INTERPLAY BETWEEN ENERGY RESERVES, PARASITISM, STRESS AND IMMUNITY IN MIGRATING SONGBIRDS

by

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(Under the Direction of Sonia Altizer)

ABSTRACT

Migrating animals likely face trade-offs in allocating resources to different physiological functions, with one potential trade-off between fat accumulation and immune defense. Here, we sampled three migratory songbird species during their fall migration through coastal Georgia and quantified body condition, fat loads and infection with blood parasites. We predicted that parasitized birds would show decreased fat scores, poorer body condition and increased stress levels. We also asked whether birds with greater immune defense would have higher or lower body condition measures and fat levels and examined the association between infection status and immune defense. Parasitized birds had elevated leukocyte counts but showed no evidence of greater stress, poorer body condition or lower fat levels. Although we found no relationship between leukocytes and body condition or fat scores, results from this study suggest that investment in immune defense might be an important cost of parasite infection for animals undertaking long-distance migrations.

INDEX WORDS: Migration, blood parasite, stress, fat, body condition, stopover, lipids, *Haemoproteus*, *Plasmodium*, warbler

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Each year, millions of animals migrate long distances to track seasonal changes in resources and climate (Bowlin et al., 2010; Dingle, 1996). These lengthy journeys can be energetically costly. For example, by combining banding efforts with laboratory manipulations, scientists have discovered that some migratory birds accrue 50% of their lean body mass prior to flight (Dingle, 1996; Owen and Moore, 2006) and expend thousands of kilojoules during their journeys (Schmaljohann et al., 2012; Wikelski et al., 2003). During migration, neotropical migrant songbirds use lipid deposits as their primary source of energy for flight (Blem, 1990). To maintain these high levels of fat, birds must stop periodically to refuel, and can also become hyperphagic, consuming high volumes of food and rapidly gaining weight in a matter of days (Holberton et al., 1996).

Many migrating animals, including many songbirds, begin their journeys infected with a parasite or pathogen or will contract one during their long-distance migrations. This is not surprising when considering that the majority of a migratory birds lifetime is spent traveling to and from its breeding site (Long and Holberton, 2004). Because infectious diseases might lower the probability of successful migration, migrating animals could experience pressure to maintain high immune defenses (Buehler et al., 2010). Yet, two decades of research show that immune defenses are themselves costly and trade-off against other functions, especially reproduction (Lochmiller and Deerenberg, 2000; Martin et al., 2003; Sheldon and Verhulst, 1996), and that an animal’s ability to mount an immune response depends in part on its energetic resources (Lochmiller and Deerenberg, 2000; Owen and Moore, 2008a). On the one hand, immune defenses may not change during migration if robust migrants or those with access to resources can invest both in immunity and in energetic reserves to fuel migration (e.g.(Buehler et al., 2010). On the other hand, the increased energetic demand required by migration might cause animals to divert resources away from immune defense, thus resulting in a trade-off between resources invested in immune response versus energy to fuel long-distance travel. In support of this idea, recent work on monarchs showed that animals sampled during the fall migration with greater measures of immune defense had lower lipid reserves (Satterfield et al., In press). Other work on migrating birds showed that actively migrating wild thrushes had lower measures of innate immunity compared to individuals tested during a non-migratory period (Owen and Moore, 2006) and that captive thrushes showed lower cell-mediated immunity during the period of migratory restlessness that immediately preceded migration (Owen and Moore, 2008a).

Although many birds will acquire a parasite or pathogen before or during migration, the cost of migrating while parasitized are not well understood. Are infected animals less capable of acquiring the energetic resources needed to successfully reach wintering grounds? Does infection during migration cause animal to become more stressed? In some migratory species, such as monarch butterflies and Bewick's swans, researchers have found evidence that parasitized animals have lower flight performance and are less likely to make the journey from their breeding grounds to wintering sites (Bartel et al., 2011; Bradley and Altizer, 2005; van Gils et al., 2007). If an animal is compromised from the costs of a parasite or pathogen it might have a reduced ability to forage due to physical damage, anemia or lethargy caused by infection (Davis, 2004). For example, two species of tanagers infected with a blood parasite, *Haemoproteus*, were found to have significantly lower fat scores than uninfected birds, which suggests that parasites pose energetic costs for migratory birds (Garvin, 2006). While the ability to gain resources for long-distance flight has been studied in great detail, how these dynamics of migration change when infection with a parasite is added to the system is relatively unknown, and may be important to host ecology (Garvin, 2006).

Due to the dynamics of migration, specifically the high density of species at stopover sites and the high diversity of species and habitats encountered by migrants, migratory animals could be at unusually high risk of contracting a pathogen or parasite (Altizer, 2011). For example, Delaware Bay has been found to be a 'hot spot' for influenza virus, with levels 17 times higher than other locations, due to the congregation of shorebirds during migration (Krauss et al., 2010). Additionally, when migratory songbirds were sampled during spring and fall migration, viremia for West Nile Virus (WNV) was detected in 19 birds, supporting the hypothesis that migratory songbirds could be spreaders of WNV (Dusek et al., 2009). While migratory birds have been shown to be vulnerable to contracting and amplifying diseases, infection with a pathogen could also diminish the likelihood of successfully reaching the wintering grounds (Altizer, 2011). As infection has been shown to be costly in terms of energetics, it is also important to investigate whether the combined effects of infection and migration might operate synergistically to increase the stress response of these birds.

It is generally believed that migration should be one of the most stressful physiological states of a bird's lifetime (Wikelski et al., 2003). Studies that examined birds during autumn or spring migration showed that the stress response of these birds can differ markedly from the stress levels experienced during the breeding season, with migration having increased baselines of stress (Holberton et al., 1996; Owen and Moore, 2006). Several studies have since addressed the differences in stress response during breeding and migration seasons in various species of birds. White-crowned sparrows (Nearctice short-distance migrants) were found to have seasonal changes in stress levels, with the breeding season having the highest baseline level of corticosterone, potentially owing to changes in physiology (Romero et al., 1997). Alternatively, while migration has been suggested to be a period of an increased baseline of stress for long-distance migrants (Holberton, 1999; Holberton et al., 1996; Owen and Moore, 2006), the cause of this increased stress is largely unknown. Corticosterone has been show to induce behaviors that increase a migrant's success in reaching the wintering grounds, such as rapid foraging and hyperawareness to surroundings (Holberton et al., 1996; Owen and Moore, 2006). Therefore, while physiological states of migrants are quite varied throughout the year, the elevated baseline and increased stress levels might actually aid long-distance migrants in reaching wintering grounds successfully. Despite this process, more work is needed to better understand variation in stress and physiology throughout the annual cycle and to identify which periods are the most stressful.

While the physiological states of long-distance migrants vary throughout the year, the ability to mount an immune response against infection remains an important aspect of survival. White blood cell

(WBC) counts are an important component of the innate immune system for vertebrate animals and have been commonly used as a proxy for immune condition and response in a number of studies (Jakubas et al., 2013; Owen and Moore, 2006, 2008a, 2008b). In the thrush example noted earlier, migrating birds had significantly lower WBC counts, especially lymphocytes, compared with conspecifics in the breeding season (Owen and Moore, 2006). Another study examining reed and sedge warblers found that adult birds had lower WBC counts during migration than during the breeding season (Jakubas et al., 2013). These studies, and others, support the notion that animals are immunosuppressed during long-distance migration (Weber, 2007). This immunosuppression might be due to the inability to allocate resources to all physiological function during a time when increasing energetic reserves is vital or to infection with a parasite or pathogen(Owen and Moore, 2006). This is further supported in a review by Weber (2007), where they show that prolonged flight leads to immunosuppression and that infection with a parasite or pathogen can negatively affect migration success (but see (Hasselquist et al., 2007). Because the ability to maintain substantial immune defense and response to infection is crucial for survival, balancing this defense with the ability to sustain other systems, like lipid deposition could be challenging.

