IMPORTANCE OF NUTRITIONAL STATUS OF PASSERINES TO IMMUNITY AND DISEASE DYNAMICS

By

Kimberly R. Fake

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ABSTRACT

IMPORTANCE OF NUTRITIONAL STATUS OF LANDBIRDS
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Host nutritional status has been long established as a mediator of host-pathogen interactions across taxa. Nutritional status of landbirds is largely determined by environmental conditions and can vary between individuals. Many landbirds by virtue of migratory movements are exposed to a variety of pathogens. Furthermore, landbirds also face conditions of limited resources and high competition, which can impact their ability to acquire nutrients. Here I explore how the host-pathogen interactions and subsequently disease dynamics among landbirds may be affected by host nutritional status. First, we review disease dynamics and effects of host nutrition on the host-pathogen interaction, highlighting the importance of exploring impacts of host nutritional status to disease dynamics among migratory landbirds. Second, we present a foundational study of the impact of host nutritional status, measured as energetic condition, on constitutive immune function of American robins (*Turdus migratorius*), a passerine which acts as a reservoir for multiple zoonotic pathogens. Together these chapters provide an ecoimmunological and conservation medicine perspective on the importance of nutritional status of passerines to immunity and disease dynamics.

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KEY TO ABBREVIATIONS

PEM	protein-energy malnutrition
PUFA	Polyunsaturated Fatty Acid
IL	Interleukin
TNF	Tumor Necrosis Factor
PRRs	Pattern Recognition Receptors
PAMPs	Pathogen Associated Molecular Patterns
ROS	Reactive Oxygen Species
cm	Centimeter
d	Days
wks	Weeks
C	Celsius
AUF	Animal Use Form
H:L	Heterophil Lymphocyte Ratio
WBC	White Blood Cells
RBC	Red Blood Cell
PCA	Principal Components Analysis
PC	Principal Component
IACUC	Institutional Animal Care and Use Committee
MI	Michigan

CHAPTER 1: IMPORTANCE OF STOPOVER REFUELING TO DISEASE DYNAMICS AMONG MIGRATING LANDBIRDS: AN ECOIMMUNOLOGY AND CONSERVATION MEDICINE PERSPECTIVE

Abstract

Movements during migration expose landbirds to a variety of pathogens. Birds also face limited resources and high competition during stopover, which can impact the ability of migratory birds to refuel and maintain optimal nutritional status. Nutritional status can affect the host-pathogen interactions and disease dynamics among migrating landbirds, ultimately impacting their fitness and survival. Furthermore, birds act as vehicles for transporting zoonotic pathogens and parasites during migration. Impaired immune function due to less than optimal nutrition may result in increased susceptibility to infection and disease, decreased pathogen resistance and tolerance, and increased transmission to susceptible hosts, including humans. Here, I review disease dynamics, effects of host nutrition on host-pathogen interactions, and highlight the importance of exploring the impacts of host nutritional status on disease dynamics among migratory landbirds.

Introduction

Energetic demands are high for birds during migration. To meet these demands, birds in migratory disposition become hyperphagic and can increase their lean body mass by 30%–50% in only a few days of foraging (Berthold 1975). Birds store energy primarily as fat in preparation for migration and often deplete these stores during bouts of migratory flight (Bairlein and Simons 1995; Blem 1990). To balance the energetic costs of migratory flights landbirds must periodically stop to forage and refuel their energy stores. The need to stop and refuel during migration poses a challenge for these birds, as stopover sites vary in quality and availability of food (Bibby and Green 1980; Moore 1995). Additionally, once birds reach a stopover site, they must compete for

resources (Moore and Yong 1991; Rappole and Warner 1976) and defend themselves against infection with pathogens (Møller and Erritzøe 1998). Migratory birds likely have higher exposure to pathogens than resident birds by virtue of their migratory movements, which cause them to interact with more populations of organisms and environments (Møller and Erritzøe 1998). Furthermore, studies have shown that stress associated with migration can cause recrudescence of infections (Applegate and Beaudoin 1970; Gylfe et al. 2000).

An animal's primary defense against pathogens is its immune system (Zuk 1994), which is comprised of both constitutive (expressed prior to an infection) and induced (expressed only in response to infection or tissue damage) immunity (Buehler et al. 2010a; Kennedy 2010). The dependence of immunity on nutritional status has been well established in humans and domestic animals. Generally, individuals with poor nutritional status have lower immune function and experience a higher incidence of infection and disease-related mortality (Cook 1991; Klasing 1998a; Klasing 2007; Møller et al. 1998). It remains comparatively less well understood how nutritional status affects the immune system of landbirds (birds that primarily use terrestrial habitats). Despite the evidence of limited food and high competition for resources at stopover (Newton 2004), impacts of nutrition on immune defenses and disease resistance of landbirds during migration have not been thoroughly studied. Moreover, many of the studies on the immune function of landbirds have primarily focused on the breeding season (Norris and Evans 2000; Sheldon and Verhulst 1996).

Land-use changes are believed to be causing declines in migrating bird species due to the loss and deterioration of stopover habitat (Newton 2004; Robbins et al. 1989). As suitable stopover sites diminish, birds may have greater difficulty meeting nutritional needs for maintaining optimal immune defenses. This may ultimately affect disease dynamics among migratory landbirds and

disease risk for landbirds, as well as other animals and humans. Here I review disease dynamics, nutritional modulation of the interaction between hosts and pathogens, nutrition of migratory landbirds, and call attention to the lack of studies that examine the nutritional effects on immunity and pathogen resistance of migrating landbirds.

Disease Dynamics

Disease dynamics are frequently illustrated using epidemiological models called SIR models. SIR models are compartmental models whereby individuals in a host population are classified depending on their infection status (Figure 1.1Figure 1.2. Epidemiological Venn diagram.). A basic model includes three compartments (state variables): susceptible (S), infected (I), and recovered (R). The rate at which individuals in a population move from the susceptible compartment to the infected compartment is the transmission rate (β), while the rate at which individuals move from the infected to the recovered compartment is the recovery rate (γ). Other parameters in the model include the population birth rate (b), non-pathogen related death rate (d), and pathogen-related death rate (d'). SIR models help predict the spread of a pathogen through a population over time. For a particular host-pathogen system, these models can also help demonstrate how changes in mortality rate, recovery rate, and transmission rate will affect the spread of disease. For example, these models can help us understand quantitatively how an increase in the infectious period, reduction in mortality rates of infectious individuals, or an increased rate of transmission will increase the probability of a disease outbreak. This is done using differential equations. These equations differ between density-dependent transmission models, which assume the contact rate between susceptible and infected individuals depends on the population density, and frequency-dependent transmission models, which assume the contact rate between susceptible and infected individuals does not depend on the population density (Anderson

and May 1991; Keeling and Rohani 2011).

Environmental differences can modify host-level factors in ways that are epidemiologically important (Hawley and Altizer 2011; Lloyd-Smith et al. 2005; Lloyd 1995). For instance, changes in stopover sites could cause changes to host-level factors, (e.g., susceptibility to infection, infectiousness, and recovery period) ultimately altering disease dynamics among migratory landbirds. Currently, disease models tend to assume homogeneity among susceptible individuals, meaning all susceptible individuals are assumed to be equally susceptible to infection, and all infected individuals are considered to be equally infectious. Illuminating the causes of individual variation in vulnerability to infection and infectiousness may help improve disease models, allowing more accurate predictions of the outbreak and spread of diseases. Studying how environmental factors influence host-pathogen interactions is necessary to understand how environmental changes will influence disease dynamics and to make better predictions of the spread of disease.

Host-pathogen Interactions

Disease results from the interaction between hosts, pathogens, and the environment (Figure 1.2). Disease can be mitigated by disrupting the links between one or more of the three factors (Mpolya et al. 2009). A common target is the host-pathogen interaction, which can vary greatly depending on the specific host and pathogen. Here I discuss variation within and among host species that can affect host-pathogen interactions, and how this variation can subsequently affect the spread of disease.

Host competence

Birds vary in host competence, the ability to sustain an infection and transmit it to susceptible individuals (Komar et al. 1999; Komar et al. 2003). Host competence can be measured as the

product of three factors: susceptibility, mean daily infectiousness, and the duration of infectiousness (Komar et al. 2003). The term "susceptibility" has been used throughout the literature to refer to either susceptibility to infection or susceptibility to disease. In the case of host competence, susceptibility refers to susceptibility to infection. Susceptibility to infection is measured as the proportion of susceptible hosts that will become infected given exposure. Mean daily infectiousness refers to the proportion of exposed susceptible individuals that will become infected per day (Komar et al. 2003). Pathogen load affects transmission as individuals with greater pathogen load have a higher probability of transmitting the pathogen to a susceptible host (i.e., mean daily infectiousness). Lastly, infectious period affects host competence, as individuals that remain infectious longer have more time to encounter susceptible hosts and transmit the pathogen (Komar et al. 2003). The infectious period may vary based on the time it takes a host to clear an infection, or it may be affected by mortality. If a host can clear an infection quickly relative to another host, its host competence is comparatively lower. Alternatively, a host may die from disease induced by the pathogen. In this case, the sooner the infectious individual dies, the shorter its infectious period and the lower its host competence. Lastly, it is important to note that reservoir competence is not universal, but pathogen-specific. For instance, a host species may have high competence for pathogen "A", but low competence for pathogen "B".

Interspecific and Intraspecific Variation in Host Competence

There is both inter- and intra-specific variation in host competence of birds. Interspecific variation is highlighted in the dilution effect and amplification effect hypotheses. The dilution effect describes a decrease in disease risk with increased biodiversity, due to the introduction of competent or less competent hosts to a disease system (Keesing et al. 2006). The amplification hypothesis states the opposite effect, that is, lower biodiversity will result in greater disease risk

(Keesing et al. 2006).

Intraspecific variation is best demonstrated by the presence of superspreaders. Superspreaders are individuals who contribute far more to secondary infection cases (i.e., those infections that occur due to transmission from the initial infected individual) than other individuals in a host population. It is recognized for many diseases that a small portion of the population (~20% of infected individuals) can be responsible for a large proportion (~80%) of new infections (Anderson and May 1991; Woolhouse et al. 1997). Intraspecific variation in host competence can be due to behavioral, physiologic, or immunologic differences between individuals that affect contact with an infected individual or vector, susceptibility to infection, pathogen amplification, or recovery rate. For example, animals that are less social within a population may have a lower susceptibility to infection due to a reduced chance of interacting with an infected individual. Hosts may vary in the number of cell receptors a virus can utilize for infection, leading to differences in cellular infection and viral shedding. Lastly, immunologic differences between hosts can lead to differences in susceptibility to infection, susceptibility to disease, infectious period, pathogen load, or pathogen clearing.

Resistance and Tolerance

Hosts have two different, although not mutually exclusive, strategies for responding to an invading pathogen: resistance and tolerance. Tolerance refers to the ability of a host to limit the effects of the pathogen on its fitness (Roy and Kirchner 2000); Figure 1.3. Each line represents the relationship between pathogen load and fitness for a group of hosts. (A) demonstrates a difference intolerance, while (B) demonstrates a difference in resistance. In each case, the group of organisms represented by the dashed line has higher tolerance/resistance than the group of organisms represented by the solid line.). Biological fitness, an organism's ability to survive and reproduce,

is often evaluated in ecological studies by measuring body mass, lifespan, reproductive output, and disease severity (Baucom and de Roode 2011). The host may utilize multiple mechanisms to limit the impact of infection on the host. Hosts may be more capable of and efficient at repairing damage to host cells or processes induced by infection. Collateral damage to host cells or processes by the host immune response may be limited. Host immune responses may not target the pathogen specifically, but pathogen produced factors that damage the host such as toxins (Råberg et al. 2009).

Resistance refers to the ability of the host to prevent or limit pathogen replication (Roy and Kirchner 2000); Figure 1.3. Each line represents the relationship between pathogen load and fitness for a group of hosts. (A) demonstrates a difference intolerance, while (B) demonstrates a difference in resistance. In each case, the group of organisms represented by the dashed line has higher tolerance/resistance than the group of organisms represented by the solid line.). When comparing tolerance and resistance between two groups of hosts, tolerance is measured as the slope of the relationship between pathogen load and fitness (the steeper the slope, the lower the tolerance), while resistance can be measured as the inverse of mean pathogen load (Figure 1.3). It is important to note that resistance strategies can reduce the impacts of infection on the host's fitness, but they do so by limiting the pathogen load (Schneider and Ayres 2008). A reservoir host, a species essential for the maintenance and transmission of an infectious agent (Aguirre et al. 2012), typically uses tolerance strategies when interacting with a pathogen, but its resistance will affect pathogen load and subsequently its host competence.

Nutritional Effects on the Host-Pathogen Interaction

Host nutrition can modulate the host-pathogen interaction through a variety of mechanisms. Host nutrition can (1) affect nourishment of the host's immune system cells (2)

modify the response of leukocytes (3) stimulate the immune system (4) protect the host from disease induced by the host's immune response, and (5) affect environment and resources for the pathogen, and subsequently pathogen growth and virulence (Figure 1.4) (Klasing 2007; Schaible and Kaufmann 2005; Smith et al. 2005). The effects of nutrition on the host-pathogen interaction are too vast and complex to be discussed in their entirety within this review, so only a general overview will be provided below; however, there are several reviews that provide an excellent survey of the subject (Klasing 2007; Kogut and Klasing 2009; Schaible and Kaufmann 2005).

Effects of Host Nutritional Status on the Host

Nutrition mainly affects the host-pathogen interaction through modulation of the immune system (see Appendix B for an overview of the avian immune system). Nearly all nutrients in the diet can affect immune function. Diets need to be balanced to ensure calories and nutrients are neither deficient nor in excess for optimal functioning of the immune system to be reached (Kogut and Klasing 2009). It is important to note that, currently most of the knowledge regarding effects of host nutritional status on host defenses, and thus shared within this review, come from studies conducted in mammalian and domestic fowl species.

