Coping with Chronic Infection: The Role of Glucocorticoid Hormones in Mediating Resistance and Tolerance to Parasites

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ABSTRACT

Parasitic infections are ubiquitous, but the consequences to hosts can vary substantially. Variation in the consequences of infection can be related to individual differences in the use of two parasite defense strategies, resistance and tolerance. Resistance entails reducing parasite burden by removing parasites or restricting parasite reproduction. Tolerance involves minimizing the costs associated with a given parasite burden. Genetic variation, environmental conditions, and life history stage can contribute to variation in resistance and tolerance, but the physiological mechanisms that underlie investment in each strategy are not well understood. I proposed that glucocorticoid hormones, which mediate responses to challenges in the physical and social environment in vertebrates, might alter host investment in resistance and tolerance (Chapter I). Glucocorticoids influence a suite of physiological processes including immune function, resource allocation, and tissue growth, all which could alter resistance and tolerance. Using a combination of observational and experimental studies, I test the hypothesis that glucocorticoids mediate resistance and tolerance to infection in red-winged blackbirds (*Agelaius phoeniceus*) infected with Haemosporidians, including malaria (*Plasmodium*) and malaria-like (*Haemoproteus* and *Leucocytozoon*) parasites. I performed a medication experiment (Chapter II) to identify the physiological consequences of Haemosporidian infection and explored the relationships between glucocorticoids and parasite resistance and tolerance in both an observational field study and a hormone manipulation experiment (Chapters III and IV). Medication treatment effectively reduced *Plasmodium* burden, increased hematocrit and

hemoglobin, and reduced the rate of red blood cell production (Chapter II). In an observational field study (Chapter III), red-winged blackbirds with higher plasma glucocorticoid concentrations maintained higher hematocrit than expected for their parasite burdens, suggesting a positive association between glucocorticoids and tolerance. In this study, I found no support for a relationship between glucocorticoids and resistance. However, experimental elevation of glucocorticoids (Chapter IV) yielded nearly opposite results: the higher of two doses of glucocorticoids increased *Plasmodium* burdens and caused a decrease in body mass with increasing parasite burden, indicative of a decrease in tolerance. I discuss possible causes of the differences in our observational and experimental studies and the implications of my work for future studies of individual variation in parasite tolerance (Chapter V).

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GENERAL AUDIENCE ABSTRACT

Why does a cold leave some people bed-ridden, while others can go about their day with only a few sniffles? We can easily see that people react differently when they encounter an infection, but it is not clear why. When faced with an infection, there are two main defense strategies: resistance and tolerance. Resisting infection means reducing the number of parasites or pathogens in the body. Tolerance, on the other hand, refers to reducing the damage or costs that occur during infection. For example, an individual could resist a cold by using the immune system to kill off viruses. If someone tolerates a cold, they might not feel very sick, despite the presence of viruses. Individuals that are more tolerant could be suppressing their own immune response, which can cause the inflammation that leads to a stuffy nose, or these individuals might be repairing damage caused by the virus. Individuals can vary in the extent to which they resist or tolerate infection, but we do not know why this variation exists. In this dissertation, I investigate how the hormones associated with physiological stress might influence resistance and tolerance to malaria and malaria-like parasite infections in red-winged blackbirds. First, I performed a study in which I treated birds infected with a blood-borne parasite with anti-malarial medication to identify the costs of chronic infection. Then I observed the relationships between glucocorticoids (the hormones associated with physiological stress) and estimates of resistance and tolerance in wild red-winged blackbirds. Finally, I used hormone implants to increase stress hormone concentrations in birds held in aviaries, and assessed whether the implants caused changes in resistance and/or tolerance. My results suggest that chronic malaria infection can

damage or destroy red blood cells and birds compensate by increasing the rate of blood cell production. In wild red-winged blackbirds, birds with higher concentrations of stress hormones were able to maintain a higher proportion of red blood cells in the blood for a given parasite burden, suggesting they were more tolerant. Stress hormone levels were not associated with the total number of parasites, and thus, we have no evidence for a relationship between the hormones and resistance. When we increased the hormone levels in the aviary experiment, we found nearly opposite results. A high dose of stress hormones caused an increase in the number of parasites and increased the cost of infection. In this dissertation, I discuss possible explanations for the different results in the observational study and the experiment and suggest avenues for future studies.

DEDICATION

To M.T., U.S., P.B., M.E., and, of course, the birds

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ATTRIBUTION

Dr. Frances Bonier, Assistant Professor, Biology Department, Queen's University, Kingston, ON, CA. Dr. Bonier is my advisor and advisory committee co-chair. She is a co-author on all publications and manuscripts (Chapters II, III, IV) resulting from this dissertation.

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Dr. Mark Haussmann, Professor and Herbert L. Spencer Chair of Biology, Department of Biology, Bucknell University, Lewisburg, PA USA. Dr. Haussmann is a co-author on Chapters II and IV. He assisted with data collection and provided laboratory supplies and equipment for this dissertation.

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CHAPTER I: INTRODUCTON

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Suffering through colds, and watching our friends and family do the same, clearly illustrates that individuals respond differently to immune challenges. Rigorous observation and experimentation yields the same conclusion: there is individual variation in the response to disease and parasites (e.g., Hayward *et al*. 2014a; b; Cai *et al*. 2016). For example, both sex and age influence how mice respond to bacterial antigens (Cai *et al*. 2016) and specific genes affect the susceptibility of wheat to the fungus that causes wheat rust (Steuernagel *et al*. 2016). When confronted with infection, individuals can employ two strategies, resistance and/or tolerance, and differential investment in these defense tactics can lead to individual differences in the consequences of infection.

Resistance and tolerance to parasites

Parasite *resistance* encompasses the idea of "fighting off" an infection, and involves limiting the number of parasites infecting a host. Before a parasite successfully infects the host, resistance can include avoidance or physically blocking infection (Best, White & Boots 2014; Kutzer & Armitage 2016a). Once infected, hosts can resist parasites by removing parasites or limiting their reproduction, often through actions of the host's immune system (Råberg, Graham & Read 2009). More resistant individuals will have fewer parasites or a faster rate of parasite clearance, and, as a result, increased host resistance has a negative impact on parasite fitness (Råberg, Sim & Read 2007). Both genetic and environmental variation can influence resistance, and an individual's ability to resist parasites can vary throughout its lifetime (Knowles, Nakagawa & Sheldon 2009; Sternberg *et al*. 2012; Hayward *et al*. 2014a; Lough *et al*. 2015).

In the field of evolutionary ecology, *tolerance* is defined as minimizing the costs of infection per parasite (Råberg *et al*. 2009). Unlike resistance, tolerance does not inherently reduce parasite fitness and serves as an important defense strategy only after a host is infected (Kutzer & Armitage 2016a). Individuals can increase tolerance by repairing damage incurred during infection or limiting the amount of damage that occurs (Schneider & Ayres 2008). Tolerance is often assessed as "range tolerance," or the change in a fitness or health metric with respect to changing parasite burden (Råberg *et al*. 2009; Little *et al*. 2010; Kutzer & Armitage 2016a). Range tolerance is usually measured as the slope of the relationship between parasite burden and fitness or health for a group of individuals, like a genotype, population, species, or treatment group in an experiment (Råberg *et al*. 2009; Little *et al*. 2010; Kutzer & Armitage 2016a). Most studies compare tolerance among groups, but range tolerance can be used to investigate among-individual variation in tolerance (e.g., Hayward *et al*. 2014b; Jackson *et al*. 2014). Individual-level range tolerance has been measured as the slope of the relationship between an individual's changing parasites burden and health, assessed at multiple time points (Hayward *et al*. 2014b). Another measure called "point tolerance" compares the average health or fitness among groups of organisms at a specific parasite burden (Little *et al*. 2010; Graham *et al*. 2011).

Resistance and tolerance to infection are not mutually exclusive strategies (Mauricio, Rausher & Burdick 1997; Restif & Koella 2004; Athanasiadou *et al*. 2015), but might trade off when activating one strategy limits or influences the other. For example, components of the immune response, such as inflammation, might increase resistance to parasites, but, at the same time, also damage host tissue, thereby reducing tolerance (Schneider & Ayres 2008). However, other contributors to tolerance, such as repairing damaged tissues, might have no impact on

resistance. Understanding the physiological mechanisms underlying individual investment in resistance and tolerance could provide insight into why the consequences of parasite infection vary among individuals.

Stress, glucocorticoids, and parasitic infection

Long-term or repeated exposure to stressors can have profound impacts on health. People faced with stressful life events (e.g., chronic illness in a family member, divorce, financial difficulties) tend to be less able to cope with diseases (Glaser & Kiecolt-Glaser 2005). For example, stress is associated with slower healing of wounds, faster progression from HIV to AIDS, and increased risk for asthma attacks (Leserman *et al*. 1999; Sandberg *et al*. 2000; Walburn *et al*. 2009). As a result, there is a strong interest in the links between physiological mediators of the stress response and the response to diseases. Glucocorticoids are highly conserved vertebrate hormones that increase in response to both psychological and physical challenges, and support a suite of physiological and behavioral changes, including alterations in immune function, metabolism, and reproductive capability (Sapolsky, Romero & Munck 2000). Thus, glucocorticoid hormones could play an important role in influencing the responses to infection, possibly through effects on resistance and tolerance.

Many studies have addressed glucocorticoids' influence on parasite resistance; however, their relationship with tolerance has rarely been explored. Acute increases in glucocorticoids can enhance immune activity, potentially preparing animals for an immune challenge, such as physical injury, and thus could increase resistance (Dhabhar & McEwen 1999; Dhabhar 2002; McCormick & Langkilde 2014). In contrast, chronic or long-term elevation of glucocorticoids can cause immunosuppression and lead to higher parasite burdens (e.g., Applegate 1970; Belden & Kiesecker 2005; Bourgeon & Raclot 2006). In fact, researchers have used treatment with

glucocorticoids to manipulate hosts' parasite burdens (LaFonte & Johnson 2013). Alternatively, glucocorticoid-driven immunosuppression could increase tolerance if it reduces aspects of immunity that also damage host tissues, like inflammation (Råberg *et al*. 1998; Medzhitov, Schneider & Soares 2012). Glucocorticoids could also improve tolerance if they support tissue repair or limit other types of damage. To my knowledge, only one published study tests the relationship between glucocorticoids and tolerance. In this laboratory-based experiment, elevating glucocorticoids had no effect on tolerance to the chytrid fungus *Batrachochytrium dendrobatidis* in captive American toads (*Anaxyrus americanus*) (Murone, DeMarchi & Venesky 2016). In my dissertation, I investigate the role of glucocorticoids in mediating resistance and tolerance to infection with avian malaria and malaria-like parasites (Haemosporidians) in red-winged blackbirds (*Agelaius phoeniceus*).

Study system

I conducted all of my studies with adult red-winged blackbirds, and almost every bird in the focal population (see below) was infected with Haemosporidian parasites. Avian Haemosporidans are insect-vectored eukaryotic parasites that include three genera: *Leucocytozoon*, *Haemoproteus*, and *Plasmodium* (the cause of avian malaria). They infect every avian order and are distributed across every continent except Antarctica (Valkiūnas 2005; Lapointe, Atkinson & Samuel 2012). Where the parasites are endemic, as in much of North America, they are thought to rarely cause immediate mortality, but chronic low intensity infections can reduce reproductive success and survival (Knowles, Palinauskas & Sheldon 2010a; Lapointe *et al*. 2012; Asghar *et al*. 2015). When a bird is first infected, it enters an acute infection phase characterized by a high parasite burden. The acute phase is followed by a chronic

phase, in which birds harbor relatively low parasite burdens for an extended period, which could last from days or weeks to years (Valkiūnas 2005; Asghar *et al*. 2012).

I conducted all studies on red-winged blackbirds that breed in southeastern Ontario, Canada, including study sites at Queen's University Biological Station (44°34'02.3" N, 76°19'28.4" W), the township of Rideau Lakes (44°36'28.8" N, 76°13'38.3" W), and the city of Kingston (44°15'04.8" N, 76°28'43.6" W). Red-winged blackbird populations in this area have a high prevalence of Haemosporidian infection, but can survive multiple years and successfully breed while infected (Weatherhead & Bennett 1991). Using blood smears to diagnose infection, Weatherhead and colleagues (Weatherhead 1990, Weatherhead and Bennett 1991, Weatherhead et al. 1993) found prevalence to be 30 – 56% in females and 35 – 71% in males in the 1980s and early 1990s. With molecular techniques, I detected prevalence of over 90% from 2013-2015 (Chapter III). As infection can reduce fitness (e.g., Marzal *et al*. 2005; Knowles *et al*. 2010; Martínez-de la Puente *et al*. 2010; Asghar *et al*. 2015), and this population has been exposed to Haemosporidians for at least 25-30 years, and likely much longer, selection might have shaped the response to the parasites in these birds. Thus, my study system provides an exciting opportunity to evaluate hormonal regulation of resistance and tolerance to Haemosporidian infection.

Approach and objectives

To assess tolerance, I first needed to understand the costs of parasite infection. Thus, I used medication to experimentally reduce chronic Haemosporidian burdens in captive red-winged blackbirds, and evaluated the physiological consequences of infection (Chapter II). I conducted an observational field study across multiple years and life history stages to identify if there are natural associations between glucocorticoids and parasite resistance and tolerance (Chapter III).

Finally, I performed a glucocorticoid manipulation experiment in captive birds to evaluate if glucocorticoids can cause changes in resistance and tolerance (Chapter IV). I also tested whether experimentally elevated glucocorticoids cause changes in immune function or tissue repair, which could underlie glucocorticoids' effects on resistance and tolerance (Chapter IV).

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CHAPTER II. AN EXPERIMENTAL TEST OF THE PHYSIOLOGICAL CONSEQUENCES OF CHRONIC INFECTION

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Abstract

Non-lethal parasite infections can reduce host fitness through impacts on reproduction and survival. As a result, these parasites can influence host ecology as well as the evolution of host morphology, behavior, and physiology. The physiological consequences of non-lethal infection can provide insight into the processes underlying parasite-driven natural selection. Here, we evaluate the physiological consequences of non-lethal, chronic infection in an avian host-parasite system, adult male red-winged blackbirds (*Agelaius phoeniceus*) infected with Haemosporidian parasites. Chronic Haemosporidian infection has been shown to reduce both reproductive success and survival in several avian species. We used anti-malarial medications to experimentally reduce Haemosporidian parasite burdens and measured the effect of treatment on body condition, hematology, immune function, physiological stress, and oxidative state. Treatment with an anti-malarial medication reduced burden for the most prevalent Haemosporidian parasites from the genus *Plasmodium*. Treatment also increased hemoglobin and hematocrit, and decreased red blood cell production. We detected no effect of treatment on body condition, immune metrics, plasma corticosterone concentrations, total antioxidant capacity, or reactive oxygen metabolites. Our results suggest that the physiological costs of chronic Haemosporidian infection could be primarily caused by the damage and replacement of red blood cells during infection. Strong links between parasite burden and the physiological consequences of infection indicate that even for chronic, low intensity infections, measuring parasite burden rather than only presence/absence

could be important when evaluating the role of infection in influencing host ecology or evolution.

Introduction

Parasites shape the evolution and ecology of their hosts (Price *et al*. 1986; Sheldon & Verhulst 1996; Hudson, Dobson & Newborn 1998). By reducing host fitness, parasites can drive host distributions and abundance, as well as influence selection on host morphology, physiology, and behavior (Schmid-Hempel 2003; Hechinger & Lafferty 2005; Ezenwa *et al*. 2016). The consequences of infection for hosts and populations are relatively easy to predict when the parasites cause direct host mortality. However, most parasitic infections are sub-lethal which are often characterized by chronic, low-intensity infections that might not produce overt clinical signs, but can still influence individuals' fitness (Anderson & May 1979; Gulland 1995). Understanding the physiological consequences of sub-lethal infections could provide insights into both the individual and population-level impacts of parasites.

Avian Haemosporidian parasites often serve as models to evaluate the ecological and evolutionary consequences of infection. For example, Haemosporidians are frequently used in studies of life history trade-offs (e.g. Karell *et al*. 2007; Arriero & Møller 2008; Knowles, Wood & Sheldon 2010b; Christe *et al*. 2012) and host-parasite coevolution (e.g. Ricklefs, Fallon & Bermingham 2004; Fallon, Bermingham & Ricklefs 2005). Avian Haemosporidian parasites provide a convenient study system because they infect species from every avian order, are found on every continent except Antarctica, and, where the parasites are endemic, rarely directly cause mortality (Valkiūnas 2005; Lapointe *et al*. 2012). In addition, infection status and parasite burdens are relatively easy to quantify using molecular techniques and microscopy (Godfrey, Fedynich & Pence 1987; Richard *et al*. 2002).

Haemosporidian parasites have complex life cycles and their effects on hosts can vary throughout the course of infection. All 3 genera of avian Haemosporidians (*Plasmodium*, *Haemoproteus*, and *Leucocytozoon)* are transmitted by insect vectors (Valkiūnas 2005). Once the parasites successfully invade their host, the bird experiences a brief acute infection phase in which the host carries high parasite burdens (Lapointe *et al*. 2012; Asghar *et al*. 2012) followed by a chronic phase of infection characterized by low parasite burdens, which could persist throughout an individual's lifetime (Manwell 1933; Bishop, Tate & Thorpe 1938). Both the acute and chronic phases of Haemosporidian infection can be costly. Acute infections can cause mortality, particularly in species that have been recently introduced to the parasites (Atkinson *et al*. 1995). During the acute phase, birds can reduce activity levels and food intake, lose body mass, and experience severe declines in both hematocrit and hemoglobin (Atkinson *et al*. 1995; Ellis *et al*. 2015; Mukhin *et al*. 2016). Less is known about the consequences of chronic infection, in part, because most previous studies are observational. However, experiments involving treatment with anti-Haemosporidian medication indicate that infection can reduce both survival and reproductive success (Merino *et al*. 2000; Marzal *et al*. 2005; Knowles *et al*. 2010a; Martínez-de la Puente *et al*. 2010). Few studies experimentally test the physiological responses to chronic infection. A long-term observational study paired with an infection experiment found chronic infection-driven reductions in fitness in the great reed warbler (*Acrocephalus arundinaceus*) were mediated through telomere attrition (Asghar *et al*. 2015). Therefore, there is evidence for a direct link between the physiological and fitness consequences of chronic infection. However, we do not know the extent of the physiological consequences of chronic infection.

Here, we experimentally reduced the parasite burden in adult male red-winged blackbirds (*Agelaius phoeniceus*) naturally experiencing chronic Haemosporidian infection. We measured changes in hematology, a hormonal mediator of the stress response, oxidative status, immune function, and body condition. We describe each metric and its plausible relationship to Haemosporidian infection in Table 2.1.

Materials and Methods

Study population, field methods, and housing

We captured 29 adult male red-winged blackbirds in April – May 2015 at Queen's University Biological Station (44°34'02.3" N, 76°19'28.4" W) and on nearby private property in Elgin, Ontario (44°36'28.8" N, 76°13'38.3" W) using mist nets with a conspecific playback and decoy or a seed-baited Troyer V-top trap. This population has been known to have high prevalence of Haemosporidian infection since the late 1980s (Weatherhead 1990; Weatherhead & Bennett 1991; Weatherhead *et al*. 1993) and from 2013-2015, prevalence was over 90% (Schoenle *et al*. unpublished data). We housed birds in semi-natural conditions using an outdoor aviary consisting of 30 large flight aviaries (6 x 2.5 x 2.5 m) located at Queen's University Biological Station. We fed the birds a diverse diet *ad libitum*, including poultry starter, seed mix, mealworms, dragonflies, hard-boiled egg, and fruit. Prior to the start of this study, the birds were included as the control group for a hormone-manipulation study (see Appendix A for details).

Experimental Design

Two weeks before the start of the experiment, we collected a blood sample and assessed the birds' Haemosporidian burden. We grouped the birds into dyads according to their parasite burden rank, and within each dyad, randomly assigned each bird to the control group (N = 14) or the anti-parasitic medication group (N = 15). One bird from each treatment group was assigned

to each aviary. Because we had an odd number of birds, one bird in the medication group was housed with a bird not included in the present study. The birds acclimated in their new aviary for one week prior to the start of the experiment.

On the first day of the study, we collected a blood sample (details below), weighed each bird, and assessed furcular fat by assigning a score on a 0-5 scale (birds in this study ranged from 0-3) (Wingfield & Farner 1978). We then orally administered 200 μL of either the control or medication treatment once per day for three days. We took blood samples and weighed each bird again 7 and 14 days later. Our protocol was modeled on treatments used in the veterinary and parasitology literature (Cranfield *et al*. 1994; Remple 2004). On each of the 3 treatment days, we gave the control group 10% sugar water. On the first treatment day, we gave the medication group 10 mg/kg Primaquine (Sigma-Aldrich 160393, St. Louis, MO, USA) and 25 mg/kg Chloroquine (Sigma-Aldrich C6628, St. Louis, MO, USA) (dose calculated for a 68 g bird) dissolved in 10% sugar water. On the second and third treatment days, we administered 10 mg/kg Primaquine and 15 mg/kg Chloroquine. Primaquine targets parasites in the blood and other tissues, and while intended for treatment of *Plasmodium* (World Health Organization 1995), has been found to be effective against avian *Plasmodium, Haemoproteus*, and *Leucocytozoon* (Graczyk *et al*. 1994; Merino *et al*. 2000; Marzal *et al*. 2005). Chloroquine targets the blood-borne *Plasmodium* parasites, but has been used effectively to treat all avian Haemosporidian genera (Graczyk *et al*. 1994; Remple 2004; Karell *et al*. 2011). To our knowledge, there are no direct effects in birds of either medication on the physiological metrics assessed during this study.

At each sampling point we collected ~500 μL of blood into heparinized capillary tubes. The first 150 μL were collected within 3 minutes (mean ± SE, 100 ± 2.2 s) of approaching the

aviaries to measure baseline corticosterone, the primary avian glucocorticoid hormone (Romero & Reed 2005). Corticosterone increases rapidly during capture and handling, but there was no relationship between the time from approaching the aviaries to collecting the blood sample and log corticosterone (LMM with individual identity as a random effect, $\beta = 0.004$, SE = 0.003, P = 0.17, N = 87), suggesting that our measures provide a reasonable estimate of baseline corticosterone. We sampled birds housed in the same flight aviary simultaneously and always at the same time of day. The start of the experiment was staggered over three days and all sampling was performed between 9:00 – 15:00, with an hour and a half between sampling aviaries.

We used the blood samples to assess the presence/absence of *Plasmodium*, *Haemoproteus*, and *Leucocytozoon*, parasite burden for each genus, white blood cell differentials, hematocrit, hemoglobin, polychromasia, corticosterone, PIT54 (a haptoglobin-like protein), nitric oxide, and oxidative stress (Table 2.1). We used a PCR/restriction enzyme-based assay to test for the presence/absence of each Haemosporidian genus pre-treatment and 7 days after the initial treatment. All other measures were assessed at each sampling point.

Blood Processing, Hemoglobin, and Hematocrit

We created two blood smears on glass slides with 5-10 μL of whole blood and measured hemoglobin concentration in ~10 μL of fresh whole blood with a Hemocue Hb 201+ (Hemocue, Ängelholm, Sweden). Blood smears were air dried and fixed in methanol within 1 hour of sample collection and stained with Giemsa within 2 months. We reserved ~20 μL of whole blood for DNA extraction and centrifuged the remaining blood sample in capillary tubes at 6,000 RPM for 10 minutes within 1 hour sample collection. We measured hematocrit for two capillary tubes with a microhematocrit card and used the average of the two measures in analyses. Then, we

separated the plasma from the red blood cells and froze the plasma, cells, and whole blood at -20 °C.

Red Blood Cell Production (Polychromasia)

On blood smears stained with Giemsa, immature red blood cells tend to be polychromatic, and can be identified by a cytoplasm stained a darker blue/purple, less densely packed chromatin, and in some cases, a more rounded shape (Campbell 1995; Mitchell & Johns 2008). A single observer (MK) who was blind to the treatment group of the samples counted the number of polychromatic cells in ~5,000 red blood cells on a compound microscope at high magnification (1000X, oil immersion). We estimated the total number of red blood cells in each field of view by comparing the spread of red blood cells to standardized photographs of known cell counts (Ricklefs & Sheldon 2007). This measure of polychromasia can be used as an estimate of the red blood cell production rate (Johns, Shooshtari & Christopher 2008; Mitchell & Johns 2008).

Haemosporidian Infection Prevalence and Intensity

We extracted DNA from whole blood or red blood cells using Qiagen DNeasy Blood and Tissue kits (Qiagen, Valencia, CA, USA). We used a restriction enyme-based assay slightly modified from Beadell and Fleischer (2005) to test for the presence/absence of *Plasmodium*, *Haemoproteus,* and *Leucocytozoon* parasites (details in Appendix A). In brief, we began with a PCR to amplify a segment of the mitochondrial genome common to all avian Haemosporidians and then used restriction enzymes to digest the PCR product into fragments that vary in size among *Plasmodium*, *Haemoproteus,* and *Leucocytozoon*. We identified the genera by visualizing the fragments after separation on a 4% agarose gel.

A single observer (LAS), who was blind to the treatment group of each sample, counted the number of infected red blood cells out of ~10,000 cells on a compound microscope at high magnification (1000X, oil immersion) and identified the genus of each parasite by morphology (Valkiūnas 2005). We estimated the number of red blood cells in each field of view in the same manner as for polychromasia.

Corticosterone Assay

We performed a double extraction of the hormone with 3 mL dichloromethane per extraction before quantifying total corticosterone in about 40 μL of plasma (mean ± SE, 39.6 ± 0.23 μL) using a single, direct radioimmunoassay (Lendvai *et al*. 2014). We corrected samples individually for extraction efficiency using a known-quantity of radiolabeled hormone that was added to each sample prior to extraction. The average extraction efficiency was 78.7%. We estimated within-assay variation from four replicates of known-concentration standards to be 9.7%, and the detection limit was ~0.97 ng/mL. Fifty-four of the 87 samples fell below the detection limit and, for these samples, corticosterone was estimated as the lowest reliable measure from the standard curve.

Immune Assays

We used a colorimetric assay from a commercial kit (TP-801, Tri-delta Diagnostics Inc., Morris Plains, NJ, USA) to measure PIT54 (a haptoglobin-like acute phase protein found in birds) and followed the manufacturer's instructions exactly. All samples were run singly across two plates and all samples from each individual were run on the same plate. Within assay variation in replicates of known-concentration standards was 2.9% and 1.5% and inter-assay variation was 4.4%.

