The Role of Corticosterone in Stress-induced Suppression of Innate Immunity in the Male House Sparrow

by

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A Dissertation Presented in Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy

Approved April 2017 by the
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ARIZONA STATE UNIVERSITY

May 2017

ABSTRACT

In wild birds, the stress response can inhibit the activity of the innate immune system, which serves as the first line of defense against pathogens. By elucidating the mechanisms which regulate the interaction between stress and innate immunity, researchers may be able to predict when birds experience increased susceptibility to infections and can target specific mediators to mitigate stress-induced suppression of innate immune activity. Such elucidation is especially important for urban birds, such as the House Sparrow (*Passer domesticus*), because these birds experience higher pathogen prevalence and transmission when compared to birds in rural regions. I investigated the role of corticosterone (CORT) in stress-induced suppression of two measures of innate immune activity (complement- and natural antibody-mediated activity) in male House Sparrows. Corticosterone, the primary avian glucocorticoid, is elevated during the stress response and high levels of this hormone induce effects through the activation of cytosolic and membrane-bound glucocorticoid receptors (GR). My results demonstrate that CORT is necessary and sufficient for stress-induced suppression of complement-mediated activity, and that this relationship is consistent between years. Corticosterone, however, does not inhibit complement-mediated activity through cytosolic GR, and additional research is needed to confirm the involvement of membrane-bound GR. The role of CORT in stress-induced inhibition of natural antibody-mediated activity, however, remains puzzling. Stress-induced elevation of CORT can suppress natural antibody-mediated activity through the activation of cytosolic GR, but the necessity of this mechanism varies inter-annually. In other words, both CORT-dependent and CORT-

independent mechanisms may inhibit natural antibody-mediated activity during stress in certain years, but the causes of this inter-annual variation are not known. Previous studies have indicated that changes in the pathogen environment or food availability can alter regulation of innate immunity, but further research is needed to test these hypotheses. Overall, my dissertation demonstrates that stress inhibits innate immunity through several mechanisms, but environmental pressures may influence this inhibitory relationship.

ACKNOWLEDGEMENTS

Since I started this dissertation, I have learned how to be comfortable with not knowing things. In fact, the more I learn, the more I feel like I know nothing. Pierre Deviche has taught me how to navigate these uncertain territories beyond our current body of knowledge through his persistence, his positive skepticism, and his open and effusive enthusiasm for everything (especially dragonflies).

I could not have accomplished anything in this dissertation without the support of the most wonderful community. I made my choice of graduate school solely based on the school's community and I have never regretted my decision. Thank you, Dale, Mike, Miles, and Kevin, for your guidance, wisdom, and endless patience. Thank you, Karen, Mike, and Susannah for your help in carrying out all of my methods. Thank you, James and your family, for allowing me to catch on your property even though the House Sparrows would just return after a few weeks. Thank you, Scott and Karla, for guiding and patiently mentoring me, especially when I asked vague questions like, "How do dissertations work???" Thank you, Clarissa, Sam, and Pierce, for your help (especially during weird field experiences) and friendship. Thank you, Shelley and Stephanie, for being there to bounce ideas off of. Thank you, Melinda, Travis, Josh, Jason, David, Alex, and Nicole, for always being supportive and making me laugh, especially during times when I was a whiny, hungry little diva.

Most importantly, thank you, Hwei, Constance, and Sherry, for showing me that life is not linear and that a biologist can wear many faces.

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CHAPTER ONE

Introduction

The immune system defends the body against elements which do not belong in the body. In other words, it is able to discern "self" from everything that is "non-self," and can then contain and eliminate "non-self" elements. The most immediate line of defense against these foreign elements is the innate branch of the immune system. It consists of the skin, mucus, a variety of antimicrobial peptides, chemicals such as cytokine and chemokines, natural antibodies, the complement system, professional antigen presenting cells (*e.g.* macrophages, dendritic cells), and natural killer-T cells. The objectives of the innate immune system are to 1) quickly contain and fight the early stages of infections, 2) to alert and activate the adaptive branch to the presence of the infection, and 3) to work with the adaptive branch in order to eliminate the infection.