Undernutrition, often referred to as protein-energy malnutrition (PEM), is defined as a lack of food (which can be either mild or severe in the case of starvation) characterized by mass loss. (Bistrian et al. 1977; Woodward 1998). Undernutrition suppresses innate and adaptive immune responses proportional to the severity of undernutrition (Gershwin et al. 2004). For example, Lymph organs (e.g., the spleen and thymus), which are involved in lymphocyte production and maturation, atrophy under undernutrition conditions. Innate non-specific immunity, in the form of physical and chemical barriers of the immune system, is also negatively affected by undernutrition. For example, mucosa atrophies and gastric secretions are reduced. In cases of severe

undernutrition, complement factors can be reduced, thereby negatively impacting non-specific immunity via impaired of complement activity. Cell-mediated immunity, such as bactericidal activity and the respiratory burst produced in heterophils, can be attenuated in cases of undernutrition. The number of B-cells tends to be unaffected by undernutrition; however, immunoglobulin A in ocular secretions and saliva are depressed. With this immunosuppression resulting from the inadequate food supply, is an increase in the probability of infection, and morbidity and mortality from infection. Furthermore, there can be a negative cycle that occurs with undernutrition and infection. For instance, when infection occurs, energy demands increase to support immune defenses and the host may lose its appetite. This can exacerbate the undernutrition leading to further reduction in host immune defenses in a negative cycle (Gershwin et al. 2004).

Specific nutrients can also modulate the functioning of the immune system based on their quantity and quality. Macronutrients such as protein and fat can influence the immune system. Protein is a crucial nutrient to immune function as immune responses are often characterized by an increase in cellular replication and production of proteins such as immunoglobulins, cytokines, and acute phase proteins. In cases where protein is deficient, the numbers of white blood cells decrease in bone marrow and circulation, the function of natural killer cells is reduced, the acute phase response is weakened, and circulating IgG is reduced (Gershwin et al. 2004). Protein deficiencies in migrating landbirds, however, may not be common as birds increase food intake when fed a low protein diet (Aamidor et al. 2011).

Although protein has traditionally received more attention, fatty acids can also be an important immune modulating macronutrient. Essential fatty acid deficiencies can lead to reduced T-cell function and reduced antibody production (Hwang 1989). Cell membranes of immune cells contain fatty acids. The ratio of fatty acids in the diet directly influence the make-up of these

cellular membranes and ultimately affect the communication between immune cells (Klasing 1998a). Studies of the effects of dietary fat on immunity have focused primarily on saturated versus unsaturated fatty acids, as well as ratios of omega-3 to omega-6 fatty acids. High polyunsaturated fatty acid (PUFA) diets are immunosuppressive, as PUFAs are anti-inflammatory and reduce the responsiveness of lymphocytes to antigens (Klasing 2007). Furthermore, omega-3 to omega-6 PUFA ratios are important, as omega-3 PUFAs are relatively more anti-inflammatory than omega-6 PUFAs (Klasing 2007). A diet high in omega-3 fatty acids can downregulate production of Interleukin 1 Beta (IL-1β) and Tumor Necrosis Factor alpha (TNF- α), cytokines that are important for inducing of an inflammatory response. Omega-3 fatty acids, can also selectively decrease some functions of lymphocytes, neutrophils, and macrophages (Gershwin et al. 2004). Thus, a diet high in PUFAs, particularly omega-3 PUFAs, can suppress defenses against pathogens for which a strong inflammatory or cell-mediated response is key (Klasing 2007). Micronutrients, such as vitamins A, D, E, zinc, and selenium, can also modulate the immune system. Deficiencies in any of these nutrients can negatively impact innate, cell-mediated, and humoral immune function. For example, vitamin A deficiency reduces leukocytes, the mass of lymphoid tissues, complement activity, T-cell number, NK cells, and IgG and IgE. For many micronutrients, supplementation beyond that which alleviates deficiency results in increased immunity. For instance, vitamin E supplementation results in increased lymphocyte proliferation, phagocytosis, antibody levels, and helper T-cell activity (Gershwin et al. 2004).

It is important to note that upregulation is not always in the best interest of the host, as in some cases the host immune system responses to a pathogen damage the host. Disease produced as a consequence of the host immune system is referred to as immunopathologic. During an inflammatory response, immune cells such as heterophils, produce and release reactive oxygen

species (ROSs) which severely damage pathogen membranes (Davison et al. 2008; Klasing 2007). ROSs, however, can damage host cell membranes and DNA (REF). Certain nutrients can, however, be anti-inflammatory or prevent oxidative damage to host cells caused by reactive oxygen species, limiting immunopathological effects (Klasing 2007). For example, as previously discussed omega-3 PUFA are anti-inflammatory. Thus, in cases where the host inflammatory response induced by a pathogen causes damage to host cells, a diet high in omega-3 PUFAs may be beneficial to limiting disease and creating tolerance to infection (Klasing 1998a).

General nutritional status of migratory landbirds is often measured in terms of energetic condition, a body size-corrected measure of energy stores (primarily as fat). Energetic condition has been shown to positively correlate with immune function of birds. For example, migrating landbirds in poor energetic condition (characterized by low fat and muscle stores) had poorer constitutive immune function than good condition conspecifics captured during spring stopover (Buehler et al. 2010a; Owen 2004; Owen and Moore 2008). Likewise, in a study in which Swainson's thrushes (*Catharus ustulatus*) and wood thrushes (*Hylocichla mustelina*) were held in captivity during stopover and fed *ad libitum*, birds gaining mass had a stronger inflammatory and cell-mediated immune response relative to birds that did not gain mass (Owen and Moore 2008). Red knots (*Calidris canutus*) arriving at a staging area in the Delaware Bay had lower constitutive immune measures compared to individuals departing the area, which suggests that as birds recover their condition at stopover habitat, their immune function improves (Buehler et al. 2010a). Similarly, Owen (2004) found that leukocyte count and cell-mediated immune responses of landbird migrants improved during stopover, regardless of a change in energetic condition. Constitutive immune function of European starlings was reduced following a 1-4hr flight in a wind tunnel and then recovered following two-days of rest and forage (Nebel et al. 2012a). It is

important to note that increased condition does not always lead to a positive outcome on disease risk. For example, Arsnoe et al. (2011) found that high body condition mallards in better body condition (maintained at capture mass) infected with avian influenza (a zoonotic pathogen) shed more virus than those in poorer body condition (maintained -20% of capture mass). Therefore, it is important that studies investigating the effects of host nutrition on host-pathogen interactions are conducted in a variety of landbird-pathogen disease systems.

These studies demonstrated the effects of energetic condition on host immune defenses, while subsequent studies have characterized the effects of nutritional status in terms of the quantity, quality, and ratios of specific nutrients acquired during migration. A recent explosion of studies investigating the impacts of dietary fatty acids and antioxidants on the health of migratory landbirds has occurred, yet these studies often do not examine the implications for host immunity, or, if they do, they lack experimentation under conditions of infection with a pathogen. It is critical that studies exploring the effect of migratory landbird nutrition to pathogen resistance do so under conditions of an active infection, as host nutrition affects not only the host but also the pathogen.

Effects of Host Nutritional Status on Pathogens

The host is essentially the environment in which a pathogen resides and, thus, host nutritional status can affect growth and pathogenesis of microorganisms. Host nutrition can affect nutrient availability to the pathogen. In some cases, pathogens may directly compete with hosts for nutrients. For example, pathogens and hosts compete for iron as it is an essential growth factor for many bacteria and parasites but also required for a variety of host functions including the production of reactive oxygen intermediates and reactive nitrogen intermediates (Schaible and Kaufmann 2005). Host nutrition can also affect a pathogens environment in ways that alter its ability reproduce or cause disease. The virulence of several viruses, including Coxsackievirus

and influenza A virus, have been shown to change in response to a nutrient deficiency of the host. The deficiencies lead to alteration of the viral genome that is thought to be brought on by increased oxidative stress of the host (Beck 2001). Moreover, Cornet et al. (2014) demonstrated that low host nutritional status was shown to increase the replication and virulence of *Plasmodium relictum,* the agent of avian malaria in domestic canaries, providing an excellent example of this type of host-pathogen interaction in a bird disease system with conservation relevance. Effects of nutritional status of migratory landbirds on pathogen growth and virulence are understudied.

Landbird Nutrition During Stopover

Birds must rely on energy stores (mainly in the form of fat but also protein) to fuel migratory flights and stopover periodically to rapidly replenish energy stores and nutrients before the next bout of flight (McWilliams et al. 2004). Food abundance and quality at stopover can affect refueling rates (Pierce et al. 2004) and subsequently the speed at which migration is completed (Lindström 1991, 2003). Furthermore, stopover itself is energetically costly, as it has been estimated that birds expend twice as much energy during stopover compared to migratory flight (Hedenström and Alerstam 1998; Wikelski et al. 2003). Birds must manage energy budgets to account for energy usage to sustain migratory flights as well as for foraging during stopover (McWilliams et al. 2004).

During migration, birds must select foods from the array available at stopover and do so based on a variety of characteristics. Birds are known to preferentially select foods based on caloric content (Johnson et al. 1985; McPherson 1987; Sorensen 1984), concentration of nutrients (Denslow 1987; Jung 1992; Levey 1987; Stiles 1993), and antioxidant content (Alan et al. 2013; Bolser et al. 2013; Catoni et al. 2011; Catoni et al. 2008), presumably to maximize

profits from foraging (Lepczyk et al. 2000). Dietary preferences of birds differ between species and individuals within a species (Levey and Rio 2001). During autumn migration, birds make an adaptive shift in feeding preferences and increase utilization of nutrients to support fattening during migration (Bairlein 1996; Bairlein and Gwinner 1994; Berthold 1976; Herrera 1984; Klasing 1998b; Levey and Karasov 1989). Birds that are primarily insectivorous during other times of the year will expand their diets to include non-insect arthropods, seeds, nectar, and fruit during the premigratory fattening and at stopover sites *enroute* (Bairlein and Gwinner 1994; Berthold 1976). Fruits make up a large portion of landbird diets during fall migration (Bairlein 1996; Herrera 1984; Klasing 1998b; Levey and Karasov 1989), and this switch is attributed to fruit abundance, distribution, and assimilation efficiency. Fruits are locally abundant and have clumped distribution, which helps the birds conserve energy while foraging (Bairlein 1990; Bairlein and Simons 1995; Smith et al. 2007). Birds consuming primarily fruit deposit fat more quickly compared to birds consuming primarily insects (Parrish 1997; Smith and McWilliams 2010). Consequently, the presence and abundance of fruit can drive habitat selection during autumn migration (Herrera 1998; Parrish 1997; Takanose and Kamitani 2003).

Current declines in migratory landbird populations are attributed to habitat loss and fragmentation (Newton 2004; Robbins et al. 1989). For example, urban development, deforestation, and agriculture fragment migratory bird habitat and have been implicated in declines of local and regional migratory birds (Faaborg et al. 2010). Historically studies have focused on breeding and wintering grounds (Moore et al. 1993). However, some evidence suggests mortality rate of landbirds are much higher during the migratory periods than other parts of the annual cycle (Sillett and Holmes 2002) and approximately 90% of the migration period is spent stationary at successive stopover sites (Hedenström and Alerstam 1998).

Anthropogenic landscape changes can alter the availability and continuity of stopover habitat, contributing to migratory bird declines (Smith et al. 2015). Some studies have shown that birds do utilize urbanized or developed areas for stopover (Smith et al. 2015); however, these sites may not provide birds with optimal foraging opportunities. Anthropogenic landscape changes can lead to the presence of non-native fruiting shrubs, which is a significant conservation concern for migrating landbirds (Catling 2005; Ewert et al. 2015). Many non-native fruiting shrubs provide low-quality fruits (Oguchi et al. 2017), and poorer refueling has been observed in birds utilizing non-native compared to native dominated shrubland (Smith et al. 2015). Furthermore, Moore and Yong (1991) showed that with increased bird density (which can occur as a consequence of habitat loss) food resources at stopover are depleted, and birds experience poorer refueling rates.

Importance of Stopover Refueling to Disease Dynamic Among Migrating Landbirds

By virtue of their migratory movements, migrating birds are more likely to encounter novel pathogens in the environment or through contact with infected individuals (Møller and Erritzøe 1998). The current changes in stopover habitat are concerning, as it is likely to increase disease risk for migratory landbirds and humans, due to lower quantity and quality of stopover habitat, increased bird density and competition, and higher prevalence of poor nutritional status birds (Figure 1.5). As suitable stopover habitat shrinks the density of migratory landbirds at stopover is expected to increase which can increase transmission of pathogens spread in a density-dependent manner. Habitat loss, competition for limited food resources, and invasion of plants that produce lower quality fruits could cause more birds to be in poor nutritional status. These birds may be less resistant or tolerant to pathogens and, thus have greater disease risk. Furthermore, migratory landbirds can act as transport vehicles for zoonotic pathogens and parasites during migration, so

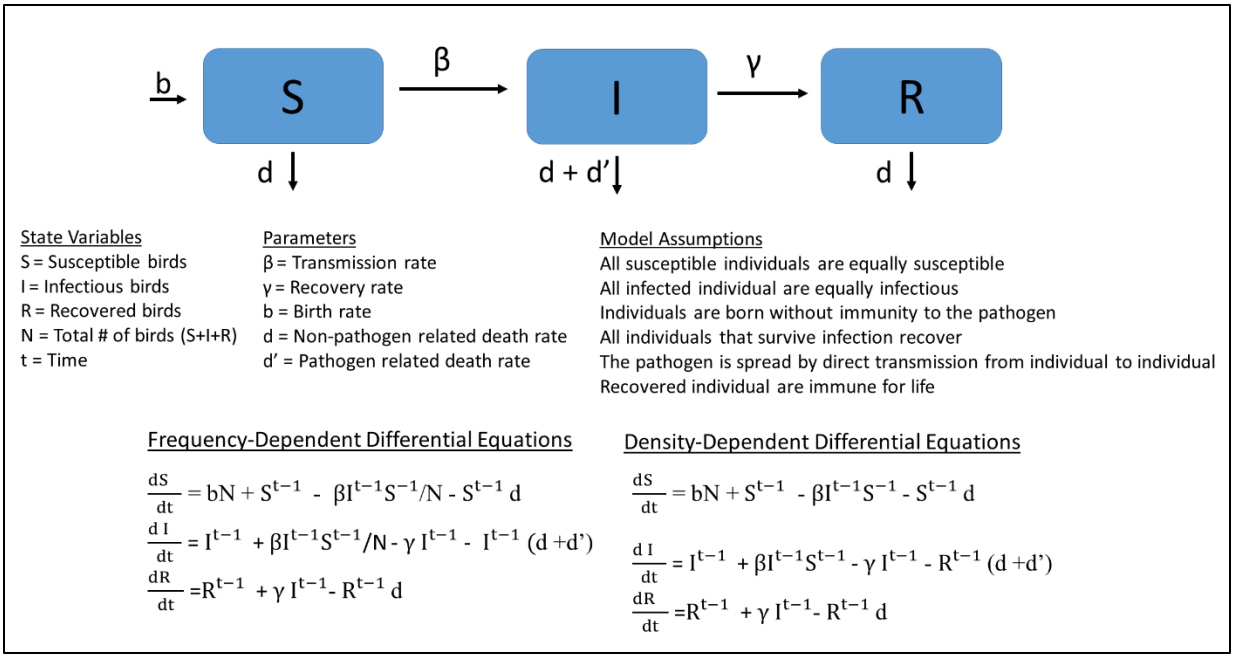
disease risk may also increase for humans as stopover habitat is lost or deteriorates in quality.