When nitric oxide oxidizes, it forms nitrate and nitrite, which can be measured in a colorimetric assay and used to quantify blood concentrations of nitric oxide (Sild & Hõrak 2009). We removed proteins from plasma samples (mean ± SE, 20.1 ± 0.37 μL) by centrifuging plasma through a 10kD spin column (Corning Spin-X UF 500, catalogue #431478, Tewksbury, MA, U.S.A.) at 10,000 x g for 10 minutes. We used a commercial kit (Abcam, ab65328, Cambridge, MA, U.S.A.) to determine the concentration of combined nitrate and nitrite in the deproteinated plasma and followed the manufacturer's instructions exactly. All samples were run in duplicate on three 96-well plates with all samples from a single individual on the same plate, and a replicate of a plasma pool on each plate. Inter-assay variation estimated from the replicate plasma pools, was 15.4%.

A single observer (MK), who was blind to the treatment group of each sample, counted the number of each white blood cell type (heterophil, lymphocyte, monocyte, basophil, and eosinophil) at high magnification (1000X, oil immersion) on a compound microscope until the total number of white blood cells tallied reached 100. We identified white blood cell types using the criteria from Campbell (1995).

Oxidative Status Assays

We measured reactive oxygen metabolites in plasma using the dROMs test (Diacron International, Grosseto, Italy) optimized for red-winged blackbirds. We added 200 μL of reagent 1 and 2 μL of reagent 2 (provided in the kit) to 2 μL of plasma, and then incubated the plate for 30 minutes at 37 °C. We then measured the absorbencies of the samples at 505 nm. We ran samples in duplicate across three 96-well plates and included all samples from a single individual on the same plate. We used two standard plasma pools per plate to calculate variation within and across assays. Average within-assay variation was 7.0% and inter-assay variation was 17.1%.

We measured total antioxidant capacity of plasma using the OXY-Adsorbent test (Diacron International, Grosseto, Italy) optimized for red-winged blackbirds. We diluted 5 μL plasma in 45 μL distilled water, then combined 5 μL of the diluted plasma with 200 μL of the reagent 1 and 2 μL of reagent 2 (reagents provided in kit), and incubated the plate for 25 minutes at 37 °C. We read the plate at 505 nm. We ran samples in duplicate across three 96-well plates including all samples from a single individual on the same plate and two standard plasma pools. As calculated from the plasma pools, average within-assay variation was 0.4% and inter-assay variation was 10.4%. Parasites could influence the concentrations of reactive oxygen species and total antioxidant capacity independently, therefore, we evaluated the effects of treatment on each oxidative status metric separately.

Statistical Analyses

We performed all analyses in R version 3.3.2 (R Core Team 2016) and set $\alpha = 0.05$. For regression models, we report the unstandardized regression coefficient as β. We used the natural log transformation of corticosterone and the natural log of parasite burden + 1 in our analyses to improve model fit.

To identify if the birds in the control and medicated group were similar prior to treatment, we tested the two treatment groups for differences (at day 0) in parasite burden for each genus and the physiological metrics shown in Table 2.1. We used a Shapiro test to determine if each measure was normally distributed, and if so, used a t-test to compare levels between treatment groups. If the measure was not normally distributed, we used the non-parametric Kruskal-Wallis one-way analysis of variance.

To assess the effectiveness of the medication treatment, we used mixed effects models with change in parasite burden as the response variable, and treatment, days post-treatment, and

the interaction of treatment and days as predictor variables. Individuals varied in their initial parasite burdens, and thus, we included pre-treatment parasite burden as a fixed effect in each candidate model, including the null model. We performed separate analyses for each of the three parasite genera (including only birds that were PCR-positive for the focal genera) because the medication's effectiveness could vary by genus.

To test the hypotheses that malaria infection alters immune function, induces a hormonal stress response, and has hematological, oxidative, and energetic costs, we compared sets of candidate linear mixed effects models predicting change in each physiological measure (Table 2.1) from pre-treatment levels. We used absolute change in the physiological metrics as response variables. Predictor variables included individual identity as a random effect and treatment, days post-treatment, the interaction between treatment and days post-treatment, and the pre-treatment value of the metric of interest as fixed effects. Testing for an interaction between day and treatment allows us to determine if there is a lag time between the application of the treatment and the response. We included the pre-treatment value of each physiological measure as a fixed effect in every candidate model, including the null model, to control for individual variation pre-treatment. We used second order Akaike's Information Criterion (AICc) to select the top model(s) (within 2 AICc of the best fit model) from the full set of candidate models, including the null model. If there were multiple top models, we used model averaging to obtain parameter estimates for each predictor variable.

To determine if the parasites cause dose-dependent changes in the physiological metrics or if the presence of parasites alone can elicit some effects (Table 2.1), we assessed the relationship between parasite burden and each physiological metric within the medication treatment group, using all samples collected from each bird. We performed mixed linear

regression models with the physiological trait as the response variable and parasite burden as a fixed effect and individual identity as a random effect. The medication treatment was only effective in reducing *Plasmodium* parasite burden (see results below), and thus analyses addressing the relationship between physiological traits and parasite burden only consider *Plasmodium* burden.

Results

Pre-treatment Measures

One control bird tested PCR-negative for Haemosporidian parasites and was excluded from all analyses. Of the remaining 28 birds, PCR indicated that all were positive for *Plasmodium*, 7 were also positive for *Haemoproteus* (3 in the control group, 4 in medicated group), and for 9 were positive for *Leucocytozoon* (3 in the control group, 6 in the medicated group). In birds infected with *Plasmodium*, the percent of infected red blood cells ranged from 0 – 2.4% (mean ± SE, 0.46 ± 0.09%, N = 28), where 0% indicates a very low infection intensity such that we observed no infected cells on the slide, but the bird was PCR-positive for the parasite. For *Haemoproteus*, we detected a single parasite on the slide for only 1 of the 7 PCR-positive birds. It is unclear which type(s) of blood cell *Leucocytozoon* infected (the parasites distort the cell and host cell types vary within the genus) (Huff 1942; Steele & Noblet 1993; Valkiūnas 2005; Zhao *et al*. 2015), and in scans of 10,000 red blood cells we observed 0-2 infected cells (mean ± SE, 0.56 ± 0.24 cells, N = 9). Birds assigned to the control (N = 13) and treatment groups (N = 15) did not significantly differ in parasite burdens of *Plasmodium*, *Haemoproteus*, or *Leucocytozoon* (all $P > 0.28$). Treatment groups were also similar for hematological (all $P > 0.38$), immunological (all $P > 0.09$), hormonal ($P = 0.78$), oxidative stress (all $P > 0.68$), and condition (all $P > 0.26$) metrics.

Effectiveness of the Medication Treatment

The anti-parasitic medication significantly reduced *Plasmodium* burden (Figure 2.1A), and the change in *Plasmodium* burden varied with days post-treatment and pre-treatment *Plasmodium* burden (Table 2.2A). Treatment had no effect on *Haemoproteus* (Table 2.2B) or *Leucocytozoon* (Table 2.2C) burden. However, the small sample of *Haemoproteus*- and *Leucocytozoon*-positive birds and their low initial parasite burden might have masked any effect, if present.

Physiological Effects of Treatment

The medication treatment increased both hematocrit (β = 2.88, SE = 0.62, P <0.0001, N = 56) (Figure 2.1B) and hemoglobin (β = 15.80, SE = 3.12 P <0.0001, N = 56) (Figure 2.1C). The effect of treatment on red blood cell production (estimated with polychromasia) varied by day (β = -14.39, SE = 5.42, P = 0.0009, N = 56) (Figure 2.1D). We conducted post-hoc linear regressions to evaluate differences in the change in polychromasia at 7 and 14 days post treatment, including both treatment and the pre-treatment polychromasia value as predictors. Birds receiving the medication treatment decreased red blood cell production more than birds in the control group both 7 (β = -124.10, SE = 58.90, P = 0.05, N = 56) and 14 (β = -201.11, SE = 49.09, P = 0.0004, N = 56) days post-treatment. The decrease in red blood cell production was greater 14 days post-treatment, suggesting there could be a time lag between the initial decline in parasite burden and maximum change in the rate of red blood cell production. The medication treatment had no effect on percent change in body mass, or change in fat score, corticosterone concentration, oxidative stress metrics, or any immune measure (Appendix A, Tables A1 and A2). However, our ability to detect change in corticosterone was limited because many samples

(62.1%) fell below the detection limit. Corticosterone concentrations were low (mean ± SE, 1.74 ± 0.16 ng/mL, N = 87) throughout the study.

Dose-dependent Effects of Plasmodium Burden

Among birds receiving the medication treatment, higher *Plasmodium* burdens were associated with lower hematocrit (β = -1.03, SE = 0.24, P = 0.0001, N = 45) and hemoglobin (β = -6.75, SE = 1.22, P = <0.0001, N = 45) and higher red blood cell production (β = 114.96, SE = 16.27, P = <0.0001, N = 45) (Figure 2.2A-C). *Plasmodium* burden was not correlated with body mass, fat score, corticosterone, oxidative stress metrics, or any immune measures (haptoglobin, nitric oxide, and white blood cell differentials) (all P > 0.11, Appendix A, Table A3).

Discussion

Our results provide experimental evidence supporting the patterns found in observational field studies (e.g. Dawson & Bortolotti 1997; Norte *et al*. 2009; Soares, Ellis & Ricklefs 2016). Treatment of chronic *Plasmodium* infection increases hematocrit and hemoglobin concentrations and reduces the rate of red blood cell production. These hematological measures were correlated with variation in *Plasmodium* burden, indicating that even with low intensity infections (<2.5% of red blood cells infected), effects of infection can depend on the number of parasites, not only the parasites' presence. Hematocrit and hemoglobin can be indicative of aerobic capacity and declines could limit individuals' ability to perform energetically demanding tasks, like parental care or flight (Calbet *et al*. 2006). Observational and experimental studies demonstrate that lower hematocrit and hemoglobin can be associated with lower reproductive performance and success, including decreases in the number and body mass of offspring (Fronstin, Christians & Williams 2015; Minias 2015). Therefore, the loss of red blood cells might be a mechanism for *Plasmodium*-caused reductions in reproductive success, and potentially lead to variation in

offspring phenotype. Increasing the rate of red blood cell production allows birds to partially compensate for the cells damaged or destroyed during infection, but cell division can also reduce telomere lengths (Allsopp *et al*. 1995). Thus higher rates of red blood cell production might underlie the recent finding that chronic *Plasmodium* infection can increase telomere attrition in red blood cells (Asghar *et al*. 2015). However, infection can also shorten telomeres in parallel body tissues, including the liver, brain, lungs, spleen, heart, and kidney (Asghar *et al*. 2016).

Treatment of chronic *Plasmodium* infection had no impact on our three measures of immune function: nitric oxide concentration, PIT54 concentration, and white blood cell differentials. Although these immune metrics can respond to acute phase avian *Plasmodium* infection (Cellier-Holzem *et al*. 2010; Bichet *et al*. 2012; Ellis *et al*. 2015), different components of the immune response might be important during chronic infection. A study by Videvall et al. (2015) found that at the peak of acute *Plasmodium* infection, birds expressed more immune genes overall, but as parasite burden declined, genes associated with mature B-cell differentiation were expressed at higher levels. Immune components that are beneficial in controlling parasite burden during acute infection, like nitric oxide, can damage host cells as well, and might be too costly to maintain at high levels during chronic infection (Bichet *et al*. 2012; de Macchi *et al*. 2013).

Treatment of chronic *Plasmodium* infection also had no effect on measures of corticosterone, oxidative status, fat, or body mass. In field studies, the relationship between corticosterone and Haemosporidian infection varies (Garvin *et al*. 2006; Cornelius, Davis & Altizer 2014a), and in many cases, the energetic costs of chronic infection might not be sufficient to cause an increase in corticosterone. Corticosterone concentrations were low throughout our study (over 50% of samples were below the assay detection limit), and as a result, we might have

been unable to detect relationships with corticosterone, if they existed. Low corticosterone concentrations could be caused by a reduction in energetic demand during captivity due to *ad libitum* food and limited activity levels, hormonal suppression due to captivity-related chronic stress, (Cyr & Michael Romero 2007; Martin *et al*. 2011), or because the birds were transitioning from the breeding life history stage to post-breeding/molt, in which birds tend to have lower corticosterone (Romero 2002). The relationship between Haemosporidian infection and pro-oxidants and/or antioxidant defenses varies among field studies and can depend on breeding stage, reproductive effort, sex, or age (van de Crommenacker *et al*. 2012; Isaksson *et al*. 2013; Delhaye, Jenkins & Christe 2016). In addition, few observational studies find relationships between infection status or parasite burden and body mass or other measures of condition (e.g. Bennett, Caines & Bishop 1988; Ots & Hõrak 1998; Shutler *et al*. 1999; Kilpatrick & LaPointe 2006a). Chronic infection might only influence oxidative stress, corticosterone, or condition metrics when individuals experience additional challenges, like food limitation or reproduction. In the aviary setting, birds were removed from such challenges and likely had sufficient resources to prevent oxidative damage, loss of body mass or fat, or increases in corticosterone.

Despite robust relationships between chronic infection and hematological measures, we found no evidence that chronic infection is a physiological stressor, increases oxidative damage, or has energetic costs. Further work is required to determine which components of the immune response are activated during chronic infection and whether immune activation has additional physiological consequences.

In summary, our results suggest that individual fitness costs associated with avian *Plasmodium* infection could be primarily driven by the damage and replacement of red blood cells. In addition, the costs of infection depend on the burden, not only the presence of parasites,

and thus hosts' ability to limit parasite burden through defenses like immune activation or infection avoidance might have important fitness consequences. Future work on non-lethal parasite infections should consider parasite burden, rather than presence/absence alone. The consequences of non-lethal infections for individuals and populations could be linked to subtle physiological changes that occur during the course of infection.

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Figures

Figure 2.1: The effect of anti-Haemosporidian medication on (A) *Plasmodium* burden, (B) hematocrit, (C) hemoglobin, and (D) red blood cell production estimated as polychromasia. The control group is represented by triangles and solid lines and the medication group is represented by open circles and dashed lines. Bars indicate the standard error around the mean at each time point.

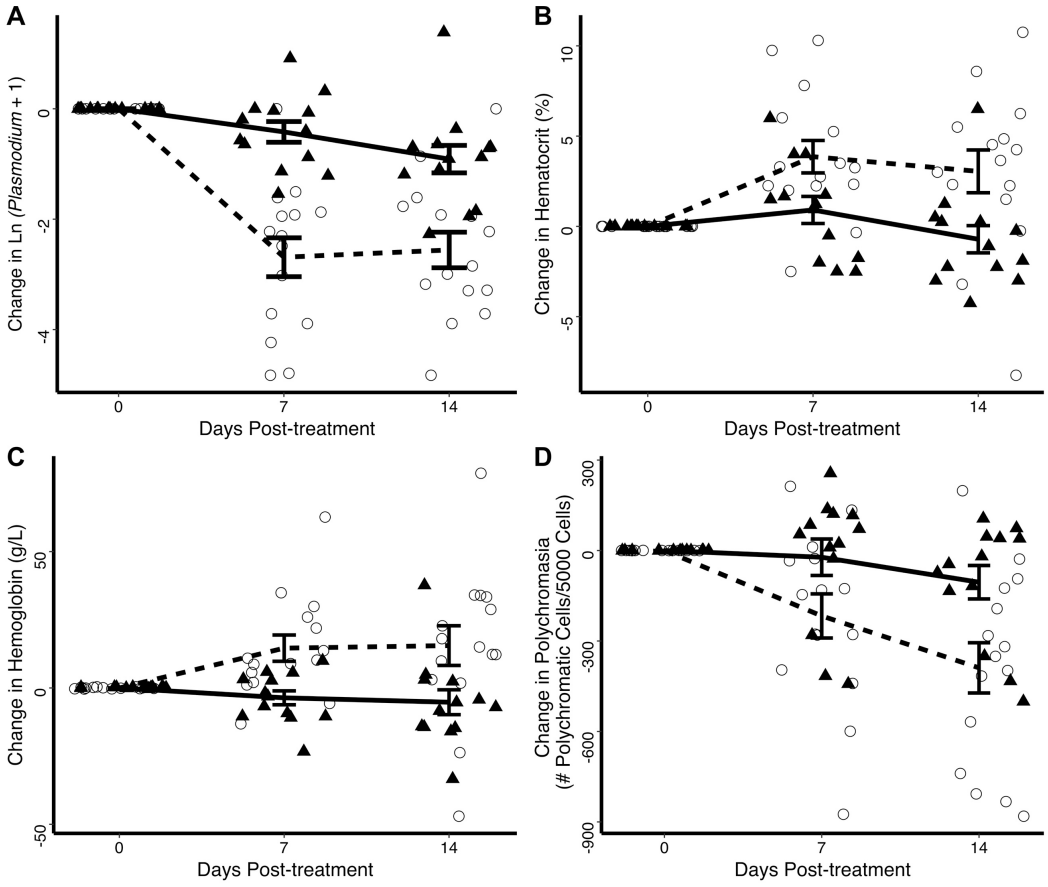
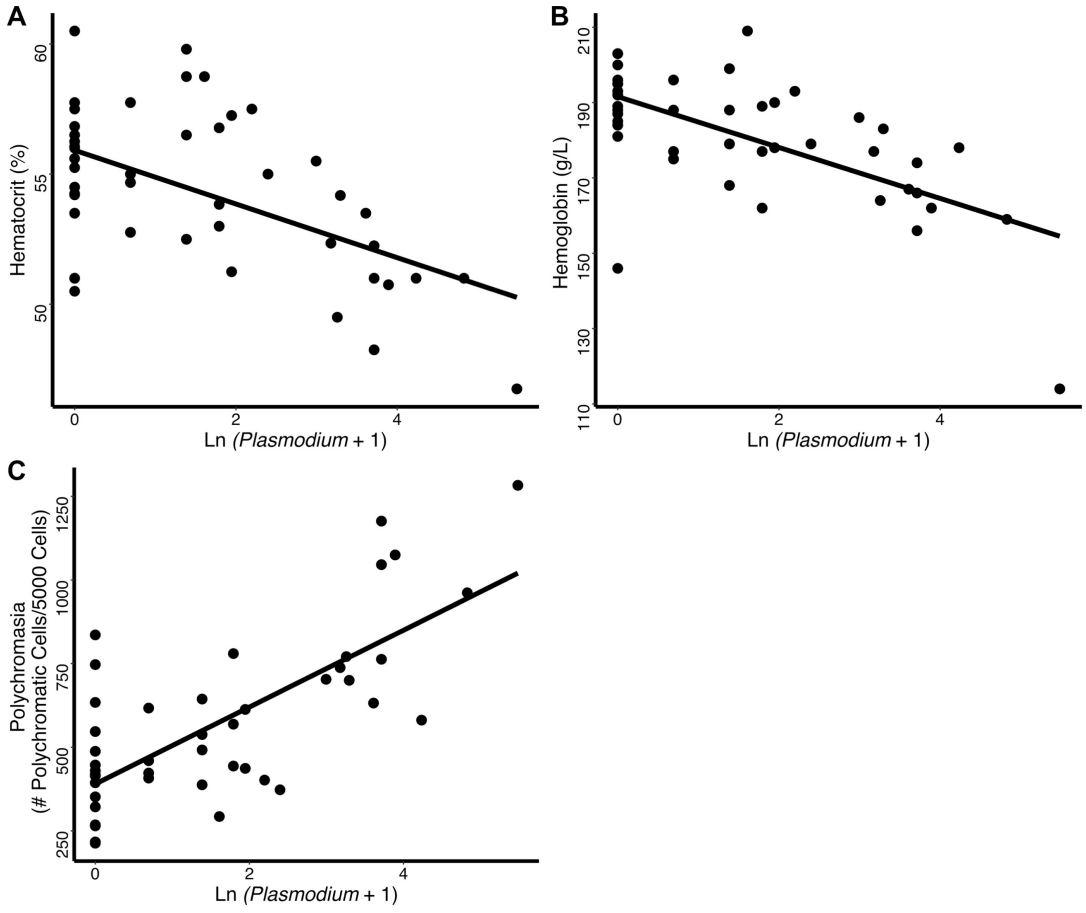


Figure 2.2. The relationship between *Plasmodium* burden and (A) hematocrit, (B) hemoglobin, and (C) red blood cell production (estimated by polychromasia) among male red-winged blackbirds in the anti-parasite medication group sampled three times throughout the experiment.



Tables

Table 2.1. Descriptions of the physiological measures assessed throughout this study in response to chronic Haemosporidian infection.

Category	Physiological Trait	Description	Connection to Haemosporidians	References
Hematology	Hematocrit	The proportion of whole blood consisting of red blood cells, by volume. A decrease in hematocrit can indicate a reduction in red blood cell numbers.	Haemosporidians and the immune response damage red blood cells during infection. Hematocrit decreases during acute infection and in observational field studies, can be correlated with infection status or burden.	(Dawson & Bortolotti 1997; Valkiūnas 2005; Cellier-Holzem *et al*. 2010; Ellis *et al*. 2015)
	Hemoglobin	An oxygen-binding protein found in red blood cells. Hemoglobin concentrations can indicate the oxygen-carrying capacity of blood.	Haemosporidians use hemoglobin as a nutrient source. Hemoglobin decreases in acute infection and can be correlated with infection status in observational field studies.	(Francis, Sullivan & Goldberg 1997; Valkiūnas 2005; Norte *et al*. 2009; Krams *et al*. 2013; Ellis *et al*. 2015)
	Polychromasia	An estimate of the proportion red blood cells that are immature. Polychromasia is indicative of the rate of red blood cell production.	A mechanism to replace cells lost during infection.	(Johns *et al*. 2008; Soares *et al*. 2016)
Physiological Stress	Corticosterone (primary avian glucocorticoid)	Corticosterone is associated with the physiological stress response. At baseline concentrations, it can mobilize resources to support changing energetic demands. Long-term elevation can suppress immune function.	Corticosterone could increase to support the metabolic requirements of responding to infection or to reduce the general inflammatory response, which can limit immunopathology during infection .	(Sapolsky *et al*. 2000; Martin 2009; Love *et al*. 2016)
Oxidative Status	Reactive Oxygen Metabolites (ROMs)	Produced by metabolic and immune activity, ROMs can damage DNA and proteins, preventing them from functioning normally.	Haemosporidians can cause oxidative damage. In some observational field studies infection correlates with ROM production.	(Becker *et al*. 2004; Monaghan, Metcalfe & Torres 2009; van de Crommenacker *et al*. 2012; Skrip & Mcwilliams 2016)
	Total Antioxidant Capacity (TAC)	A measure of the antioxidants available to prevent oxidative damage from ROMs.	In some observational field studies, infection correlates with antioxidant production.	(Isaksson *et al*. 2013; Delhaye *et al*. 2016; Skrip & Mcwilliams 2016)
Immune Function	Nitric Oxide	An inflammatory, immune signaling molecule that can damage invading parasites.	Involved in regulating *Plasmodium* burden, but could also damage healthy red blood cells.	(Sild & Hõrak 2009; Bichet *et al*. 2012; de Macchi *et al*. 2013)
	PIT54 (haptoglobin-like protein in birds)	A pro-inflammatory acute phase protein that can scavenge extra-cellular hemoglobin and limits the production of reactive oxygen metabolites.	Increases during acute infection and in observation field studies, can be correlated with infection status.	(Wicher & Fries 2006; Cellier-Holzem *et al*. 2010; O'Reilly & Eckersall 2014; Ellis, Kunkel & Ricklefs 2014b; Ellis *et al*. 2015)
	White blood cell differential	The proportion of white blood cells consisting of heterophils, lymphocytes, monocytes, basophils, and eosinophils.	White blood cell differentials and total counts shift during acute infections and in observational field studies, can by associated with infection status.	(Ricklefs & Sheldon 2007; Campos *et al*. 2014; Cornelius *et al*. 2014a; Ellis *et al*. 2014, 2015)
Condition	Body mass & fat score	Both can indicate available resources. Fat score is a measure of the fat stored in the furculum.	Mounting an immune response and repairing damage during infection may be energetically costly, or infection may reduce food intake.	(Atkinson *et al*. 2000; Cellier-Holzem *et al*. 2010; Campos *et al*. 2014; Ellis *et al*. 2015)

Table 2.2. Parameter estimates from linear mixed effects models predicting change in parasite burden[1] from pre-treatment to 7 and 14 days post-treatment. Bolded values highlight statistically significant fixed effects.

Parameter	β [2]	SE[3]	P value
(A) Change in *Plasmodium* (N = 56)			
Treatment (Medication)	**-2.83**	**0.52**	**<0.0001**
Days Post-Treatment	**-0.07**	**0.03**	**0.038**
Treatment*Days Post-Treatment	0.09	0.04	0.053
Pre-treatment Burden	**-0.58**	**0.09**	**<0.0001**
(B) Change in *Haemoproteus* (N = 14)			
Treatment (Medication)	0.007	1.23	1.0
Days Post-Treatment	0.17	0.08	0.06
Treatment*Days Post-Treatment	0.02	0.11	0.86
Pre-treatment Burden	-0.35	0.89	0.70
(C) Change in *Leucocytozoon* (N = 18)			
Treatment (Medication)	0.12	0.43	0.79
Days Post-Treatment	-0.03	0.03	0.32
Treatment*Days Post-Treatment	0.01	0.04	0.81
Pre-treatment Burden	**-0.59**	**0.18**	**0.01**

[1]Parasite burden is the natural log(parasite burden + 1). [2]β is the unstandardized regression coefficient. [3]SE is the standard error for the coefficient.

CHAPTER III: HIGHER PLASMA CORTICOSTERONE IS ASSOCIATED WITH REDUCED COSTS OF INFECTION IN RED-WINGED BLACKBIRDS

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Abstract

Glucocorticoid hormones allow individuals to rapidly adjust their physiology and behavior to meet the challenges of a variable environment. An individual's baseline concentration of glucocorticoids can reflect shifts in life history stage and resource demands while mediating a suite of physiological and behavioral changes that include immune modulation and resource allocation. Thus, glucocorticoids could facilitate a response to parasites that is optimized for an individual's specific challenges and life history stage. We investigated the relationship between endogenous circulating glucocorticoids and measures of resistance and tolerance to Haemosporidian parasites (including those that cause avian malaria) in red-winged blackbirds (*Agelaius phoeniceus*). We found that higher endogenous concentrations of circulating glucocorticoids were associated with reduced costs of parasite infection, which is indicative of higher tolerance, but were unrelated to parasite burden in free ranging, breeding male birds. Post-breeding, both males and females with higher glucocorticoid concentrations had higher measures of tolerance to Haemosporidian infection. Our findings suggest a potentially adaptive role for glucocorticoids in shifting the response to parasites to align with an individual's current physiological state and the challenges they face.