This main goal of this thesis work was to investigate trade-offs between physiological responses required for successful migration, including lipid deposition and immune defenses necessary for countering parasite infection. Specifically, my goals were to determine (1) whether decreased fat levels and body condition and increased stress were indicative of infection with a blood parasite and (2) whether immune measures depended on infection status and showed a negative relationship with fat levels and body condition. To do this I captured wild migrating songbirds at a stopover site in coastal Georgia and obtained blood samples to determine the presence/absence infection. I also measured the weight, wing chord, and assigned a fat score of each bird to calculate the body condition index and level of current fat reserves. I expected to find evidence of an energetic trade-off between a bird’s ability to successfully accumulate fat and maintain baseline levels of innate immune defense, as might be indicated by a negative relationship between current subcutaneous fat scores and body condition and WBC counts. I also expected to find that parasitized birds would have greater immune cell counts and would be in poor condition with lower fat reserves, indicating that if a bird was infected with blood parasites, it would have less energy to allocate to successful migration. Findings of this work are important for understanding how animals balance the demands of energetically costly and highly seasonal activities such as migration or reproduction against immune defenses against response to parasite infection. Importantly, the relationship between migration, infection and immunity is not likely to be straightforward, and could further depend on resource availability and other risks factors such as predation or environmental stress (Buehler et al. 2008). Finally, as many songbird populations are currently declining, future work should focus on the long-term implications of parasitism, especially those that can occur as lasting subclinical infections, like avian malaria, on the migration success and lifetime fitness of these songbirds.

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CHAPTER 2

HAEMOPARASITE INFECTED BIRDS HAVE HIGHER LEUKOCYTE CONCENTRATIONS, BUT INFECTION IS NOT ASSOCIATED WITH STRESS OR CONDITION IN MIGRATING SONGBIRDS.[1]

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Abstract

Long-distance migrations are energetically expensive for many animals, including migratory songbirds. During these demanding journeys, animals likely face trade-offs in allocating resources to different physiological functions, with one potential trade-off between lipid reserves to fuel the migration and costly immune defense against pathogens. We sampled three species of long-distance migratory songbirds during their fall migration through coastal Georgia and quantified their body condition, fat loads and infection with blood parasites (*Hemoproteus* and *Plasmodium*). We also quantified cellular immunity based on total and differential white blood cell counts, and measured hematological stress from the heterophil:lymphocyte ratio. We tested whether birds infected with blood parasites had decreased fat scores, poorer body condition and increased stress levels. We also examined relationships between immune cell profiles and the following variables: body condition, fat levels, infection status, age and species. Infected birds did not show greater stress, poorer body condition or lower fat levels, but infected birds for one species showed significantly elevated leukocyte counts. Although we found no evidence for a tradeoff between immune cell counts and body condition or fat scores, results from this study suggest that investment in immune defense might be an important cost of parasite infection for animals undertaking long-distance migrations.

Keywords

Blood parasite, body condition, *Dendroica palmarum palmarum*, *Dumetella carolinensis, Geothlypis trichas*, leukocyte, lipid reserves, migration, white blood cells

Introduction

Long-distance migration is a strenuous undertaking can require immense energy expenditures (Wilcove, 2008). Many bird species travel upwards of 10,000 kilometers round-trip, and must acquire and store energy to fuel this flight (Alerstam et al., 2003; Blem, 1990), with some animals investing up to 50% of their lean body mass in lipid reserves to prepare for migrations (Dingle, 1996). These energetic demands can cause birds to atrophy organs and reduce investment in functions that are not necessary for flight (Piersma, 1998), leading some physiological systems, such as immune defense, to suffer as a result (Owen and Moore, 2006). Indeed, a large body of past work showed that immune defense itself can be energetically expensive to maintain, and these defenses can trade off against other activities such as reproduction (Demas et al., 2012). In support of the idea that migration might lower the resource pool available for immune defense, several studies showed that adult songbirds (warblers and thrushes) had lower baseline measures of several components of innate immunity (including leukocyte counts) during migration than during the breeding season (Jakubas et al., 2013; Owen and Moore, 2006). In captive experiments, Owen and Moore (2008b) showed that cell-mediated immunity declined with the onset of migratory restlessness (the agitated behavior of birds that precedes their migratory departure), suggesting that changes in immunity coincide with preparation for long-distance flight. Aside from a small number of studies, however, the potential trade-offs between energy expenditure during long-distance flight and mounting immune defense against pathogens are not well understood (Buehler et al., 2010).

Many animals harbor parasite infections while migrating (Altizer, 2011), and these parasites can have negative consequences for migratory success. Parasites are known to be costly for wild animals, and can lower survival, slow development, reduce reproduction, and alter behaviors important for fitness (Wobeser, 2006). In terms of impacts on migration success, captive studies on monarch butterflies showed that individuals infected with a protozoan parasite flew shorter distances and with reduced flight speeds (Bradley and Altizer, 2005), and field studies showed that parasite prevalence decreased as monarchs moved southwards during their fall migrations (Bartel et al., 2011), consistent with the idea that infected animals migrate less well than healthy ones. Other work on Bewick's swans (*Cygnus* *columbianus bewickii*) showed that infection by low-pathogenic avian influenza (LPAI) viruses delayed the onset of migration and reduced travel distances (van Gils et al., 2007). In addition to causing direct damage to host tissues, parasites could also have indirect costs for migrating animals in the form of elevated immune defense (Hawley and Altizer, 2011). Thus, infectious agents could negatively affect migration success, through both direct damage and by causing animals to invest in costly defenses.

Although migration is physically demanding, increasing the need for energy to fuel flight, much of what is known about competing energetic demands in the context of parasitism and immunity in birds comes from studies conducted during the breeding season. Like migration, the breeding season is a period of increased physical demand due to reproduction and parental activity. While it is unknown which season has the greatest energetic demand, information can be drawn from the breeding season to develop hypotheses about the costs of energetic demands during migration. Past research on birds shows that increased work associated with breeding tends to decrease immune defense. For example, cellular immunity in barn swallows (*Hirundo rustica*) declined in response to experimentally increased brood size (Pap and Markus, 2003). When brood size was increased in great tits (*Parus* major) the numbers of lymphocytes and total white blood cells decreased (Horak, 1998). Zebra finches (*Taeniopygia guttata)* with experimentally increased brood size similarly showed suppressed antibody production (Deerenberg et al., 1997), although recovered some responsiveness when given a fortified diet. Collectively, evidence supports that increased physical demand has negative physiological implications for immune functions; leading to the possibility that migratory hosts might be suffer high fitness costs when long-distance flight is combined with a parasite infection.

Neotropical migrant songbirds are a model system to explore the potential interactions that occur between infection, immunity and energy reserves during long distance migration. These songbirds typically breed in the northern United States and Canada during the spring and summer, and winter in Mexico, the Caribbean or South America. To complete this long-distance flight, most birds stop along their migration paths to rest and replenish fat reserves. Birds at stopover sites are in variety of conditions, ranging from no fat to high amounts of fat deposits (Owen and Moore, 2008a). In this study, we captured neotropical migrant songbirds at a stopover site in coastal Georgia to investigate the physiological profiles (ie. levels of stress, fat and body condition) associated with infection by blood parasites and to test for evidence of trade-offs between immune measures and energetic reserves. Specifically, we predicted that: (1) decreased fat reserves, poorer body condition and increased stress levels would be predictive of infection with a blood parasite; and (2) immune defense would associate negatively with fat reserves and either positively or negatively with body condition, depending on energetic demands, and (3) immune defense would be higher among infected individuals. Thus, we aimed to advance scientific understanding of the physiological processes associated with infection that are crucial to migration success, and to quantify the potential effects of haemoparasites during a time of heightened physical activity.