Conclusion

Further studies are needed to characterize how current changes occurring in stopover habitat will affect disease dynamics involving migratory landbirds. As stopover habitats are destroyed or altered, there may be far-reaching effects on avian disease, biodiversity, conservation, and human disease. Microorganisms that are pathogenic to migratory landbirds may increase in prevalence and impact the fitness and survival of migratory birds. The resistance of landbirds may be reduced leading to greater competence of reservoir hosts for a variety of zoonotic pathogens. In conclusion, more study is needed to determine how changes in stopover habitat, specifically food availability and quality, affect disease dynamics among migratory landbirds in a variety of disease systems. With more information, perhaps stopover habitat can be better protected and managed in ways that will mitigate disease and pathogen transmission among migratory landbirds.

APPENDICES

APPENDIX A: Figures



State Variables
S = Susceptible birds
I = Infectious birds
R = Recovered birds
N = Total # of birds (S+I+R)
t = Time

Parameters
β = Transmission rate
γ = Recovery rate
b = Birth rate
d = Non-pathogen related death rate
d' = Pathogen related death rate

Model Assumptions
All susceptible individuals are equally susceptible
All infected individual are equally infectious
Individuals are born without immunity to the pathogen
All individuals that survive infection recover
The pathogen is spread by direct transmission from individual to individual
Recovered individual are immune for life

Frequency-Dependent Differential Equations

$$\frac{dS}{dt} = bN + S^{t-1} - \beta I^{t-1} S^{-1}/N - S^{t-1} d$$

$$\frac{dI}{dt} = I^{t-1} + \beta I^{t-1} S^{t-1}/N - \gamma I^{t-1} - I^{t-1} (d + d')$$

$$\frac{dR}{dt} = R^{t-1} + \gamma I^{t-1} - R^{t-1} d$$

Density-Dependent Differential Equations

$$\frac{dS}{dt} = bN + S^{t-1} - \beta I^{t-1} S^{-1} - S^{t-1} d$$

$$\frac{dI}{dt} = I^{t-1} + \beta I^{t-1} S^{t-1} - \gamma I^{t-1} - R^{t-1} (d + d')$$

$$\frac{dR}{dt} = R^{t-1} + \gamma I^{t-1} - R^{t-1} d$$

Figure 1.1. Compartmental SIR model and density-dependent and frequency-dependent differential equations used to determine the amount of susceptible (S), infected (I), and recovered (R) individuals at a given time (t).

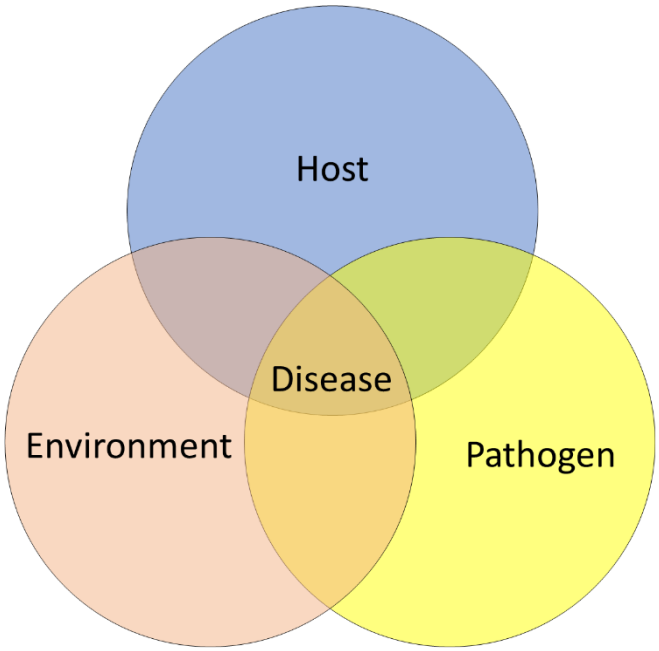


Figure 1.2. Epidemiological Venn diagram.

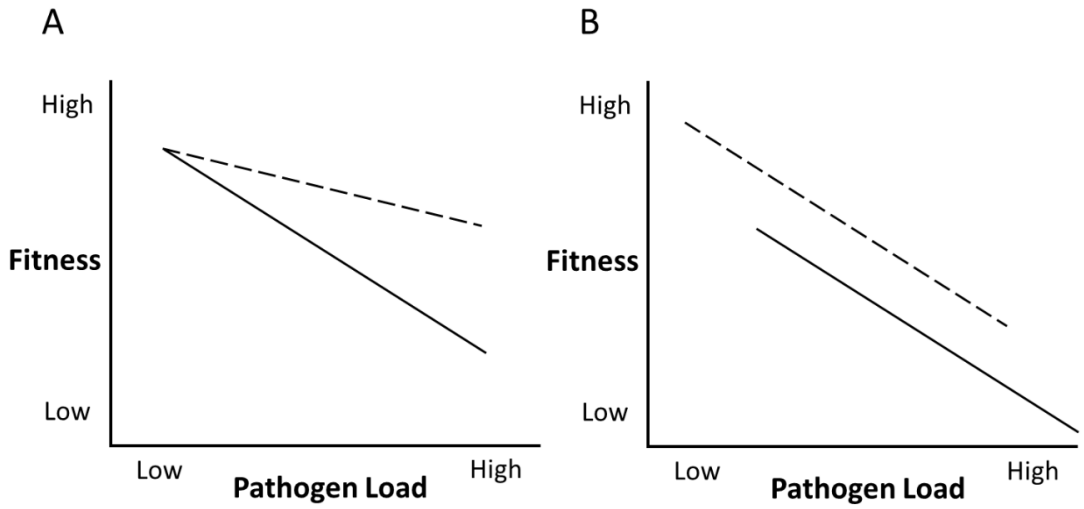


Figure 1.3. Each line represents the relationship between pathogen load and fitness for a group of hosts. (A) demonstrates a difference intolerance, while (B) demonstrates a difference in resistance. In each case, the group of organisms represented by the dashed line has higher tolerance/resistance than the group of organisms represented by the solid line.

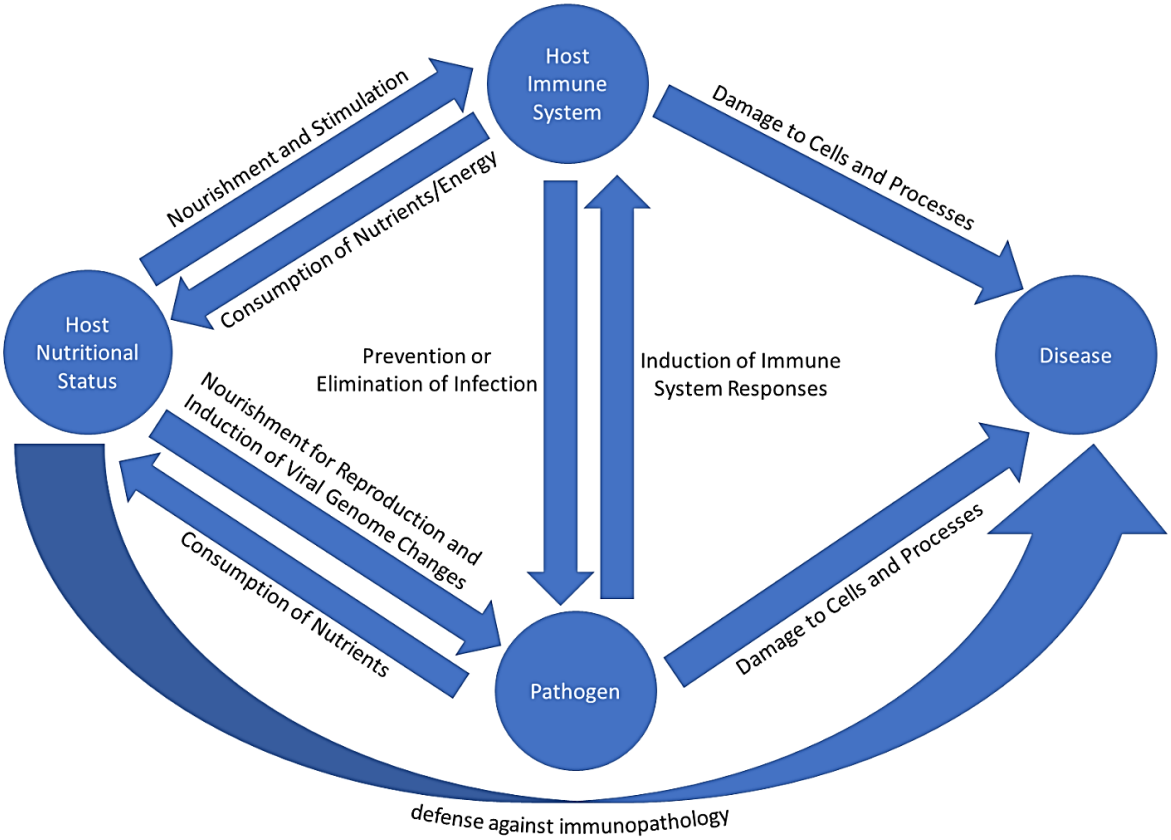


Figure 1.4. Descriptive figure of the mechanisms by which host nutrition can affect the pathogen, the host immune system, and the (potential) disease outcome.

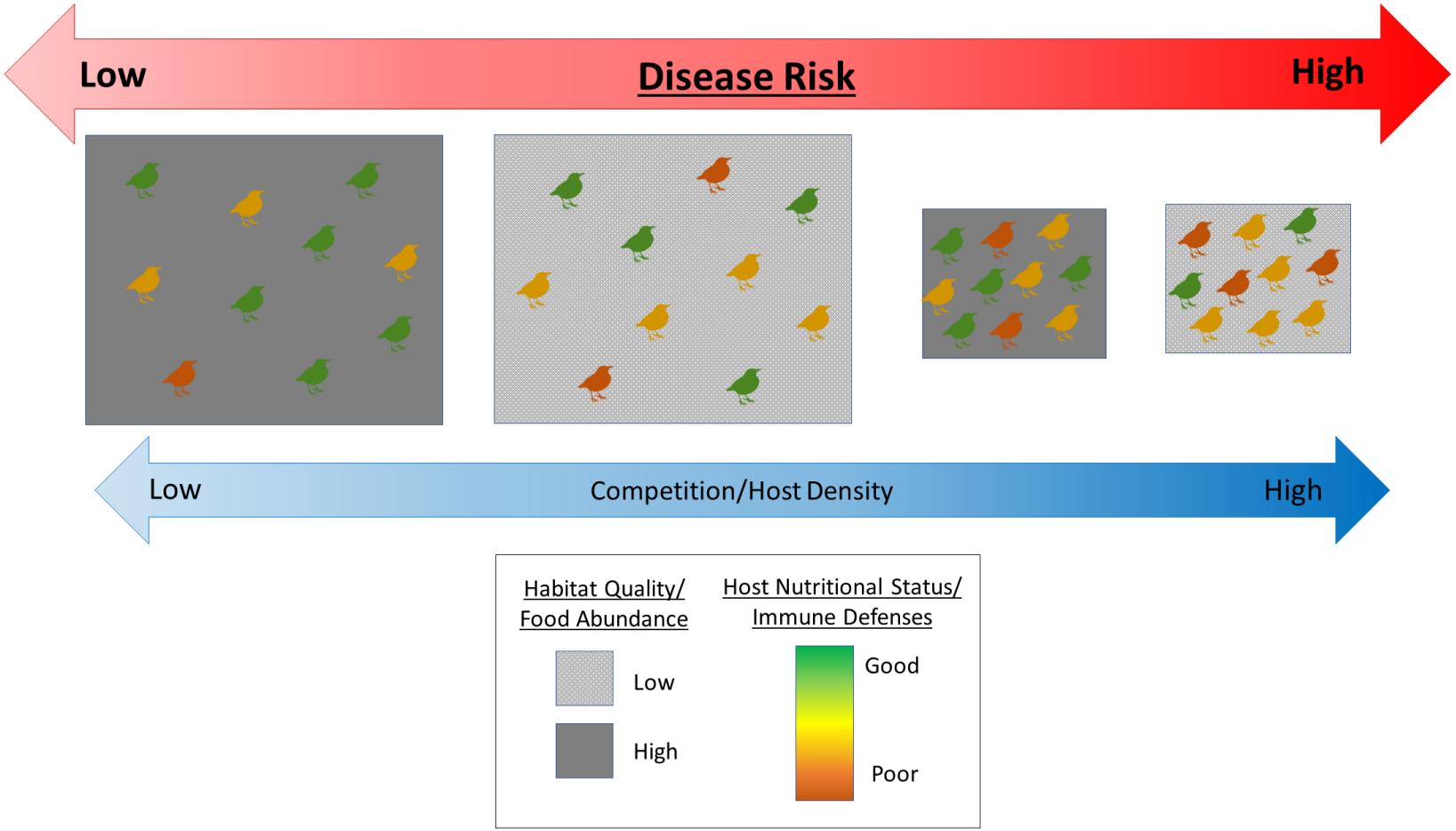


Figure 1.5. Disease risk of migratory landbirds and humans relative to quantity and quality of stopover habitat due to differences in bird competition and density and nutritional status of migrating landbirds.