Introduction

Once infected with a parasite, individuals have two defense strategies at their disposal: resistance and tolerance (Råberg, Sim & Read 2007; Råberg, Graham & Read 2009). Resistance is an individual's ability to limit their parasite burden (the number of parasites), and can be achieved by removing parasites, or limiting parasite replication (Best, White & Boots 2008). Tolerance involves reducing the damage incurred per parasite, or in other words, minimizing the costs of infection (Råberg *et al*. 2007; Ayres & Schneider 2012). Infection costs can be assessed through health or performance measures (e.g., loss of body mass, tissue damage, locomotor activity), survival probability, or relative reproductive output (Kutzer & Armitage 2016). More tolerant individuals prevent or limit the damage accrued during infection and/or repair damage as it occurs (Medzhitov, Schneider & Soares 2012).

Although resistance and tolerance are not mutually exclusive strategies (Mauricio, Rausher & Burdick 1997; Restif & Koella 2004; Athanasiadou *et al*. 2015), trade-offs between the defenses could be unavoidable if activating one strategy is detrimental to the other (Råberg *et al*. 2007). For example, activating an inflammatory response can increase resistance by increasing parasite clearance, but, at the same time, compromise other host tissues and functions, like reproduction and survival (Ilmonen, Taarnal & Hasselquist 2000; Libert *et al*. 2006), ultimately decreasing tolerance (Schneider & Ayres 2008). In addition, the relative advantages of investing in resistance and/or tolerance could vary with environmental conditions or host ecology and life history. Sears et al. (2011) hypothesized that species with a faster pace-of-life (i.e., have quick growth and development, reproduce early in life, and have a short lifespan) would invest more in parasite resistance, whereas those with a slower pace-of-life (i.e., develop more slowly, reproduce later, and have a longer lifespan) would invest more in tolerance. Studies in birds and

rodents have demonstrated that a faster pace-of-life correlates with the pro-inflammatory immune responses associated with resistance, whereas a slower pace-of-life correlates with the anti-inflammatory responses that might facilitate tolerance (Lee, 2006; Martin et al., 2006; Previtali et al., 2012; but see Martin et al., 2007; Versteegh et al., 2012). Among individuals, variation in life history strategies and environment can also influence resistance and/or tolerance (Festa-Bianchet 1989; Knowles, Nakagawa & Sheldon 2009; Sternberg *et al*. 2012; Lutermann, Bodenstein & Bennett 2012). For example, increases in reproductive effort can reduce both immune function (Saino *et al*. 2002; Ardia, Schat & Winkler 2003; Hanssen *et al*. 2005) and parasite resistance (Festa-Bianchet 1989; Nordling 1998; Knowles *et al*. 2009).

Glucocorticoid hormones have the potential to influence investment in both resistance and tolerance. Glucocorticoids mediate reversible changes in physiology and behavior in response to both predictable (e.g., reproduction) and unpredictable (e.g., food availability, predation risk) challenges (Landys, Ramenofsky & Wingfield 2006). Experimental elevation of glucocorticoid concentrations tends to reduce parasite resistance (e.g., Applegate, 1970; Belden and Kiesecker, 2005; Wang et al., 2014), but in observational studies, the relationships between glucocorticoids and parasite burden are varied (e.g., Bortolotti et al., 2009; Cornelius et al., 2014; Garvin et al., 2006). Glucocorticoids could decrease resistance by suppressing components of the immune response, which are frequently associated with extended elevation of glucocorticoids (e.g., Bourgeon and Raclot, 2006; Dhabhar and McEwen, 1997). However, effects of glucocorticoid on immune activity are not always inhibitory, and their effects can vary among different parts of the immune system (Sapolsky, Romero & Munck 2000; Martin 2009), as well as with the timing, duration, and amplitude of hormone secretion (Dhabhar 2009; McCormick, Shea & Langkilde 2015).

Glucocorticoids might influence tolerance via their effects on immune function, tissue repair, and behavior (Råberg *et al*. 1998; Medzhitov *et al*. 2012; Adelman & Hawley 2016). Although the immune system is critical for the recognition and removal of parasites as well as tissue repair and regeneration, a robust immune response is potentially dangerous to the host's own tissues and could increase the risk of immunopathology (Råberg *et al*. 1998). If glucocorticoids suppress the components of the immune response and inflammation that damage host tissue or cause sickness behaviors, they could increase tolerance (Apanius 1998; Råberg *et al*. 1998; Adelman, Moore & Hawley 2015). Furthermore, glucocorticoids could promote tolerance by enhancing tissue repair through interactions with the immune system and other physiological systems. For example, some parasites, including malaria, blood-borne nematodes, and biting insects, can damage and/or reduce numbers of red blood cells (Meagher 1998; Valkiūnas 2005; Brommer *et al*. 2011). Glucocorticoids can induce increased red blood cell production (Golde, Bersch & Cline 1976; Voorhees *et al*. 2013) and, by replacing the lost tissue, could increase tolerance to these parasites.

Few empirical studies have tested the hypothesis that glucocorticoids mediate tolerance to infection. A recent laboratory experiment tested the effect of exogenous glucocorticoid treatment on resistance and tolerance to the chytrid fungus *Batrachochytrium dendrobatidis* in the American toad (*Anaxyrus [Bufo] americanus*) (Murone, DeMarchi & Venesky 2016). High-dose glucocorticoid treatment reduced survival, but the effect was independent of chytrid exposure, suggesting that glucocorticoid treatment had no effect on tolerance. By manipulating both glucocorticoids and chytrid exposure in the laboratory, this experiment tested for a causal relationship between the hormones and response to infection (Murone *et al*. 2016). However, such manipulations do not account for variation in the hosts' behaviors that can influence

parasite exposure and burden, and they move animals away from their own endogenous and potentially optimal hormone concentrations. Observational field studies are required to describe the relationships of resistance and tolerance to infection with glucocorticoid hormones under natural conditions, and can therefore be critical to validating experimental work. To our knowledge, no studies have described the relationship between glucocorticoids and tolerance to infection in a free-living population. Here, we evaluate the relationship between glucocorticoids and measures of resistance and tolerance to infection in free-living red-winged blackbirds (*Agelaius phoeniceus)* infected with Haemosporidian parasites. We also assess whether glucocorticoids might influence tolerance via effects on tissue repair.

Haemosporidian parasites, which include malaria, infect several vertebrate taxa (e.g., birds, reptiles, and mammals), and are geographically widespread (Valkiūnas 2005; Martinsen, Perkins & Schall 2008). Avian Haemosporidians are vector-transmitted parasites and include the genera *Plasmodium* (avian malaria), *Haemoproteus*, and *Leucocytozoon* (Valkiūnas 2005). Haemosporidians have complex life cycles, including multiple life stages in different host tissues. Soon after the parasites successfully invade a host, the host faces an acute stage infection, characterized by high numbers of parasites in the blood (Valkiūnas 2005; Lapointe, Atkinson & Samuel 2012; Asghar *et al*. 2012). This stage is followed by a chronic phase of the infection characterized by low parasite burdens. Although the length each stage of infection varies with the host and parasite species (Valkiūnas 2005), the acute stage tends to be shorter, often lasting for weeks (Cellier-Holzem *et al*. 2010; Asghar *et al*. 2012; Ellis *et al*. 2015), whereas chronic infections can persist for months or years (Manwell 1933; Bishop, Tate & Thorpe 1938; Asghar *et al*. 2012). Where Haemosporidians are endemic, the parasites' primary effects tend to be sub-lethal and few observational studies have found costs of chronic infection

in birds (Lapointe *et al*. 2012). However, anti-malarial medication experiments and long-term monitoring studies demonstrate that Haemosporidian infection can reduce survival (Martínez-de la Puente *et al*. 2010; Asghar *et al*. 2015), reproductive success (Merino *et al*. 2000; Marzal *et al*. 2005; Knowles, Palinauskas & Sheldon 2010), and have physiological consequences (Coon *et al*., 2016; Ellis *et al*., 2015), even in species with a long history of coexistence with these parasites. Thus, resistance and tolerance to malaria during the chronic stage of infection could be important to birds' fitness and subject to selection.

In this study, we assessed measures of resistance and tolerance to Haemosporidians in birds naturally infected with the parasites. As an indicator of resistance, we measured individuals' parasite burdens at the time of capture. Assessing parasite burden at a single time point does not account for the variation caused by differences in host exposure to parasites or in the phase of infection. However, we expect that most birds captured during our study were experiencing the chronic stage of Haemosporidian infection because we observed relatively low parasite burdens (results below), the chronic stage is longer than the acute phase (Bishop *et al*. 1938; Asghar *et al*. 2012), and we are less likely to capture birds during the acute phase of infection because they tend to have reduced mobility (Mukhin *et al*. 2016). As a measure for individual tolerance, we used the residuals from a regression of a health measure against parasite burden. We considered individuals with a health measure greater than the expected value for a given parasite burden for that population to be more tolerant (Fig. 1). As a health measure, we selected hematocrit, the proportion of whole blood consisting of red blood cells by volume, because Haemosporidian parasites destroy and damage red blood cells (Dawson & Bortolotti 1997; Valkiūnas 2005; Ellis *et al*. 2015). In addition, reductions in hematocrit can decrease the quality and number of offspring (Fronstin, Christians & Williams 2015), suggesting that

hematocrit could represent a true cost of infection. Tolerance is usually compared among groups of organisms (e.g., genotypes, species) rather than individuals, and is most often assessed as range tolerance, or the slope of the relationship between a health measure and parasite burden (Kutzer & Armitage 2016). This method of measuring tolerance can be logistically challenging when applied at the individual level. In some study systems, it can be difficult or even impossible to collect multiple samples from an individual that span substantial variation in parasite burden (but see Hayward *et al*., 2014; Jackson *et al*., 2014). A recent study by Burgan et al. (2016) demonstrated that individual-level range tolerance is highly correlated with tolerance estimated as residual health controlling for parasite burden, similar to the approach that we employed.

We used an observational study to test the hypothesis that corticosterone, the primary avian glucocorticoid, is associated with resistance and tolerance to infection. We also investigated whether there are relationships between either corticosterone or measures of tolerance and red blood cell production (erythropoiesis), because replacing cells damaged during infection could be a mechanism underlying tolerance to Haemosporidians. We predicted that birds with higher plasma corticosterone concentrations would have higher malaria burdens (indicating lower resistance) and higher hematocrit relative to their parasite burden (indicating higher tolerance).

Materials and Methods

Study species and population

We studied adult, breeding red-winged blackbirds at two sites in southeastern Ontario, Canada: Queen's University Biological Station (44°34'02.3" N, 76°19'28.4" W) and outside the city of Kingston (44°15'04.8" N, 76°28'43.6" W). Red-winged blackbirds breeding in this area have high prevalence of Haemosporidian parasites. In the late 1980s and early 1990s, prevalence

as detected on blood smears ranged between 30 – 56% in females and 35 – 71% in males (Weatherhead 1990, Weatherhead and Bennett 1991, Weatherhead et al. 1993). However, actual prevalence might have been higher, as detection on blood smears tends to be lower than with PCR (Garamszegi 2010). Between 2013-2015, Haemosporidian prevalence as detected by PCR was over 90% (Supplementary Material, Table S1).

Field Methods

All procedures were approved by the Virginia Tech Institutional Animal Care and Use Committee and the Queen's University Animal Care Committee and complied with US and Canadian national standards for the use of animals in research.

During May – June 2013, we captured 32 female and 28 male birds on their territories using mist nets between 5:00 – 11:00 AM. We lured males into nets with playback of conspecific song and calls accompanied by a taxidermied mount of a conspecific, and we flushed females from their nest into the net. We individually marked each bird with a numbered aluminum band from the Canadian Wildlife Service and a unique combination of color bands to allow individual identification. From each individual we measured body mass (to the nearest 0.5 g) with a Pesola spring scale, tarsus length (to the nearest 0.1 mm) with calipers, and wing chord (to the nearest 0.5 mm) with a wing rule.

We collected blood samples from each individual to measure: 1) corticosterone levels, 2) the presence/absence and burden of avian Haemosporidians by genus (*Plasmodium*, *Haemoproteus*, and *Leucocytozoon*), 3) hematocrit, and 4) polychromasia. Quickly after capture, we punctured the brachial vein with a 26 gauge ½ inch needle and collected ~350 μL of blood into heparinized capillary tubes. We recorded the time between capture and the completion of blood sampling, and only samples collected within three minutes of the bird entering the net

were included in analyses (Romero & Reed 2005). In samples collected within three minutes, we found no significant relationship between corticosterone concentrations and either time between capture and blood sampling or the time between net set-up and capture for males or females (all $P \geq 0.18, N = 43$), therefore, we considered these samples to be representative of baseline corticosterone. We measured baseline corticosterone in 25 females and 18 males.

We prepared blood smears from each sample by spreading a drop of blood (approximately 5 – 10 μL) on glass slides, and immediately air-dried the smears and fixed each in absolute methanol. We stained smears with Diff-Quick (IMEB INC., San Marcos, CA, USA) within four months of sample collection.

Blood was initially stored on ice (for no more than 6 hours) before being centrifuged at 6,000 RPM for 10 minutes. After centrifugation, we measured hematocrit for the first two capillary tubes collected from each bird using a microhematocrit card. The average of the two hematocrit measures was used in the final analysis. We separated the plasma and red blood cells and stored both at -20 °C until analysis.

Corticosterone Assays

We quantified total corticosterone in plasma samples in a single direct radioimmunoassay in which samples were run in duplicate (Wingfield *et al*., 1992) using the corticosterone antibody from Esoterix Endocrinology (catalogue number B3-163) and labeled corticosterone from New England Nuclear Research Products (catalogue number NET-399). We extracted steroid hormones from 20 – 40 μL (mean $\pm$ SE, 32.6 $\pm$ 1.0) of plasma using a single extraction with 5 mL dichloromethane. We corrected for extraction efficiency for each sample individually, and the average extraction efficiency was 70%. Within-assay variation in replicates of known

standards was 7.5% (4 known standards). The detection limit was ~1.5 ng/mL and 1 sample fell below that threshold.

Haemosporidian Infection Prevalence

DNA was extracted from whole blood or red blood cells using Qiagen DNeasy Blood and Tissue kits (Qiagen, Valencia, CA, USA). We then screened for the presence of Haemosporidians and identified the genera of the parasites using a restriction enzyme-based assay designed by Beadell and Fleischer (2005). The assay begins with a PCR to detect the presence of Haemosporidians and then uses restriction enzymes to digest the PCR product into fragments that based on their size, can be used to identify the presence of the genera *Plasmodium*, *Haemoproteus,* and *Leucocytozoon*. In a 25 μL reaction, we combined 4.5 μL DNA with final concentrations of 0.2 mM dNTPs, 3 mM $MgCl_2$, 0.6 μM of each primer, 0.5 U *Taq* polymerase (New England BioLabs, Ispwich, MA), 1X *Taq* buffer (New England BioLabs, Ispwich, MA ms), 10X BSA. PCR cycling conditions were identical to Beadell and Fleischer (2005). We then digested 10 μL of PCR product in a total volume of 20 μL at 37° C overnight (minimum 12 hours) with 1 U of each of the restriction enzymes XmnI and XbaI and 1X CutSmart Buffer (New England BioLabs, Ispwich, MA). We separated the digested PCR product by electrophoresis on a 4% agarose gel and identified the presence of each Haemosporidian genera based on fragment size as described in Beadell and Fleischer (2005). To reduce the risk of false negatives, any samples that tested negative for Haemosporidians were re-extracted and re-tested by PCR three times.

Haemosporidian Burden

A single observer (LAS) counted the number of parasite-infected cells per 10,000 red blood cells at high magnification (1000X, oil immersion) as recommended by Godfrey et al.

(1987) and identified the infecting genera by parasite morphology (Valkiūnas 2005). We estimated the number of red blood cells by comparing the spread of red blood cells across each field of view to standardized photographs of known cell counts (Ricklefs & Sheldon 2007). To measure parasite burden, we counted the number of infected red blood cells out of 10,000. In each case that we detected Haemosporidians on a slide, the corresponding PCR was also positive for Haemosporidians. In 9 out of 66 individuals, the PCR was positive for Haemosporidians, but we were unable to detect parasites in the corresponding slide.

Polychromasia: A Measure of Red Blood Cell Production

Immature red blood cells can be identified using traditional stains (e.g., Wright-Giemsa) as the cells stain a lavender or light blue-red color termed polychromatophilic, due to an increase in cytoplasmic organelles and RNA (Mitchell & Johns 2008). In addition, compared to the normal elliptical shape and densely packed chromatin of a mature erythrocyte, less mature red blood cells are more rounded and the nuclei contain loosely packed chromatin, which both become more pronounced with increasing immaturity. We counted the number of polychromatic cells per 1,000 red blood cells, and used this number as a measure of red blood cell production rate.

Post-breeding plasma corticosterone and tolerance (2016)

In a separate study, we repeated the investigation of the relationship between corticosterone and tolerance to *Plasmodium* and *Haemoproteus*. From mid-June to July 2016, we captured adult, non-breeding red-winged blackbirds occupying the same study site, many of which were in molt (one female was caught in both 2013 and 2016). As the birds were no longer territorial, we captured individuals using Potter and Troyer V-top traps baited with sunflower seeds. We observed traps from a minimum of 15 m away, and removed individuals immediately

after they entered the trap. We collected measurements and blood samples following the procedures described above. We sampled 27 individuals, including 9 females and 18 males. As in 2013, there was no relationship between time from capture (entering the trap) to blood sampling and corticosterone concentration among blood samples obtained within 3 minutes (β = -0.002, SE = 0.003, R^2 = 0.01 P = 0.57, N = 27).

We used blood samples to measure corticosterone, parasite burden of each genus by microscopy, and hematocrit. We did not use molecular techniques to determine parasite presence/absence. We assessed hematocrit and parasite burden as previously described, but microscope slides were analyzed by a different person (IS). We quantified total corticosterone using enzyme immunoassay (EIA) kits (catalogue #501320, Cayman Chemical Company, MI, USA) according to the manufacturer's instructions. We validated the assay by performing serial dilutions (eight dilutions ranging from 1:2 to 1:256, ranging from 6.87 – 21.75 ng/mL) of pooled red-winged blackbird plasma and found that serially diluted samples paralleled a series of standards (ranging from 1.90 – 10.03 ng/mL). We found the optimal plasma dilution to maintain sample values within the range of the standard curve to be 1:16 plasma:EIA buffer. We analyzed all samples in duplicate across 2 plates. Intra-assay variability among 2 plasma pools was 4.51% and inter-assay variability among 4 plasma pools (2 per plate) was 6.50%. The minimum assay detection limit was 1.90 ng/ml and no samples fell below this limit.

Statistics

All analyses were performed in R version 3.3.2 (R Core Team 2016) and we set $\alpha = 0.05$. To improve model fit, we used the natural log transformation of plasma corticosterone concentration and the natural log of parasite burden + 1. For all regression analyses, we initially summarized model results using Type III sums of squares, and if there were no significant

interaction terms in the model, we used Type II sums of squares. The Type II sum of squares is more statistically powerful than Type III in the absence of significant interactions (Hector, von Felten & Schmid 2010; Smith & Cribbie 2014). If a categorical variable (e.g., sex, study site) was a significant predictor in a model, we compared the effects among levels within the variable using pairwise contrasts. We assessed model fit by visually examining plots of the residuals by the predicted values and for linear models, checked the residuals for normality. Model fit was good unless otherwise reported.

Breeding Season Study (2013)

To determine if there were differences in plasma corticosterone concentration, the probability of infection, or parasite burden between the sexes or study sites, we ran regression models with sex, study site, and their interaction as predictor variables and either plasma corticosterone, infection status, or parasite burden as the response variable. We used linear regression for models predicting plasma corticosterone concentration and parasite burden, and logistic regression for models predicting infection status. We performed separate analyses for infection status and burden for each of the three parasite genera. Analyses of parasite burden included only birds that were PCR-positive for the focal genus. We also tested if plasma corticosterone was associated with body mass in either sex using linear regression models with plasma corticosterone as the response variable and body mass as the predictor variable.

To test the hypothesis that glucocorticoids are associated with parasite burden, a measure of resistance, we performed linear models with parasite burden as the response variable and plasma corticosterone concentration, sex, and the corticosterone-sex interaction as predictor variables. We included sex in the models because parasite burdens often vary by sex, due to sex differences in exposure and physiology (Zuk & Mckean 1996). We ran two models, one

predicting *Leucocytozoon* parasite burden, and one predicting the combined *Haemoproteus* and *Plasmodium* burden. Combining *Haemoproteus* and *Plasmodium* burdens in analyses is a common practice (e.g., Asghar *et al*. 2015) because the two parasites utilize red blood cells in their hosts in a similar way and can have similar consequences to the host, whereas *Leucocytozoon* is more phylogenetically distant and can infect different cell types (Valkiūnas 2005). Only birds that were PCR-positive for the genus/genera of the response variable were included in the analysis.

To test the hypothesis that plasma corticosterone concentration is associated with a measure of tolerance to Haemosporidian infection, we first used a simple linear regression to determine if hematocrit correlated with Haemosporidian burden in our population. We included hematocrit as the response variable and natural log of parasite burden, sex, and the interaction between parasite burden and sex as predictor variables. As described above, we conducted analyses separately for *Leucocytozoon* and combined *Plasmodium* and *Haemoproteus* and only included birds that were PCR-positive for the genus/genera in the analysis. If parasite burden was negatively correlated with hematocrit, we used residual hematocrit from the model as a quantitative measure of tolerance (Fig. 1). We then performed a second linear regression with tolerance as the response variable and natural log of plasma corticosterone, sex, and the interaction between corticosterone and sex as the predictor variables.

To evaluate if increasing red blood cell production underlies variation in tolerance, we performed a simple linear regression with tolerance as the response variable and polychromasia, sex, and their interaction as predictors. To determine if plasma corticosterone is linked to polychromasia, we performed an additional linear regression including polychromasia as the

response variable and plasma corticosterone, parasite burden, sex, and their interactions as predictors.

Post-breeding plasma corticosterone and tolerance (2016)

We performed statistical analyses of the 2016 investigation of the plasma corticosterone-tolerance relationship separately from the 2013 study because we did not use the PCR/restriction enzyme assay to identify parasite infection status by genus in 2016. We used the same analyses to test for tolerance as described above, except that we included all birds in the analysis rather than excluding individuals based on molecular identification of the presence/absence of a specific genus. We used the natural log transformation of plasma corticosterone and the natural log (*Plasmodium* and *Haemoproteus* burden + 1) to improve model fit. We used a t-test to determine if plasma corticosterone varied between the sexes.

Results

Haemosporidian infection prevalence and intensity (2013)

Ninety-five percent of birds were infected with at least one genus of Haemosporidian parasite, and the most common genus was *Plasmodium*, with 83.3% prevalence, followed by *Leucocytozoon*, and finally *Haemoproteus* (Table 1). Average parasitemia, or the percent of infected red blood cells (mean $\pm$ SE), was 0.34 $\pm$ 0.08% for *Plasmodium*, 0.09 $\pm$ 0.08% for *Haemoproteus*, and 0.01 $\pm$ 0.003% for *Leucocytozoon*. Thus, all birds were likely in the chronic phase of infection at the time of sampling.

Birds captured at Queen's University Biological Station were infected with *Plasmodium* more often than those captured in Kingston (Queen's, 97.1% infected, Kingston, 64.0% infected ; $F = 15.91, P = 0.0002, N = 60$), but infection status did not vary by sex ($F = 0.16, P = 0.69, N = 60$), and the sex-site interaction was not significant ($F = 1.61, P = 0.21, N = 60$). Among

Plasmodium-infected individuals, there was a tendency for *Plasmodium* burden to vary by site in males (sex-site interaction, $F = 3.13, P = 0.08, N = 50$). Pairwise comparisons demonstrated that males captured at Queen's University Biological Station had higher *Plasmodium* burdens than males captured in Kingston ($t = 2.66, P = 0.01, N = 24$), and *Plasmodium* burden did not vary by sex at either site (Queen's, $t = -1.19, P = 0.24, N = 34$; Kingston, $t = 1.33, P = 0.19, N = 16$). Males were more likely to be infected with *Leucocytozoon* than females (males, 57.1% infected females, 21.9% infected; $F = 7.57, P = 0.008, N = 60$), but neither site ($F = 0.13, P = 0.72, N = 60$) nor the interaction of sex and site correlated with infection status ($F = 0.16, P = 0.69, N = 60$). *Leucocytozoon* burden and *Haemoproteus* infection status and burden were similar across sexes and sites, and the sex-site interactions were not significant (all $P > 0.17$).

Plasma corticosterone and parasite resistance (2013)

Plasma corticosterone did not correlate with burdens of either *Plasmodium* and *Haemoproteus* ($F = 0.31, P = 0.58, N = 40$) or *Leucocytozoon* ($F = 0.56, P = 0.47, N = 16$), providing no evidence for a relationship between plasma corticosterone and resistance. In addition, plasma corticosterone concentrations did not vary by sex ($F = 0.67, P = 0.42, N = 43$; mean $\pm$ SE, females: 11.54 $\pm$ 2.06 ng/mL, males: 12.31 $\pm$ 1.86 ng/mL), study site ($F = 1.12, P = 0.30, N = 43$), or their interaction ($F = 0.61, P = 0.44, N = 43$). Plasma corticosterone was not associated with body mass in females ($F = 1.32, P = 0.26, N = 25$) or males ($F = 0.03, P = 0.87, N = 18$).

Plasma corticosterone and tolerance (2013 & 2016)

Breeding Season Study (2013)

Birds with higher *Plasmodium* and *Haemoproteus* burdens had lower hematocrit ($F = 27.90, P < 0.0001, N = 53$) (Fig. 2A). Females had lower hematocrit than males ($F = 21.99, P <$

0.0001, $N = 53$), but the interaction between sex and parasite burden was not significant ($F = 0.22$, $P = 0.64$, $N = 53$) (Fig, 2A). The sex difference in hematocrit was also present in birds infected with *Leucocytozoon* ($F = 4.7$, $P = 0.04$, $N = 22$). However, *Leucocytozoon* burdens were not associated with hematocrit ($F = 0.32$, $P = 0.58$, $N = 22$), suggesting that reduced hematocrit is not a cost of *Leucocytozoon* infection. As a result, tolerance analyses were performed for only *Plasmodium* and *Haemoproteus* infection.