Materials and Methods

Sampling location. This study was conducted at the Jekyll Island banding station (JIBS) located off the coast of Georgia, USA (figure 1), where mist netting has occurred every Oct, coinciding with the timing of fall migration, since 1978. While a large number of songbird species are typically captured each year at this site, in this study we specifically focused on three neotropical migrants, Gray Catbirds (GRCA), *Dumetella carolinensis*, Common Yellowthroats (COYE), *Geothlypis trichas*, and Western palm warblers (WPWA), *Dendroica palmarum palmarum,* due to their high abundance during the time of our sampling. Birds were sampled during a two-week period from October 7-21, 2012. We used 18 mist nets (Avinet, Dryden, NY) located in areas with little or no overhead tree cover. Nets were open from 20 minutes before sunrise until noon each day and checked at least every 20 minutes. All captured birds were placed in individual paper or cloth bags, and were carried to a central banding facility. Sampling procedures were approved by the University of Georgia (AUP #A2011 08-006).

Data collection. For the purposes of this project, all birds were classified as hatch-year (HY) or after hatch-year (AHY) following Pyle (1997). Sex determination was only possible with COYEs, due to similar plumage between sexes of the other species (Pyle, 1997), and thus sex was excluded from analyses below. Birds were weighed to the nearest 0.01 g using a standard electronic scale and wing chord (an index of bird size) was measured to the nearest mm using a wing ruler. The level of subcutaneous fat in the furculum of each bird was visually scored on a six-point scale (from 0-5), with a score of 5 denoting the highest fat load (fat bulging from furculum), following Helms and Drury (1960). A blood sample was taken from the brachial vein (no more than 50µl) using a 26 ½ -gauge needle and collected into a capillary tube, from which a blood smear was made. The remaining blood was frozen at -20 °C for polymerase-chain reaction (PCR) detection of haemoparasites.

Leukocyte profiles. Dried blood smears were stained with a Giemsa-Wright (Sigma-Aldrich) stain (Owen, 2011) and examined under a compound microscope at 1000x magnification. Slides were examined until 100 white blood cells (WBC) were counted or until 150 fields of view had been viewed. Within each field we recorded the presence of five major leukocyte types: lymphocytes (L), heterophils (H), eosinophils (E), monocytes (M) and basophils (B) (Appendix A). Full descriptions and functions of these cells are provided in (Clark et al., 2009; Davis et al., 2008). Briefly, lymphocytes are the most common WBC type in passerines (60-73% of WBCs depending on species) and are differentiated into B-cells and T-cells that are morphologically indistinguishable and that are involved in the adaptive immune response and immune memory. Heterophils are phagocytic cells that are the next most common WBC type (7-20% of WBCs; (Davis, 2009). Monocytes are generally rare, are the largest WBC type, and are also phagocytic. Eosinophils are involved in defense against multicellular parasites, and basophils are important for the inflammation response (although their precise function is unclear). Collectively, lymphocytes and heterophils make up 80% of passerine WBCs (Davis et al., 2008). In our study 98.4% of the cells were made up of L, H and B (Appendix B).

From the counts of heterophils and lymphocytes, we calculated H:L ratios as a hematological index of stress (Davis et al., 2008). This measure has been shown to scale positively with plasma levels of corticosterone, the primary vertebrate stress hormone (Davis et al., 2008). Unlike plasma corticosterone, which can increase within 2 min of acute stress associated with animal capture (Romero & Romero, 2002), prior research has shown that H:L ratios in songbirds increase more slowly, and remain at baseline levels during routine handling for up to one hour after capture (Davis et al., 2008).

Detecting avian malaria parasites. Nested PCR was used to identify infection status with avian malaria parasites (*Hemoproteus* and *Plasmodium*). This method has been shown to be more sensitive than blood smear analysis for parasites (Bradley, 2009; Waldenstrom et al., 2004), with the potential to detect one infected erythrocyte per 10,000 cells. Methods and conditions for determining the presence of avian malarial parasites were adapted from the protocol developed by Waldenstrom et al. (2004). This protocol amplifies a 520-bp segment of the mitochondrial cytochrome-b gene. After DNA extraction from whole RBC using the Qiagen DNEasy blood and tissue kit (Valencia, CA, USA), we conducted a nested PCR procedure. For the first round of PCR primers HAEMF (5'-ATGGTGCTTTCGATATATGCATG-3') and HAEMR2 (5'-GCATTATCTGGATGTGATAATGGT-3') were used. All samples were run in 27µl volumes with 12.5 µl GoTaq Green Master Mix (M7122, Promega), 8.5 µl deionized water, 1 µl of each primer and 4 µl of the extracted DNA. The second round of PCR used the primers HAEMNF (5'-CATATATTAAGAGAATTATGGAG-3') and HAEMNR2 (5'-AGAGGTGTAGCATATCTATCTAC-3'). Nested reactions were run using the same total volumes, with 4 µl of the PCR product from round 1. Each set of PCR reactions was run in duplicate with positive and negative controls, and results were visualized by separating on a 2% agarose gel using GelRed (Biorad, Hayward, CA) under UV light. Bands in the 520bp region were considered positive for parasites.

Statistical Analyses. Data were analyzed using STATISTICA version 6.1 (Statsoft, 2003). All leukocyte count data and H:L ratios were $\log_{10}$-transformed to normalize the error variance. For each bird we calculated a size-corrected body condition index following (Owen and Moore, 2008a) by regressing weight (g) onto wing chord (mm) and extracting standardized residual values. Regressions were performed separately for each species; positive values denote birds that weigh more than expected given their body size (indexed by wing chord) and negative values denote birds that weigh less than expected. Condition indices were normalized to account for differences in magnitude across the three species (i.e., because Gray catbirds had a broader range of condition scores, we divided their scores by 3 to place them within the same range as the other two species). Analyses described below did not control for the effects of sample date, as all birds were captured over the same two-week time interval.

To examine whether species, age, H:L ratio, body condition or fat scores predicted haemoparasite infection status (0/1 based on PCR detection), we first used logistic regression, treating species identity and age as categorical predictors, and H:L ratio, body condition and fat score as continuous variables. Owing to sample size limitations, interaction effects were not included in the logistic regression model. Secondly, we conducted a multivariate analysis of variance (MANOVA) to determine whether our immune measures (total leukocyte counts, and individual counts of the three most common leukocyte types, excluding monocytes and eosinophils owing to their low numbers) were associated with species identity, age, and infection status (as categorical variables) and with body condition and fat scores (as continuous variables). In particular, energetic tradeoffs faced by animals might be evidenced by a negative association between leukocyte counts and body condition or fat scores. To account for potential differences in baseline leukocyte counts between species, we included the interaction effects between species identity and all categorical and continuous predictor variables. After testing for overall model significance we conducted univariate ANOVAs to determine which individual WBCs (lymphocytes, heterophils or basophils) contributed most strongly to significant effects, and used Bonferonni correction ($\alpha/4=0.013$) for multiple comparisons. After running the above analyses, poc-hoc student’s t-tests were completed to compare the difference in means between uninfected and infected birds on the species level.