APPENDIX B: Overview of Avian Immune System

Hosts have a variety of defenses to prevent and eliminate infection, which are collectively referred to as the immune system. These defenses can be separated into two categories: nonspecific and specific immunity. Nonspecific immune defense, also known as the innate immune response, is the first defense against invading pathogens; its response to an invading pathogen begins within minutes to hours of infection and relies on recognition of characteristics shared by broad classes of pathogens. If the innate immune response is unsuccessful at preventing infection or eliminating a pathogen, it initiates the specific or adaptive immune response. The adaptive immune response, unlike the innate immune response, takes days to weeks to activate, forms an attack tailored to the specific invading pathogen, and produces a memory of the pathogen (Janeway et al. 1997).

The innate immune response consists of physical, physiological, chemical, inflammatory, and phagocytic barriers. Many of these immune defenses may be expressed constitutively (continuously regardless of infection) or are induced in response to invasion by a pathogen. Physical barriers include skin and mucus. Pathogens that make it beyond physical barriers are next faced with physiological and chemical barriers such as temperature (i.e., fever response), pH, oxygen levels, digestive enzymes, and antimicrobial peptides (Kennedy 2010). The innate immune response can destroy or eliminate pathogens via cell-mediated and soluble components (see below) (Juul-Madsen et al. 2008) and is responsible for activating the adaptive immune response (Janeway et al. 1997; Kennedy 2010).

The cell-mediated component of the innate immune response is the more complex part of the innate branch of the immune system, in which host immune cell pattern recognition receptors (PRRs) and highly conserved molecular structures of pathogens, termed pathogen-associated molecular patterns (PAMPs), interact (Juul-Madsen et al. 2008; Kennedy 2010). Cellular components of innate immunity include many phagocytic and cytotoxic cells, such as

macrophages, heterophils, and dendritic cells, which express PRRs mainly in the form of toll-like receptors. When PRRs interact with PAMPs, the inflammatory response is induced through a cascade of cytokines that triggers the infiltration of phagocytes and complement proteins to the site of infection. Heterophils, which are granulocytes and the avian equivalent of mammalian neutrophils, are among the first white blood cells (WBCs) in circulation to infiltrate the site of infection (Campbell and Dein 1984b). These cells have two methods of eliminating pathogens: (1) consuming and destroying them using enzymatic activity of their granules, and (2) producing and releasing reactive oxygen species (ROSs) which severely damage the pathogens' membranes (Davison et al. 2008). Monocytes, another circulating WBC, differentiate into macrophages in tissues. Macrophages are phagocytic cells that live longer than heterophils and are antigen-presenting cells (APCs). After destroying a phagocytized pathogen, APCs display the pathogen's antigens on their cell surface to stimulate cells of the adaptive immune response. Dendritic cells, like macrophages, are APCs, but these cells migrate to the lymph nodes to present antigens to cells of the adaptive immune response. Lastly, natural killer (NK) cells, lymphocyte WBCs, recognize and destroy virus-infected and cancerous cells as part of innate immunity (Kennedy 2010).

Soluble components of the innate immune response consist of the acute phase proteins and the complement system. The acute phase response is characterized by a change in the concentration of many plasma proteins, particularly by the production and secretion of proteins by hepatocytes in the liver. Proteins that increase or decrease by 25% or more are called acute phase proteins. These acute response proteins functions include pathogen recognition, pathogen elimination, inflammatory response, and coagulation. C-reactive protein and mannose-binding lectin can bind the surface of pathogens, activating the complement system (Parham 2014). The complement system, made up of serum proteins, can respond to and destroy a pathogen in multiple ways. First,

complement proteins can opsonize (coat) the pathogen, which facilitates phagocytosis by infiltrating white blood cells. Second, they can form an attack complex that kills a pathogen by punching a hole in its membrane. Third, complement proteins can facilitate the destruction of the pathogen by the adaptive immune response (Davison et al. 2008).

Like the innate immune response, the adaptive immune response is composed of soluble (humoral) and cell-mediated components; however, it differs in that it relies on the recognition of unique molecular structures of pathogens (i.e., antigens), takes longer to activate, and creates a lasting memory of pathogens. Most lymphocytes, including B-cells, helper T-cells, and cytotoxic T-cells, are responsible for the adaptive immune response. T-cells are responsible for the cell-mediated portion of the adaptive immune response. Helper T-cells are directors of the immune response and stimulate and activate other lymphocytes via cytokine production when they detect a pathogen through the binding of antigens presented by APCs. B-cells are responsible for the humoral response, mainly through the production of immunoglobulins (Ig), also known as antibodies, which are proteins that recognize and bind to antigens. B-cells are typically activated when helper T-cells present an antigen and produce stimulating cytokines. Once activated, B-cells differentiate into memory and secretory cells. Memory cells present antibodies on their cell surface and will produce secretory B-cells when encountering the same antigen in the future; hence, they provide a lasting memory of an invading pathogen. Secretory B-cells produce and secrete antibodies, which may then bind to pathogen antigens, engulfing the pathogen to prevent it from attacking host cells, and marking it for destruction by cytotoxic T-cells and phagocytic cells. Cytotoxic T-cells, part of the cell-mediated immune response, are activated by APCs and helper T-cells during infection and seek out and destroy pathogen-infected cells (Janeway et al. 1997; Kennedy 2010).

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CHAPTER 2: EFFECTS OF ENERGETIC CONDITION ON CONSTITUTIVE IMMUNE FUNCTION OF AMERICAN ROBINS (TURDUS MIGRATORIUS)

Abstract

The immune system acts as the primary defense against parasites and pathogens and is influenced by nutritional status. Birds must allocate energy among competing needs (including immunity) in the face of seasonally changing resources. Yet, little is known about how differences in energetic condition (a measure of nutritional status) affect immune defenses of songbirds. Herein, the effects of energetic condition on constitutive immunity of American robins as examined. In this 60-day study, wild-caught American robins (*Turdus migratorius*) were acclimated to captivity and split into two treatment groups: control (n = 5; maintained at capture mass) and poor condition (n = 6; food restricted to depletion of subcutaneous fat stores). Blood samples, collected after birds acclimated to captivity and following step-wise food restriction of the poor condition group, were used to assess constitutive measures of immune function (i.e., leukocytes, acute phase protein, natural antibody activity, and complement activity). Results showed that leukocyte counts and acute phase proteins positively correlated with size-corrected condition and that constitutive immunity decreased with decreasing photoperiod. Furthermore, we determined that < 23% of the observed variation in constitutive immunity correlated with size-corrected condition, whereas 54% of the variation correlated with photoperiod. This indicates that American robins have suppressed immunity and may be more vulnerable to infection when in poor energetic condition or during periods of short day-length.

Introduction

An animal's primary defense against parasites and pathogens is its immune system (Zuk 1994); however, maintenance and use of the immune system is energetically costly. Under limited

energy conditions, energy must be allocated between competing needs to maximize fitness. Energetic trade-offs between immunity and other life-history components, such as reproduction and sexual selection, have been well established (Norris and Evans 2000; Sheldon and Verhulst 1996). The energy available to be allocated among these competing needs is largely influenced by an animal's external environment. Animals, including birds, use ultimate cues, such as food availability, and proximal cues, such as photoperiod, to time investment in physiological processes with seasonal changes in resources and energy demands (Nelson et al. 1990; Nelson and Demas 1996; Sinclair and Lochmiller 2000). In fact, previous studies in a variety of taxa have shown that immune defenses vary seasonally (Lochmiller et al. 1994; Martin et al. 2003; Nelson and Demas 1996; Nelson et al. 2002; Owen and Moore 2006a; Sinclair and Lochmiller 2000). The effects of season on immunity in birds is unclear and appears to vary among species and specific immune parameters. For instance, cellular and humoral immunity have been shown to increase or remain unchanged from summer to winter (Lee 2006; Martin et al. 2008). Total immunoglobulin and heterophil levels of great tits (*Parus major*) were shown to be highest during the summer, while lymphocyte counts were higher in winter (Pap et al. 2010). Constitutive innate immunity of skylarks was lower during fall migration than during the breeding season (Hegemann et al. 2012a).

The dependence of immunity on nutritional status has been well established in domestic and wild animals, as well as humans. Generally, individuals in optimal nutritional status have greater immunity and incur fewer infections than individuals in poor nutritional status (Cook 1991; Klasing 1998a; Klasing 2007; Møller et al. 1998). However, it remains incompletely understood how differences in energetic condition, a measure of nutritional status of birds that characterizes mainly fat stores but also muscle stores, affects immune defenses of passerines (perching birds of the order Passeriformes). This is particularly important as passerines act as reservoir hosts for a

variety of zoonotic pathogens (Tsiodras et al. 2008). Birds across and within species demonstrate variable resistance to pathogens and, ultimately, reservoir competence (Ginsberg et al. 2005; Kilpatrick 2007). Yet the underlying differences between individual birds that lead to variation in vulnerability to infection have yet to be fully investigated. Currently, zoonotic disease models tend to assume homogeneity among susceptible individuals, meaning all susceptible individuals are assumed to be equally susceptible to infection. Illuminating the causes of individual variation in vulnerability to infection may help improve zoonotic disease models, allowing more accurate predictions of the outbreak and spread of diseases.

Herein the effects of energetic condition on constitutive immunity of American robins is examined as constitutive immunity is the first defense against infection. We tested the hypothesis that birds in poor energetic condition have lower white blood cell counts, plasma haptoglobin, natural antibody activity, and complement activity compared to birds in good energetic condition. To test this hypothesis, we conducted a captive study in which wild-caught American robins were separated into two experimental groups, control, and poor condition, where the control birds were fed to maintain capture mass and condition, while the poor condition birds were food restricted to reduce energetic condition until subcutaneous fat stores were depleted. The energetic condition of the birds was assessed at capture and throughout the experiment and constitutive measures of immune function were assessed post acclimation to captivity and following reduction of energetic condition of the poor condition group.

Thus, in order to conduct this study and to lay the groundwork for future infection studies characterizing the effects of energetic condition on pathogen infection of American robins, we needed to determine 1) the required food intake to maintain capture mass in captivity, 2) percent food restriction required to significantly reduce energetic condition, and 3) percent food reduction

tolerated (neither causing morbidity or mortality) by captive-wild American robins.

Methods

Focal Species Capture and Housing

The American robin was used as the study species, as it is a migratory passerine that has been implicated as a reservoir for a variety of zoonotic pathogens, including West Nile virus and *Borrelia burgdorferi* (Hamer et al. 2008; Tsiodras et al. 2008). Thirteen American robins were captured mid to late October 2016 using mist nets in a state-managed wildlife area near Bath, MI, USA (Rose Lake Wildlife Research Area, N 42.811972˚, W 84.384917˚). One bird died within 24hrs in captivity, and another was released at the capture site, as it did not eat within 48 hours in captivity. Upon capture, the birds were aged using plumage and skull characteristics, sexed using morphological and plumage characteristics (Pyle and Howell 1997), and condition of the birds was estimated via the methods detailed below. Birds were then immediately transported to the Michigan State University Research Containment Facility (URCF). All birds were individually housed in 30 x 38 x 38 cm cages and given water *ad libitum*. Throughout captivity, the natural photoperiod for Michigan was maintained by adjusting the light cycle 1-2 times per week. Following the study, the birds were euthanized via CO_2 asphyxiation. All work was performed under the following permits: USGS Master Banding (#23629), USFWS Scientific Collection (#MB194270), MI Scientific Collection (#SC1386), Rose Lake Special Use, and Michigan State University Institutional Animal Care and Use Committee protocol (#12/16-211-00).

Experimental Design (Supplemental Figure 2.1)

The birds were fed a semisynthetic diet consisting of mealworms, blueberries, cottage cheese, malted barley cereal, and canola oil. The birds were also given two live mealworms each day for enrichment. Following capture, food was adjusted to maintain capture body mass. After body mass

began to stabilize within approximately 10% of capture mass (post acclimation) the birds were randomly assigned to two treatment groups: (1) control and (2) poor condition. Treatment groups were age and sex-stratified. Birds in the control group were fed to maintain body mass for the remainder of the experiment, while the poor condition group had their food gradually restricted to reduce their condition to a poor state in which subcutaneous stores of fat were depleted (fat score of <1; see below; post treatment; Supplemental Figure 2.1). Food intake by each individual bird was monitored daily during the experiment by subtracting the dry mass of remaining food from the food given the previous day.

Measures of Condition

The condition of birds was characterized by measuring fat score, muscle score, and size-corrected condition at capture, post acclimation (21-30 days), and post food restriction (xx days; Supplemental Figure 2.1). Visible subcutaneous fat was quantified using modified versions of the fat scale developed by Helms and Drury (1960) (Supplemental Table 2.2). Muscle mass was quantified using breast muscle scoring developed by Bairlein (1995) (Supplemental Table 2.3). Size-corrected condition was measured using a modified version of the protocol detailed by Owen and Moore (2006a). Fat-free mass was determined using a linear regression of head-length in mm (as a measure of body size) against the mass of robins with a 0-fat score, using data collected by the Burke Lake Banding Station over spring and fall of 2015. Total head-length was chosen over typical measures of body size, such as unflattened wing-chord, tarsus length, or keel length, as it showed the strongest correlation with fat-free body mass of American robins as determined using data collected by the Burke Lake Banding Station in Fall of 2015 (Supplemental Figure 2.2). Size-corrected condition was expressed as percent difference from predicted fat-free mass (see below for details).

Sample Collection and Storage

Blood samples were collected via the brachial vein (wing vein) puncture and collected via heparinized capillary tubes. Approximately 450ul of blood was collected from all birds post acclimation and post treatment. Additionally, blood samples (~450 ul) were taken from the control birds 19 days (d) following acclimation to captivity (post acclimation) and from the poor condition birds 30 d post acclimation (Supplemental Figure 2.1). Blood smears were created at the time of blood collection, air dried, fixed with 100% methanol, and stained with Wright-Giemsa stain. Following collection, blood samples were stored on ice. The bulk of the blood (approximately 250-330 ul) collected was centrifuged for 5 minutes (min) at 10,000 x g to separate plasma. Two capillary tubes of blood (approximately 120-200 ul) collected from each bird were centrifuged for 15 min at 12,000 x g, the packed red blood cell volume (hematocrit), was determined as a general measure of bird health. All separated plasma was combined and then aliquoted for subsequent assays and stored at -80ºC (Owen 2011).