We used residual hematocrit controlling for *Plasmodium* and *Haemoproteus* burden and sex as a quantitative measure of tolerance. The interaction of plasma corticosterone and sex was a significant predictor of tolerance ($F = 9.94$, $P = 0.003$, $N = 39$, Fig. 3A). To determine how the relationship between plasma corticosterone and tolerance varied between the sexes, we ran post-hoc linear regressions for each sex with tolerance as the response variable and log plasma corticosterone as the predictor variable. Male birds with higher plasma corticosterone were more tolerant of *Plasmodium* and *Haemoproteus* infection than males with lower plasma corticosterone ($F = 12.25$, $P = 0.004$, $N = 16$) (Fig. 3A). Among females, plasma corticosterone was not related to the measure of tolerance ($F = 1.07$, $P = 0.31$, $N = 23$) (Fig 3A).

Post-breeding plasma corticosterone and tolerance (2016)

Hematocrit was negatively correlated with parasite burden ($F = 5.00$, $P = 0.04$, $N = 27$) indicating that, as seen in the breeding season, individuals with the highest number of parasites had the lowest hematocrit (Fig. 2B). However, unlike the 2013 breeding study, neither sex ($F = 0.29$, $P = 0.59$, $N = 23$) nor the interaction between sex and parasite burden ($F = 2.34$, $P = 0.13$, $N = 23$) were associated with hematocrit in non-breeding birds. Thus, post-breeding, sexes did not differ in hematocrit or the relationship between parasite burden and hematocrit. We used

residual hematocrit controlling for parasite burden to quantify tolerance to *Plasmodium* and *Haemoproteus*.

As seen in the 2013 breeding season, birds with higher plasma corticosterone were also more tolerant to *Plasmodium* and *Haemoproteus* infection ($F = 7.02, P = 0.01, N = 27$) (Fig. 2B). However, neither sex ($F = 0.71, P = 0.41, N = 27$) nor the plasma corticosterone by sex interaction ($F = 0.54, P = 0.47, N = 27$) were significant predictors of the tolerance measure, indicating that the relationship between plasma corticosterone and tolerance was similar in both sexes. As in 2013, we found no difference in plasma corticosterone between sexes ($t = 0.74, P = 0.46, N = 27$; mean $\pm$ SE, females: 6.43 $\pm$ 0.69 ng/mL, males: 6.1 $\pm$ 0.78 ng/mL).

Red blood cell production, tolerance, and plasma corticosterone (2013)

Red blood cell production (polychromasia) was not correlated with tolerance ($F = 1.54, P = 0.23, N = 32$). Individuals with higher *Plasmodium* and *Haemoproteus* burdens produced more red blood cells ($F = 6.00, P = 0.02, N = 32$) (Supplementary Material, Figure B1), but red blood cell production was not associated with plasma corticosterone ($F = 1.65, P = 0.21, N = 32$).

Discussion

To our knowledge, our study provides the first empirical description of a relationship between endogenous glucocorticoids and a measure of tolerance to infection in a free-ranging animal. During the 2013 breeding season, male red-winged blackbirds with higher plasma corticosterone concentrations maintained higher hematocrit than males with similar parasite burdens but lower corticosterone, suggesting that male birds with higher plasma corticosterone concentrations were more tolerant to infection. After the 2016 breeding season, plasma corticosterone concentration was positively correlated with the tolerance measure in both males and females. Our ability to replicate this finding in a different year and at different life history

stages suggests a robust relationship between plasma corticosterone and this measure of tolerance. In addition, this broad measure of tolerance could be biologically significant because a bird's ability to maintain hematocrit can be associated with reproductive performance (Fronstin *et al*. 2015). An experimental reduction of hematocrit in European starlings (*Sturnus vulgaris*) to levels similar to those observed in red-winged blackbirds with the highest parasite burdens, reduced reproductive performance during some years (Fronstin *et al*. 2015). However, it is important to consider that we assessed tolerance as residual health controlling for parasite burden. Although this tolerance measure can be correlated with tolerance when estimated as the slope of the relationship between health and parasite burden (Burgan 2016), caution must be taken whenever comparing different types of tolerance measures across studies. We observed no relationship between plasma corticosterone and Haemosporidian parasite burden, and thus have no evidence for a relationship between corticosterone and resistance to parasites.

Corticosterone could be mediating tolerance to Haemosporidian infection in red-winged blackbirds by supporting tissue repair or reducing the damage incurred during infection (Apanius 1998; Råberg *et al*. 1998). However, neither tolerance nor plasma corticosterone was associated with red blood cell production in males, suggesting that tolerance to the parasites is not achieved by replacing damaged cells. Instead, birds might increase tolerance by reducing the damage caused during infection, potentially by dampening immune activity that harms healthy tissue as well as invading parasites. In both human and rodent malaria (*Plasmodium spp*.), the parasites do not destroy enough red blood cells to account for the extent of anemia experienced by hosts, and there is evidence that T-cell activity could contribute to the loss of uninfected red blood cells (Price *et al*. 2001; Evans *et al*. 2006). Glucocorticoids could influence the extent of red blood cell destruction during malaria infections because they shift the balance between two classes of

T-helper lymphocytes, Th1 and Th2 (Elenkov 2004; Sears *et al*. 2011; Cain & Cidlowski 2017). Strong Th1 (pro-inflammatory) and lower Th2 (anti-inflammatory) responses are associated with severe anemia during human malaria infections (Kurtzhals *et al*. 1998; Biemba *et al*. 2000), suggesting that activating the Th2 response and suppressing Th1 could increase tolerance. In addition, corticosterone can inhibit nitric oxide signaling, which can increase tolerance to *Plasmodium*: when the signaling pathway is blocked, birds have an increased parasite burden, but do not suffer the expected decrease in hematocrit (Szabó *et al*. 1994; Bichet *et al*. 2012; Soriano *et al*. 2013; de Macchi *et al*. 2013). We did not measure immune function in this study, and thus further work is required to determine the importance of immune activation in influencing red-winged blackbirds' tolerance to Haemosporidians.

Alternatively, corticosterone might not directly influence tolerance, but instead be linked to tolerance via a third, unmeasured factor. Glucocorticoids can support reproductive effort (e.g. CORT-adaptation hypothesis; Bonier et al., 2009; Bonier and Martin, 2016), and high quality birds and/or birds in better condition might have higher plasma corticosterone because they are investing heavily in reproduction. In this case, higher quality individuals could be more tolerant to infection and also have higher plasma corticosterone concentrations. However, this appears unlikely given that the relationship between plasma corticosterone and tolerance was maintained in both the breeding and non-breeding season. In our study, we found no link between plasma corticosterone and body mass, suggesting that condition might not underlie the link between plasma corticosterone and tolerance, but body mass is not always a reliable indicator of condition (Hayes & Shonkwiler 2001). In addition, we assessed a single tolerance measure based on hematocrit, but avian Haemosporidian infections can induce other physiological or behavioral changes, such as telomere attrition (Asghar *et al*. 2015, 2016) or reduced song quality (Gilman,

Blumstein & Foufopoulos 2007), that could also be used to estimate tolerance. The relationship between plasma corticosterone and tolerance might vary depending on the performance or health trait used to assess tolerance.

Glucocorticoid-mediated tolerance could be an adaptive response to infection when an individual is faced with other environmental challenges. For example, higher glucocorticoids are thought to support the energetic challenges of reproduction (Romero & Wingfield 1998; Moore *et al*. 2000; Bonier *et al*. 2009) and, if they promote tolerance, glucocorticoids could decrease the need for the pro-inflammatory parasite defenses associated with sickness behaviors that can reduce reproductive success (Adelman & Martin 2009). Glucocorticoids also influence life history stage transitions (Wada 2008; Ramenofsky & Wingfield 2016), and it might be beneficial to animals to alter investment in resistance and tolerance depending on the life history stage. Both plants and animals can shift their defense strategies from more resistant to more tolerant as they move from the juvenile to reproductive stage (Boege *et al*. 2007; Jackson *et al*. 2014). However, studies investigating the relationships between glucocorticoids, tolerance, and fitness metrics will be required to determine if glucocorticoid-mediated tolerance can be adaptive.

We observed sex differences in both hematocrit and the relationship between plasma corticosterone and a measure of tolerance during the 2013 breeding season, but not among non-breeding birds sampled in 2016. In 2013, plasma corticosterone concentration correlated with a measure of tolerance in male, but not female breeding red-winged blackbirds, and females tended to have lower hematocrit. During reproduction, male and female red-winged blackbirds engage in very different activities (males primarily defend territories and females provide nearly all parental care) (Beletsky 1996), and thus, corticosterone might influence their physiology differently. In addition, the estrogens required for egg production can suppress red blood cell

production, resulting in lower hematocrit and as a result, females' hematocrit is likely to be variable across the breeding season (Wagner *et al*. 2008a; Wagner, Stables & Williams 2008b). Here, the range of hematocrit was greater for females than males (Fig. 2A), and the additional variation in hematocrit could mask an effect of plasma corticosterone on tolerance, if present. After the conclusion of the breeding season, both sexes enter molt and prepare for migration, and fewer differences between males and females during this life history stage might explain the lack of differences in both hematocrit and the plasma corticosterone-tolerance relationship in the 2016 study. Alternatively, year-to-year environmental variation, such as differences in resource availability or parasite exposure, could differentially affect male and female physiology, resulting in sex differences in 2013, but not 2016.

We found no evidence for a relationship between plasma corticosterone and parasite burden, an indicator of resistance. Experimental elevation of plasma corticosterone concentration increased parasite burden in house sparrows (*Passer domesticus*) (Applegate 1970; Applegate & Beaudoin 1970), however other observational studies have also found no link between the hormone and parasite burden (Garvin *et al*. 2006; Sorensen *et al*. 2016). In observational studies, variation in individual quality or environmental influences (e.g., seasonal variation, co-infections) on both plasma corticosterone and parasite burden could obscure patterns. Alternatively, studies manipulating hormones might yield different results than observational studies because: 1) manipulations might not closely replicate endogenous hormone release patterns or concentrations or 2) the manipulation shifts an animal away from an endocrine phenotype matched to the current conditions (e.g., social and physical environment, physiology) and as a result, the manipulation does not cause the same response seen in observational studies. Regardless of its influence on resistance post-infection, corticosterone could still be important

for within-host infection dynamics by influencing parasite exposure and, and thereby infection risk. For example, house mosquitos (*Culex quinquefasciatus*), which can vector several pathogens including some species of avian malaria (Van Riper *et al*. 1986; Bartholomay *et al*. 2010), preferentially feed on birds with higher plasma corticosterone (Gervasi *et al*. 2016). Thus, birds with higher plasma corticosterone might be at higher risk of infection with mosquito-vectored parasites.

While hormonal regulation of parasite resistance has been studied for decades, very few studies have addressed hormonal regulation of tolerance to infection. Glucocorticoids influence immune function, as well as other aspects of physiology and behavior, and thus, are a particularly promising candidate for a mediator of tolerance. Given that the relationship between plasma corticosterone concentrations and tolerance varied between the breeding and non-breeding seasons in female birds, considering life history stage and strategy (e.g., variation in reproductive effort) could provide additional insight into how individuals balance investment in resistance and tolerance. If our findings generalize to other host-parasite relationships, glucocorticoids could be important regulators of potentially adaptive parasite defenses.

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Figures

Figure 3.1. An illustration of the method we used for estimating a measure of tolerance. Each point represents an individual sampled at a single time point for both parasite burden and a measure of health or performance that declines as the number of parasites increases. The regression line is the expected change in health or performance with increasing parasite burden. Individuals performing better than expected for a given parasite burden are more tolerant to the infection, and those performing worse than expected are less tolerant. The residual cost of infection (e.g., *a* and *b*) for each point can be used as a quantitative measure of tolerance.

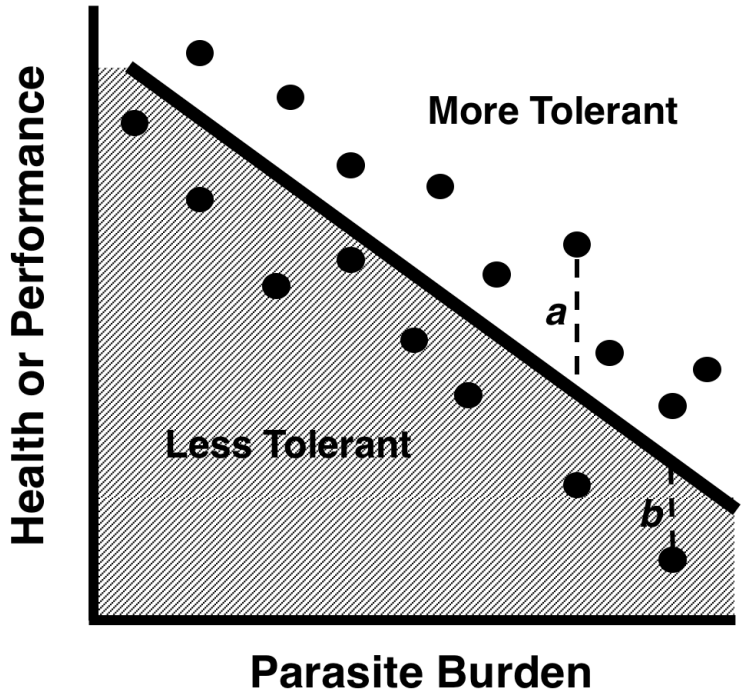

Figure 3.2. (A) Breeding birds with higher *Plasmodium* and *Haemoproteus* burdens had lower hematocrit in 2013 (β = -1.08, SE = 0.25, $P < 0.0001$, $N = 53$). Females tended to have lower hematocrit than males (β = -2.85, SE = 1.11, $P < 0.0001$, $N = 53$). (B) Birds infected with higher *Plasmodium* and *Haemoproteus* burdens also had lower hematocrit levels in 2016, post-breeding. However, hematocrit was similar in both females and males.

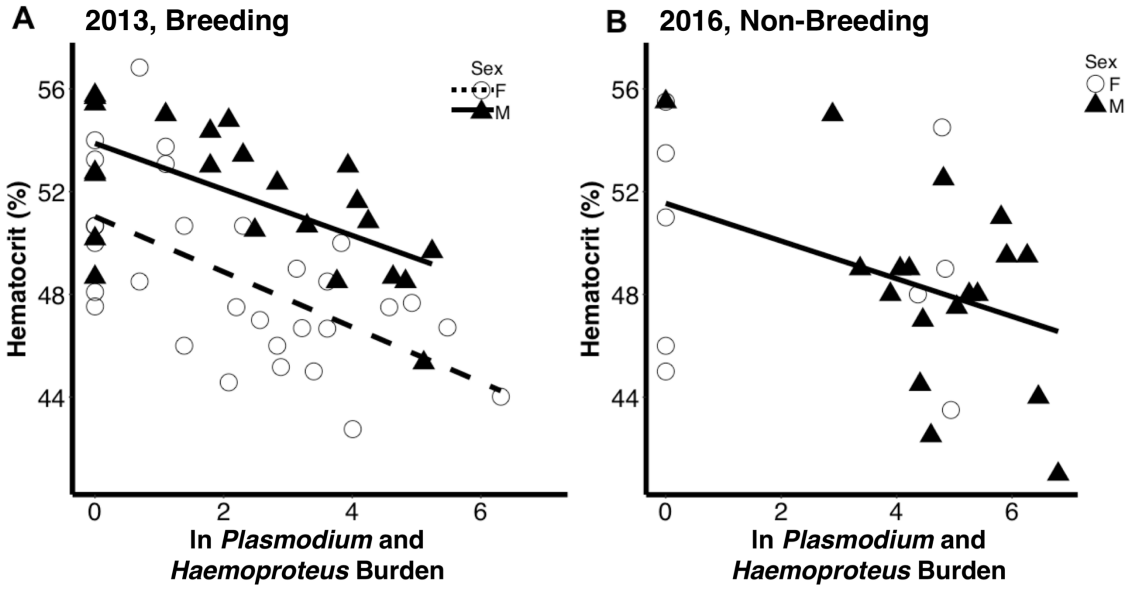

Figure 3.3. (A) In 2013, breeding males with higher plasma corticosterone were more tolerant to *Plasmodium* and *Haemoproteus* infection than males with lower plasma corticosterone ($\beta = 2.43$, SE = 0.69, $P = 0.004$, $R^2 = 0.43$, $N = 16$). In females plasma corticosterone was not associated with tolerance ($\beta = -0.60$, SE = 0.58, $P = 0.31$, $R^2 = 0.003$, $N = 23$). Tolerance is estimated as residual hematocrit controlling for *Plasmodium* and *Haemoproteus* burden and sex. (B) In 2016, birds with higher plasma corticosterone, regardless of sex, were also more tolerant to *Plasmodium* and *Haemoproteus* infection post-breeding. Tolerance is estimated as residual hematocrit controlling for *Plasmodium* and *Haemoproteus* burden.

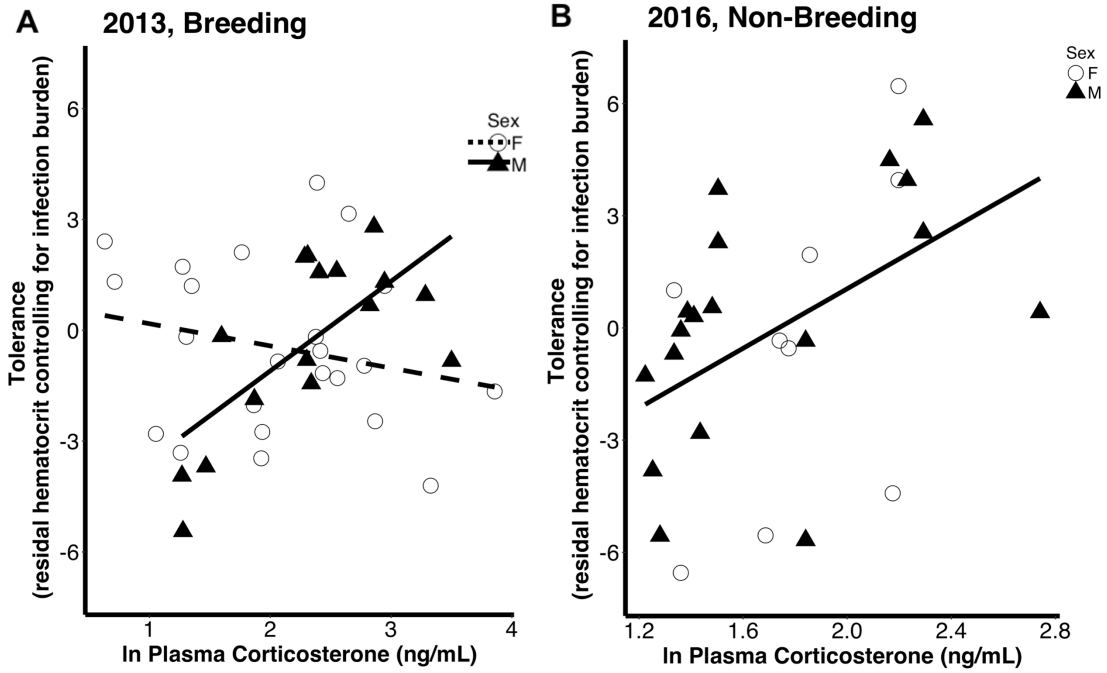

Table 3.1 The prevalence of Haemosporidian infection by genus and sex based on molecular methods.

Sex	Haemosporidians (any genera)	*Plasmodium*	*Haemoproteus*	*Leucocytozoon*
All (N = 60)	57 (95.0%)	50 (83.3%)	15 (25.0%)	23 (38.3%)
Female (N = 32)	30 (93.8%)	26 (81.3%)	9 (28.1%)	7 (21.9%)
Male (N = 28)	27 (96.4%)	24 (85.7%)	6 (21.4%)	16 (57.1%)

CHAPTER IV: EXOGENOUS GLUCOCORTICOIDS AMPLIFY THE COSTS OF INFECTION BY INCREASING PARASITE BURDEN AND REDUCING TOLERANCE

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Abstract

The consequences of parasite infection vary across individuals, populations, and species, and some of these differences could be associated with variation in the use of two defense strategies: resistance and tolerance. Resistance involves reducing the total parasite burden, whereas tolerance involves minimizing the costs of infection for a given parasite burden. Individuals' ability to resist and tolerate parasites could ultimately influence how diseases move through populations, but the individual-level physiological mechanisms that underlie variation in these defenses remain unclear. Glucocorticoid hormones mediate changes in a suite of traits including immune function, resource allocation, and tissue growth and repair, and thereby provide a means for individuals to rapidly adjust their physiology to meet the challenges of a variable environment. Thus, glucocorticoids have the potential to facilitate resistance and tolerance in response to changing environmental conditions. In this study, we experimentally increased glucocorticoid concentrations in red-winged blackbirds (*Agelaius phoeniceus*) that were naturally infected with Haemosporidian parasites and assessed changes in resistance and tolerance to infection. The higher of two doses of glucocorticoids increased the burdens of *Plasmodium*, the parasite causing avian malaria, but the effect only occurred in the absence of co-infection with another Haemosporidian, *Haemoproteus*. Birds receiving the high dose treatment also had lower body mass at high parasite burdens than birds in the low dose treatment group, suggesting that the high dose treatment reduced tolerance of infection. However, the relationship of parasite burden with both hemoglobin and hematocrit did not vary across treatment groups, indicating that corticosterone influences tolerance estimated with body mass,

but not hematocrit or hemoglobin. We found no evidence for an effect of treatment on metrics of the inflammatory immune response or red blood cell production. Our findings suggest that exogenous glucocorticoids can increase the costs of infection by both causing increases in parasite numbers and reducing an individual's ability to cope with infection.

Introduction

Parasite infections are universal, but hosts' responses to infection can vary widely across individuals, populations, or species. When faced with infection, hosts can invest in two defense strategies: resistance and tolerance (Råberg, Graham & Read 2009; Kutzer & Armitage 2016a). Resistance entails killing parasites and/or limiting their reproduction to reduce the total parasite burden. Tolerance involves minimizing the costs of infection for a given parasite burden by either limiting or repairing damage (Medzhitov, Schneider & Soares 2012). Importantly, tolerance allows a host to cope with infection without reducing the parasites' fitness (Råberg, Sim & Read 2007; Råberg *et al*. 2009). Resistance and tolerance are not mutually exclusive strategies (Mauricio, Rausher & Burdick 1997; Restif & Koella 2004; Athanasiadou *et al*. 2015); however, a trade-off could occur if activating one strategy forces a reduction in the other (Råberg *et al*. 2007). For example, up-regulating the inflammatory immune response could kill parasites, improving resistance, but inflammation can damage the host's tissues, decreasing tolerance (Ilmonen, Taarnal & Hasselquist 2000; Libert *et al*. 2006). Investment in resistance and tolerance could change parasite burdens and the costs of infection for the host, and as a result, influence not only host fitness, but also parasite transmission (Miller, White & Boots 2006; Gopinath *et al*. 2014). However, the physiological mechanisms underlying individual variation in resistance and tolerance remain unclear.

Resource limitation and other challenging environmental conditions are associated with variation in resistance and tolerance (Ould & Welch 1980; Guivier *et al*. 2014; Clough, Prykhodko & Råberg 2016; Kutzer & Armitage 2016b); therefore, the hormonal stress response might be influencing parasite defenses. Glucocorticoid hormones are highly conserved vertebrate hormones that mediate responses to both extended and acute challenges, such as low food availability, increased predation risk, and the demands of caring for offspring (Landys, Ramenofsky & Wingfield 2006; Bonier *et al*. 2009a). Changes in circulating concentrations of these hormones can alter immune activity, energy mobilization, and tissue growth and repair, all of which could influence resistance and tolerance to infection (Sapolsky, Romero & Munck 2000). In addition, plasma glucocorticoid concentration often increases in response to an infection or immune challenge, and therefore might be important to regulating the physiological response to parasites and pathogens (Martin *et al*. 2011; Love *et al*. 2016). In addition, many of the challenges associated with altered resistance or tolerance are also associated with increases in glucocorticoids. For example, protein deficiencies, which can increase glucocorticoid concentrations (Jacobson, Zurakowski & Majzoub 1997), also depress immune responses, reducing resistance (Ing *et al*. 2000; Taylor *et al*. 2013), and amplify the effects of infection on body mass, lowering tolerance (Clough *et al*. 2016). Plasma glucocorticoid concentrations tend to increase during reproductive life history stages, facilitating elevated reproductive effort (Romero 2002; Bonier *et al*. 2009b; Ouyang *et al*. 2013), and, during the same periods, individuals can also be less resistant to parasites (Barger 1993; Knowles, Nakagawa & Sheldon 2009). Thus, glucocorticoids have the potential to mediate investment in both resistance and tolerance to infection.

The effects of glucocorticoids on parasite resistance have been well investigated, but the relationship between glucocorticoids and tolerance to infection has been largely unexplored. Experimental increases in glucocorticoids tend to reduce resistance and the effect is primarily attributed to immunosuppression caused by extended elevation of glucocorticoids (Applegate 1970; Belden & Kiesecker 2005; Martin 2009; LaFonte & Johnson 2013). Conversely, glucocorticoid-driven immunosuppression might increase tolerance because some components of the immune response, such as inflammation, damage not only the parasite, but also host tissues (Apanius 1998; Råberg *et al*. 1998). Glucocorticoids could also increase tolerance by promoting tissue repair. For example, glucocorticoids can increase red blood cell production (Golde, Bersch & Cline 1976; Voorhees *et al*. 2013), which could repair the damage caused by parasites that consume or damage blood cells, like nematodes, malaria, or biting insects (Meagher 1998; Valkiūnas 2005; Brommer *et al*. 2011). In an observational field study, we previously found that red-winged blackbirds (*Agelaius phoeniceus*) with higher circulating concentrations of corticosterone, the primary avian glucocorticoid, had higher physiological tolerance of Haemosporidian parasites, including avian malaria (Schoenle *et al*. in revision). Birds with higher corticosterone maintained higher hematocrit for a given parasite burden than birds with lower corticosterone, suggesting corticosterone might increase tolerance. However, we do not know if corticosterone causes individual differences in tolerance to Haemosporidians, and if so, whether the mechanisms underlying its effects are associated with limiting damage, potentially by suppressing host immune function, and/or repairing damage incurred during infection.