Results

A total of 86 birds across the three focal species were sampled (28 COYE, 38 GRCA and 20 WPWA). Among all birds captured, a total of 13.95% (n=12) were infected with either *Hemoproteus* or

Plasmodium based on PCR detection, and most positive samples were from HY birds (8) relative to AHY birds (4). Because all birds that tested positive for parasites via PCR detection showed no signs of parasitemia on blood smears, we were unable to assign levels of parasite intensity. Of the birds that tested positive for blood parasites, 7 (25%) were common yellowthroats, 3 (7.9%) were gray catbirds and 2 (10%) were western palm warblers. The prevalence of haemoparasite infection measured here was generally lower than values reported for passerine birds (including the three focal species) sampled during the breeding season (Appendix C). Leukocyte profiles (including H:L ratios) of birds sampled here during the fall migration were within the ranges reported from other published studies of passerine birds sampled during the breeding season (Appendix B).

Logistic regression analysis provided no support for effects of any categorical or continuous predictor variables measured here, including species, age, body condition, fat score and H:L ratio, on hemoparasite infection status (Table 2.1). MANOVA analysis provided support for differences in leukocyte profiles (across all WBC measures) in relation to infection status, species, and the species*infection status interaction (Table 2.2). Univariate ANOVAs focused on individual WBC measures as dependent variables showed that species identity, species*infection, and infection status were all significant predictors of total WBC count, lymphocyte count and heterophil count (Table 2.3). These three measures were all higher in infected than uninfected birds for GRCA, but not for COYE or WPWA (Table 2.3 and Figure 2.3). This result was comparable to the post-hoc student t-tests, which showed that the differences in mean cell counts (total, lymphocyte, heterophil and basophil) between uninfected and infected birds were significant only for GRCAs (Appendix D).

Discussion

This study showed no evidence for differences in subcutaneous fat, body condition or hematological stress between hemoparasite-infected and uninfected birds across the three migrating songbird species. Moreover, we found no evidence to support a negative association between leukocyte measures and body condition or energy reserves as might be predicted by resource-based energetic tradeoffs during periods of active migration. We also found no evidence for a positive association between immune measures and body condition, as might be expected if birds in better condition overall are able to maintain higher levels of defense. Our results did show that for one species (Gray catbirds, which had the highest numbers of WBCs overall), birds with haemoparasite infections had higher total WBC counts, and higher counts of lymphocytes and heterophils than uninfected birds.

WBCs are an important aspect of the innate immune system, serving to phagocytize extracellular parasites and destroy infected host cells, and some WBCs further play a role in adaptive immunity (Clark et al., 2009; Figuerola et al., 1999; Ricklefs and Sheldon, 2007). Infection by a range of pathogens can cause increases in observed leukocyte levels, as demonstrated by prior ecological (Davis, 2004; Ricklefs and Sheldon, 2007) and clinical veterinarian research (Clark et al., 2009; Latimer et al., 1988). To the author’s knowledge, this is one of only a handful of studies to show higher WBC counts in sub-acute haemoparasite infected birds during migration, indicating that subclinical or latent infections can elicit long-term activation of innate immune defense (see also (Arizaga et al., 2009; Arizaga et al., 2010; Wojczulanis-Jakubas et al., 2012). Indeed, blood smear scans from our study showed no signs of haemoparasite infection, indicating that birds that tested positive for haemoparasites via PCR were not suffering from acute parasitemia. Thus, our data demonstrate that birds with subclinical blood parasite infections have higher leukocyte profiles than uninfected bird’s long-after initial infection; however the long-term costs of this immune activation are unknown.

The higher level of white blood cells in birds infected with blood parasites was found to be substantial in one species, Gray catbirds, but not in the two other species, Common yellowthroats or Western palm warblers. This difference could be caused by biological traits of species, additional infections, or the ability of some birds to better tolerate infection. For example, it could be that Common yellowthroats and Western-palm warblers are more tolerant to subclinical infections than Gray catbirds due to an inherent difference in immunology or foraging or behavioral ecology, and thus do not maintain high immune defense following initial blood parasites infections. It might also be that the presence of additional infections in one species (e.g., Gray catbirds) could have further elevated their immune response to blood parasites, although we did not record the presence of other infections here. Another possible explanation is that WBC counts were overall much higher in GRCAs relative to the other two species, allowing us to more readily detect differences in immune cell counts arising from infection status. Finally, Gray catbirds had the lowest levels of parasitism observed in this study, yet were the only bird to have higher WBC counts when infected. This result suggests that future work should examine why the most highly parasitized species, Common Yellowthroat, showed no effect of infection on WBC count.

We predicted that a decreased ability to procure or store energy reserves infection would result from infection by blood parasites (Wikelski et al., 2003). Despite the higher WBC levels in parasitized birds, we found no evidence that lower fat scores, lower body condition indices or higher stress levels in migrating songbirds predicted haemoparasite infection. However, it is important to note that all birds examined here were subclinically infected. Despite this, our results are consistent with a prior study showing that *Plasmodium* infection did not alter fat deposition or fat levels in migratory blackcaps (Arizaga et al., 2009) that were captured and later recaptured at a stopover site in northern Iberia. From the previous study, about 35% of the captured birds were infected with either *Haemoproteus* or *Plasmodium* based on PCR, but body condition, age and fat levels did not depend on parasite infection (Arizaga et al., 2009). Another study by the same researchers again found no effects of parasite infection on fat scores (Arizaga et al., 2010). Collectively, this work demonstrates that migrating with a blood parasite, especially in the absence of parasitemia, might not impede a bird’s ability to acquire the lipid reserves necessary for long-distance flight.

We also expected to find evidence in support of physiological trade-offs between immune defense and energy reserves, consistent with the idea that when resources are limited and in the presence of other demanding activities, such as long-distance flight, measures of immune defense might be reduced(reviewed by (Norris and Evans, 2000). Alternatively, we might have found that birds in better overall condition had both greater fat scores and higher numbers of WBCs, as expected if more robust individuals had greater resources to invest in immunity. However, our analysis showed no relationships between leukocyte profiles and fat levels or body condition in any species examined here. This finding diverges somewhat from previous work, where researchers showed positive associations between leukocyte counts and fat score or body condition in wild songbirds (Hatch et al., 2010; Owen and Moore, 2008a). In other work, Hasselquist et al. (2007) found that experimental long-distance flight in red knots (*Calidris canutus*) did not cause changes in cell-mediated immunity (when compared to control birds), which further suggests that migratory flight might not lower resources available for immune defense.

In sum, results from this study contribute to scientific understanding of the interactions between parasitism, avian physiology and migration. We found that that blood parasites induced a subclinical immune response that was evident in one of the three species of migratory birds, suggesting that further work should focus on the long-term implications of subclinical infections and chronically activated innate immune measures (WBCs) for migratory animals. Although migration is thought to be energetically costly, the absence of evidence for a tradeoff between immune defense and fat reserves could suggest that birds for which migration is a regular part of life can maintain stable physiological processes during these journeys (Jakubas et al., 2011; Jakubas et al., 2013). We found no effects of parasitism on fat reserves or body condition, although this does not preclude long-term implications of parasitism on migratory success or fitness. However, our lack of evidence for immune trade-offs should be interpreted with caution, as our small samples size might have limited our ability to detect underlying trade-offs that occur in these species. In summary, the results of this study provide a starting point for further exploration of physiological trade-offs during periods of increased energetic demand, and for understanding the consequences of parasitism during different periods of migratory species' annual cycles.

Acknowledgements

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Table 2.1: Logistic regression analysis of infection with a blood parasite (*Haemoproteus* or *Plasmodium);* using fat score, body condition index (residuals of weight regressed on wing chord) and stress (H:L ratio) as predictor variables.