Measures of Constitutive Immune Function

Total Leukocyte Counts and Heterophil Lymphocyte Ratio

The cellular component of the constitutive immune function was measured using total leukocyte counts (Buehler et al. 2010b; Campbell and Dein 1984a; Nebel et al. 2012b; Owen and Moore 2006b). In addition, long-term stress can result in elevated heterophil lymphocyte ratio (H:L) (Davis et al. 2008; Maxwell 1993; Owen and Moore 2006b). Both total leukocytes and H:L were measured using the methods described by Owen and Moore (2006c).

Natural Antibody and Complement Activity: Hemolysis and Hemagglutination

A hemolysis-hemagglutination assay, described by Oguchi (2015), was used to measure natural antibody and complement activity in plasma. This technique characterizes the ability of natural

antibodies to agglutinate foreign red blood cells (hemagglutination) and to activate the complement system. Activated complement proteins then form pores in foreign red blood cells, lysing them (hemolysis), which results in the visible clearing of the blood cell suspension (Matson et al. 2005; Oguchi et al. 2017).

Acute Phase Protein: Haptoglobin

Haptoglobin, an acute phase protein, binds to free circulating heme (iron) to prevent its use as a nutrient to pathogens. High blood levels of haptoglobin indicate greater fever response readiness. (Matson et al. 2012). Haptoglobin was measured using a commercially available kit (Tridelta Development #TP801, Maynooth, County Kildare, Ireland) following the methods described in the manufacturer's manual with two modifications: 1) the volumes of all samples and reagents were halved so that the assay could be run using only 3.75 ul of plasma; and 2) we took a background absorbance before addition of reagent 2 to correct for initial differences in plasma color.

Spleen Mass

The spleen is a secondary organ of the avian immune system and one of the major sites of production, differentiation, and storage of lymphocytes, including those responsible for cell-mediated immune responses (Glick 2000). Spleen size is assumed to positively reflect an individual's ability to mount an immune response (Møller and Erritzøe 1998). Post treatment, birds were weighed just prior to euthanasia. Spleens were collected from the birds immediately following euthanasia and weighed to the nearest milligram. Spleen size was then corrected for bird size and reported as a percentage of total body mass of the bird.

Data Analyses

All analyses were performed using R version 3.3.1. All tests were 2-tailed with critical $\alpha = 0.05$.

Measures of body size were compared to fat-free mass using linear regressions. The regression model with total head length accounted for the most variation (R^2= 0.6021), and thus was used to calculate predicted fat-free body mass for each individual bird:

Predicted Fat-free Mass (g) = 2.58g/mm x Head Length (mm) - 46.84 g

Size-corrected condition for each bird was expressed as % difference from its predicted fat-free mass:

Current Mass (g) - Predicted Fat-free Mass (g)/ Predicted Fat-free Mass (g) x 100%

Percent change in body mass per day (% change in mass/days since initial mass measurement) was regressed against food intake (average grams of food intake per day since initial mass measurement) for all measurements taken between capture and post acclimation. The y-intercept was determined to be the approximate mass of food needed to maintain capture mass.

Effects of the food treatments on measures of condition were determined using a linear mixed model in which treatment group and time point were fixed factors and bird was a random effect. This allowed for non-independence to be resolved by assuming a different "baseline" of condition for each bird. Size-corrected condition, total leukocytes (leukocytes/10,000 red blood cells (Log_{10}) H:L (Log_{10}), plasma haptoglobin (square root) and hematocrit at capture, post acclimation, and post treatment were compared using a Type II Wald Chi-square test with a post-hoc Tukey's multiple comparison tests. Fat score and muscle score, at capture, post acclimation and post treatment were compared using a non-parametric factorial permutation test, and a non-parametric post-hoc Dunn's test with p-values adjusted using the Bonferroni method. Likewise, hemolysis score and hemagglutination score were compared at post acclimation and post treatment using a non-parametric permutation test, and post-hoc Dunn's test with p-values adjusted using the Bonferroni method. Nonparametric tests were used for all score variables as they are ordinal data

that was not normally distributed. Spleen size was compared between treatment groups post treatment using a two-sample t-test.

As a significant difference in size-corrected condition was not observed between the treatment groups, a linear regression analysis of data from all time points (including data gathered using blood samples collected between post acclimation and post treatment time points) was used to determine if there were relationships between size-corrected condition and each individual measure of constitutive immunity (leukocytes/10,000 red blood cells (Log_{10}), plasma haptoglobin, and hemagglutination and hemolysis score). Each measure of constitutive immunity was regressed against size-corrected condition. Four measures of constitutive immunity (leukocytes/10,000 red blood cells (Log_{10}), plasma haptoglobin, and hemagglutination and hemolysis score) were entered into a principal component analysis (PCA) to examine collective variation in constitutive immunity. Each PC was regressed against size-corrected condition and date to determine if the variation in constitutive immunity captured by the PC correlated with size-corrected condition or photoperiod. Date was used as a proxy for photoperiod as the length of daily light exposure was not explicitly recorded throughout the experiment but was adjusted 1-2 times per week to match the natural photoperiod of Michigan.

Results

Approximately 18.5 g of food intake per day was needed to maintain capture mass.

Body mass fluctuated over the course of the study, as the robins' body mass was easily affected by small changes (~1g) in food (Supplemental Figure 2.3). After 21-30d of acclimation to captivity (post acclimation), most birds (9 of 11) began maintaining body mass within 10% of capture mass. Based on food intake and mass changes recorded between capture and post acclimation, we determined the birds needed approximately 18.5g of food to maintain capture

mass of the birds (Supplemental Figure 2.4).

Body composition changed in captivity.

Size-corrected condition of the experimental groups was statistically similar at capture and post acclimation (Figure 2.1A). Although muscle score of the experimental groups was statistically similar at capture and post acclimation, both groups trended towards reduction, each dropping approximately half a score post acclimation (Good: $p = 1.892x10^{-1}$, Poor: $p = 5.172x10^{-1}$; Figure 2.1B). Fat score was not significantly different between the treatment groups at capture or post acclimation. however, fat score of the control group marginally significantly increased by approximately 3 scores following acclimation to captivity ($p = 0.0668$) while fat score of the poor condition group significantly increased by approximately 3.5 scores following acclimation to captivity ($p = 0.0063$; Figure 2.1C).

Birds tolerated 22-53% food restriction.

Due to the change in body composition (increased fat), it took 30 days of gradual food reduction for the poor condition group to lose their subcutaneous stores. At this point, the poor condition birds were food restricted by 30-64%, and 2 of 6 birds in the poor condition group became lethargic. Food was gradually increased by 2.7-5.8 g for the poor condition birds, and by post acclimation none of the birds were lethargic. At post acclimation, the poor condition birds were food restricted by only 22-53%.

Condition of the poor condition group was not significantly reduced post treatment.

Size-corrected condition of the poor condition group significantly decreased between capture and post treatment by approximately 25% of predicted fat-free mass ($p = 0.0044$), but as size-corrected condition dropped slightly by approximately 6% of predicted fat-free mass post acclimation, there was not a significant difference in size-corrected condition between post acclimation and post

treatment despite an approximately 4.5 fold change ($p = 0.1122$). Post treatment size-corrected condition of the poor condition group was significantly lower compared to the control group at capture, with a difference of approximately 5.5% of predicted fat-free mass ($p = 0.0002$). Likewise, post treatment size-corrected condition of the poor condition group was significantly lower compared and to the control group post-acclimation, with a difference of approximately 6% of predicted fat-free mass ($p = 0.0026$). Post treatment, the poor condition group had a marginally significantly lower size-corrected condition compared to the control group post treatment with a difference of 27% of predicted fat-free mass ($p = 0.0610$). Post treatment, size-corrected condition of the control group was significantly reduced by approximately 2.5% of predicted fat-free mass from capture ($p = 0.0022$); however, size-corrected condition of the control group was similar at the post acclimation and post treatment time points ($p = 0.1122$; Figure 2.1A). Subcutaneous fat of the poor condition group decreased by approximately 2 scores between post acclimation and post treatment, but was not significantly reduced ($p = 0.8651$), nor did it significantly differ from the control group post treatment ($p = 1.000$). Post acclimation, the control group maintained subcutaneous fat comparable to the post-acclimation time point ($p = 1.000$; Figure 2.1C). Muscle score of the poor condition group significantly decreased by approximately 1.6 scores between capture and post treatment ($p = 1.960 \times 10^{-4}$) but was not significantly reduced compared to post acclimation ($p = 3.719 \times 10^{-1}$; Figure 2.1B), nor the control group post treatment ($p = 4.852 \times 10^{-1}$; Figure 2.1B).

No significant effect of treatment on constitutive immunity.

There was no significant effect of the food treatment on any of the constitutive immunity measures (total leukocytes, plasma haptoglobin, hemolysis score, and hemagglutination score). There was, however, a significant decrease in total leukocytes ($X^2 = 21.2167$, $p = 4.102 \times 10^{-6}$), plasma

haptoglobin (X^2 = 4.1461, p = 0.0417), hemolysis score (p = 0.0112), and hemagglutination score (p = 0.0050) with time point. The poor condition (p = 0.0045) and control (p = 0.0045) groups had significantly reduced total leukocyte count post treatment and did not significantly differ from each other post acclimation or post treatment (Figure 2.2A). Total leukocyte count of the poor condition group dropped by half post treatment, while the control group dropped by two-thirds post treatment. Plasma haptoglobin did not vary significantly between treatment groups or time points (Figure 2.3). There was no significant difference in hemolysis score between treatment groups post acclimation or post treatment; however, the poor condition group had a marginally significant 1.5 score reduction in the hemolysis score post treatment (p = 0.0572; Figure 2.4A). Hemagglutination score of the poor condition group was significantly higher (by 1.2 scores) than the control group post acclimation, and there was no significant difference in hemagglutination score between the treatment groups post treatment; however, the poor condition hemagglutination score marginally significantly decreased by approximately 1 score post treatment (p = 0.0570; Figure 2.4B).

Significant effect of treatment on measures of stress and health.

There was a significant effect of the food treatment on H:L (X^2 = 7.3802, p = 0.0065946) and hematocrit (X^2 = 13.225, p = 0.0002763). Additionally, similar to the measures of immune function, there was a significant effect of time point on H:L (X^2 = 11.0747, p = 0.0008751) and hematocrit (X^2 = 16.733, p = 4.302×10^{-5}). The H:L of the poor condition group significantly increased 5.5 fold post treatment (p = 0.0120) and was significantly elevated compared to the control group post treatment (p = 0.0274; Figure 2.2B). The hematocrit of the poor condition group did not differ from the control group post acclimation. Post treatment hematocrit of the poor condition group was significantly reduced by approximately a third post treatment (p = 0.0001)

when compared to the control group post treatment ($p < 0.0001$; Figure 2.5). Spleen size did not differ significantly between good and poor condition groups ($p = 0.7042$; Supplemental Figure 2.5).

Leukocytes and plasma haptoglobin had significant relationships with size-corrected condition.

Birds in the poor condition group could not tolerate food restriction to the extent that a significant difference in energetic condition between the treatment groups post treatment could be reached; however, variation in size-corrected condition among all the birds was produced, and thus the correlation between size-corrected condition and each measure of constitutive immunity was investigated. Total leukocytes and plasma haptoglobin had a significantly positive relationship with size-corrected condition (total leukocytes $p = 0.04815$; plasma haptoglobin $p = 0.005002$; Figure 2.6A and 2.7B). All other measures of immunity did not have a significant relationship with size-corrected condition (hemolysis score, $p = 0.6769$; hemagglutination score, $p = 0.3606$; Figure 2.6C and 2.7D.

Variation in constitutive immunity correlated with both size-corrected condition and date.

Each principal component (PC), PC1, PC2, and PC3 explained 54.01%, 23.07%, and 19.35% of the variation in constitutive immunity respectively. The fourth component accounted for a very small proportion of the variation in constitutive immunity (3.57%) and thus was not investigated further for a relationship with size-corrected condition or date. Loadings of the variables and percentage of variance for PC1-PC4 are shown in Table 2.1. PC1 significantly correlated with date ($p = 0.0008087$; Figure 2.8A), but not size-corrected condition ($p = 0.08936$; Figure 2.7A); however, PC2 significantly correlated with both size-corrected condition ($p = 0.009042$; Figure 2.7B) and date ($p = 0.04797$; Figure 2.8B) yet correlated most strongly with the former. PC3 did not significantly correlate with size-corrected condition ($p = 0.9464$) or date ($p = 0.2853$Figure

2.7C and Figure 2.8C).

Discussion

To test the hypothesis relating energetic condition with immunity, variation in energetic condition first needed to be created. Thus, we aimed to determine 1) the food intake required to maintain capture mass in captivity, 2) the percent food restriction required to reduce energetic condition significantly, and 3) percent food reduction tolerated (neither causing morbidity or mortality) by captive-wild American robins. We determined that American robins need approximately 18.5 g of the previously described semisynthetic diet to maintain capture mass and that 22-53% food restriction was tolerated. The birds did not, however, tolerate the food restriction required to significantly reduce size-corrected condition, in that the poor condition birds began to exhibit signs of morbidity (lethargy) prior to complete depletion of subcutaneous fat and a significant reduction in energetic condition. However, the moderate decline in energetic condition that was produced successfully created enough variation to investigate the effects of energetic condition on constitutive immunity. There was no significant effect of the food treatment on any of the four constitutive immunity measures (e.g., total leukocyte count, plasma haptoglobin, natural antibody activity, and complement activity). This may have been due to the lack of significant difference in the energetic condition of the treatment groups post treatment. Thus, we investigated the correlation between each measure of constitutive immunity and size-corrected condition. Total leukocytes and plasma haptoglobin were significantly positively related to size-corrected condition. Interestingly all measures of constitutive immunity were significantly affected by time point. Moreover, we demonstrated that approximately a quarter of the variability in constitutive immunity observed was explained by differences in size-corrected condition, while the majority of variation in constitutive immunity was explained by date (a proxy for photoperiod). This likely

indicates that a seasonal change, due to the natural photoperiod maintained during the experiment, caused a decrease in constitutive immunity.

Our study shows that birds in poor energetic condition, due to limited food, experience suppressed constitutive immunity. Our findings also indicate that decreases in constitutive immunity may occur in the fall mainly due to a seasonal change in photoperiod, rather than nutritional status. Consequently, passerines in poor condition or during short-day periods may be more likely to be infected by pathogens, including zoonotic pathogens. Furthermore, this study provides the foundation for designing further studies of the effects of energetic condition on immunity and pathogen resistance of American robins.

