mu$ L of PCR product must be used on a 1% agarose in 1XTAE gel ran for 40-60 min to visualize the banding pattern. CHID-Z shows a 520 bp band and CHID-W shows a 319 bp band. Males should show CHID-Z band, and females should show both bands. Some individuals will show a single CHID-W band, these individuals are classified as unknown and sexed by plumage.

\*Reagent used: DreamTaq Green DNA Polymerase; DreamTaq buffer already contains 20mm of MgCl<sub>2</sub>. Concentration on the table refers to final master mix concentration of MgCl<sub>2</sub>, which include both MgCl<sub>2</sub> from buffer and from MgCl<sub>2</sub> stock.

Reference:

Morbey, Y.E., C.G. Guglielmo, P. Taylor, I. Maggini, J. Deakin, S. Mackenzie, J.M. Brown, and L. Zhao. 2018. Evaluation of sex differences in the stopover behavior and postdeparture movements of wood-warblers. *Behav. Ecol.* 29:117-127. doi: 10.1093/beheco/axx123.

## Appendix E: Model Selection

Models predicting the coefficient of variation (a proxy for activity) of 40 captive Yellow-rumped Warblers (*Setophaga coronata*) in an inoculation experiment using *Plasmodium cathemerium*. Predictor variables were Response (Infected, Exposed but Uninfected, Sham), Day (days since the experiment began); Room (aviary room), Individual (individual bird), and Sex (Male or Female). Shaded row is the final model.

### CV before Inoculation, Day

Variables	K	AICc	$\Delta$ AICc
Day + Room	4	-165.78	0
Response + Room	5	-162.93	2.85
Response + Day + Room	6	-156.42	9.36
Response + Day + Response/Day(interaction) + Room	7	-147.06	183.71
Response + Day + Response/Day(interaction) + Room + Individual	8	-144.59	21.19
Response + Day + Response/Day(interaction) + Room + Individual + Sex	9	-141.33	24.45

### CV before Inoculation, Night

Variables	K	AICc	$\Delta$ AICc
Day + Room	4	-129.55	0
Response + Room	5	-126.57	2.98
Response + Day + Room	6	-116.64	12.93
Response + Day + Response/Day(interaction) + Room	7	-108.76	20.79
Response + Day + Response/Day(interaction) + Room + Individual	9	-102.26	27.29
Response + Day + Response/Day(interaction) + Room + Individual + Sex	8	-105.62	23.93

### CV after Inoculation, Day

Variables	K	AICc	$\Delta$ AICc
Day + Room + Individual	5	-747.57	0
Response + Day + Room + Individual	7	-730.75	16.81
Response + Day + Response/Day(interaction) + Room + Individual	9	-704.95	42.62
Response + Day + Response/Day(interaction) + Room + Individual + Sex	10	-702.72	44.85

## CV after Inoculation, Night

Variables	K	AICc	$\Delta$ AICc
Day + Room + Individual	5	-745.06	0
Response + Day + Room + Individual	7	-731.41	13.66
Response + Day + Response/Day(interaction) + Room + Individual	9	-708.96	36.11
Response + Day + Response/Day(interaction) + Room + Individual + Sex	10	-707.03	38.03

Model predicting the coefficient of variation (a proxy for activity) of 40 released Yellow-rumped Warblers (*Setophaga coronata*) in an inoculation experiment using *Plasmodium cathemerium*. Predictor variables were Response (Infected, Exposed but Uninfected, Sham), Sex (Male or Female), Hour (hour since release), and Individual (individual bird). Shaded row is the final model.

## Linear Model of CV

Variables	K	AICc	$\Delta$ AICc
Hour + Individual	4	-690.75	0
Response + Individual	5	-649.22	41.53
Response + Hour + Individual	6	-675.65	15.1
Response + Sex + Hour + Individual	7	-670.51	20.24
Response + Sex+ Hour + Response/Hour(interaction) + Individual	9	-652.1	38.65

Model predicting the detection frequency of 40 released Yellow-rumped Warblers (*Setophaga coronata*) in an inoculation experiment using *Plasmodium cathemerium*. Predictor variables were Time Interval (time since release), Sex (Male or Female), Individual (individual bird), Treatment (Inoculation or Sham), Response (Infected, Exposed but Uninfected, Sham), and Room (aviary). Shaded row is the final model.

### Generalized Linear Model with Binomial Error Distribution of Detection Frequency

Variables	K	AIC	$\Delta$ AICc
Time Interval + Response + Room	5	2335.6	0
Time Interval + Treatment + Response + Room	6	2335.6	0
Time Interval + Sex + Treatment + Response + Room	7	2322.3	13.3
Time Interval + Sex + Individual + Treatment + Response + Room	8	2319.6	16



## Curriculum Vitae

**Name:** Rebecca Howe

**Post-secondary Education and Degrees:** University of Guelph  
Guelph, Ontario, Canada  
2009-2013 B.S. in Environmental Science, Honors Program

Cambrian College of Applied Arts and Technology  
Sudbury, Ontario, Canada  
2014-2015 Graduate Certificate in Environmental Monitoring and Impact Assessment

**Conference Posters and Presentations:** Presentation- Canadian Society for Ecology and Evolution  
Annual Meeting  
2021 Meeting

Presentation- American Ornithological Society and the Society of Canadian Ornithologists  
2021 Meeting

Presentation- Canadian Society of Zoologists  
60<sup>th</sup> Annual Meeting, 2021

Poster- 29<sup>th</sup> Comparative Physiology and Biochemistry Workshop  
2019

**Related Work Experience:** Ecologist  
RiverStone Environmental Solutions Inc.  
2021- Present

Contract- As Needed/Hourly  
North-South Environmental  
2021- Present

Programming Committee- Biology Graduate Research Forum,  
Current

University of Western Ontario  
London, Ontario, Canada

Volunteer Moderator- American Ornithological Society and the Society of Canadian Ornithologists 2021 Meeting, 2021  
Online

Volunteer Moderator- Canadian Society for Ecology and Evolution  
Annual Meeting, 2021

Online

Ontario Breeding Bird Atlas Primary Atlasser, 2021

Birds Canada

Woodstock, Ontario, Canada

Canadian Nightjar Survey, 2019-2021

Birds Canada

Woodstock, Ontario, Canada

Teaching Assistant, 2018-2021

The University of Western Ontario

London, Ontario, Canada

Common Nighthawk and Eastern Whip-poor-will Field  
Technician, 2018

The University of Guelph

Guelph, Ontario, Canada

Grassland Bird Field Technician, 2017

Bird Conservancy of the Rockies

Beach, North Dakota, United States of America

Avian Field Technician, 2016-2017

Bird Ecology and Conservation Ontario

Creemore, Ontario, Canada

Bat Banding Volunteer, 2015

Awenda Provincial Park, Ontario, Canada

Assistant Resource Management Technician

Ministry of Natural Resources and Forestry

Midhurst, Ontario, Canada

Barn Swallow Research Technician, 2015

Bird Ecology and Conservation Ontario  
Guelph, Ontario, Canada