Predictor variables	**df**	**Wald**	**p**
Species	2	1.163661	0.558875
Age	1	2.618551	0.105621
Fat	1	0.689286	0.406407
Body condition	1	0.281145	0.595952
Log H:L	1	0.241584	0.623064

Table 2.2: MANOVA results testing whether species identity, age, infection status, fat score, or body condition index were associated with leukocyte counts (Total WBC count, and counts of lymphocytes, heterophils, and basophils).

Predictor variables	**Wilk's λ**	**F**	**Effect df**	**Error df**	**p**
Species	**0.680**	**3.674**	**8**	**138**	**0.001**
Age	0.912	1.666	4	69	0.168
Infection Status	**0.739**	**6.102**	**4**	**69**	**0.000**
Fat	0.951	0.889	4	69	0.475
Body condition	0.875	2.466	4	69	0.053
Species*Age	0.954	0.408	8	138	0.914
Species*Infection Status	**0.603**	**4.963**	**8**	**138**	**0.000**
Species*Fat	0.935	0.585	8	138	0.789
Species* Body condition	0.885	1.090	8	138	0.374

Table 2.3: Univariate ANOVA results to examine associations between single WBC dependent variables and each dependent variable (following from MANOVA results in Table 2.2). Bonferroni correction was applied (α/4) with significance at the p<0.013 level.

		Log total WBC/FOV		**Log L/FOV**		**Log H/FOV**		**Log B/FOV**	
Predictor variables	**df**	**F**	**p**	**F**	**p**	**F**	**p**	**F**	**p**
Species	2	11.018	**0.000**	12.585	**0.000**	3.923	0.024	0.175	0.840
Age	1	0.659	0.419	0.056	0.814	3.153	0.080	2.989	0.088
Infection Status	1	11.142	**0.001**	10.327	**0.002**	10.813	**0.002**	2.478	0.120
Fat	1	0.031	0.861	0.010	0.921	0.491	0.486	2.686	0.106
Body condition	1	1.942	0.168	1.231	0.271	0.003	0.960	2.642	0.108
Species*Age	2	0.175	0.840	0.121	0.886	1.072	0.348	0.031	0.970
Species*Infection Status	2	7.435	**0.001**	7.057	**0.002**	5.323	**0.007**	4.603	**0.013***
Species*Fat	2	0.013	0.988	0.060	0.942	0.202	0.817	1.038	0.359
Species*Body condition	2	1.413	0.250	0.880	0.419	1.450	0.241	1.572	0.215

Figure legends

Figure 2.1: Map showing the location of the field sampling site. Sampling was completed on the south end of Jekyll Island, Georgia (31.015°,-81.432°) from October 7-21, 2012. Red dotted line represents southern limit of estimated breeding range and the blue dashed lines represents estimated wintering range.

Figure 2.2: Three focal species of this study. (left to right) Gray catbird, Common yellowthroat, and Western-palm warbler. Gray catbirds and Common yellowthroats breed in the northern United States and into Canada during the summer month, while the Western-palm warbler breeds typically only in small portions of the northern U.S. and mostly mid to southern Canada. All species winter in the Caribbean and Central America, and very rarely in the southern U.S. Photos taken by Dr. Richard Hall.

Figure 2.3: Differences in leukocyte measures based on infection status for the three focal bird species sampled here and examples of cell types as seen at 1000x under a compound microscope. (A) Total leukocyte counts, (B) Numbers of lymphocytes, (C) Numbers of heterophils and (D) Numbers of basophils. Abbreviations are as follows: GRCA= Gray catbird, COYE = Common yellowthroat, and WPWA=Western-palm warbler.

Figure 2.1.

Figure 2.2.

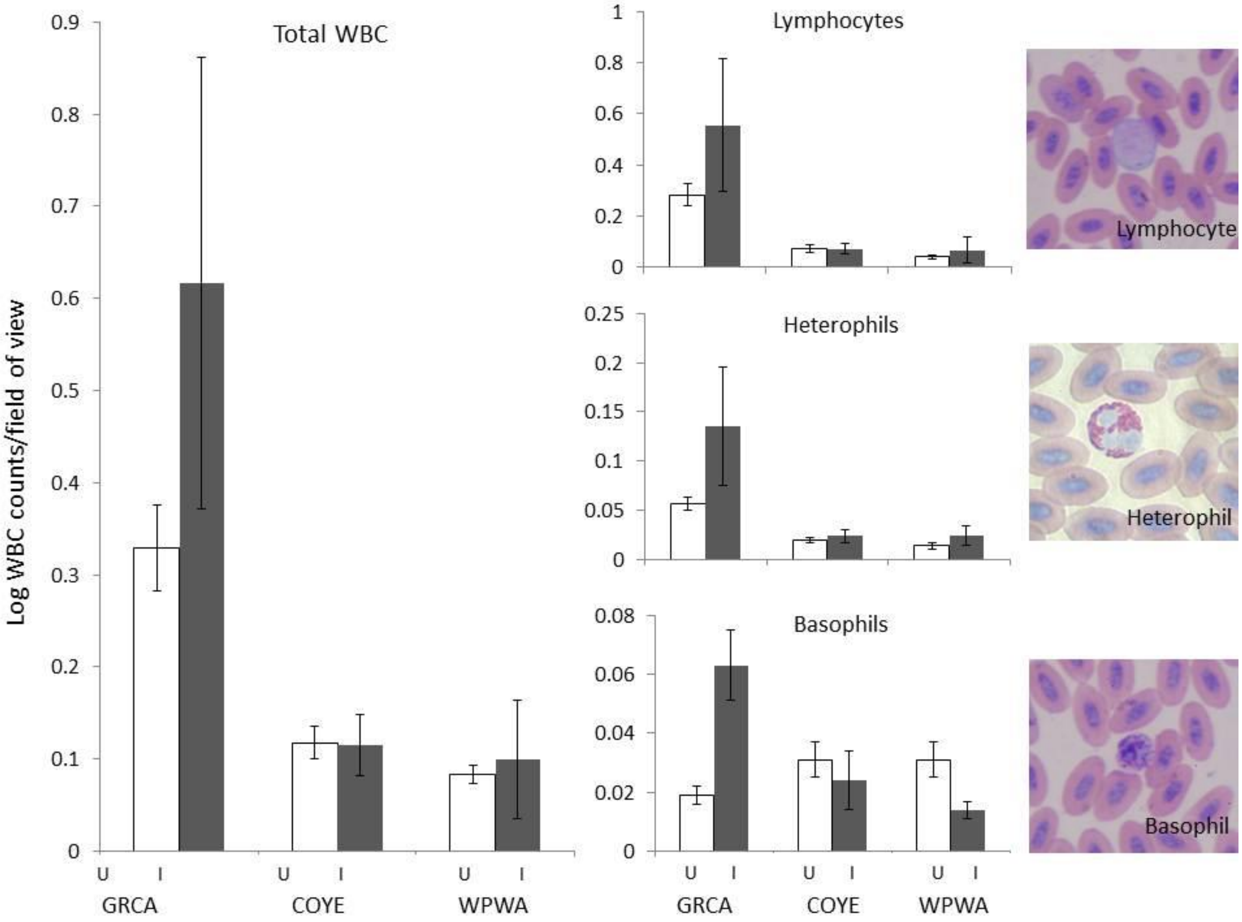
Total WBC
Log WBC counts/field of view
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
U
I
GRCA
COYE
WPWA
Lymphocytes
1
0.8
0.6
0.4
0.2
0
Lymphocyte
Heterophils
0.25
0.2
0.15
0.1
0.05
0
Heterophil
Basophils
0.08
0.06
0.04
0.02
0
Basophil

Figure 2.3

CHAPTER 3

MAJOR FINDINGS AND CONCLUSIONS

Migration is an energetically costly phenomenon that many animal species, including a large number of passerine birds, undergo annually. Here, I demonstrated that blood parasite infections can elevate immune profiles in one of three species of songbirds during their fall migration. This could represent a subtle cost of subclinical, latent infections for migrating animals, provided that defenses are costly in some way. Aside from elevated leukocyte profiles, other measures such as fat score, body condition and stress levels did not indicate infection status, suggesting that these subclinical blood parasite infections are not actively costly to other physiological systems (i.e., fat deposition). Alternatively, infections detected here were subclinical (in the absence of parasitemia), and it could be that certain physiological traits only respond to active infections. Further, as these blood parasite infections are found to be long-lasting and infect birds throughout their annual cycle, it could be that the virulence of these malarial blood parasites is relatively low to maintain transmission to other mosquitos (and birds), although other work has suggested that intermediate levels of virulence of blood parasites could be evolutionarily favored to maintain transmission (Mackinnon and Read, 1999). Regardless of the dynamics of these malarial parasites, long-distance migration is energetically expensive and could alter the investment in the physiological systems of these birds.