Our findings show similar results to previous studies investigating the relationship between energetic condition and constitutive immunity (Buehler et al. 2010a; Killpack et al. 2013; Owen and Moore 2008; Palacios et al. 2009). For example, Buehler et al. (2010a) found that red knots (*Calidris canutus*) recovering protein at a staging area in the Delaware Bay had lower total leukocyte counts and plasma haptoglobin compared to individuals storing fat in preparation for departing the area. Likewise, Owen and Moore (2008) showed that for free-living Swainson's thrushes (*Catharus ustulatus*) and wood thrushes (*Hylocichla mustelina*) total leukocyte counts positively related to energetic condition. Buehler et al. (2010a), however, found complement activity to be higher in individuals storing fat, which is counter to our results. Similar to our study, Killpack et al. (2013) found no effect of food restriction on natural antibody activity and complement activity of free-living altricial house sparrows (*Passer domesticus*). Likewise, Palacios et al. (2009) conducted a study with free-living tree swallows (*Tachycineta bicolor*) in which they found no association between size-corrected condition and either natural antibody activity, complement activity, or leukocyte proliferation.

Our results are likewise similar to previous studies investigating seasonal changes in immunity (Lochmiller et al. 1994; Nelson and Demas 1996; Owen and Moore 2006a; Schultz et al. 2017; Sinclair and Lochmiller 2000). Hegemann et al. (2012b) found that three measures of constitutive immunity (complement activity, natural antibody activity, and plasma haptoglobin) were lower in the fall than during the breeding season for skylarks (*Alauda arvensis*). Owen and Moore (2006c) found that three migratory species of thrush had lower leukocyte counts during the fall migratory period compared to the non-migratory period. Lastly, Schultz et al. (2017) found that photoperiod rather than energetic condition governed changes in constitutive immunity of red crossbills (*Loxia curvirostra*). Red crossbills kept on long-daylength had higher bacterial killing and total leukocytes than those kept on short-daylength. However, no significant effect of photoperiod on plasma haptoglobin or natural antibody and complement activity was found. However, our results differ from those of Pap et al. (2010), who found leukocytes of great tits (*Parus major*) were higher in the winter than in the summer.

Interpretation of the current findings may be limited, as observations were made using a small sample size under sedentary captive conditions, and birds only varied between mid to low energetic condition. Studies in humans and animals have shown that obesity can lead to suppressed immunity (Nieman et al. 1999; Tanaka et al. 1993). Our study did not include a high energetic condition group; therefore, we cannot draw any conclusions regarding the effects of high energetic condition (characterized by large fat stores similar to obese subjects) on constitutive immunity. Additionally, the movements of birds, particularly flight, were limited due to individual housing in 30 x 38 x 38 cm cages; meaning the conditions of captivity restricted the birds to a relatively sedentary activity level and thus differing energetic demands compared to conditions in the wild. This also likely explains the observed change in body composition between capture and post-

acclimation time points, as we observed a decrease in muscle and an increase in fat following acclimation to captivity. Ultimately, body composition observed post-acclimation was not representative of the body composition observed in wild American robins, which is typically lower in fat and higher in muscle (unpublished data from the Burke Lake Banding Station). Lastly, captivity-induced stress may have affected absolute measures of constitutive immunity. Despite the known negative effect of stress on immunity (Apanius 1998; Martin 2009), Dickens et al. (2009) showed that stress-induced cortisol levels dropped significantly by day 5 and day 9 in captivity, so it is unlikely that length of time in captivity contributed as much to changes in constitutive immunity and health parameters as the food treatments and change in photoperiod.

There are several explanations for the lack of detection of a difference in constitutive immunity between treatment groups in our study, while total leukocytes and plasma haptoglobin significantly positively related to size-corrected condition. First, the relatively small sample size may not have been large enough to detect a difference in response variables between the treatment groups. Second, as a significant difference in energetic condition was not reached, the effect of the treatments may not have been large enough to produce a detectable difference in leukocytes or plasma haptoglobin between treatment groups. Third, significant effects of the treatments on immunity may have been unobservable due to the decrease in constitutive immunity with decreasing photoperiod.

Future studies are necessary to determine how energetic condition affects immunity of wild passerines. We suggest that future studies modulate energetic condition over a wider range (possibly through the addition of a treatment group fed *ad libitum*), allow birds to exercise via flight to maintain body composition comparable to wild birds, and either keep photoperiod constant or account for photoperiod-induced changes in immunity in the study design and analysis.

Furthermore, the effects of energetic condition on constitutive immunity should be investigated during different seasons. As host nutrition can also affect pathogen virulence (Cornet et al. 2014), future studies should include an immunologic challenge with a pathogen. Our study sets the groundwork for further future research testing the effects of energetic condition on pathogen resistance of American robins through immunologic challenges. More study is needed to characterize how energetic condition affects susceptibility to pathogen infections and how this may affect disease transmission, particularly for zoonotic diseases of concern.

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APPENDICES

APPENDIX C: Tables and Figures

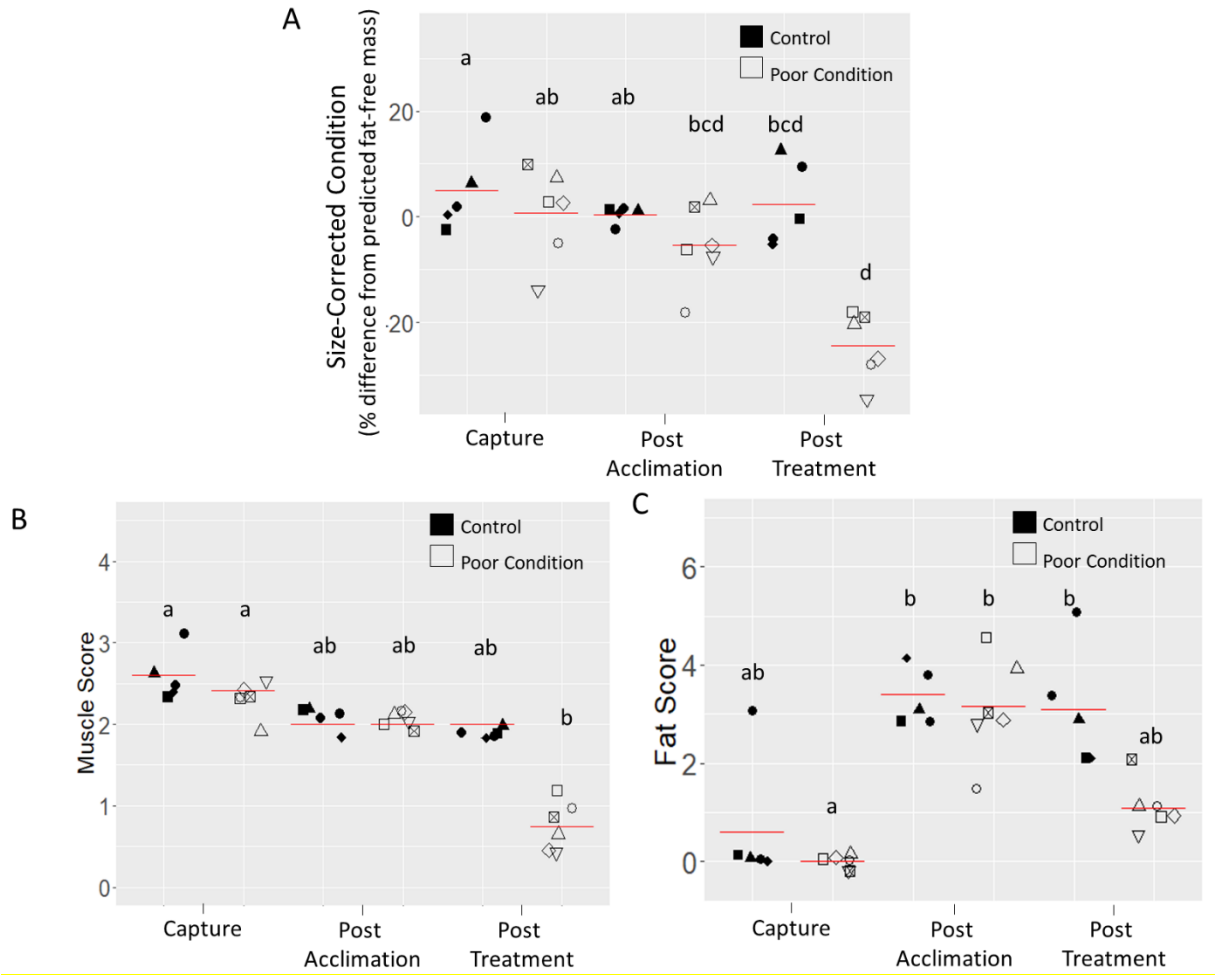


Figure 2.1. Timepoint means (red lines) of size-corrected condition (A), subcutaneous fat (B) score, and breast muscle score (C), at capture, post acclimation, and post treatment of the treatment groups: control (solid shapes) and poor condition (unfilled). Unique symbols indicate individual birds. Different letters indicate significantly different groups ($p<0.05$).

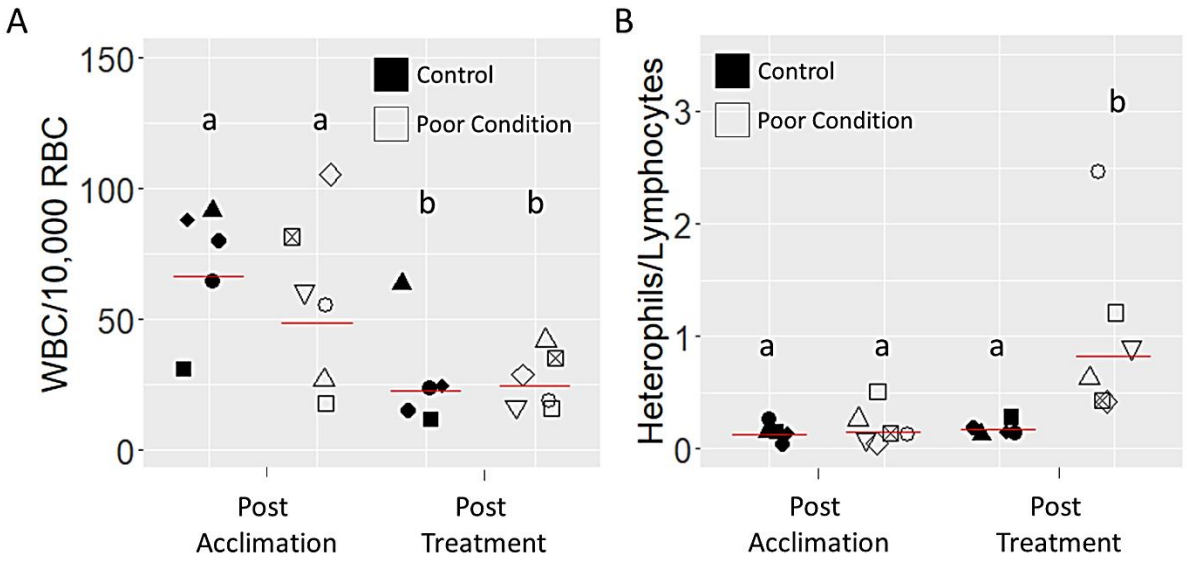


Figure 2.2. Time point means (red lines) of total leukocytes (Log_{10} white blood cells per 10,000 red blood cells) (A) and heterophil to lymphocyte ratio (B) of the treatment groups: control (solid shapes) and poor condition (unfilled). Unique symbols indicate individual birds. Different letters indicate significantly different groups ($p<0.05$).

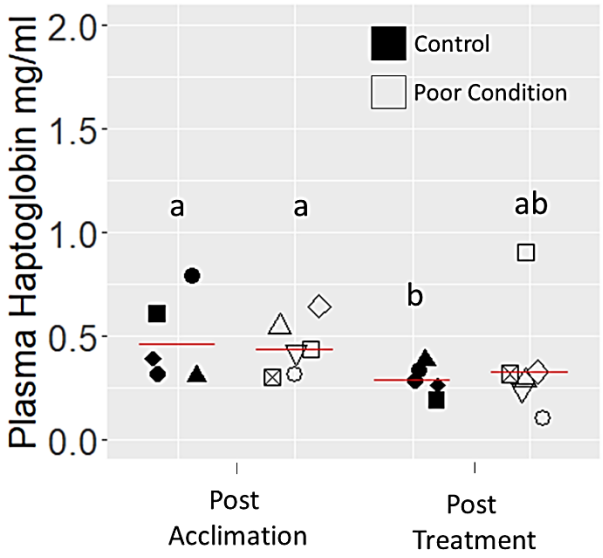


Figure 2.3. Time point means (red lines) of plasma haptoglobin (mg/mL) of the treatment groups: control (solid shapes) and poor condition (unfilled). Unique symbols indicate individual birds. Different letters indicate significantly different groups ($p<0.05$).

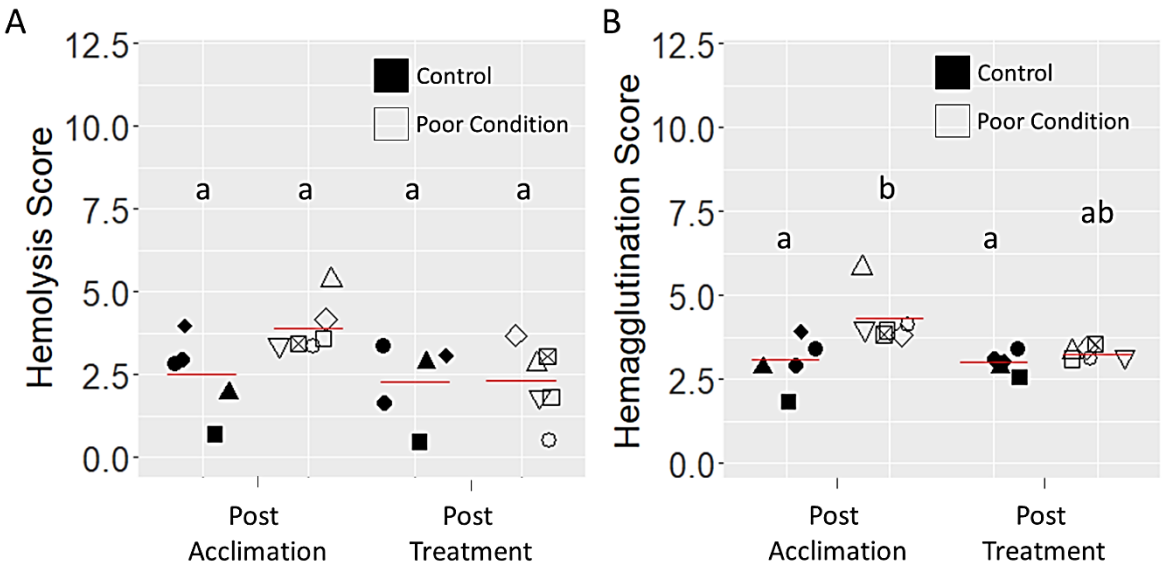


Figure 2.4. Time point means (red lines) of hemolysis score (A) and hemagglutination score (B) of the treatment groups: control (solid shapes) and poor condition (unfilled). Unique symbols indicate individual birds. Different letters indicate significantly different groups ($p<0.05$).