In this study, we experimentally increased corticosterone in red-winged blackbirds infected with Haemosporidian parasites, including malaria (*Plasmodium*) and malaria-like parasites (*Haemoproteus* and *Leucocytozoon*), to (i) investigate the effects of corticosterone on

resistance and tolerance to infection and (ii) test whether any effects of corticosterone are mediated through immunosuppression and/or tissue repair. Because our focal population has a very high prevalence (over 90%; see below) of Haemosporidian infection, we manipulated corticosterone levels in with birds that were already naturally infected with one or more genera of Haemosporidian parasites. This experiment allowed us to additionally ask whether co-infection with multiple Haemosporidian genera correlated with resistance and tolerance or influenced the effects of corticosterone. We predicted that exogenous corticosterone would increase parasite burden, indicating a reduction in resistance, and reduce the rate at which measures of host health declines as parasite burden increases, indicating an increase in tolerance. If corticosterone reduces measures of immune function, immunosuppression could be a mechanism for reduced resistance and/or increased tolerance. If corticosterone increases a measure of tissue repair, tissue repair could be a mechanism for corticosterone-driven increases in tolerance.

Materials and Methods

Study population

Red-winged blackbirds breeding in the marshes at Queen's University Biological Station (44°34'02.3" N, 76°19'28.4" W) and in the surrounding Rideau Canal region of southeastern Ontario, Canada, have high Haemosporidian infection prevalence. In the late 1980s to early 1990s, blood smear analyses revealed that 30 – 71% of birds were positive for the parasites (Weatherhead 1990, Weatherhead and Bennett 1991, Weatherhead et al. 1993). From 2013-2015, PCR-based screening indicated that infection prevalence was well over 90% and no less than 40% of birds sampled were infected with at least two genera of Haemosporidians (Schoenle *et al*. In revision).

Experimental design and data collection

In April – May 2015, we captured 89 adult males either using V-top Troyer traps baited with seeds and conspecifics or mist nets paired with conspecific playback at Queen's University Biological Station and on nearby private property in Elgin, Ontario (44°36'28.8" N, 76°13'38.3" W). We housed birds in groups of three in large outdoor flight aviaries (2.4 x 6.1 x 2.4 m, 30 aviaries in the complex; see Appendix D, for detailed information about bird capture and husbandry). Birds acclimated to the aviaries and their aviary-mates for a minimum of 16 d before the start of the experiment (mean $\pm$ SE, 27 $\pm$ 0.55 d, range 16-33 d).

We randomly assigned birds to one of three treatment groups, such that each aviary contained one bird from each treatment group, with the exception of one aviary containing only two birds. Treatment groups included: low-dose corticosterone (0.1 mg implant, $N = 30$), high-dose corticosterone (0.5 mg implant, $N = 30$), and control (vehicle-only implant, $N = 29$) (60-day slow release pellets, Innovative Research of America, Sarasota, FL, USA). We selected these implants following a pilot study, which demonstrated that the 0.5 mg implant significantly increases plasma corticosterone concentration above both the control implants and no surgery controls (Appendix C). We sampled birds once pre-treatment, then immediately implanted the pellets subcutaneously on the birds' backs. We sampled each bird again 7, 14, and 21 d post-treatment. At each sampling event, we collected 500 µL of blood and measured body mass. We used blood samples to assess each individual's parasite presence and burdens, health, immune function, and tissue repair. We evaluated host health using four metrics that can be impaired by Haemosporidian infection: hematocrit (Cellier-Holzem *et al*. 2010; Ellis *et al*. 2015), hemoglobin (Francis, Sullivan & Goldberg 1997; Ellis *et al*. 2015), body mass (Atkinson *et al*. 2000; Cellier-Holzem *et al*. 2010), and oxidative balance (Becker *et al*. 2004; Isaksson *et al*. 2013; Delhaye,

Jenkins & Christe 2016). We assessed two immune metrics that can increase in response to Haemosporidian infection: (i) nitric oxide, an inflammatory signaling molecule and anti-parasite defense (Sild & Hõrak 2009; Bichet *et al*. 2012; de Macchi *et al*. 2013) and (ii) PIT54 (avian analogue of haptoglobin), an acute phase, anti-inflammatory protein and hemoglobin scavenger (Wicher & Fries 2006; Quaye 2008; Ellis, Kunkel & Ricklefs 2014; Ellis *et al*. 2015). We assessed red blood cell production rate via polychromasia as a measure of tissue repair because both Haemosporidian parasites and the immune response to malarial parasites are known to damage red blood cells (Valkiūnas 2005). Parasite presence/absence and burden were assessed pre-treatment and 7 d post-treatment and all other measures were assessed at every sampling point.

After sample collection, we immediately measured hemoglobin in ~5 μL of fresh blood using a HemoCue HB 201+ (HemoCue AB, Ängelholm, Sweden) and we created blood smears in duplicate. We stained the blood smears with Giemsa stain and used the blood smears to assess Haemosporidian parasite burdens (number of infected cells per ~10,000 red blood cells), and the degree of red blood cell production (polychromasia). We centrifuged the remaining blood samples in capillary tubes and measured hematocrit using the average from two capillary tubes of blood. We then separated the plasma from the red blood cells and froze both at -20 °C. We used the blood cells to assess the presence/absence of *Plasmodium*, *Haemoproteus*, and *Leucocytozoon* via a PCR-restriction enzyme assay (modified from (Beadell & Fleischer 2005)). We used the plasma to measure plasma corticosterone concentration via radioimmunoassay (Wingfield, Vleck & Moore 1992) and PIT54, nitric oxide, reactive oxygen species, and total antioxidant capacity via commercial colorimetric assay kits. See Appendix D, for details of blood sampling, blood processing, and laboratory assays.

Statistical analyses

We performed all data analyses in R version 3.3.2 (R Core Team 2016). Before conducting any analyses we transformed plasma corticosterone concentration values with a natural log function and parasite burdens as ln(burden + 1) to improve model fit. For all regression analyses, we initially summarized model results using Type III sums of squares. However, if there were no significant interaction terms, we used Type II sums of squares, which are more statistically powerful in the absence of significant interactions (Hector, von Felten & Schmid 2010; Smith & Cribbie 2014). We compared the effects among levels within categorical factors (e.g., levels of corticosterone treatment) with pairwise contrasts adjusted with Tukey's multiple comparisons.

We determined whether the implants effectively elevated corticosterone by performing a linear mixed effect model predicting change in the corticosterone from pre-treatment. As predictor variables, we included individual identity as a random effect and pre-treatment corticosterone, treatment, day of the experiment, and the interaction between treatment and day as fixed effects.

Our pilot study indicated that parasite burden peaked 7 d post-implantation with the high dose implant, and therefore, we assessed treatment effects on resistance and tolerance at that time point (Appendix C, Figure C2). We assessed resistance as the change in parasite burden after treatment. Using a separate analysis for each parasite genus, we tested the effect of corticosterone treatment on change in parasite burden, including only individuals that were PCR-positive for the focal genus. We performed linear models including change in parasite burden as the response variable and corticosterone treatment, pre-treatment parasite burden, the presence/absence of each non-focal parasite genus, and the interactions between treatment and

the presence/absence of each non-focal parasite genus as explanatory variables. Because we found an effect of the high dose implant and co-infection status on *Plasmodium* burden (see Results below) we conducted two post-hoc analyses. First, we used an analysis of variance and Tukey's multiple comparisons to assess how the change in *Plasmodium* burden varied with overall co-infection status within the high dose treatment. Then we tested if there was a relationship between co-infection status and *Plasmodium* burden pre-treatment using a simple linear regression with *Plasmodium* burden as the response variable and the presence/absence of both *Haemoproteus* and *Leucocytozoon*, and their interaction, as explanatory variables.

For *Plasmodium*, only one parasite life stage, the gametocyte, can transmit the infection to an insect vector (Valkiūnas 2005). Therefore, we also tested the effect of treatment on the change in *Plasmodium* gametocyte number and the probability that a *Plasmodium*-infected cell contains a gametocyte. We performed a linear model including change in gametocytes as the response variable and corticosterone treatment, pre-treatment parasite burden, the presence/absence of *Haemoproteus* and *Leucocytozoon*, and the interactions between treatment and the presence/absence of both *Haemoproteus* and *Leucocytozoon* as explanatory variables. We performed a logistic regression with the ratio of gametocytes: non-gametocytes among *Plasmodium* infected cells as the response variable and treatment, day (0 or 7), and the interaction between day and treatment as fixed effects, and individual identity as a random effect. We were unable to include presence/absence of *Haemoproteus* and *Leucocytozoon* in the logistic regression because of uneven distributions among co-infection status (Appendix D, Table D1).

We measured tolerance as the slope of the relationship between parasite burden and proxies for host fitness or health (Råberg *et al*. 2007). A sharper decline in fitness or health as parasite burden increases (a steeper slope) indicates lower tolerance. To test the effect of

corticosterone on tolerance, we performed linear regressions predicting measures of health (hematocrit, hemoglobin, body mass, and oxidative balance) including the following predictor variables: treatment group, total Haemosporidian parasite burden, and the interaction between treatment group and parasite burden. A significant interaction between treatment and parasite burden indicates a difference in tolerance among treatments. We included all birds that were PCR-positive for Haemosporidians and used total parasite burden in the analyses because all Haemosporidian genera can influence health.

We performed separate analyses to determine if co-infection correlated with tolerance or influenced any treatment effects. Only a subset of all possible combinations of co-infections were observed in our sample (Appendix D, Table D1), and, as a result, we did not have the statistical power to include the presence/absence of each genus and their interactions in a regression model. Instead, we included infection status as a categorical variable where the levels were the combination of infecting genera. We excluded birds with any infection status where $n < 5$ within any of the three treatment groups, which allowed us to test for effects of co-infection among three infection statuses: infection by *Plasmodium* only, *Plasmodium* and *Haemoproteus*, and *Plasmodium* and *Leucocytozoon*. The models testing the effect of co-infection and corticosterone treatment on tolerance included measures of health as the response variables, and treatment, total Haemosporidian parasite burden, infection status, and their pairwise interactions as predictor variables. Because of the large number of interactions, we performed model selection from candidate models including all possible combinations of the predictor variables. We identified the best-fit model as having the lowest second order Akaike information criterion (AICc); we present the top five models and the null model in Appendix D, Table D2. We used

maximum likelihood model fitting to obtain model ranks, and then apply restricted maximum likelihood to obtain parameter estimates from the best-fit model.

We assessed whether corticosterone treatment was associated with change in red blood cell production and/or immune metrics using linear mixed models. We included individual identity as a random effect, and pre-treatment value of the response variable, day, treatment, change in parasite burden, and pairwise-interactions of day, treatment, and change in parasite burden as fixed effects. As in the analyses of tolerance, we tested for the additional effect of co-infection using a subset of the data including birds infected with either *Plasmodium*, *Plasmodium* and *Haemoproteus*, or *Plasmodium* and *Leucocytozoon*, and added the following fixed effects to the model: co-infection status and the pairwise interactions between co-infection status and day, treatment, and parasite burden. For all analyses of immune function and red blood cell production, we performed AICc model selection from a set of candidate models including all possible combinations of the fixed effects. Model selection tables including the top five models and the null model are presented in Appendix D, (Tables D3 and D4) and we present the *t* and *p*-values from the best fit model below.

Results

Effectiveness of corticosterone treatment

The high corticosterone treatment elevated plasma corticosterone concentrations throughout the experiment above both the control implant ($t = 2.58$, $P = 0.03$, 257 observations of 88 birds) and the low corticosterone implant ($t = 3.18$, $P = 0.006$). However, corticosterone concentrations of birds in the low dose treatment were no different than birds receiving the sham implant ($t = -0.62$, $P = 0.81$) (Appendix D, Figure D1). Seven days after implantation, corticosterone concentrations of birds in the high dose implant group had corticosterone

concentrations of (mean ± SE) 8.79 ± 1.89 ng/mL, which is similar to concentrations in free-living, breeding males, which we previously measured as 12.31 +/- 1.86 ng/mL (Schoenle et al. In revision). Across all treatments, the change in corticosterone from the initial value decreased over time ($F = 14.66$, $P = 0.0002$).

Effect of glucocorticoids and co-infection on resistance

The high dose corticosterone treatment increased *Plasmodium* burden, but the effect depended on co-infection status. There were significant interactions between treatment and both *Haemoproteus* ($F = 16.74$, $P < 0.0001$, $N = 82$) and *Leucocytozoon* ($F = 4.15$, $P = 0.02$, $N = 82$) infection status, and the three-way interaction between treatment, *Haemoproteus*, and *Leucocytozoon* approached significance ($F = 2.93$, $P = 0.06$, $N = 82$). If birds were infected with *Plasmodium* but not *Haemoproteus*, the high dose implant increased *Plasmodium* burdens more than the control implant (Figure 4.1, A and B), but the effect disappeared in the presence of a *Haemoproteus* co-infection (Figure 4.1, C and D). The high dose implant also increased *Plasmodium* burden more than the low dose implant if a bird was only infected with *Plasmodium* (Figure 4.1A), but not when co-infected with *Leucocytozoon* and/or *Haemoproteus* (Figure 4.1B-D). In birds co-infected with *Plasmodium* and *Haemoproteus* (Figure 4.1C), the low dose implant increased *Plasmodium* burden more than the high dose implant, but that effect was partially driven by a single bird with a large increase in parasite burden, and if that outlier is removed from the analysis, the effect is no longer significant. Within the high dose treatment, change in parasite burden was associated with co-infection status ($F = 15.62$, $P < 0.0001$, $N = 28$). Birds infected with *Plasmodium* alone experienced greater increases in *Plasmodium* burden than those with co-infections, and none of the birds co-infected with *Haemoproteus* increased *Plasmodium* burden (Figure 4.2). The relationship between *Haemoproteus* co-infection and

Plasmodium burden also existed pre-treatment. Pre-treatment, lower *Plasmodium* burdens were associated with *Haemoproteus* co-infection ($F = 54.9$, $P < 0.0001$, $N = 81$), but there was no relationship with *Leucocytozoon* co-infection ($F = 0.84$, $P = 0.36$, $N = 81$) or the interaction between *Haemoproteus* and *Leucocytozoon* ($F = 1.84$, $P = 0.18$, $N = 81$) (Appendix D, Figure D2).

Corticosterone treatment increased the overall number of *Plasmodium* gametocytes, but not the proportion of *Plasmodium*-infected cells containing gametocytes. As with total *Plasmodium* burden, the effect of corticosterone treatment on the number of *Plasmodium* gametocytes depended on co-infection. The high dose implants caused an increase in gametocytes relative to birds receiving both the control and low dose implants in the absence of *Haemoproteus* (vs. control, $t = 4.00$, $P = 0.0005$, $N = 28$; vs. low, $t = 3.35$, $P = 0.004$, $N = 28$) and *Leucocytozoon* (vs. control, $t = 2.92$, $P = 0.01$, $N = 28$; vs. low, $t = 3.06$, $P = 0.009$, $N = 28$). See additional details in Appendix D. Post-treatment, the likelihood that a *Plasmodium*-infected cell contained a gametocyte was lower for the high dose treatment than either the control ($t = -4.14$, $P = 0.001$, 167 observations of 87 birds) or low dose treatment ($t = -2.34$, $P = 0.05$), but there was no difference between the low dose and control treatments ($t = -1.86$, $P = 0.15$). There were no differences among the treatment groups pre-treatment (all $P > 0.56$).

Corticosterone treatment had no effect on change in *Haemoproteus* ($F = 1.95$, $P = 0.17$, $N = 23$) or *Leucocytozoon* ($F = 0.92$, $P = 0.41$, $N = 37$) burdens. Because of the limited sample sizes of some co-infection statuses present in our study (Appendix D, Table D1), we could not test for an effect of co-infection on change in these genera.

Effect of glucocorticoids and co-infection on tolerance

Corticosterone treatment reduced tolerance to infection with Haemosporidian parasites as estimated by body mass (interaction between treatment and parasite burden; $F = 5.98$, $P = 0.004$, $N = 87$). Body mass of birds in the high dose group declined more steeply as parasite burden increased than birds in the low dose group ($t = -3.35$, $P = 0.004$, $N = 87$) (Figure 4.3A). However, the slope of the mass-parasite burden relationship did not differ between the high dose and control groups ($t = -1.60$, $P = 0.25$, $N = 87$) or the low dose and control groups ($t = 0.62$, $P = 0.81$, $N = 87$) (Figure 4.3A). The range in parasite burden in the control group (2 – 174 parasites/10,000 red blood cells) is much smaller than the range in the low dose (1 – 2,143 parasites/10,000 red blood cells) or high dose (0 – 4,346 parasites/10,000 red blood cells) groups. Thus, we might not have been able to detect a difference between the high dose and control groups, if present.

Corticosterone treatment had no effect on tolerance when estimated using hematocrit or hemoglobin. Although hematocrit was affected by both treatment ($F = 11.78$, $P < 0.0001$, $N = 87$) and parasite burden ($F = 31.40$, $P < 0.0001$, $N = 87$), the slope of the relationship between hematocrit and parasite burden was similar across corticosterone treatments ($F = 0.59$, $P = 0.55$, $N = 87$) (Figure 4.3B). Hemoglobin declined with increasing parasite burden ($F = 13.19$, $P = 0.0005$, $N = 87$), but did not differ among treatments ($F = 1.40$, $P = 0.25$, $N = 87$), and there was no interaction between treatment and parasite burden ($F = 0.37$, $P = 0.69$, $N = 87$) (Figure 4.3C). Oxidative balance was not associated with parasite burden ($F = 0.46$, $P = 0.50$, $N = 84$), and thus, could not be used to assess tolerance. The interaction between co-infection status and parasite burden was not retained in any of the top models predicting costs of infection (Appendix D, Table D2), suggesting that co-infection does not influence tolerance.

The effects of corticosterone and co-infection on tissue repair and immune function

Corticosterone treatment was not associated with change in red blood cell production. Although treatment was included in two of three top models predicting change in red blood cell production (Appendix D, Table D3), pairwise contrasts revealed no differences among treatment groups or significant treatment-interactions (all $P > 0.24$, 240 observations of 81 birds). Co-infection status was included in the two top models predicting change in red blood cell production (Appendix D, Table D4). Birds infected with *Plasmodium* alone ($t = 3.85$, $P = 0.0005$, 210 observations of 71 birds) or both *Plasmodium* and *Leucocytozoon* ($t = 4.68$, $P < 0.0001$) had greater increases in red blood cell production than birds infected with both *Plasmodium* and *Haemoproteus*. However, parasite burden was associated with both co-infection status (Figure 4.1) and red blood cell production (parasite burden, $F = 10.33$, $P = 0.002$; parasite burden-day interaction, $F = 21.85$, $P < 0.0001$), and thus, we cannot determine whether co-infection influences red blood cell production beyond the strong effect of parasite burden. Neither corticosterone treatment nor co-infection status was linked to the immune measures and they were not retained in any top models predicting change in the two immune metrics, haptoglobin and nitric oxide (Appendix D, Tables D3, D4).

Discussion

Our results suggest that treatment with exogenous glucocorticoid hormones can amplify the costs of Haemosporidian infection for a host by both increasing parasite burden and reducing tolerance to infection. In addition, exogenous glucocorticoids might increase the risk of disease transmission. The high dose treatment caused an increase in avian malaria (*Plasmodium*) burden and in the total number of gametocytes, the malaria life stage responsible for transmission to the insect vector. However, the hormone's effect on malaria was absent in the presence of co-infection with a related parasite, *Haemoproteus*. Corticosterone treatment also reduced infection

tolerance such that birds in the high dose treatment experienced steeper declines in body mass with increasing parasite burden. We found no evidence supporting an immunosuppressive role for either corticosterone or co-infection, suggesting that suppression of the inflammatory response might not underlie glucocorticoids' effects on resistance and tolerance and/or other components of immunity might be more important to controlling malaria infection in this species. Individual variation in both glucocorticoid concentration and co-infection status can influence parasite dynamics within hosts and, as a result, might alter infection risk in a population.

Our study confirms that increases in corticosterone can cause increases in parasite burden (Applegate 1970; Belden & Kiesecker 2005), and also demonstrates that treatment increases the total number, but not the proportion, of malaria gametocytes. Researchers often assume that glucocorticoids' effects on parasite burden are the result of immunosuppression, however parasites might also respond directly to changes in hormone concentrations or to other physiological changes associated with glucocorticoid increases. *In vitro* experiments with malaria as well as other protozoan parasites indicate that glucocorticoids can increase parasite replication in the absence of a host immune system and, thus, the parasites might be detecting the hormones directly (Maswoswe, Peters & Warhurst 1985; Escobedo *et al*. 2005; Li, Leatherland & Woo 2013). The increase in the total number of gametocytes is likely a function of the overall increase in the number of parasites. However, we found the higher dose of corticosterone also caused a decrease in the proportion of gametocytes, whereas an *in vitro* study of a human malaria species, *Plasmodium falciparum*, indicated that glucocorticoids can increase the proportion of gametocytes (Maswoswe *et al*. 1985). Avian malaria parasites can modulate their life history strategy with changes in the host environment (Cornet *et al*. 2014), and changing their

reproductive strategy in the presence of glucocorticoids, when hosts might be experiencing a substantial or potentially life-threatening challenge, might be an adaptive transmission strategy. Moreover, elevated glucocorticoids can increase host attractiveness to mosquito vectors (Gervasi *et al*. 2016), and increasing the total number of gametocytes could further improve transmission success (Drakeley *et al*. 1999).

If malaria-infected birds were co-infected with *Haemoproteus*, the high dose treatment did not increase malaria parasite burden or the number of gametocytes. Similarly, another study of co-infections demonstrated that the presence of one avian Haemosporidian species can reduce the probability of infection with a different species (Clark *et al*. 2016). Competitive interactions among parasites can yield negative relationships between parasite infection intensities (Lafferty 2010). For example, competition for shared resources, like blood cells or hemoglobin in the case of Haemosporidians, could result in higher burdens of the more competitive parasite (Graham 2008). Alternatively, infection with a parasite could result in cross immunity, in which a host has increased resistance to a different parasite (Cox 2001; Fenton & Perkins 2010). However, our study found no support for differences in immune function with co-infection using the immune metrics we measured. Regardless of the mechanism, co-infections with multiple Haemosporidian parasites could contribute to parasite dynamics within bird populations.

In our previous field study of red-winged blackbirds, higher endogenous plasma corticosterone concentrations were associated with maintaining higher hematocrit for a given parasite burden (Schoenle et al. In Revision), but in the present study, corticosterone treatment had no effect on tolerance when measured using hematocrit or hemoglobin and instead, reduced tolerance estimated with body mass. Our results also differ from a study in toads (*Anaxyrus americanus*), where corticosterone manipulation had no effect on tolerance to a chytrid fungus

(Murone, DeMarchi & Venesky 2016). Energy regulation is a central role of glucocorticoids (Sapolsky *et al*. 2000), and corticosterone-driven changes in metabolism coupled with the challenge of parasite infection might have led to the decline in tolerance estimated with body mass. The discrepancy between the observational and experimental study could be a result of an unknown factor underlying the observational correlation in the field, differing effects of endogenous and exogenous hormones, or differences in the captive and natural environment (e.g., diet, social interactions, activity levels). Slow-release hormone implants do not match the pulsatile, cyclic release of endogenous hormones, which could result in different physiological outcomes (Fusani 2008; Sopinka *et al*. 2015). In addition, individuals' regulation of hormone concentrations have been shaped by selection. Thus any hormone manipulation that shifts individuals away from their endogenous levels could have negative effects, like the reduction in tolerance seen here. Furthermore, physiological responses to stimuli can vary in captive and wild settings (Martin *et al*. 2011), and corticosterone manipulations might not be good representations of individual variation in the hormones.

In summary, individual variation in glucocorticoid concentrations and co-infection status can contribute to among individual differences in the two key host defense strategies, resistance and tolerance. To our knowledge, this is the first study to demonstrate glucocorticoid-driven reduction in tolerance of parasites and change in parasite burden that is modulated by co-infection. Elevated glucocorticoids had two-fold costs: birds suffered higher parasite burdens and the associated reductions in hemoglobin and hematocrit, and experienced higher costs of infection, in terms of reduced body mass. The increase in *Plasmodium* burden could also increase malaria transmission rates in the population, suggesting a mechanism by which individual glucocorticoid levels could link to population-level processes. Moving forward, we

need to address whether factors underlying variation in glucocorticoids, including life history strategy or the physical and social environment, are also associated with changes with resistance and tolerance to parasites. Through its effects on an individual's physiology, environmental variation could lead to variation in host-parasite dynamics at the individual and population level.

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Figures

Figure 4.1. The effect of treatment on *Plasmodium* burden by co-infection status with (A) *Plasmodium* only, (B) *Plasmodium* and *Leucocytozoon,* (C) *Plasmodium* and *Haemoproteus*, and (D) *Plasmodium*, *Haemoproteus*, and *Leucocytozoon*. Letters (a, b) represent significant differences ($P < 0.05$) among treatments within the same panel. In the absence of *Haemoproteus*, (A and B), high dose corticosterone causes an increase in *Plasmodium* burden, but the effect disappears with *Haemoproteus* co-infection (C and D).