Although migration is physiologically demanding, very few studies have quantified the energetic trade-offs associated with infection and immunity that are faced by migrating songbirds. Those studies to date have generally found that birds infected with a pathogen delay the onset of migration and are able to fly shorter distances, and that there are indirect costs of infection in terms of elevated immune defenses. This study is in agreement with previous work, because our results showed that infected birds had higher levels of white blood cells, but also diverges from prior work, in that we found no other evidence for infected birds being in poorer condition. In addition to immune trade-offs with pathogen infection, there is a growing interest in the way that infectious diseases interact and respond to long-distance migration. Specifically, an interesting question is whether infection decreases an animal's ability to deposit fat, and therefore gain the fuel necessary to reach the wintering grounds. Studies completed to date have not been able to provide evidence for infection decreasing investment in other physiological systems, like fat deposition, including the study conducted here.

Results from this study showed that immune profiles did not appear to trade-off against other physiological functions like fat score or body condition. This result, combined with the observation that H:L ratios for birds sampled here were similar to levels reported for birds outside of the migratory season, might suggest that migratory species can undertake these long-distance journeys without becoming stressed or sacrificing physiological performance. An alternative explanation is that some animals in the poorest condition might have already perished earlier in the migration, as coastal Georgia is a mid-point along the birds' migratory journey, leaving only those birds in the best condition to complete the migration south.

Findings from this study point to several directions for future research. First, one goal is to expand field monitoring of birds at migratory stopover sites to examine a greater diversity of pathogens and multiple components of immune defense. Further, recording and making public the morphometric data (such as weight, wing chord and fat scores) will allow for more long-term studies of bird migration and the physiological changes over the annual cycle. Another avenue is to examine infection processes in birds throughout their annual migratory cycle, and consider the longer-term implications of parasitism and activated immune systems on migratory success and fitness. Our study was conducted at one site in coastal Georgia and therefore to make further conclusions about the energetics of migratory songbirds, more studies sites, over longer periods of time should be included. Conclusively, as more and more migratory songbird populations are declining, it will be important to outline how parasitism influences migration physiology and how the energetic demands of migration influence overall migration success.

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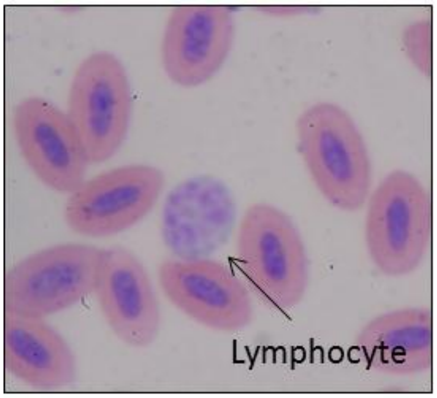
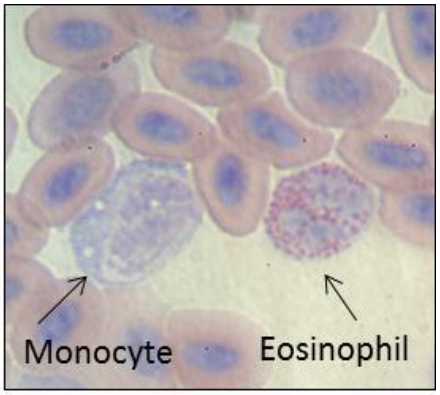
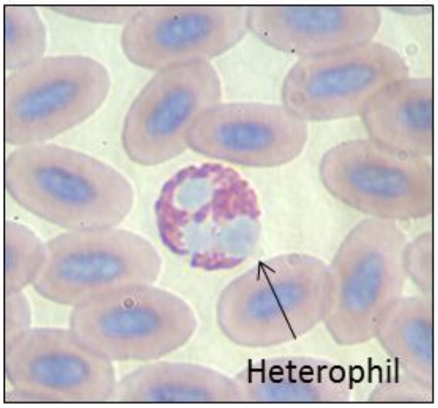
APPENDICES

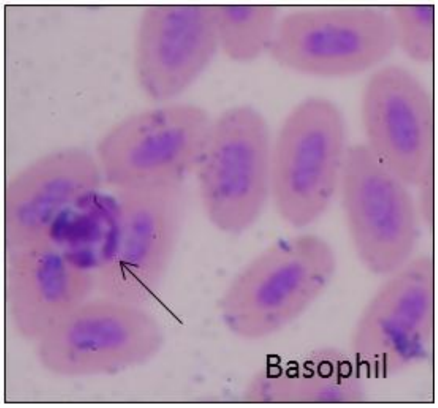
APPENDIX A- IMAGES OF EACH LEUKOCYTE CELL TYPE

Blood smears were scanned for all leukocyte types, including: lymphocyte, monocyte, eosinophil, heterophil and basophil (left to right, from top to bottom. All pictures are from gray catbird samples, except for the basophil which was from a common yellowthroat sample.