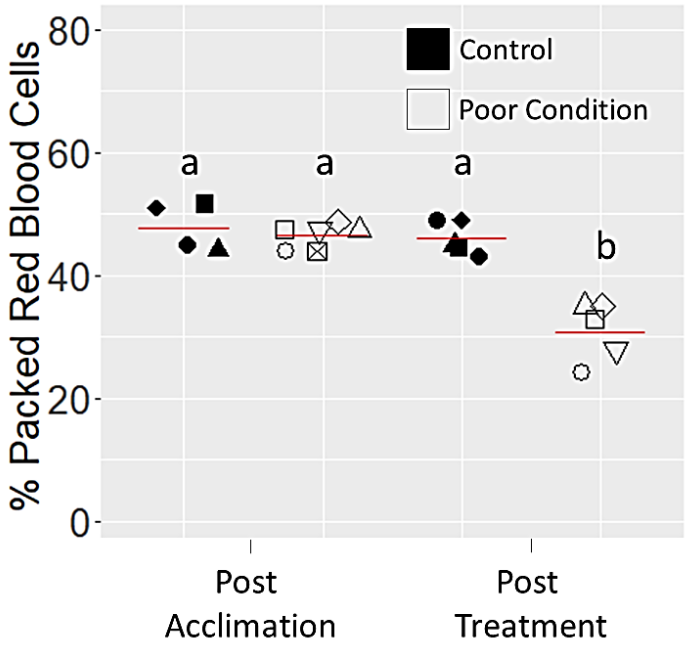


Figure 2.5. Time point means (red lines) of hematocrit (% packed red blood cells) of the treatment groups: control (solid shapes) and poor condition (unfilled). Unique symbols indicate individual birds. Different letters indicate significantly different groups ($p<0.05$).

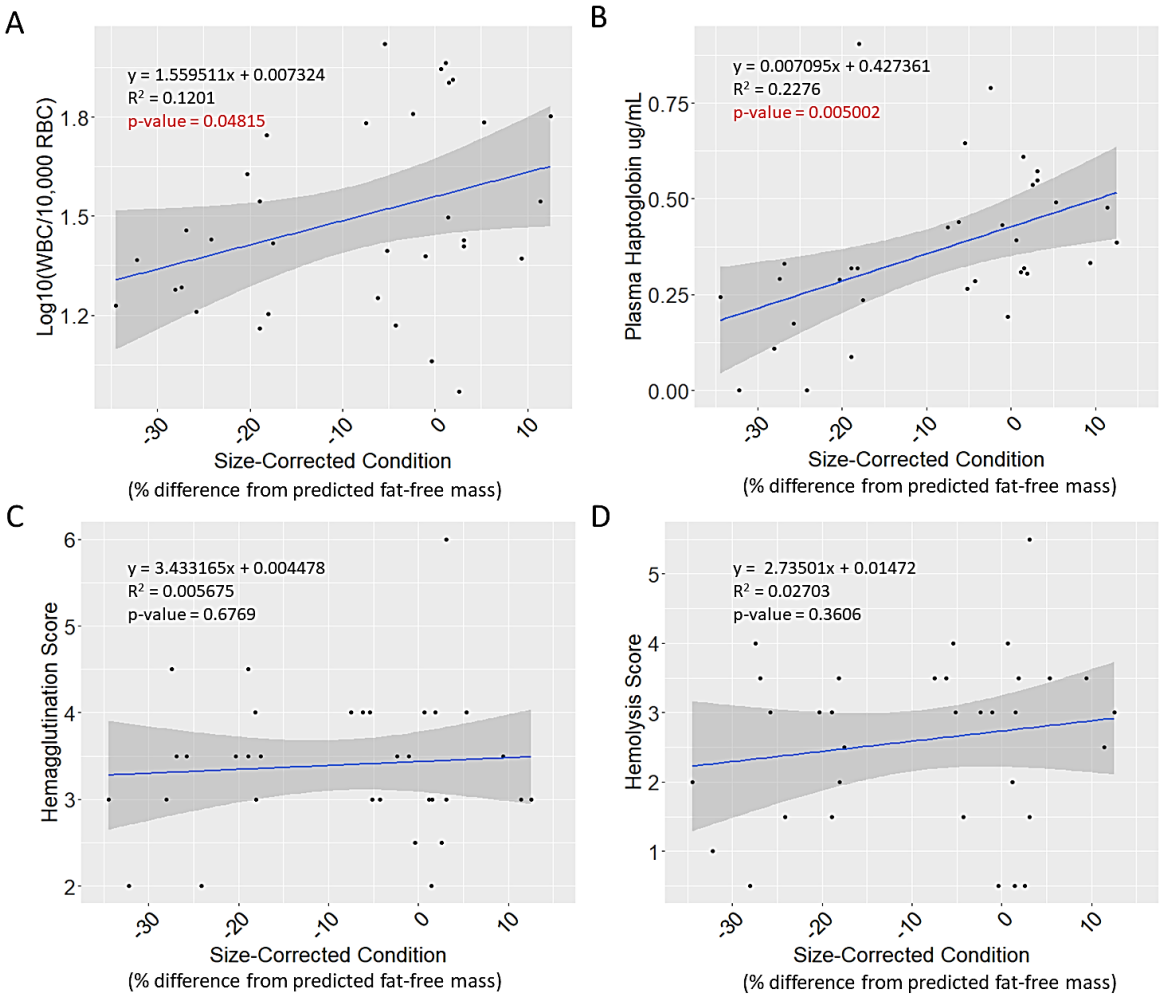


Figure 2.6. Regressions of size-corrected condition against measures of constitutive immunity, total leukocytes (A), plasma haptoglobin (B), hemagglutination score (C), and hemolysis score (D). Gray shading indicates 95% confidence intervals.

Table 2.1. Loadings of the constitutive immunity variables (leukocytes, plasma haptoglobin, hemagglutination score, and hemolysis score) and percentage of variance for principal components 1-4.

Principal Component	Variable Loadings				Percent of Variance
	Leukocytes	Plasma Haptoglobin	Hemolysis Score	Hemagglutination Score	
PC1	0.3299806	0.5755984	0.5725042	0.4817035	54.01%
PC2	0.84176415	0.06316576	-0.10658359	-0.52543614	23.07%
PC3	0.4119486	-0.3937323	-0.4305152	0.6999499	19.35%
PC4	-0.11333250	0.71391281	-0.68959088	0.04414367	3.57%

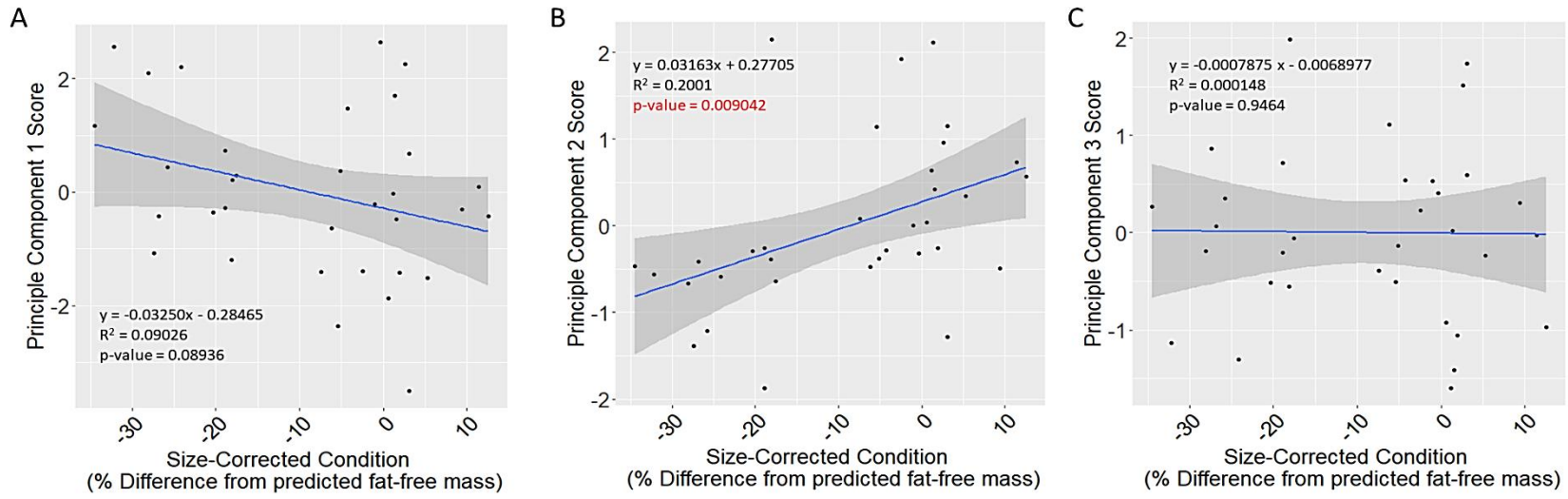


Figure 2.7. The relationship between size-corrected condition and constitutive immunity principal component 1 (A), principal component 2 (B), and principal component 3 (C). Gray shading indicates 95% confidence intervals.

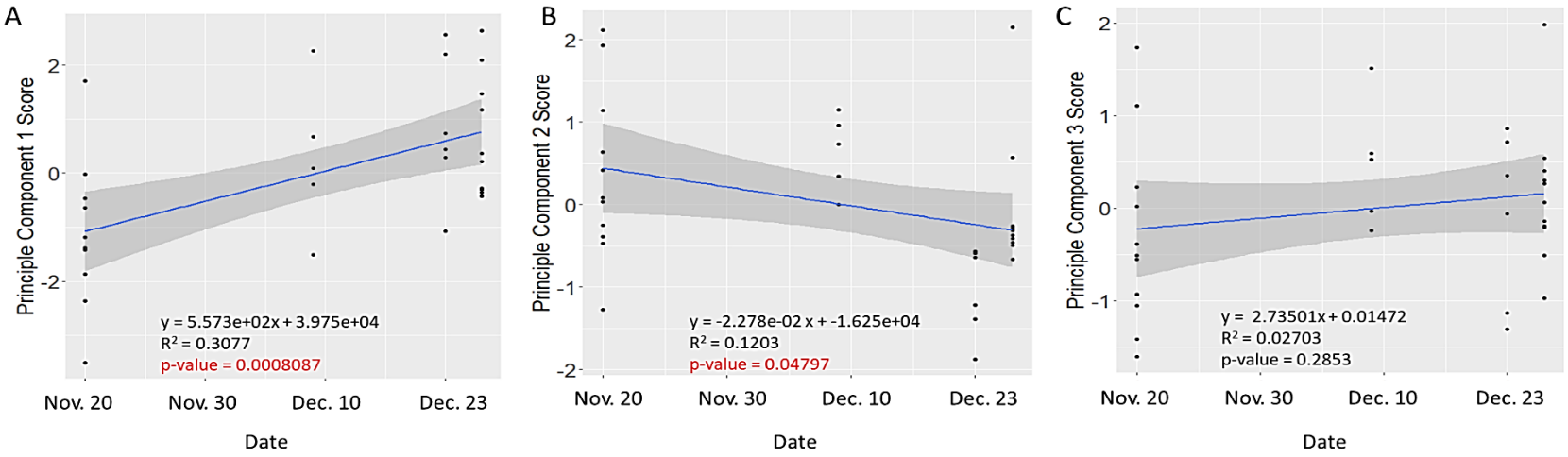
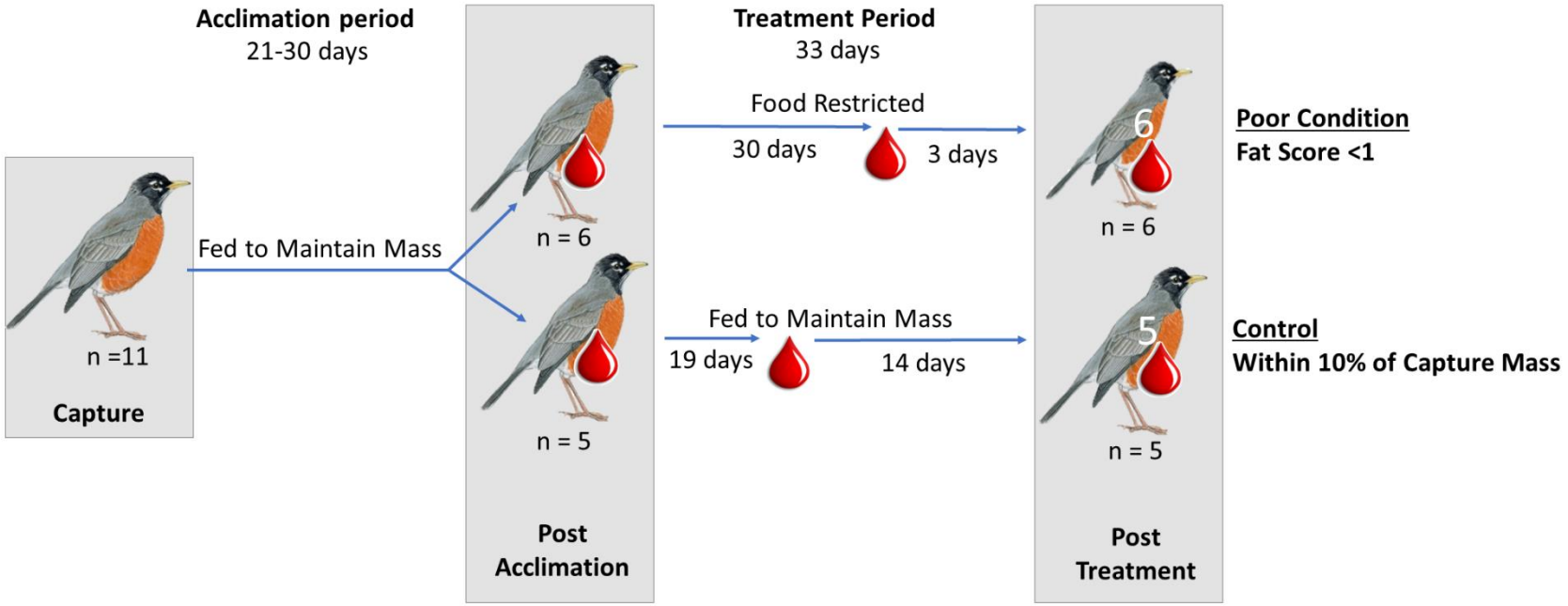


Figure 2.8. The relationship between date and constitutive immunity principal component 1 (A), principal component 2 (B), and principal component 3(C). Gray shading indicates 95% confidence intervals.