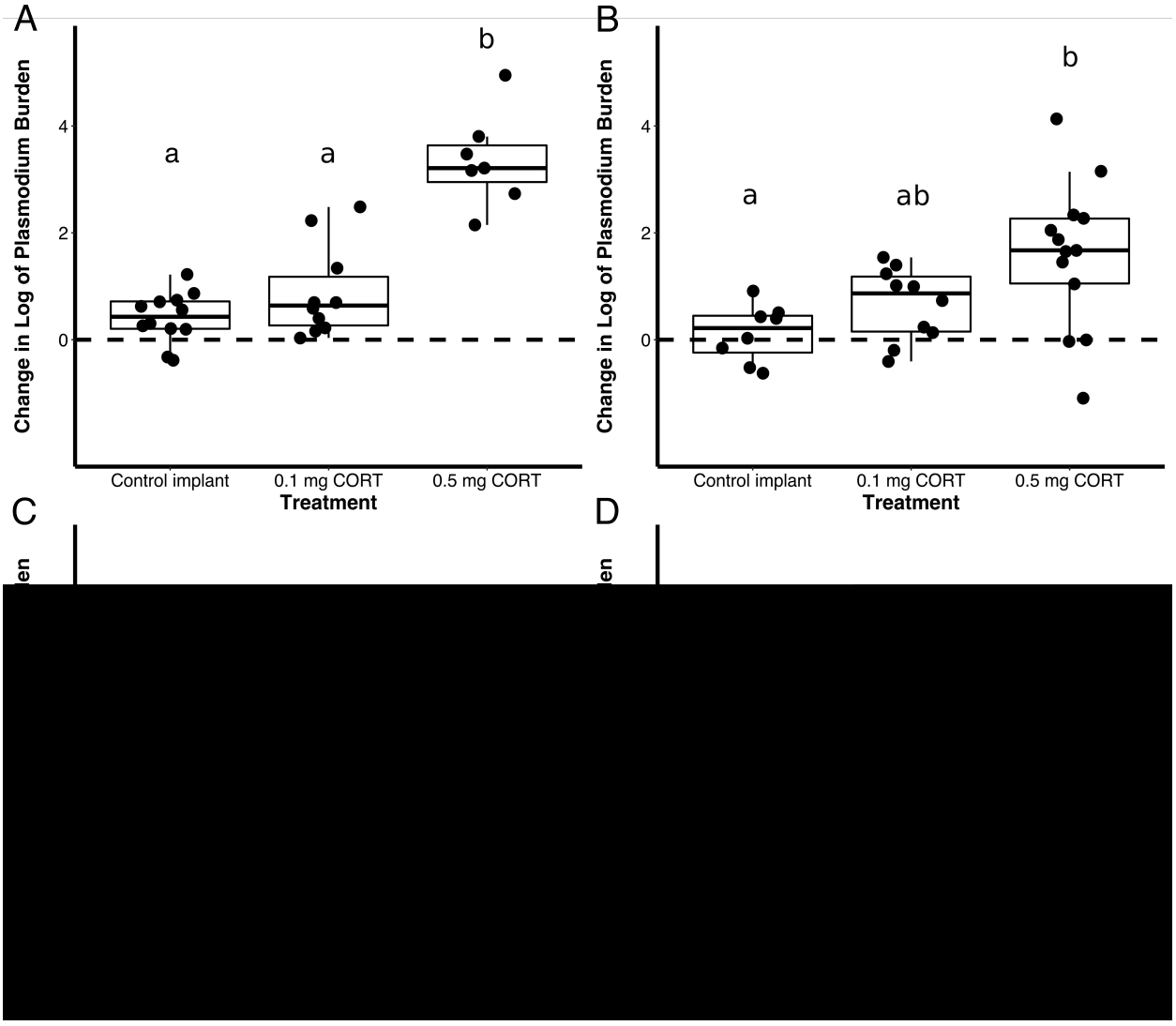


Figure 4.2. The relationship between co-infection status and change in parasite burden in birds receiving the high dose corticosterone treatment. Letters (a, b, c) represent significant differences ($P < 0.05$) among co-infection statuses. Co-infection with either or both *Haemoproteus* and *Leucocytozoon* limits the effect of corticosterone on *Plasmodium* burden.

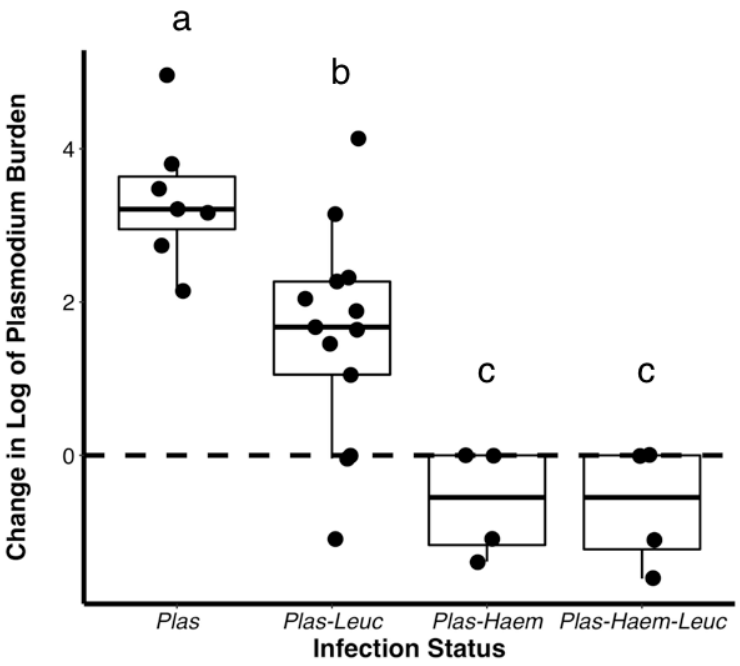
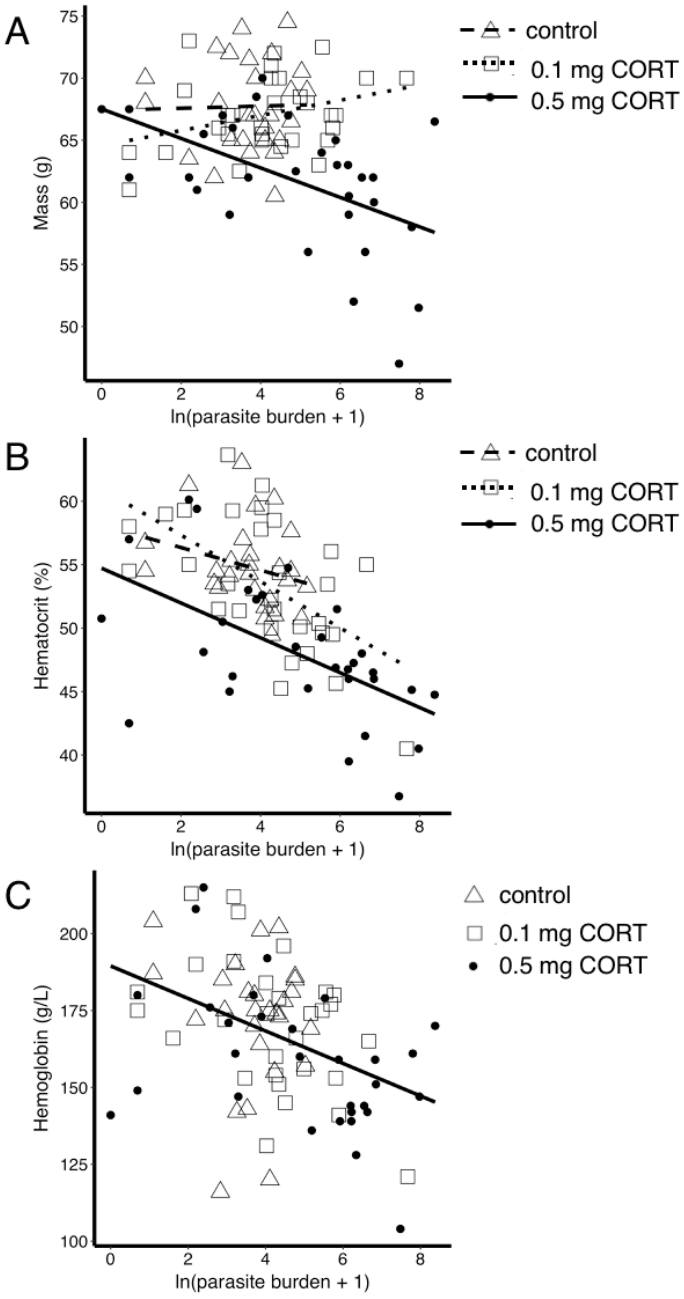


Figure 4.3. The effect of corticosterone treatment on tolerance to infection estimated using (A) body mass, (B) hematocrit, and (C) hemoglobin. Open triangles represent birds in the control group, open squares represent the low dose group, and solid circles represent the high dose group. (A) Among birds implanted with the high dose, body mass was lower in birds with higher parasite burdens, and the slope was significantly different from the low dose group or the low and control groups combined, indicating lower tolerance. (B) Birds in the high corticosterone group had lower mean hematocrit than birds in the other treatment groups. There was no difference in the slope of the relationship between hematocrit and parasite burden among treatment groups, indicating no difference in tolerance. (C) There was no difference in mean hemoglobin concentrations or the slope of the hemoglobin-parasite burden relationship among treatment groups.


A
Mass (g)
ln(parasite burden + 1)
control
0.1 mg CORT
0.5 mg CORT
B
Hematocrit (%)
ln(parasite burden + 1)
control
0.1 mg CORT
0.5 mg CORT
C
Hemoglobin (g/L)
ln(parasite burden + 1)
control
0.1 mg CORT
0.5 mg CORT

CHAPTER V. CONCLUSIONS

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My dissertation research investigates a hormonal mechanism that could underlie variation in parasite defenses. Here, I review and synthesize the results of that research, discuss their implications, and propose directions for future research.

Overview: Parasite burden, co-infection, and corticosterone influence the consequences of chronic malaria infection

Once infected with a parasite, individuals can invest in two defense tactics: resistance and tolerance (Råberg *et al*. 2007, 2009). Resistance is an individual's ability to limit their parasite burden (the number of parasites), and can be achieved by killing or removing parasites, or limiting parasite replication (Best *et al*. 2008; Råberg *et al*. 2009). Tolerance involves minimizing the costs of infection for a given parasite burden by either limiting or repairing damage (Medzhitov *et al*. 2012). The relative costs and advantages of these defense strategies vary, and might change when an animal is facing other challenges, such as limited food availability or inclement weather, or engaging in resource demanding activities, such as provisioning young. Glucocorticoid hormones facilitate the response to challenges by mediating a suite of physiological and behavioral changes, including energy mobilization, tissue growth and repair, and immune function (Sapolsky *et al*. 2000; Landys *et al*. 2006). As a result, glucocorticoid hormones might also influence parasite resistance and tolerance. My dissertation presents some of the first studies to investigate the role of glucocorticoids in mediating infection tolerance and contributes to the literature assessing the influence of glucocorticoids on parasite resistance.

I used a series of observational and experimental studies to investigate the role of glucocorticoids in influencing resistance and tolerance to parasites in a population of red-winged blackbirds with high prevalence of Haemosporidian parasites. The study population, located in southern Ontario, Canada, is exposed to all three genera of avian Haemosporidians: *Plasmodium*, *Haemoproteus*, and *Leucocytozoon* and over 90% of birds are infected with at least one genus. I conducted a medication study to identify physiological consequences of infection (Chapter II), an observational study to identify relationships between corticosterone, the primary avian glucocorticoid, and parasite defenses in a natural setting (Chapter III), and a corticosterone manipulation experiment to test if the hormones mediate changes in resistance and tolerance, and if so, by what mechanisms (Chapter IV). In the first study, I found that anti-malarial medication effectively reduced *Plasmodium* burden, increased both hematocrit and hemoglobin, and decreased red blood cell production. There were no effects of the medication on multiple immune metrics, body condition, oxidative status, or corticosterone. These results suggest that the primary physiological costs of *Plasmodium* infection might be hematological: the damage of red blood cells and compensatory up-regulation of red blood production. In the second study, I tested the hypothesis that glucocorticoids are associated with indicators of resistance and tolerance to infection. Breeding male red-winged blackbirds with higher corticosterone concentrations maintained higher hematocrit for a given parasite burden, suggesting improved tolerance to Haemosporidian parasites. However, corticosterone was not associated with parasite burden. I replicated this finding three years later during a different life history stage, post-breeding, and found that both males and females with higher corticosterone appeared to be more tolerant to infection, having higher hematocrit relative to their parasite burdens. In my final study, when manipulating corticosterone in an aviary setting, the relationships between

corticosterone and parasite defenses were nearly opposite to those seen in the field study. Corticosterone treatment increased *Plasmodium* parasite burden, but only in the absence of co-infection with *Haemoproteus*. Corticosterone also reduced tolerance to Haemosporidians in terms of body mass loss, but did not influence tolerance when estimated using hematocrit or hemoglobin. I found no evidence of effects of corticosterone on either immune response or tissue repair.

Does corticosterone influence resistance and tolerance to parasite infection?

In the observational field study (Chapter III) and the hormone manipulation experiment (Chapter IV), I observed relationships between corticosterone and both parasite burden and costs of infection, suggesting that the hormone is likely to influence resistance and tolerance. However, the nature of corticosterone's effects remains unclear because the direction of the observed relationships differed across the two studies. An additional complication involves the possibility that the parasites, not only the hosts, could be responding directly to corticosterone. High dose corticosterone treatment caused birds' parasite burdens to increase, however, we found no evidence for change in host immune responses using the measures examined in the experiment. Rather than reducing host resistance, the treatment might have directly caused the parasites to increase their rate of replication within the host. Haemosporidian parasites can respond to subtle changes in their hosts, including detecting and responding to the host's response to a mosquito bite (Cornet *et al*. 2014), and studies *in vitro* have shown that glucocorticoids increase the production of gametocytes in a related species of human malaria, *Plasmodium falciparum* (Maswoswe *et al*. 1985). Therefore, I cannot determine if changes in parasite burden were caused by responses by the host or the parasites to corticosterone.

Dissimilarities in natural and captive environments, differences in the effects of manipulated, exogenous hormone concentrations and endogenous hormone concentrations, or heterogeneity in individual quality could contribute to the different outcomes I observed in the observational (Chapter III) and experimental study (Chapter IV). In an aviary setting, birds might mount a different physiological response to infection and glucocorticoid treatment because they are facing a different set of challenges than free-ranging birds. In captivity, birds are freed from food limitation and the energetic and physical demands of breeding, but are experiencing a different physical and social environment as well as more frequent encounters (visual and physical contact) with humans, which could be perceived as predation risk. Any of these differences could alter the hypothalamic-pituitary-adrenal (HPA) axis activity at various levels (e.g., receptors, hormone concentrations, negative feedback) or change various components of the immune response (Sopinka *et al*. 2015). For example, a study on house sparrows (*Passer domesticus*) demonstrated that captivity caused a hyper-inflammatory state and, unlike in free-living birds, captive sparrows did not increase corticosterone concentrations after exposure to an immune challenge (lipopolysaccharide, a component of bacterial cell walls) (Martin *et al*. 2011). Thus, captivity could result in priming of the HPA axis and components of immunity, leading to different responses to corticosterone.

Exogenous hormone implants, like those used in my experiment, cannot mirror the cyclic (daily) and pulsatile release of natural hormone cycling, and they elevate circulating concentrations above an individual bird's endogenous set-point. Even if the implants increase corticosterone within a natural physiological range, the secretion pattern and total volume of hormone released is likely different from individuals with similar endogenous concentrations at a single time point. As a result, experimental elevations of corticosterone could affect the HPA

axis differently than endogenous corticosterone concentrations even if plasma levels of the hormone appear equivalent (e.g., binding to different receptor types or altering negative feedback), which could alter the downstream effects of corticosterone. In addition, manipulating hormone concentrations disrupts individuals from their homeostatic set-point (Fusani 2008). Natural selection has shaped animals to match their hormone concentrations to their internal and external environments (e.g., energy reserves, life history stage, social environment), and experimental manipulations are moving them away from their perceived optima. As a result, manipulations might have negative effects simply because individuals' hormone concentrations are no longer close to their individual optima. In our experiment, elevation of corticosterone caused two negative outcomes: higher parasite burdens and lower tolerance.

If variation in the quality of individuals is associated with plasma corticosterone and either or both resistance and tolerance, we would also expect to observe differences in our observational and experimental studies. We often assume that there is heterogeneity in the quality of individuals that can underlie differences in their fitness or their ability to respond to challenges, like parasitic infections (Cam, Aubry & Authier 2016). If plasma corticosterone concentrations and tolerance of Haemosporidians are both positively correlated with quality, then we expect plasma corticosterone to be positively associated with tolerance, as we observed in our field study. However, when we manipulated plasma corticosterone, we did so independent of individual quality, thereby minimizing the effect of variation in quality on the relationship between plasma corticosterone concentration and tolerance. In that case, the negative effect of the high dose corticosterone implant on tolerance would reflect the true effects of plasma corticosterone on tolerance of Haemosporidians.

It is also important to consider the differences in the methods used to estimate tolerance in the observational (Chapter III) and experimental (Chapter IV) studies. In the observational study, I assessed tolerance as residuals from a regression of a health metric (hematocrit) against parasite burden (Chapter III, Figures 1 and 2). This tolerance metric is dependent both on the individuals' vigor (their hematocrit at a parasite burden of zero) and within that individual, the slope of the regression of hematocrit against parasite burden. In the corticosterone manipulation experiment, I assessed the relationship between parasite burden and measures of health (including hematocrit, hemoglobin, and mass) as an indicator of tolerance for an entire treatment group (Chapter IV, Figure 3). We used different estimates of tolerance because the data structure varied across the two studies. In the observational study (Chapter III), we assessed individual variation in tolerance, whereas in the experimental study (Chapter IV) we compared tolerance across treatment groups. Currently, it is not entirely clear when these different tolerance measures provide similar information about hosts' ability to cope with infection. A recent study demonstrated a strong, positive correlation among two different measures of individual-level tolerance: the slope of the relationship of health against parasite burden within an individual and the residuals from a regression of a health measure against parasite burden (Chapter III, Figure 1) (Burgan 2016). The positive correlation between the two tolerance measures suggests that both provide similar information, however, whether or not the differences in measurement type are truly comparable will depend on traits of hosts (e.g., how variable is host vigor). In our corticosterone manipulation experiment, birds receiving the larger, high dose corticosterone implant had lower hematocrit regardless of parasite burden, but the slope of the relationship between hematocrit and parasite burden did not vary among treatments (Chapter IV, Figure 3B). If we had assessed tolerance as residual hematocrit as we did in the observational field study

(Chapter III), we would have found that birds in the high corticosterone treatment group had lower residual hematocrit controlling for infection intensity than birds in the control or low dose treatment groups. Thus, we would still find that the relationship between tolerance and corticosterone varies across our observational and experimental studies.

Future directions

Drivers of tolerance to avian Haemosporidians: a comparative approach

Avian Haemosporidians are widespread and studied broadly, and thus, it might be possible to conduct a comparative analysis of variation in tolerance to Haemosporidians. A comparative analysis could reveal the relative contributions of host and parasite phylogenetic history, geographic distribution, and host life history to tolerance. Hematocrit is frequently measured in studies of Haemosporidians, and could serve as a common health metric to calculate tolerance across studies. Using a meta-analytic approach, one could determine which covariates influence the relationship between parasite burden and hematocrit, a metric of range tolerance. Although the relationship between hematocrit and parasite burden is not often reported in publications, one might be able to obtain data directly via the Avian Malaria Research Coordination Network, which includes people who have been collecting this type of data for many years (e.g., Carter Atkinson, Staffan Bensch, Bob Ricklefs, Ravinder Sehgal, Gediminas Valkiūnas). Although much of the variation in tolerance might be due to differences in virulence among parasite species or lineages, we might also be able to identify which host traits are associated with tolerance.

Continuing in the red-winged blackbird – Haemosporidian system

The uncertainty that remains regarding the link between corticosterone and the response to infection is unlikely to be resolved with additional hormone manipulations or captive studies,

for the reasons discussed above (e.g., physiological differences between captive and free-living birds, hormone implants do not mirror natural hormone secretion patterns, hormone implants move animals from their homeostatic set-points). However, by manipulating birds' stressors, one could determine if stressors affect tolerance, and, by simultaneously measuring corticosterone, identify if changes in corticosterone correlate with changes in tolerance. I expect that manipulating stressor exposure will change several aspects of physiology and behavior, and thus, could elicit different responses than manipulating a single hormone. One could manipulate social stress by altering males' epaulet size or color, or energetic costs by clipping wing feathers. It is also interesting to consider how changing reproductive investment, by manipulating clutch size, might affect tolerance in females. Because reproductive effort can reduce resistance to Haemosporidians (Knowles *et al*. 2009), I would hypothesize that individuals increasing their reproductive effort might shift their parasite defenses towards tolerance. This work might be most successful if conducted in a system where recapture and assessing reproductive success is more manageable, like in a box nesting population of tree swallows (*Tachycineta bicolor*) or European starlings (*Sturnus vulgaris*).

My work demonstrated that co-infection with multiple Haemosporidian genera could influence the costs to the host and the likelihood of parasite transmission. Future work is required to address how co-infection and infection burdens influence reproductive success and survival, and the mechanisms underlying the suppressive effect of *Haemoproteus* on *Plasmodium*. A multi-year, observational field study assessing Haemosporidian presence/absence and burden, as well as measures of reproductive effort and success and return rates, could begin to address this question. If sample size were sufficient, such a study would also be able to address whether infection with one parasite influences the probability of infection with other parasites. In

addition, the effects of co-infection on the host and parasite-parasite interactions can depend on the order and timing of infection with each parasite. Artificial infection studies on previously unexposed birds (perhaps, hand-reared) that manipulate the order, dose, and timing of infections with *Plasmodium* and *Haemoproteus* could be used to determine how co-infection processes influence parasite burdens. Assessing immune metrics throughout the experiment could indicate whether immune priming by *Haemoproteus* is important to limiting *Plasmodium* burdens. For any future work on co-infection in this system, I recommend identifying Haemosporidian parasites to the species and lineage level by sequencing parasite DNA (cytochrome C) as well as extending parasite surveys beyond Haemosporidans to include gastrointestinal parasites, ectoparasites, and other blood parasites (e.g. *Trympanosoma*).

Conclusions

Individual variation in hosts' responses to parasites could scale up to population-level processes, including host and parasite population dynamics (Hawley & Altizer 2011; Adelman & Hawley 2016). Thus, understanding how individuals alter their investment in resistance and tolerance to infection could contribute to our understanding of how diseases spread through populations. The research described in my dissertation suggests that glucocorticoid hormones could contribute to individual variation in resistance and tolerance. As a result, changes in energetic demands, environmental challenges, or life history stage that induce increases in glucocorticoids could ultimately influence individual responses to parasites. I hope that this dissertation serves as a foundation for further investigations into individual variation in infection tolerance and the physiological bases of hosts' responses to parasites.

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APPENDIX A
SUPPLEMENTARY METHODS AND RESULTS, CHAPTER II

Overview of Hormone-Implant Experiment

We conducted a hormone-manipulation experiment from May – June 2015. We housed the birds in groups of three males in an outdoor flight aviary (2.4 x 6.1 x 2.4 m, 30 aviaries in the complex), implanted each with a subcutaneous pellet implant (Innovative Research of America SC-111, Sarasota, FL, USA), and collected a small blood sample once per week for 3 weeks. All birds included in the present study received a control implant containing only vehicle. Birds were given a one-week recovery period before the start of the malaria-treatment study. While in captivity, birds had *ad libitum* access to water (in elevated dishes and bird baths) and food (poultry starter, seed mix, fruit, corn, hard-boiled egg, and meal worms).

Haemosporidian Presence/Absence: PCR and Restriction Enzyme Analysis

We used a modified restriction enzyme assay from Beadell & Fleischer (2005) to identify the presence/absence of each Haemosporidian genus. In a 25 μL reaction, we combined 4.5 μL DNA with final concentrations of 0.2 mM dNTPs, 3 mM $MgCl_2$, 0.6 μM of the 213F and 372R primers (Beadell & Fleischer 2005), 0.5 U *Taq* polymerase (New England Biosystems), 1X *Taq* buffer (New England Biosystems), 10X BSA. We followed the PCR cycling conditions of Beadell and Fleischer (2005) exactly. In a total reaction volume of 20 μL, we digested 10 μL of the PCR product with 1 U of each of the restriction enzymes XmnI and XbaI (New England BioLabs, Ispwich, MA) and CutSmart Buffer at a final concentration of 1X (New England BioLabs, Ispwich, MA). We incubated the restriction enzyme reaction at 37° C overnight (minimum 12 hours). We electrophoresed the digested PCR product on a 4% agarose gel and used fragment size to identify the presence of each Haemosporidian genus as described in

Beadell and Fleischer (2005). To minimize the probability of false negatives, we re-extracted DNA and reran the PCR three times for any samples that tested negative for Haemosporidians.

Table A1. Comparison of candidate models predicting the effect of treatment and the number of days post-treatment on physiological metrics over time. All models, including the null model, include the pre-treatment value for the physiological measure. Sample sizes vary across physiological metrics because of sample availability or quality.

Model Rank	K[1]	AICc[2]	Effects[3]	ΔAICc[4]
Change in Hematocrit (N = 56)				
1	4	265.6	Treatment + Day	0
2	3	266.7	Treatment	1.13
3	5	267.8	Treatment + Day + Treatment*Day	2.18
4	3	279.9	Day	14.34
5	2	280.8	Null	15.25
Change in Hemoglobin (N = 56)				
1	3	444.3	Treatment	0
2	4	446.8	Treatment + Day	2.51
3	5	449.2	Treatment + Day + Treatment*Day	4.96
4	2	461.4	Null	17.09
5	3	463.8	Day	19.50
Change in Polychromasia (N = 56)				
1	5	711.3	Treatment + Day + Treatment*Day	0
2	4	712.3	Treatment + Day	1.03
3	3	720.0	Day	8.75
4	3	729.9	Treatment	18.64
5	2	737.8	Null	26.45
Change in Body Mass (N = 56)				
1	2	188.5	Null	0
2	3	189.2	Treatment	0.69
3	3	190.0	Day	2.41
4	4	191.7	Day + Treatment	3.20
5	5	194.3	Treatment + Day + Treatment*Day	5.78

Table A1 continued				
Rank	**K[1]**	**AICc[2]**	**Effects[3]**	**ΔAICc[4]**
Change in Fat Score (N = 56)				
1	3	110.0	Day	0
2	2	110.6	Null	0.63
3	4	112.3	Treatment + Day	2.34
4	3	112.8	Treatment	2.86
5	5	113.6	Treatment + Day + Treatment*Day	3.59
Change in Reactive Oxygen Molecules (N = 52)				
1	3	491.2	Day	0
2	4	493.1	Treatment + Day	1.88
3	5	495.7	Treatment + Day + Treatment*Day	4.52
4	2	509.1	Null	17.84
5	3	511.0	Treatment	19.82
Change in Total Antioxidant Capacity (N = 52)				
1	3	404.0	Day	0
2	4	405.8	Treatment + Day	1.88
3	2	406.2	Null	2.26
4	4	408.1	Treatment	4.09
5	5	408.4	Treatment + Day + Treatment*Day	4.43
Change in Corticosterone (N = 56)				
1	2	201.2	Null	0
2	3	202.8	Treatment	1.67
3	3	203.5	Day	2.39
4	4	205.3	Treatment + Day	4.16
5	5	207.7	Treatment + Day + Treatment*Day	6.53
Change in Haptoglobin (N = 48)				
1	3	-7.0	Day	0
2	4	-4.4	Treatment + Day	2.60
3	5	-1.7	Treatment + Day + Treatment*Day	5.35
4	2	4.6	Null	11.66
5	3	7.1	Treatment	14.15

Table A1 continued				
Rank	**K[1]**	**AICc[2]**	**Effects[3]**	**ΔAICc[4]**
Change in Nitric Oxide (N = 34)				
1	1	372.0	Null	0
2	2	374.4	Day	2.35
3	2	374.7	Treatment	2.65
4	3	377.2	Treatment + Day	5.18
5	4	380.4	Treatment + Day + Treatment*Day	8.38
Change in Heterophils (N = 52)				
1	2	341.0	Null	0
2	3	342.2	Day	1.15
3	3	343.2	Treatment	2.16
4	4	344.4	Treatment + Day	3.36
5	5	347.0	Treatment + Day + Treatment*Day	5.95
Change in Lymphocytes (N = 52)				
1	2	418.4	Null	0
2	3	419.7	Day	1.33
3	3	420.4	Treatment	2.05
4	4	421.8	Treatment + Day	3.45
5	5	424.4	Treatment + Day + Treatment*Day	6.00
Change in Monocytes (N = 52)				
1	3	383.0	Treatment	0
2	2	383.5	Null	0.49
3	4	384.9	Treatment + Day	1.93
4	3	385.4	Day	2.43
5	5	387.5	Treatment + Day + Treatment*Day	4.55
Change in Eosinophils (N = 52)				
1	2	332.0	Null	0
2	3	334.4	Treatment	2.45
3	3	334.4	Day	2.45
4	4	337.0	Treatment + Day	5.01
5	5	338.9	Treatment + Day + Treatment*Day	6.92

Table A1 continued				
Rank	**K[1]**	**AICc[2]**	**Effects[3]**	**ΔAICc[4]**
Change in Basophils (N = 52)				
1	4	149.5	Treatment + Day	0
2	5	150.8	Treatment + Day + Treatment*Day	1.22
3	3	152.2	Day	2.63
4	3	153.2	Treatment	3.64
5	2	155.6	Null	6.11

[1]K indicates the number of parameters in the model.