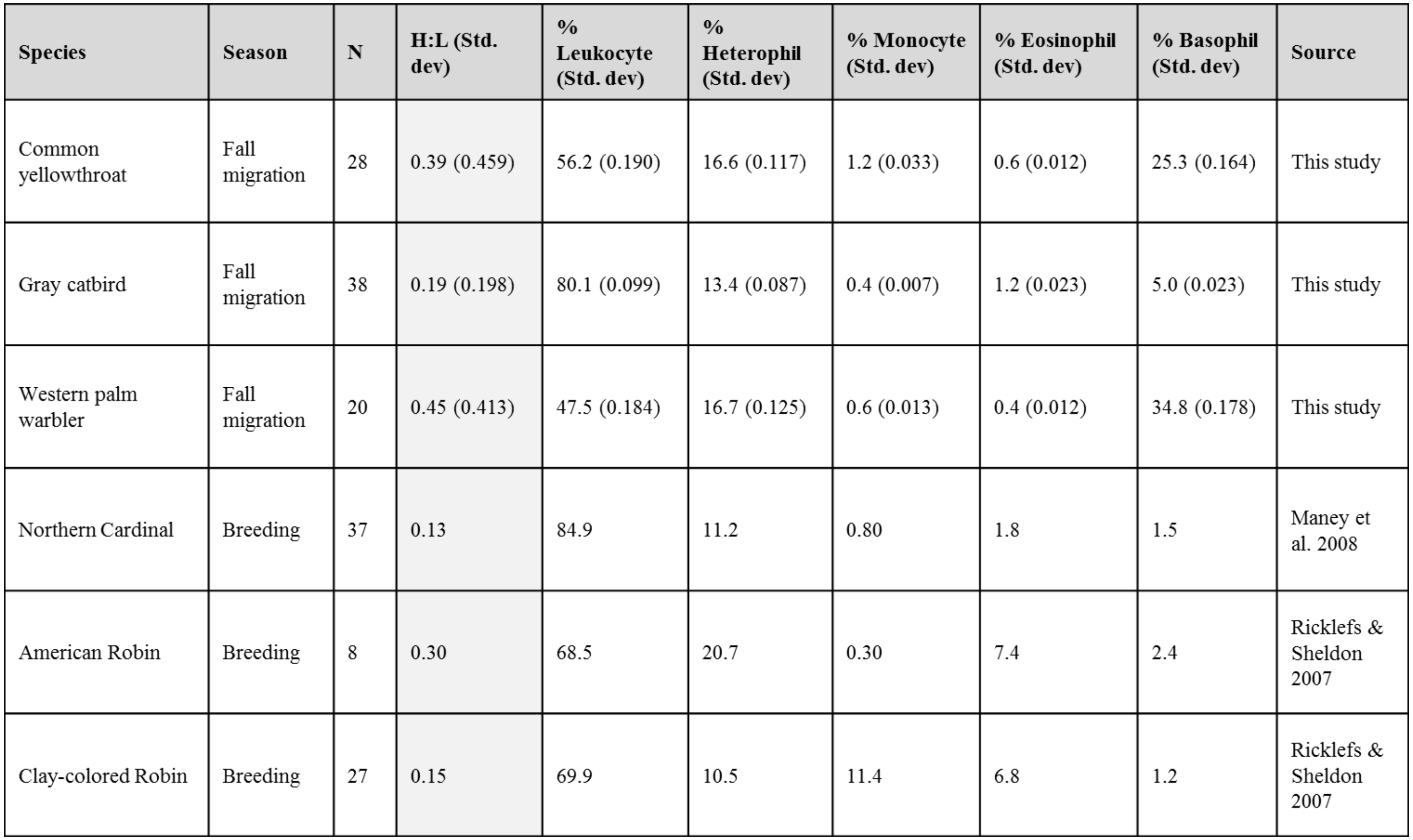






APPENDIX B- LEUKOCYTE PROFILES OF OUR THREE FOCAL SPECIES DURING MIGRATION VERSUS OTHER PASSERINES DURING THE BREEDING SEASON.

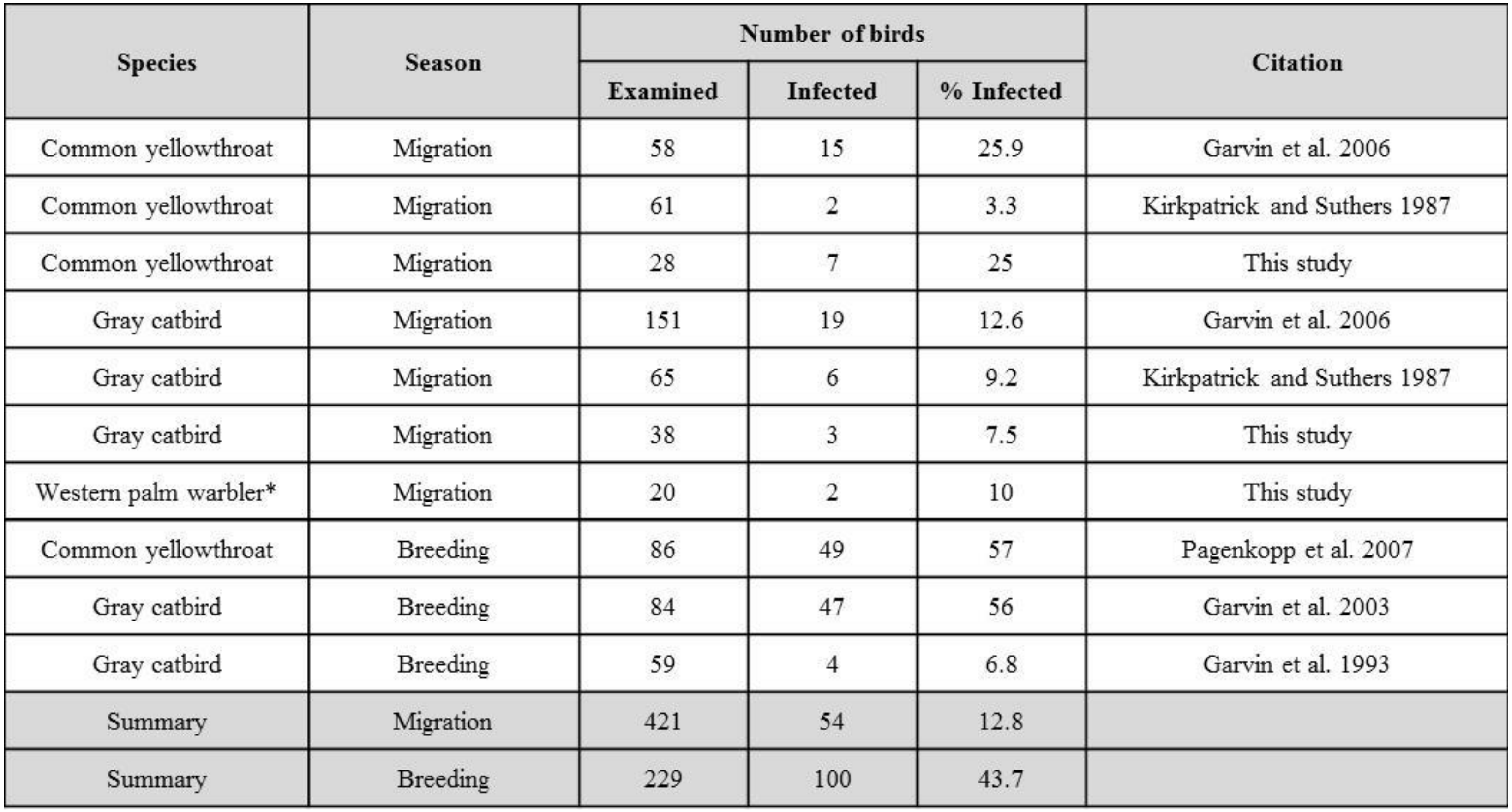
Leukocyte profiles of the three focal species in this study, captured during migration in Georgia. These are compared to published records of passerine species, captured during the breeding season. When comparing our sample species to that of other passerines during the breeding season, the H:L ratios fall within the same range, indicating that H:L profiles might be increased due to migration. Similarly, birds with a H:L ratio of below 1.0 is regarded by some to not indicate stress (Davis, Owen, communications).



Species	Season	N	H:L (Std. dev)	% Leukocyte (Std. dev)	% Heterophil (Std. dev)	% Monocyte (Std. dev)	% Eosinophil (Std. dev)	% Basophil (Std. dev)	Source
Common yellowthroat	Fall migration	28	0.39 (0.459)	56.2 (0.190)	16.6 (0.117)	1.2 (0.033)	0.6 (0.012)	25.3 (0.164)	This study
Gray catbird	Fall migration	38	0.19 (0.198)	80.1 (0.099)	13.4 (0.087)	0.4 (0.007)	1.2 (0.023)	5.0 (0.023)	This study
Western palm warbler	Fall migration	20	0.45 (0.413)	47.5 (0.184)	16.7 (0.125)	0.6 (0.013)	0.4 (0.012)	34.8 (0.178)	This study
Northern Cardinal	Breeding	37	0.13	84.9	11.2	0.80	1.8	1.5	Maney et al. 2008
American Robin	Breeding	8	0.30	68.5	20.7	0.30	7.4	2.4	Ricklefs & Sheldon 2007
Clay-colored Robin	Breeding	27	0.15	69.9	10.5	11.4	6.8	1.2	Ricklefs & Sheldon 2007

APPENDIX C- MIGRATION AND BREEDING SEASON HAEMOPARASITE PREVALENCE IN THE THREE FOCAL SPECIES

Summary of published records showing the prevalence of haemoparasites (*Haemoproteus* and *Plasmodium*) in the focal species studied here. These birds were determined positive by using microscopy methods, whereas our individuals were determined positive via polymerase chain reaction (PCR).