APPENDIX D: Supplemental Tables and Figures



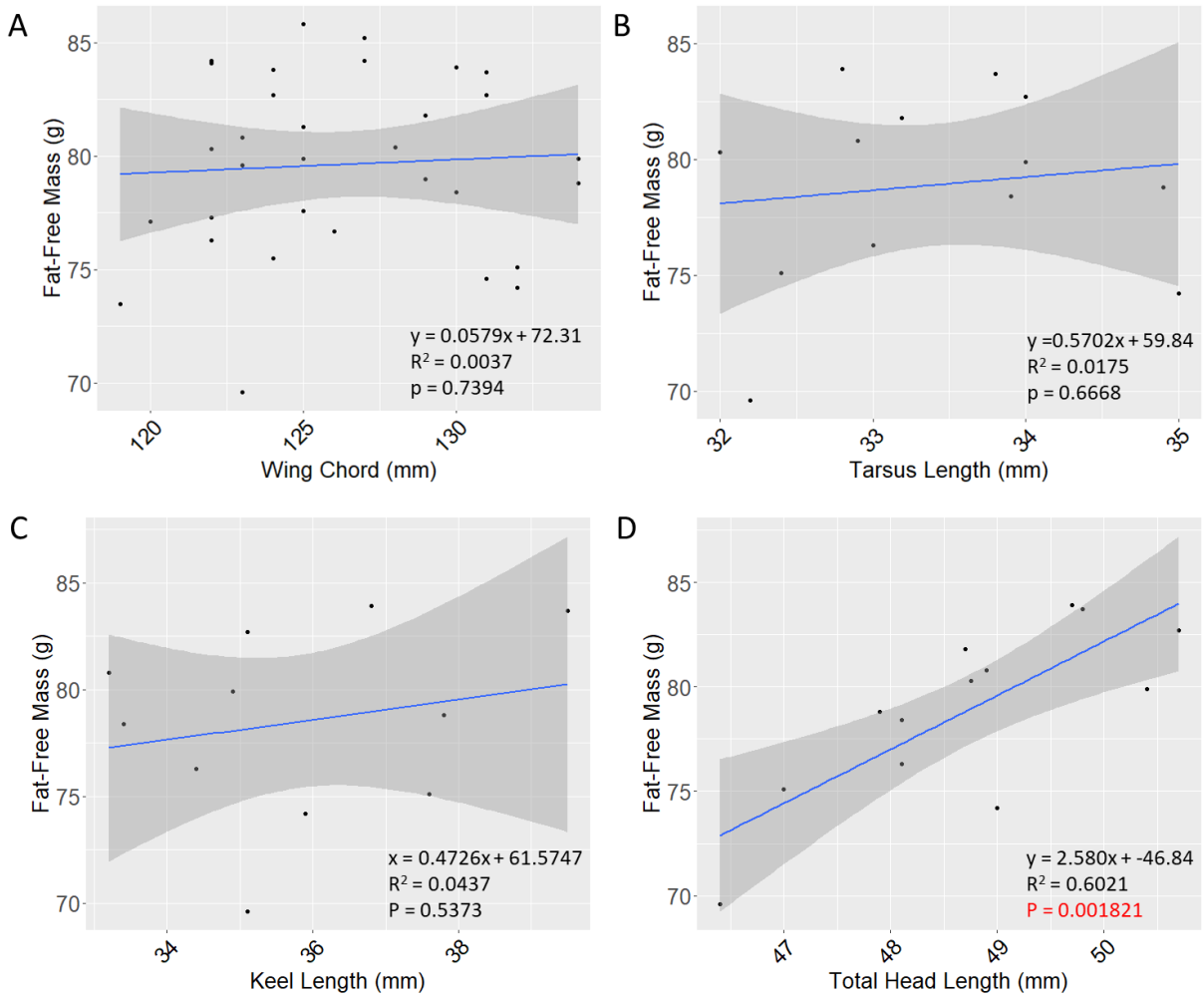
Supplemental Figure 2.1. Depiction of the study timeline. Shade boxes indicate key time points in the experiment, capture, post acclimation, and post treatment. Red droplets indicate that blood samples (~450 ul) were collected from all birds 3-4 weeks after capture (post acclimation) and 33 days following the start of the food treatments (post treatment), and additional blood samples (~450 ul) were collected from only the poor condition group birds after the first 30 days of food restriction, and from only the control birds 19 days following acclimation to captivity.

Supplemental Table 2.2. Scoring system used as a measure subcutaneous fat (Helms and Drury 1960).

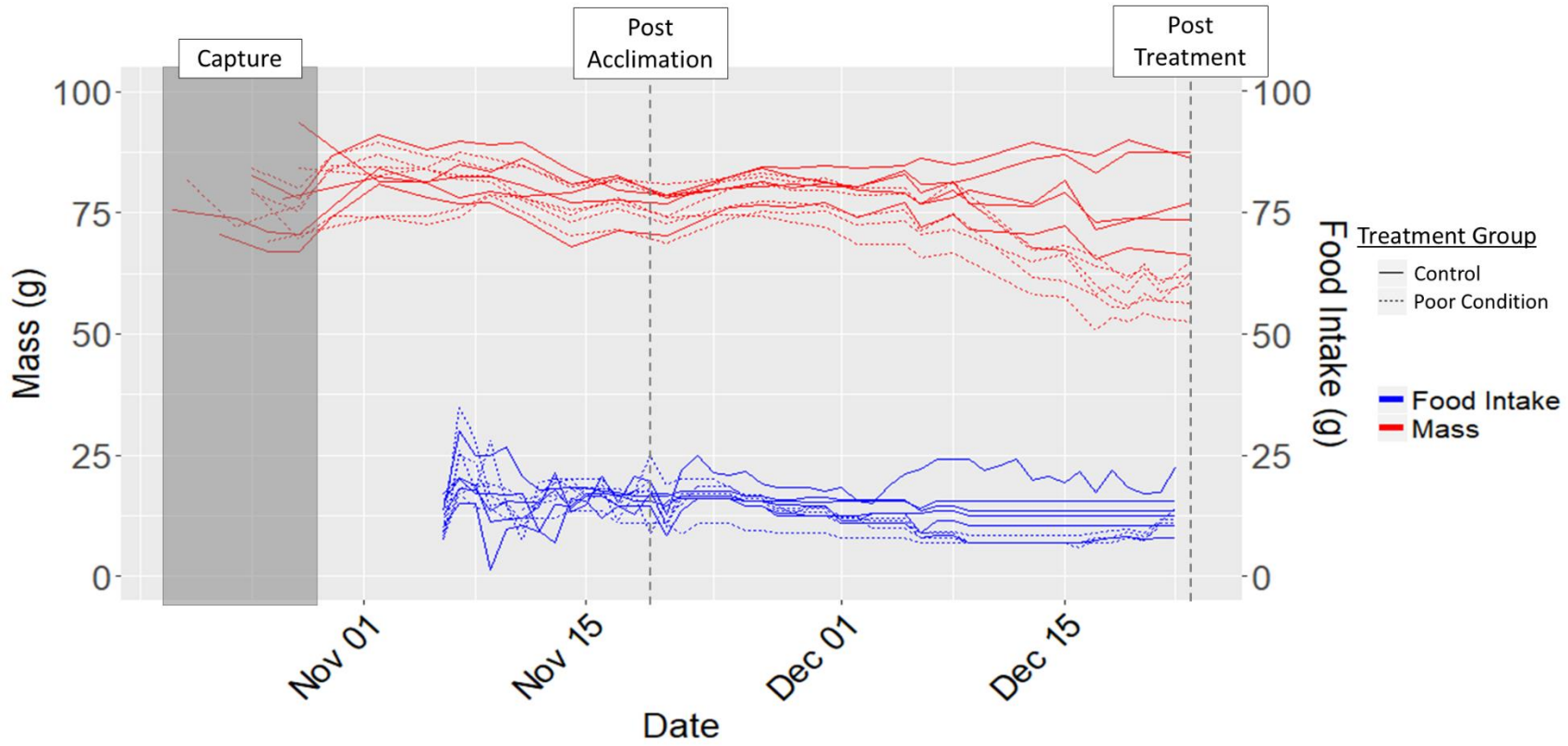
0	No visible fat
1	Traces of fat in the furcular fossa
2	Continuous sheet of fat that does not fill fossa
3	Continuous sheet of fat beginning to fill fossa, but still concave
4	Considerable stores filling furcular fossa, no longer concave
5	Furcular fat deposits conspicuously mounded and convex

Supplemental Table 2.3. Scoring system used as a measure of breast muscle (Bairlein 1995).

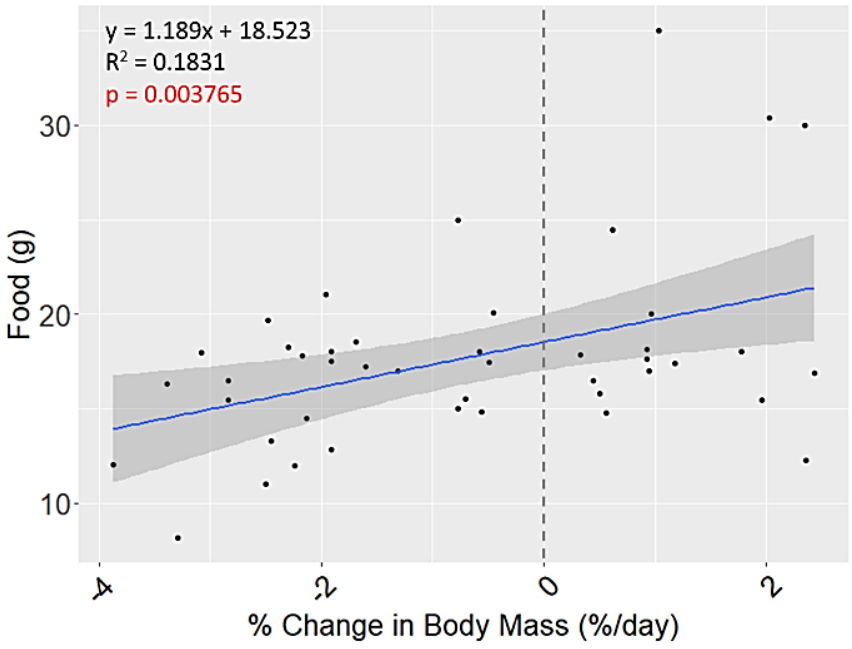
0	The edge of the keel is rough, sharp, and prominent. Very little breast muscle can be felt, and the breast on either side of the keel feels hollow or concave.
1	The keel is prominent but doesn't feel sharp. There is some breast muscle, and the breast on either side of the keel feels flat. This bird is thin.
2	The keel is less prominent, and the edge is smoother. The breast muscle is well developed. The breast on either side of the keel is rounded or convex.
3	The keel feels smooth and not very prominent. Feeling the edge of the keel may be difficult through the plump, rounded breast muscles.



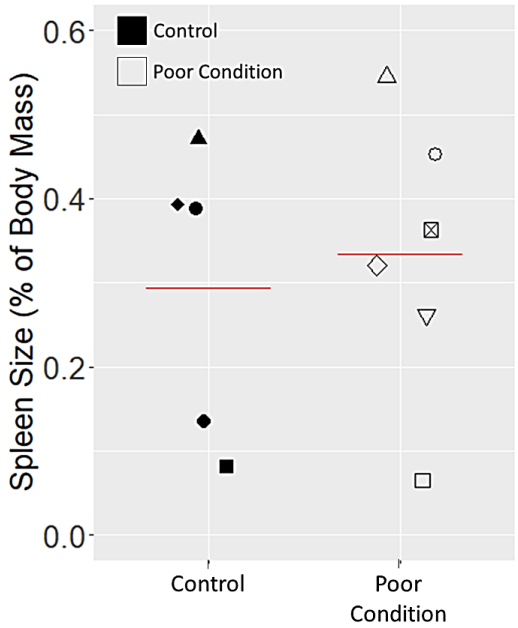
Supplemental Figure 2.2. Four measures of body size regressed against fat-free body mass of American robins captured in September-October, 2015 in Bath, MI by the Burke Lake Banding Station: (A) wing chord, (B) tarsus length, (C) keel length, and (D) total head length, Gray shading indicates 95% confidence intervals.



Supplemental Figure 2.3. Body mass (red lines) and food intake (blue lines) of individual birds from the control group (solid lines) and poor condition group (dotted lines) over the course of the study. The capture time point is different for each individual bird and so the capture time point is indicated by the gray shaded area, while the post acclimation and post treatment time points (which were the same day for all birds) are indicated with gray dashed lines.



Supplemental Figure 2.4. Food intake in grams regressed percent change in body mass per day. Gray shading indicates 95% confidence intervals. The dotted line indicates that there is 0% change in mass at 18.523g of food intake.



Supplemental Figure 2.5. Spleen size (% of body mass) means (red lines) of the treatment groups: control (solid shapes) and poor condition (unfilled). Unique symbols indicate individual birds.

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