[2]AICc is the second order Akaike's Information Criterion.

[3]Variable abbreviations of Effects: *Treatment* is the control or anti-malarial medication, *Day* is a continuous variable of the number of days since the start of the experiment, and * indicates an interaction between variables.

[4]ΔAICc is the difference in AICc from the best-fit candidate model.

Table A2. Parameter estimates from the top model or conditional model averaging of top (within 2 AICc) mixed linear regression models predicting change in physiological measures in response to anti-malarial treatment over time. Significant fixed effects are in bold.

Response Variable	Parameter	β[1]	SE[2]	*P*
Change in Hematocrit (N = 56)				
	Intercept	54.76	6.77	<0.0001
	Treatment	**2.88**	**0.62**	**<0.0001**
	Day	-0.17	0.09	0.06
	Initial Hematocrit	**-1.02**	**0.13**	**<0.0001**
Change in Hemoglobin (N = 56)				
	Intercept	150.95	16.04	<0.0001
	Treatment	**15.80**	**3.12**	**<0.0001**
	Initial Hemoglobin	**-0.89**	**0.09**	**<0.0001**
Change in Polychromasia (N = 56)				
	Intercept	569.50	83.17	<0.0001
	Treatment	-78.68	96.08	0.42
	Day	**-14.39**	**5.42**	**0.009**
	Treatment*Day	-12.76	6.46	0.054
	Initial Polychromasia	**-0.73**	**0.08**	**<0.0001**
Change in Mass (N = 56)				
	Intercept	9.91	4.47	0.03
	Treatment	0.52	0.39	0.19
	Initial Mass	**-0.16**	**0.07**	**0.02**
Change in Fat Score (N = 56)				
	Intercept	0.72	0.28	0.01
	Day	-0.04	0.02	0.08
	Initial Fat Score	**-0.50**	**0.11**	**<0.0001**
Change in Reactive Oxygen Molecules (N = 52)				
	Intercept	4.46	12.17	0.72
	Treatment	-5.69	6.85	0.42
	Day	**4.82**	**0.95**	**<0.0001**
	Initial ROMs	**-1.24**	**0.17**	**<0.0001**
Change in Total Antioxidant Capacity (N = 52)				
	Intercept	326.82	99.46	0.001
	Treatment	2.46	2.96	0.42

Table A2 continued				
	Day	**-0.93**	**0.42**	**0.03**
	Initial TAC	**-0.65**	**0.20**	**0.002**
Response Variable	**Parameter**	***β*[1]**	**SE[2]**	***P***
Change in Corticosterone (N = 56)				
	Intercept	0.29	0.25	0.27
	Treatment	-0.30	0.36	0.42
	Initial corticosterone	-0.53	0.37	0.16
Change in Haptoglobin (N = 48)				
	Intercept	0.02	0.11	0.89
	Day	**0.03**	**0.008**	**0.0002**
	Initial haptoglobin	**-1.01**	**0.21**	**0.0001**
Change in Nitric Oxide (N = 34)				
	Intercept	157.03	40.11	0.001
	Initial nitric oxide	**-0.90**	**0.25**	**0.002**
Change in Heterophils (N = 52)				
	Intercept	3.51	2.58	0.18
	Day	0.22	0.19	0.26
	Initial Heterophils	**-0.29**	**0.12**	**0.02**
Change in Lymphocytes (N = 52)				
	Intercept	44.38	10.21	<0.0001
	Treatment	-2.96	3.87	0.45
	Day	-0.34	0.36	0.35
	Initial lymphocytes	**-0.67**	**0.18**	**0.0002**
Change in Monocytes (N = 52)				
	Intercept	7.27	4.35	0.10
	Treatment	4.25	2.41	0.09
	Day	0.27	0.35	0.44
	Initial monocytes	**-0.61**	**0.15**	**<0.0001**
Change in Eosinophils (N = 52)				
	Intercept	2.95	1.31	0.03
	Initial eosinophils	**-0.67**	**-0.14**	**<0.0001**
Change in Basophils (N = 52)				
	Intercept	2.06	0.52	0.0001
	Treatment	-0.96	0.64	0.14
	Day	**-0.10**	**0.05**	**0.02**
	Treatment*Day	0.03	0.06	0.61
	Initial basophils	**-0.70**	**0.19**	**0.0004**

[1]β is the unstandardized regression coefficient.

[2]SE is the standard error for the coefficient.

Table A3. Parameter estimates from linear mixed models testing the relationship between physiological metrics and *Plasmodium* burden in birds that received the anti-malarial medication. Significant fixed effects are bolded.

Response Variable	Parameter	β[1]	SE[2]	*P*
Hematocrit (N = 45)				
	Intercept	55.92	0.54	<0.0001
	***Plasmodium* Burden**	**-1.03**	**0.24**	**0.0001**
Hemoglobin (N = 45)				
	Intercept	191.57	2.71	<0.0001
	***Plasmodium* Burden**	**-6.75**	**1.22**	**<0.0001**
Polychromasia (N = 45)				
	Intercept	390.15	36.09	<0.0001
	***Plasmodium* Burden**	**114.96**	**16.27**	**<0.0001**
Mass (N = 45)				
	Intercept	64.41	0.66	<0.0001
	Plasmodium Burden	0.18	0.11	0.11
Fat Score (N = 45)				
	Intercept	1.14	0.18	<0.0001
	Plasmodium Burden	0.01	0.06	0.85
Reactive Oxygen Molecules (N = 44)				
	Intercept	43.08	5.53	<0.0001
	Plasmodium Burden	-1.73	2.55	0.50
Total Antioxidant Capacity (N = 44)				
	Intercept	496.12	1.98	<0.0001
	Plasmodium Burden	0.44	0.91	0.63
Corticosterone (N = 45)				
	Intercept	0.25	0.11	0.03
	Plasmodium Burden	0.01	0.04	0.77
Haptoglobin (N = 39)				
	Intercept	0.35	0.05	<0.0001
	Plasmodium Burden	-0.004	0.02	0.88
Nitric Oxide (N = 35)				

Table A3 continued				
	Intercept	170.83	12.78	<0.0001
	Plasmodium Burden	-5.75	5.01	0.26
Heterophils (N = 43)				
	Intercept	14.17	1.96	<0.0001
	Plasmodium Burden	-0.48	0.47	0.32
Lymphocytes (N = 43)				
	Intercept	58.51	3.08	<0.0001
	Plasmodium Burden	-0.72	1.15	0.54
Monocytes (N = 43)				
	Intercept	20.89	2.27	<0.0001
	Plasmodium Burden	0.42	0.90	0.64
Eosinophils (N = 43)				
	Intercept	11.45	2.13	<0.0001
	Plasmodium Burden	0.72	0.50	0.15
Basophils (N = 43)				
	Intercept	0.37	0.10	0.0006
	Plasmodium Burden	-0.03	0.06	0.64

[1]β is the unstandardized regression coefficient.
[2]SE is the standard error for the coefficient.

References

Beadell, J.S. & Fleischer, R. (2005) A Restriction Enzyme – Based Assay to Distinguish Between Avian Hemosporidians. *Journal of Parasitology*, **91**, 683–685.

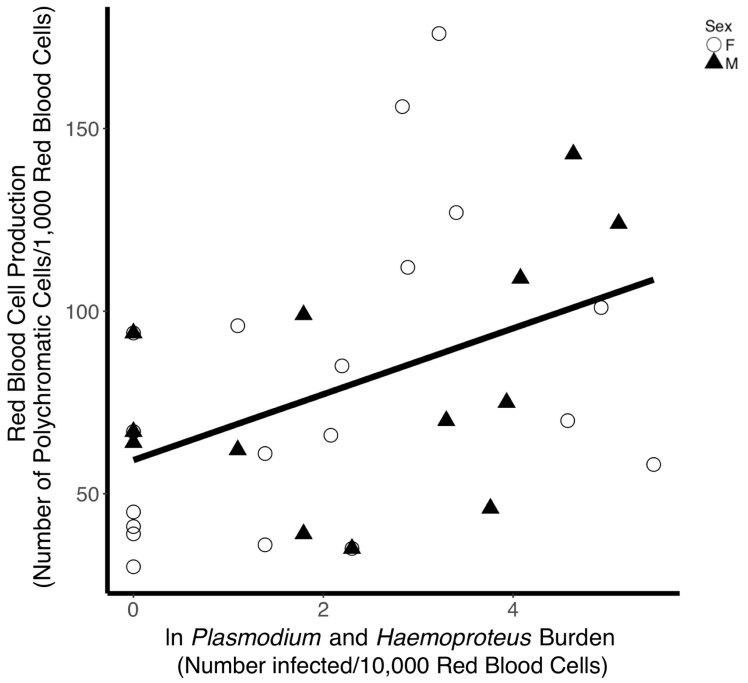
APPENDIX B

SUPPPLEMENTARY DATA, CHAPTER III

Table B1. The proportion of birds infected with each genus of Haemosporidian parasite, specifying single, double, or triple infection from 2013 - 2015. *Plasmodium*, *Haemoproteus*, and *Leucocytozoon* are abbreviated as *Plas*, *Haem*, and *Leuc*, respectively.

		Single Infections			Double Infections			Triple Infections	Total Infected
Year	Sex	*Plas*	*Haem*	*Leuc*	*Plas /Haem*	*Plas /Leuc*	*Haem/ Leuc*	*Plas/Haem/ Leuc*	
2013	All (N = 60)	27 (45%)	4 (6.7%)	2 (3.3%)	3 (5%)	13 (21.7%)	1 (1.7%)	7 (11.7%)	57 (95.0%)
	Female (N = 32)	18 (56.2%)	3 (9.4%)	0 (0%)	2 (6.3%)	3 (9.4%)	1 (3.1%)	3 (9.4%)	30 (93.8%)
	Male (N = 28)	9 (32.1%)	1 (3.6%)	2 (7.1%)	1 (3.6%)	10 (35.7%)	0 (0%)	4 (14.3%)	27 (96.4%)
2014	Male (N = 20)	3 (15.0%)	0 (0%)	0 (0%)	7 (35.0%)	6 (30%)	0 (0%)	4 (20%)	20 (100%)
2015	Male (N = 89)	24 (27.0%)	2 (2.2%)	0 (0%)	17 (19.1%)	40 (44.9%)	0 (0%)	5 (5.1%)	88 (98.9%)

Figure B1. Birds with higher *Plasmodium* and *Haemoproteus* burdens produce greater numbers of red blood cells (from a model with parasite burden as the sole predictor: $\beta = 9.02$, SE = 3.49, $P = 0.01$, $R^2 = 0.16$, $N = 32$).



APPENDIX C

SUPPLEMENTARY MATERIAL, CHAPTER IV, CORTICOSTERONE IMPLANT PILOT STUDY

Materials and Methods

Experimental Design

From April – May 2014, we captured 18 adult males on their territories with mist nets paired with playback or with V-top Troyer traps baited with seed and a taxidermy decoy. At capture, we took morphological measures as described in the main text and then collected 150 µL blood into heparinized capillary tubes, immediately created a blood smear, and saved the rest of the sample for use in a malaria PCR. Birds were then housed singly in outdoor flight aviaries (2.4 x 6.1 x 2.4 m) at Queen's University Biological Station and provided with food (poultry starter, mixed seed, corn, meal worms, and hard-boiled egg) and water *ad libitum*. Each bird had a minimum of one week to acclimate to the aviaries prior to the start of the experiment.

We assigned birds to one of four treatments: 1.5 mg corticosterone implant (N = 5), 0.5 mg corticosterone implant (N = 5), control implant (N = 4), and no surgery control (N = 4). Each implant was a 60-day slow release corticosterone pellet from Innovative Research of America (Sarasota, FL, USA). To ensure that malaria parasite burdens were approximately equally distributed across treatments, we used blood smears to identify parasite burden (see main text for details) and ranked the birds by burden. We determined the 4 birds with the highest parasite burden and randomly assigned each to one of 4 treatment groups. We then repeated this with each subsequent set of 4 birds ranked by parasite burden, such that initial infection burden was similarly distributed across treatment groups.

We staggered the start of the experiment so that we were sampling no more than 5 birds at a time. Within the group of birds sampled together (4 groups total), there was at least one individual from each treatment group. Blood samples were always collected at the same time, either 20:00 (3 groups) or 16:00 (1 group). We took a pre-treatment (day 0) blood sample (100 μL) and the following morning at 9:00, implanted the birds with the pellets subcutaneously (or handled the birds in the case of the no surgery control). We then took additional blood samples from each bird at the evening sampling time on days 1 (~8 hours post-treatment, 100 μL), 2 (~32 hours post-treatment, 100 μL), 7 (300 μL), 14 (300 μL), and 21 (300 μL). Each blood sample was used to create a blood smear, and the rest of the sample was kept on ice until processing.

In April - May 2015, we piloted an additional, low-dose corticosterone implant, 0.1 mg (Innovative Research of America, Sarasota, FL, USA) in 4 adult males. We followed the exact procedures described above, and all blood sampling took place at 16:00.

We used the blood samples to measure corticosterone and parasite burden. We assessed parasite burden as the number of infected cells in 10,000 red blood cells for pre-treatment samples and on days 2, 7, and 14. Corticosterone was measured at all sample points. Laboratory methods are described in the main text and Appendix D.

Statistical Analyses

We conducted all analyses in R version 3.3.2 (R Core Team 2016). We set alpha at 0.05 and for all regressions, report the unstandardized regression coefficient (β) relative to the no surgery control group. To improve model fit, we use the natural log of corticosterone. For all regression analyses, we fit the models with restricted maximum likelihood and initially summarized model results using Type III sums of squares. However, if there were no significant

interaction terms, we used Type II sums of squares, which are more statistically powerful in the absence of significant interactions (Hector *et al*. 2010; Smith & Cribbie 2014).

To test if the implants successfully increased corticosterone, we used a linear mixed effects model with the change in corticosterone from the pre-treatment as the response variable and as predictor variables, individual identity as a random effect, and treatment, day, the treatment-day interaction, and pre-treatment corticosterone as fixed effects. To identify if and when corticosterone manipulation influenced parasite burden, we used a linear mixed effects model with change in ln(parasite burden + 1) as the response variable, where parasite burden is the sum of all Haemosporidian parasites detected on a slide (methods in main text). As predictor variables, we used individual identity as a random effect and treatment, day, the treatment-day interaction, and pre-treatment ln(parasite burden + 1) as fixed effects.

Results

Treatment significantly influenced corticosterone concentrations ($F = 3.71$, $P = 0.03$, 99 observations of 20 birds). The 0.5 mg corticosterone implant caused the greatest increase in corticosterone ($t = 2.56$, $P = 0.02$, 99 observations of 20 birds) and parasite burden ($t = 1.88$, $P = 0.07$, 59 observations of 20 birds) relative to the no surgery control, and therefore we selected it as the high dose treatment for the larger study and the 0.1 mg implant as the low dose treatment (Appendix C, Figures C1 and C2, Tables C1 and C2). The control implant, no surgery control, and 0.1 mg implant did not affect corticosterone (Table C1, Figure C1) and corticosterone was similar for birds in the no surgery control and control implant treatments ($t = 0.58$, $P = 0.56$, 99 observations of 20 birds). The 1.5 mg corticosterone implant, the highest dose used in the pilot, increased circulating corticosterone to similar concentrations as the 0.5 mg implant 8 h after implantation (Appendix C, Figure C1), but did not significantly increase corticosterone

throughout the study ($t = 1.83$, $P = 0.08$, 99 observations of 20 birds). The change in corticosterone decreases with day of sampling ($F = 14.66$, $P = 0.0002$), indicating that the effects of the implant decrease over time, indicating a waning effect of the implants (Table C1, Figure C1). Although there was not a statistically significant treatment effect ($F = 1.51$, $P = 0.25$, 59 observations of 20 birds), birds receiving the 0.5 mg implants tended to increase in parasite burden, particularly 7 days post-treatment (Table C2, Figure C2). Therefore, we selected 0.5 mg as the high dose corticosterone treatment, 0.1 mg as the lose dose corticosterone treatment, and examined parasite burden 7 days post-treatment in the main study.

Figure C1. Variation in corticosterone over time from a pilot study testing the effectiveness of corticosterone implants. We collected a pre-treatment sample on day 0, gave birds their respective treatments on day 1, and collected blood samples at ~8 h (day 1), ~32 h (day 2), 7 days, 14 days, and 21 days. Points and error bars represent means and standard errors.

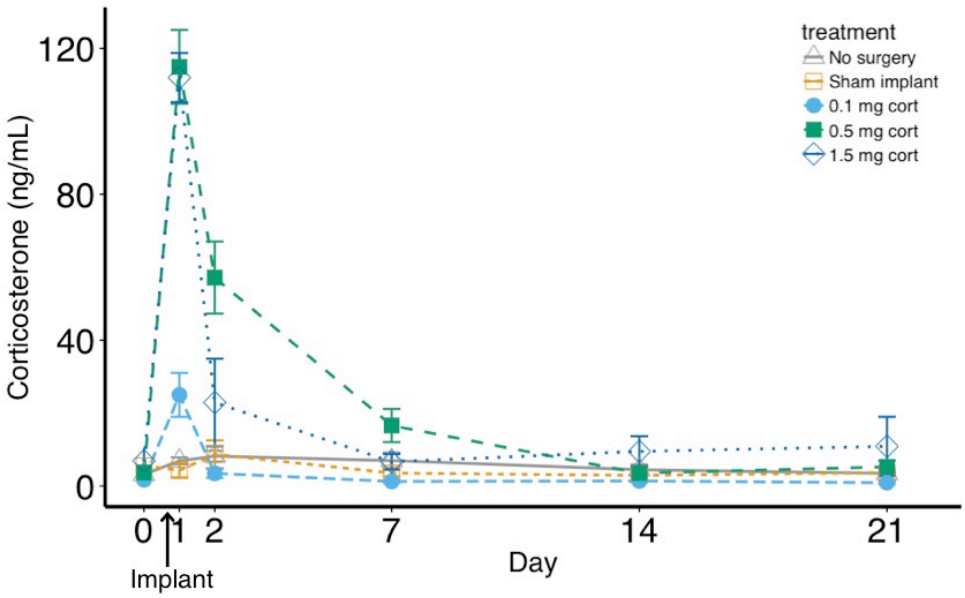


Figure C2**.** Variation in change in parasite burden over time in the corticosterone implant pilot study. Points and error bars represent means and standard errors.

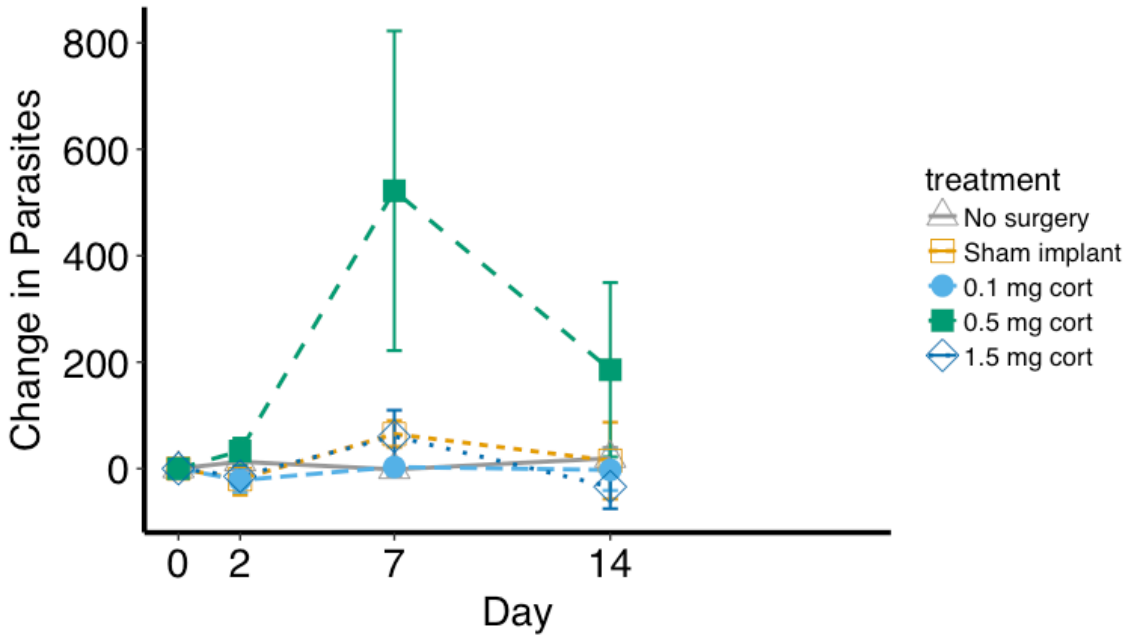


Table C1. Parameter estimates for change in corticosterone in response to a pilot corticosterone manipulation (99 observations of 20 birds). Significant fixed effects are in bold.

Parameter	β^1	**SE**[2]	**P value**
Pre-treatment CORT	**-0.73**	**0.21**	**0.003**
Treatment (relative to no surgery control)			
Control implant	0.34	0.59	0.56
0.1 mg CORT	-0.73	0.57	0.21
0.5 mg CORT	**1.46**	**0.57**	**0.02**
1.5 mg CORT	1.06	0.58	0.08
Day	**-0.09**	**0.03**	**0.002**
Treatment-Day interaction (relative to no surgery control–day)			
Control implant-Day	0.009	0.04	0.82
0.1 mg CORT-Day	0.04	0.04	0.34
0.5 mg CORT-Day	-0.04	0.04	0.34
1.5 mg CORT-Day	-0.03	0.04	0.51

β^1 is the unstandardized regression coefficient, SE[2] is the standard error for β.

Table C2. Parameter estimates for change in parasite burden in response to a pilot corticosterone manipulation (59 observations of 20 birds). Significant fixed effects are in bold.

Parameter	β^1	**SE**[2]	**P value**
Pre-treatment CORT	**-0.29**	**0.11**	**0.02**
Treatment (relative to no surgery control)			
Control implant	0.49	0.79	0.54
0.1 mg CORT	0.37	0.80	0.65
0.5 mg CORT	1.47	0.78	0.07
1.5 mg CORT	0.87	0.77	0.27
Day	0.07	0.05	0.12
Treatment-Day interaction (relative to no surgery control–day)			
Control implant-Day	-0.08	0.07	0.26
0.1 mg CORT-Day	-0.05	0.07	0.47
0.5 mg CORT-Day	-0.05	0.07	0.49
1.5 mg CORT-Day	-0.11	0.07	0.12

β^1 is the unstandardized regression coefficient, SE[2] is the standard error for β.

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APPENDIX D

SUPPLEMENTARY METHODS AND RESULTS, CHAPTER IV

Materials and Methods

Bird capture and husbandry

We captured adult male red-winged blackbirds from April – May 2015. We banded each bird with a unique combination of color bands and measured their tarsus (with calipers to the nearest 0.1 mm), wing cord (with a wing rule to the nearest 0.5 mm), and body mass (with a Pesola spring scale to the nearest 0.5 g). We assigned a fat score from Wingfield and Farner's (1978) 0-5 scale. We housed birds in groups of three in large outdoor flight aviaries (each 2.4 x 6.1 x 2.4 m, 30 aviaries in the complex). We provided the birds with *ad libitum* food (poultry starter, mixed seed, fruit, corn, egg, and mealworms) and water (in both elevated dishes and bird baths on the aviary floors).

Blood sampling and processing

All sampling was conducted between 9:00 – 16:30. To facilitate the sampling of multiple birds for baseline corticosterone, we sampled an entire aviary (three birds) at a time, including one bird from each treatment group. Care was taken that sequentially sampled aviaries were as far away from one another as possible to avoid additional disturbance. The sampling regime allowed for a nearly balanced design, such that a similar number of birds from each treatment group was sampled at each time point.

At each sampling event, we collected 500 μL of blood into heparinized capillary tubes after puncturing the brachial vein with a 26 gauge ½ inch needle. We aimed to collect at least 150 μL of blood within 3 minutes for measuring baseline corticosterone. Of the 353 blood

samples used to measure baseline corticosterone, only three samples were collected in over 3 minutes (mean +/- SE, 122 +/- 1.6 seconds, range: 59 – 257 seconds). We created two blood smears by smearing approximately 5 – 10 μL of blood on glass slides. We air-dried the slides and fixed them in absolute methanol within 1 h. Within 3 months, we stained the smears for one hour in Giemsa solution (stock solution #22050291, Fisher Scientific, Hampton, NH, USA) (Valkiūnas 2005). We stored the rest of the blood samples on ice until processing.