Species	**Season**	**Number of birds**			**Citation**
		Examined	**Infected**	**% Infected**	
Common yellowthroat	Migration	58	15	25.9	Garvin et al. 2006
Common yellowthroat	Migration	61	2	3.3	Kirkpatrick and Suthers 1987
Common yellowthroat	Migration	28	7	25	This study
Gray catbird	Migration	151	19	12.6	Garvin et al. 2006
Gray catbird	Migration	65	6	9.2	Kirkpatrick and Suthers 1987
Gray catbird	Migration	38	3	7.5	This study
Western palm warbler*	Migration	20	2	10	This study
Common yellowthroat	Breeding	86	49	57	Pagenkopp et al. 2007
Gray catbird	Breeding	84	47	56	Garvin et al. 2003
Gray catbird	Breeding	59	4	6.8	Garvin et al. 1993
Summary	Migration	421	54	12.8	
Summary	Breeding	229	100	43.7	

* Western palm warbler is a subspecies of Palm warbler

APPENDIX D- STUDENT'S T-TEST FOR COMPARISON OF INFECTION STATUS BY SPECIES IDENTITY

Results from the MANOVA revealed that species identity and the species*infection status interaction were significant. Therefore poc-hoc student t-tests were completed to determine whether the immune measures (all four response variables) were significantly different between infected birds (1) and uninfected birds (0), for each species.

Species	Response variable	Mean 0	Std.dev. 0	Mean 1	Std.dev. 1	t-value	df	p	F-ratio	P variances
Common yellowthroat (n=28)	Total WBC	0.114	0.058	0.111	0.062	0.122	26	0.904	1.160	0.731
	Lymphocyte	0.071	0.053	0.070	0.047	0.029	26	0.977	1.256	0.832
	Heterophil	0.020	0.015	0.023	0.017	-0.545	26	0.590	1.276	0.625
	Basophil	0.030	0.024	0.024	0.023	0.619	26	0.541	1.050	1.000
Gray catbird (n=38)	Total WBC	0.310	0.130	0.602	0.134	-3.719	36	0.001	1.065	0.712
	Lymphocyte	0.266	0.123	0.534	0.173	-3.515	36	0.001	1.963	0.312
	Heterophil	0.056	0.035	0.130	0.080	-3.206	36	0.003	5.419	0.018
	Basophil	0.023	0.018	0.063	0.019	-3.791	36	0.001	1.152	0.656
Western-palm warbler (n=20)	Total WBC	0.083	0.037	0.089	0.061	-0.289	20	0.776	2.723	0.153
	Lymphocyte	0.041	0.026	0.060	0.050	-1.061	20	0.301	3.605	0.070
	Heterophil	0.014	0.011	0.022	0.011	-1.256	20	0.224	1.170	1.000
	Basophil	0.031	0.024	0.012	0.005	1.523	20	0.143	24.725	0.022

APPENDIX E-THE WORMSLOE HISTORIC SITE AND JEKYLL ISLAND: DIFFERENCES IN HABITATS AS LOCATIONS FOR BANDING STATIONS

INTRODUCTION

The purpose of this appendix is to inform researchers and stakeholders about the differences in our study sites and how those differences might have influenced songbird capture ability. We faced difficulties when attempting to mist net songbirds during migration at the Wormsloe Historic site. At a nearby site, Jekyll Island, trapping was much more efficient, largely owing to site characteristics.

THE WORMSLOE HISTORIC SITE

Figure 1 & 2: (left and right) Northern-most part of the Wormsloe property (privately owned). The underbrush is much thicker here than the salt-marsh areas of the property.

The Wormsloe Historic Site (WHS) is located on the coast of Savannah, GA just north of Skidaway Island, GA. WHS is an undisturbed, and until recently, privately owned properties in southern Georgia. It is also one of the oldest of Georgia’s tidewater estates. In the past, WHS has served as a silkworm plantation, as well as other agricultural functions. WHS would be characterized as a maritime climax forest. The majority of the tree species are mature live oak and southern magnolias. Undergrowth is dominated by saw palmetto plants.

All nets were placed about 200m from the researcher’s cabin. Netting areas did not extend any further than the drainage ditch area (between the cabin and the library). Nets were equally distributed near the marsh and closer to the main allay. Eleven mist nets were operated for the majority of the season.

WHS is in the prime location to serve as a ‘stopover’ habitat for migrating songbirds. While there appeared to be many migratory species on the property (Northern parulas, common yellowthroats, gray catbirds, palm warblers, etc.), many of these birds were captured in small quantities. This result may be due to the lack of undergrowth near the marsh areas or because birds are stopping at habitats that are more coastal (such as Skidaway Island) or because birds are utilizing the higher canopy areas. Further research should be completed to examine the property for point counts and migratory bird surveys.

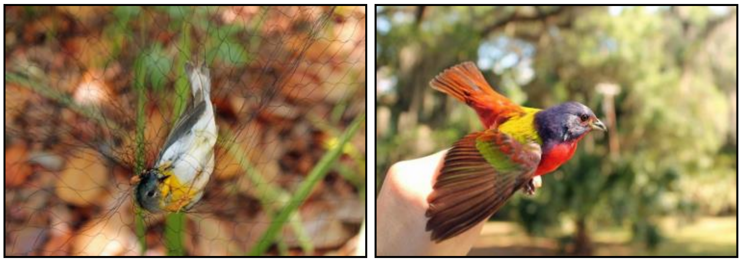


Figure 3 & 4: Northern parula caught in mist net and Painted bunting (left and right) caught at WHS in 2012.

JEKYLL ISLAND BANDING STATION

Figure 1 & 2: (left and right) Northern section of beach on Jekyll Island and the banding station set-up with the surrounding undergrowth on the southern section of beach.

Jekyll Island, GA is one of four Georgia barrier islands and is about 5,700 acres. The Jekyl Island Banding Station (JIBS) has been located on both the north and south ends of the island, but is currently being operated at the southern end. This banding station has been operating since 1978, by a group of volunteers. JIBS opens for around three weeks every fall (usually in October).

The southern end of Jekyll would be characterized as a primary dune ecosystem. The soil was almost complete sand and was located in between two marshes and the ocean at the southern-most end. The area had no upper canopy that overshadowed the station, and had a large amount of undergrowth throughout. This past fall, the station had 18-23 mist nets of varying lengths open for the majority of the season.

JIBS is known for the large number of migratory birds that they capture each fall, usually in the thousands. This station also catches large numbers of a few species including: gray catbirds, common yellowthroats, and palm warblers. The productivity of this station is probably due to its location right on the Atlantic flyway and the island-habitat it provides (water and undergrowth-which migratory birds seek).

Figure 3 & 4: Black-and-white warbler and Common yellowthroat (left and right) caught at JIBS in 2012.

CONCLUSION

While both areas are located in coastal Georgia, there are distinct differences between the habitats of both sites. These differences in vegetation, and potentially the distance inland, could be driving the ability to capture migratory songbirds on the properties. In both areas the same species of migratory songbirds were captured, pointing towards the potential for each site to be used during stopover, but the quantity of each bird species caught varied. Therefore, while WHS has the potential to be used as a stopover site by migratory songbirds, the differences in each habitat might be driving either the ability to capture or the total numbers of birds that utilize this area.