Within 1 h of sample collection, we centrifuged the remaining blood samples in capillary tubes at 6,000 RPM for 10 minutes. We immediately measured hematocrit for the first two capillary tubes collected with a microhematocrit card, using the average to estimate hematocrit. We then separated the plasma from the red blood cells with a Hamilton syringe and froze both at -20 °C in multiple aliquots to avoid freeze-thaw cycles when conducting multiple assays.

Blood smear analyses

For all blood smear analyses, the observer was blind to the treatment group of the samples.

Malaria Infection Intensity

A single observer (LAS) counted the number of parasite-infected cells per 10,000 red blood cells at high magnification (1000X, oil immersion) as recommended by Godfrey *et al*. (1987) and identified the infecting genera by parasite morphology (Valkiūnas 2005). We estimated the number of red blood cells by comparing the spread of red blood cells across each field of view to standardized photographs of known cell counts (Ricklefs & Sheldon 2007).

Red blood cell production (polychromasia)

Polychromasia is a measure of the degree of red blood cell production, estimated by counting the proportion of total red blood cells that are immature (Campbell 1995). Young or immature red blood cells are identifiable on slides because they tend to have less densely packed

chromatin in the nucleus and stain darker purple/blue (are polychromatic) because they have a higher affinity for stains like the Diff-Quick and Giemsa (Mitchell & Johns 2008). A single observer (A. Dudek) counted the number of polychromatic cells per 5,000 red blood cells.

Heterophil: lymphocyte ratio

Using high magnification (1000X) on a light microscope, a single observer (M. Mays) counted the number of each white blood cell type (monocytes, heterophils, lymphocytes, basophils, or eosinophils) using the criteria provided in Campbell (1995) and calculated the ratio of heterophils: lymphocytes.

Haemosporidian presence/absence

We extracted DNA from red blood cells with Qiagen DNeasy Blood and Tissue kits (Qiagen, Valencia, CA, USA). We screened for the presence of Haemosporidians and identified the genera of the parasites using a restriction enzyme-based assay designed by Beadell and Fleisher (2005). The assay begins with a PCR to amplify a fragment of mitochondrial DNA and uses restriction enzymes to digest the PCR product into fragments. The fragment size can be used to identify the presence of the genera *Plasmodium*, *Haemoproteus,* and *Leucocytozoon* as well as co-infection with these genera. In a 25 μL reaction (PCR reagents from New England BioLabs, Ispwich, MA), we combined 4.5 μL DNA and 0.5 U Taq with final concentrations of 0.2 mM dNTPs, 3 mM $MgCl_2$, 1X Taq buffer, 10X BSA, and 0.6 μM of 213F and 372R primers (Beadell & Fleischer 2005). PCR cycling conditions were identical to Beadell and Fleischer (2005). Then, we digested 10 μL of PCR product in a total volume of 20 μL at 37° C overnight (minimum 12 hours) using 1 U of each of the restriction enzymes XmnI and XbaI and 1X CutSmart Buffer (New England BioLabs, Ispwich, MA). We electrophoresed the digested PCR product on a 4% agarose gel and identified the presence of each haemosporidian genus based on

fragment size as described in Beadell and Fleischer (2005). To reduce the risk of false negatives, any samples that tested negative for Haemosporidians were re-extracted and re-tested by PCR three times.

Plasma Assays

Corticosterone

We quantified total corticosterone in plasma using two direct radioimmunoassays (Wingfield et al., 1992), with all samples from an individual within a single assay but individuals distributed randomly across assays. We extracted the hormones from plasma using a double extraction with 3 mL dichloromethane per extraction. We ran samples singly to maximize the likelihood of detection (20 – 60 μL, mean $\pm$ SE, 42.9 $\pm$ 0.62). Average extraction efficiencies were 78% and 82%. The average detection limits were ~0.55 and ~0.71 ng/mL. Thirty-six of 350 samples were below the detection limit and were assigned the lowest reliable value from the standard curve, and all samples were individually corrected for extraction efficiency. Within-assay variation in replicates of 6 known-concentration standards were 10.8% and 14.9%. Inter-assay variation was 14.8%.

PIT54 (avian analogue of haptoglobin)

We used a colorimetric assay kit (TP-801, Tri-delta Diagnostics Inc., Morris Plains, NJ, USA) to measure PIT54 and followed the manufacturer's instructions exactly. Samples were run singly on five 96-well plates and within assay variation in replicates of known-concentration standards (5 per plate) was 3.5%, 4.8%, 1.8%, 4.3%, and 3.9% and inter-assay variation was 8.6%.

Nitric oxide

We centrifuged plasma samples through a 10kD spin column (Corning Spin-X UF 500, #431478, Tewksbury, MA, USA) to remove proteins. Nitric oxide is oxidized into nitrate and nitrite in plasma, and by assessing combined nitrate and nitrate we can quantify plasma concentrations of nitric oxide. We used a colorimetric assay kit (ab65328, Abcam, Cambridge, MA, USA) to assess the combined nitrate and nitrite in plasma and followed the manufacturer's instructions exactly. Samples were run singly on five 96-well plates with all samples from a single individual on the same plate, and a replicate of a plasma pool on each plate. Inter-assay variation was 15.4%.

Reactive oxygen metabolites and total antioxidant capacity

We used the dROMs test (Diacron International, Grosseto, Italy) to measure reactive oxygen metabolites after optimizing the assay for red-winged blackbirds. We diluted 2 µL of plasma in 200 µL of reagent 1 and 2 µL of reagent 2 (provided in the Diacron kit). We incubated the plate for the next 30 minutes at 37 °C and measured the absorbencies of the samples at 505 nm on a plate reader (BioTek ELx800, VT, USA). Samples were run in duplicate across nine 96-well plates with all samples from a single individual on the same plate, and with two standard plasma pools per plate to calculate variation within and across assays. Average within-assay variation was 8.2% and inter-assay variation was 11.5%.

We used the OXY-Adsorbent test (Diacron International, Grosseto, Italy) to measure the total antioxidant capacity after optimizing the assay for red-winged blackbirds. We diluted 5 µL of plasma in 45 µL of distilled water, then combined 5 µL of the diluted plasma with 200 µL of the reagent 1 and 2 µL of reagent 2 (reagents provided in kit). We incubated the plate for 25 minutes at 37 °C, then measured the samples' absorbencies at 505 nm on a plate reader (BioTek ELx800, VT, USA). Samples were run in duplicate across nine 96-well plates with all samples

from a single individual on the same plate, and with two standard plasma pools per plate to calculate variation within and across assays. Average within-assay variation was 0.4% and inter-assay variation was 3.5%. We used the ratio of reactive oxygen metabolites: total antioxidant capacity as a measure of oxidative balance.

Results

Effect of glucocorticoids and co-infection on resistance

The effect of corticosterone treatment on the number of *Plasmodium* gametocytes depended on co-infection with *Haemoproteus* ($F = 4.55, P = 0.01, N = 82$) and *Leucocytozoon* ($F = 3.27, P = 0.04, N = 82$), but the three-way interaction between treatment and the presence/absence of both *Haemoproteus* and *Leucocytozoon* was not significant ($F = 0.92, P = 0.40, N = 82$). The change in gametocytes was similar among treatment groups in the presence of co-infection with either genus (all $P > 0.34$), but the high dose implants caused an increase in gametocytes relative to both the control and low dose implants in the absence of *Haemoproteus* (vs. control, $t = 4.00, P = 0.0005, N = 28$; vs. low, $t = 3.35, P = 0.004, N = 28$) and *Leucocytozoon* (vs. control, $t = 2.92, P = 0.01, N = 28$; vs. low, $t = 3.06, P = 0.009, N = 28$) (Figure D3). To assess how the change in *Plasmodium* gametocyte numbers varied with overall co-infection status in the high corticosterone treatment, we performed a post-hoc analysis of variance, adjusting *p*-values with Tukey's multiple comparisons. Change in gametocyte burden was associated with co-infection status within the high corticosterone group ($F = 4.15, P = 0.02, N = 28$). Individuals infected with *Plasmodium* alone experienced greater increases in *Plasmodium* burden than those with *Plasmodium-Haemoproteus* or *Plasmodium-Haemoproteus-Leucocytozoon* co-infections (Figure D4).

Figure D1. Corticosterone concentrations of birds in each treatment group throughout the experiment. The 0.5 mg corticosterone implant increased corticosterone (CORT) throughout the experiment (relative to the control implant, $t = 2.22$, $P = 0.03$, 257 observations of 88 birds). The points and bars represent means and standard errors.

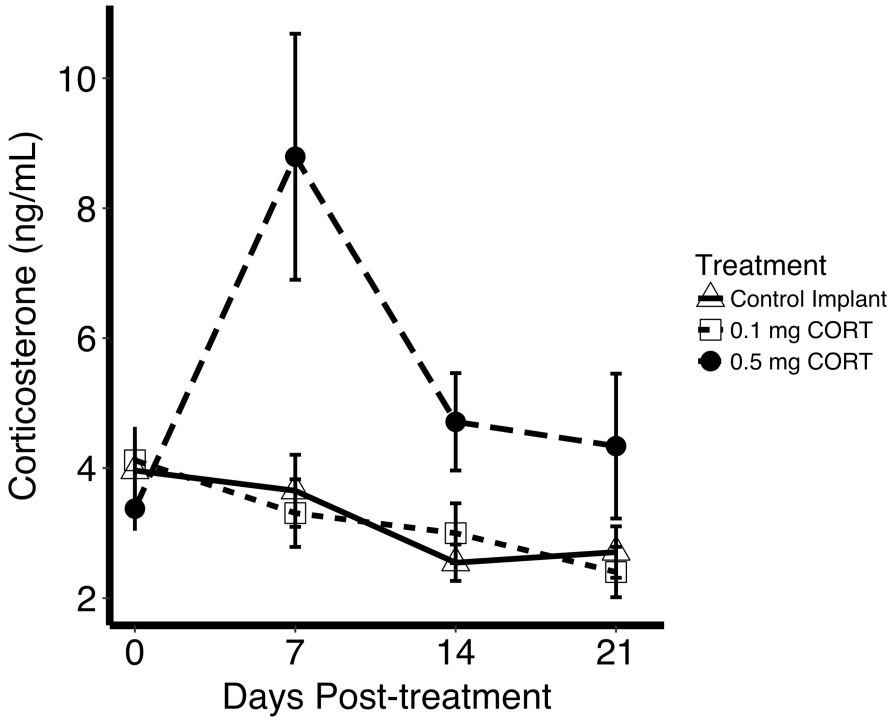

Figure D2. *Plasmodium* burden in birds of varying co-infection statuses before treatment. Birds co-infected with *Haemoproteus* had lower parasite burdens than those without *Haemoproteus* ($F = 54.9, P < 0.0001, N = 81$). Co-infection with *Leucocytozoon* had no effect ($F = 0.84, P = 0.36, N = 81$) and the interaction between *Haemoproteus* and *Leucocytozoon* was not significant ($F = 1.84, P = 0.18, N = 81$).

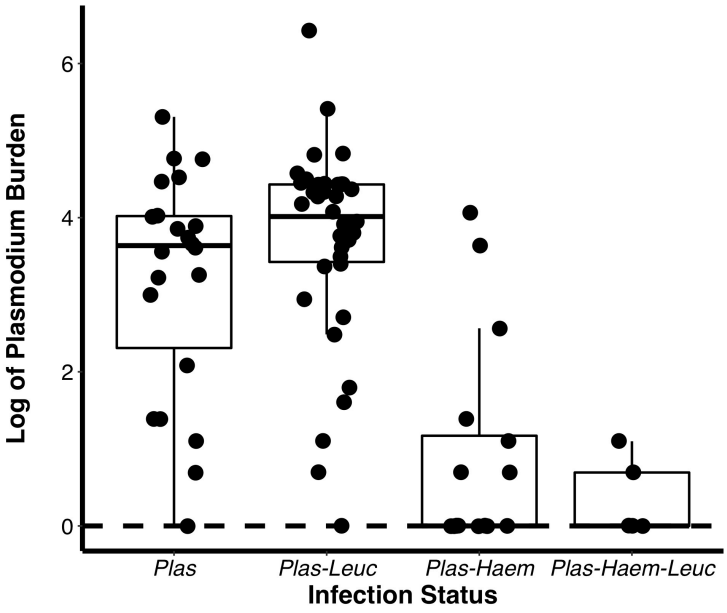

Figure D3. The effect of treatment on the change in number of *Plasmodium* gametocytes by co-infection status: (*A*) *Haemoproteus* negative, (*B*) *Haemoproteus* positive, (*C*) *Leucocytozoon* negative, (*D*) *Leucocytozoon* positive. Letters (a,b) represent significant differences ($P < 0.05$) among treatments within the same panel. Only in the absence of co-infection with *Haemoproteus* or *Leucocytozoon*, high dose corticosterone treatment increases the number of gametocytes.

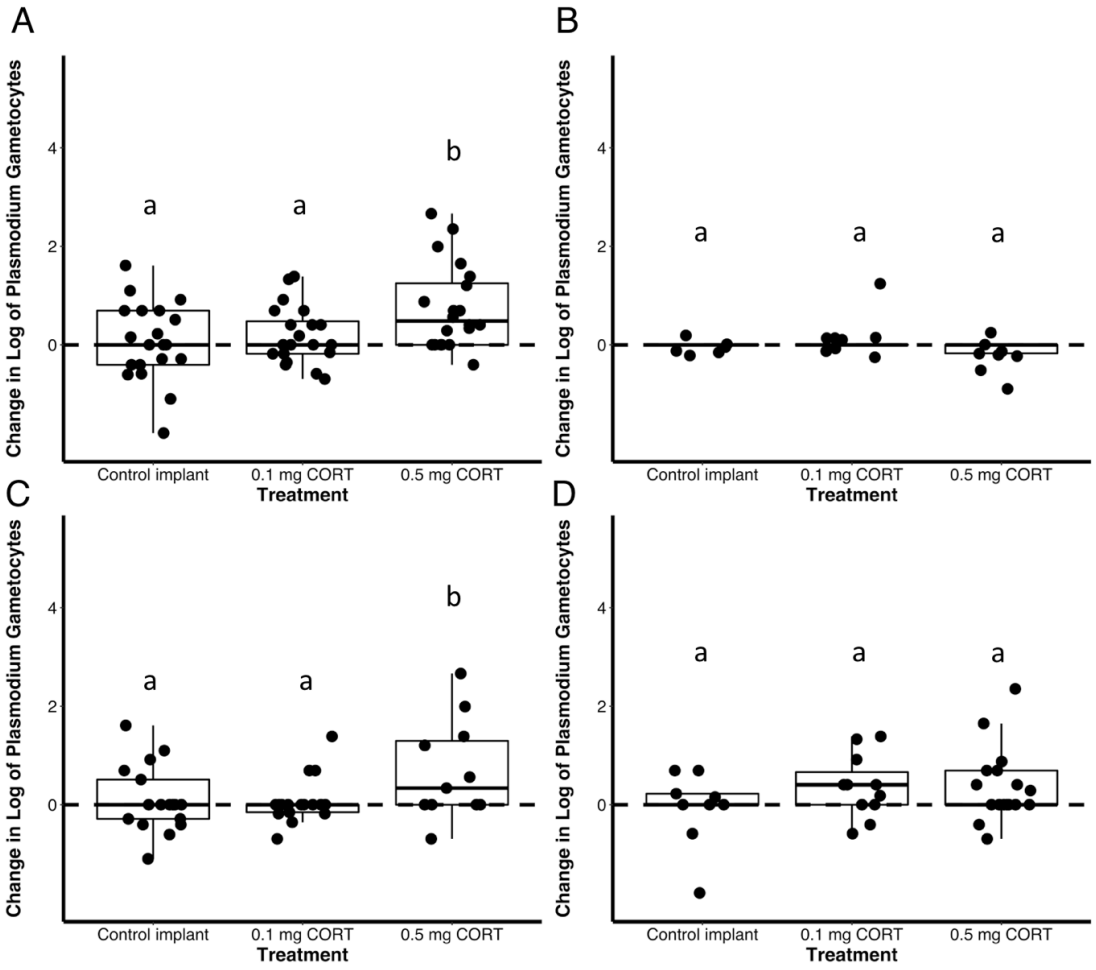

Figure D4. Change in *Plasmodium* gametocytes within the high corticosterone treatment. Birds infected with *Plasmodium* alone had greater increases in gametocytes than individuals that were co-infected with *Haemoproteus* and/or *Leucocytozoon*. Letters (a,b) represent significant differences ($P < 0.05$) among co-infection statuses.

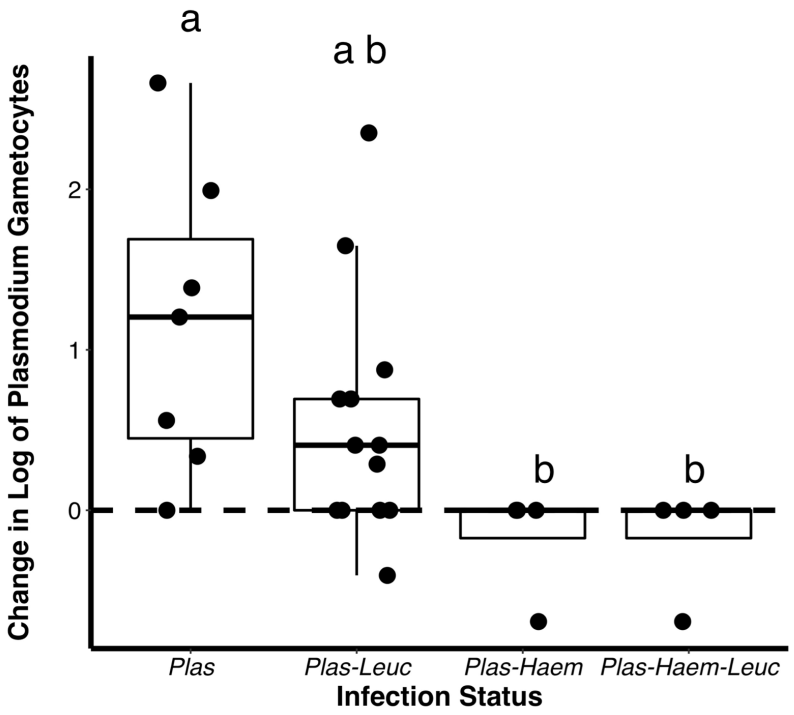

Table D1. The proportion of birds infected with each Haemosporidian genus pre-treatment, organized by treatment group and co-infection status (single, double, triple). *Plasmodium*, *Haemoproteus*, and *Leucocytozoon* are abbreviated as *Plas*, *Haem*, and *Leuc*, respectively.

Treatment	Single Infections			Double Infections			Triple Infections	Total Infected
	Plas	*Haem*	*Leuc*	*Plas/ Haem*	*Plas/ Leuc*	*Haem/ Leuc*	*Plas/Haem/ Leuc*	
Control (N = 29)	8 (27.6%)	0 (0%)	0 (0%)	6 (20.7%)	13 (44.8%)	0 (0%)	1 (3.5%)	28 (96.6%)
Low (N = 30)	8 (26.7%)	1 (3.3%)	0 (0%)	6 (20.0%)	13 (43.3%)	0 (0%)	1 (3.3%)	29 (96.7%)
High (N = 30)	7 (23.3%)	1 (3.3%)	0 (0%)	5 (16.7%)	14 (46.7%)	0 (0%)	3 (10.0%)	30 (100%)
All (N = 89)	23 (25.8%)	2 (2.2%)	0 (0%)	17 (19.1%)	40 (44.9%)	0 (0%)	5 (5.6%)	87 (97.8%)

Table D2. The top five candidate linear regression models and the null model testing the effect of corticosterone and infection status on tolerance seven days post treatment. Tolerance was assessed for four potential costs of infection: hematocrit, hemoglobin, mass, and oxidative balance.

Model Rank	K[1]	AICc[2]	Effects[3]	ΔAICc[4]
(A) Hematocrit (N = 80)				
1	4	453.5	Treatment + Burden + Genera	0
2	3	454.3	Treatment + Burden	0.81
3	5	455.9	Treatment + Burden + Genera + Treatment*Burden	2.38
4	5	456.6	Treatment + Burden + Genera + Burden*Genera	3.09
5	5	457.3	Treatment + Burden + Genera + Treatment*Genera	3.80
19	1	507.2	Null	53.65
(B) Hemoglobin (N = 80)				
1	2	719.2	Burden	0
2	3	720.0	Treatment + Burden	0.79
3	4	720.9	Treatment + Burden + Genera	1.66
4	3	720.9	Burden + Genera	1.73
5	5	724.0	Treatment + Burden + Genera + Burden*Genera	4.75
18	1	732.4	Null	13.16
(C) Mass (N = 80)				
1	4	447.4	Treatment + Burden + Treatment*Burden	0
2	5	450.4	Treatment + Burden + Genera + Treatment*Burden	3.04
3	6	452.9	Treatment + Burden + Genera + Treatment*Burden + Burden*Genera	5.48
4	6	455.6	Treatment + Burden + Genera + Treatment*Genera + Burden*Genera	8.24
5	5	455.9	Treatment + Burden + Genera + Burden*Genera	8.54
18	1	485.7	Null	38.34
(D) Oxidative Balance (N =78)				
1	1	-109.1	Null	0
2	2	-107.5	Burden	1.59
3	2	-107.2	Genera	1.90
4	3	-105.6	Treatment	3.50
5	3	-105.5	Burden + Genera	3.57

[1]K indicates the number of parameters in the model. [2]AICc is the second order Akaike's Information Criterion. [3]Variable abbreviations of Effects: *Treatment* is the control implant, low dose (0.1 mg) corticosterone implant, or the high dose (0.5 mg) corticosterone implant, *Burden* is the total number of Haemosporidian-infected cells in 10,000 red blood cells, *Genera* is the genus(era) infecting an individual (in this analysis, limited to *Plasmodium*, *Plasmodium-Haemoproteus*, and *Plasmodium-Leucocytozoon*, and * indicates an interaction between variables. [4]ΔAICc is the difference in AICc from the best fit candidate model.

Table D3. The top five candidate linear mixed models and the null model testing the effect of corticosterone on red blood cell production and immune metrics.

Model Rank	K[1]	AICc[2]	Effects[3]	ΔAICc[4]
(A) Change in red blood cell production (*N* = 240)				
1	5	3201.1	Initial + Day + Burden + Day*Burden	0
2	6	3201.4	Initial + Day + Burden + Treatment + Day*Burden	0.34
3	8	3202.2	Initial + Day + Burden + Treatment + Day*Burden + Burden*Treatment	1.17
4	7	3204.5	Initial + Day + Burden + Treatment + Day*Burden + Day*Treatment	3.43
5	8	3205.4	Initial + Day + Burden + Treatment + Day*Burden + Day*Treatment + Burden*Treatment	4.34
38	1	3240.1	Null	39.00
(B) Change in haptoglobin (*N* = 237)				
1	3	-26.2	Initial + Day	0
2	4	-24.2	Initial + Day + Burden	1.98
3	4	-23.2	Initial + Day + Treatment	3.03
4	5	-22.3	Initial + Day + Burden + Day*Burden	3.87
5	5	-21.1	Initial + Day + Burden + Treatment	5.07
30	1	87.1	Null	113.30
(C) Change in nitric oxide (*N* = 139)				
1	2	1929.8	Initial	0
2	3	1931.3	Initial + Day	1.49
3	3	1931.7	Initial + Burden	1.89
4	4	1933.2	Initial + Day + Burden	3.39
5	3	1933.9	Initial + Treatment	4.04
20	1	1980.5	Null	50.65

[1]K indicates the number of parameters in the model. [2]AICc is the second order Akaike's Information Criterion. [3]Variable abbreviations of Effects: *Treatment* is the control implant, low dose (0.1 mg) corticosterone implant, or the high dose (0.5 mg) corticosterone implant, *Burden* is the total number of Haemosporidian infected cells in 10,000 red blood cells, *Day* is the number

of days post-treatment, *Initial* is the pre-treatment value of the response variable, and * indicates an interaction between variables. [4]ΔAICc is the difference in AICc from the best fit candidate model.

Table D4. The top five candidate linear mixed models and the null model testing the effect of coinfection and corticosterone on red blood cell production and immune metrics.

Model Rank	K[1]	AICc[2]	Effects[3]	ΔAICc[4]
(A) Change in red blood cell production (*N* = 210)				
1	6	2803.3	Initial + Day + Burden + Genera + Day*Burden	0
2	7	2804.7	Initial + Day + Burden + Genera + Treatment + Day*Burden	1.45
3	7	2806.1	Initial + Day + Burden + Genera + Day*Burden + Burden*Genera	2.82
4	7	2806.6	Initial + Day + Burden + Genera + Day*Burden + Day*Genera	3.34
5	7	2807.4	Initial + Day + Burden + Genera + Treatment + Day*Burden + Burden*Genera	4.07
	1	2855.2	Null	51.9
(B) Change in haptoglobin (*N* = 210)				
1	3	-32.2	Initial + Day	0
2	4	-30.3	Initial + Day + Burden	1.96
3	5	-28.8	Initial + Day + Burden + Day*Burden	3.45
4	4	-28.5	Initial + Day + Treatment	3.73
5	4	-28.1	Initial + Day + Genera	4.12
	1	67	Null	99.27
(C) Change in nitric oxide (*N* = 123)				
1	2	1713.4	Initial	0
2	3	1715.1	Initial + Day	1.70
3	3	1715.2	Initial + Burden	1.79
4	4	1716.9	Initial + Day + Burden	3.52
5	3	1717.0	Initial + Genera	3.63
18	1	1761.1	Null	38.34

[1]K indicates the number of parameters in the model. [2]AICc is the second order Akaike's Information Criterion. [3]Variable abbreviations of Effects: *Treatment* is the control implant, low dose (0.1 mg) corticosterone implant, or the high dose (0.5 mg) corticosterone implant, *Genera* is

the genus(era) infecting an individual (in this analysis, limited to *Plasmodium*, *Plasmodium-Haemoproteus*, and *Plasmodium-Leucocytozoon*, *Burden* is the total number of Haemosporidian infected cells in 10,000 red blood cells, *Day* is the number of days post-treatment, *Initial* is the pre-treatment value of the response variable, and * indicates an interaction between variables. [4]ΔAICc is the difference in AICc from the best fit candidate model.

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