In this dissertation, I investigate the activity of natural antibodies and the complement system. Natural antibodies bind to a broad range of antigens and can force foreign cells to agglutinate. Through this method, natural antibodies contain early infections and tag foreign cells for removal via opsonization by phagocytes or via lysis by the complement system. The complement system consists of a variety of proteins and their regulators. To lyse foreign cells, complement proteins can work with and without the help of natural antibodies through three different pathways (*i.e.* the natural antibody-dependent classical pathway, the natural antibody-independent alternative pathway, and the natural antibody-independent lectin pathway) in order to form membrane attack complexes that latch onto

and puncture holes in the foreign cell's plasma membrane. In these chapters, I use a hemolysis-hemagglutination assay to assess complement-mediated and natural antibody-mediated activity, respectively. I also assess complement-mediated activity with a bacterial killing assay against *Escherichia coli*.

Because the innate immune system is the first line of defense against pathogens, any inhibition of innate immune activity may increase susceptibility to infections. The stress response, which is characterized and coordinated by a transient elevation of plasma catecholamines and glucocorticoids, is known to alter innate immunity in many wild avian species (Matson et al., 2006; Merrill et al., 2012; Zylberberg, 2015; Davies et al., 2016). In birds, the primary glucocorticoid is corticosterone (CORT). At low non-stressed levels, plasma CORT exerts effects through the activation of cytosolic mineralocorticoid receptors while at high stressed levels, it exerts effects through the activation of cytosolic glucocorticoid receptors and/or through membrane-bound glucocorticoid receptors. Many studies have implicated plasma CORT to be the primary mediator between stress and immune function in wild birds (Lindström et al., 2005; Matson et al., 2006; Cyr et al., 2007; Buehler et al., 2008; Zylberberg, 2015). However, none have demonstrated that the elevation of this hormone during the stress response is necessary or sufficient for stress-induced changes in innate immunity and none have clarified the receptors which are involved. Elucidation of the role of plasma CORT during stress-induced changes in innate immunity may aid in predicting when innate immune activity will change under other circumstances which are also known to elevate plasma CORT (Romero, 2002; Koolhaas et al., 2011). Furthermore, understanding the mechanisms which regulate innate

immunity may identify important mediators to target for conservation, agricultural, and veterinary sciences—all areas which may need to develop treatments to reduce an animal's susceptibility to infection under stressful circumstances.

Understanding how innate immune activity changes during the stress response and the mechanisms which underlie this interaction are especially important for urban birds, which are exposed to a variety of anthropogenic stressors (Chace and Walsh, 2006) and demonstrate increased prevalence, abundance, and transmission of pathogens when compared to rural counterparts (Bradley et al., 2007; Giraudeau et al., 2014; Gil and Brumm, 2013; Galbraith et al., 2016). Increased exposure to pathogens may be due to reduced biodiversity (Schmidt and Ostfeld, 2001), reduced seasonality (Bradley et al., 2007), and/or the increased presence of water (Fokidis et al. 2008). Reliable, anthropogenic food sources like bird feeders may increase pathogen transmission directly by increasing the density and frequency of aggregating birds (Dhondt et al., 2005), and indirectly by supplementing sickly birds that would have otherwise perished (Gil and Brumm, 2013). Therefore, when investigating the mechanisms underlying stress-induced changes in innate immunity, I have used the House Sparrow, *Passer domesticus*, as my model. Furthermore, I have only used males in reproductive condition to control for any effects of sex and life history stage.

In order to elucidate the role of CORT on innate immunity during stress in male House Sparrows, I first characterized the interaction between stress and innate immunity (Chapter Two) by testing three assumptions: 1) stress-induced changes in immune activity remain the same throughout the duration of the stress response, 2) the stress

response influences all measurements of innate immunity in a similar manner, and 3) the transient elevation of plasma CORT is necessary for stress-induced changes in innate immune activity. To accomplish these assessments, I used two different durations of experimental stress such that effects observed within the shorter duration (10 mins) could only be explained by non-genomic effects (Haller et al., 2008), and evaluated stress-induced effects on both natural antibody- and complement-mediated activity. I also used mitotane treatment to perform pharmacological adrenalectomy in order to temporarily inhibit endogenous corticosterone production. The results of this experiment suggested that plasma CORT may act on immune activity through the activation of the cytosolic glucocorticoid receptor. To test this hypothesis, I utilized the glucocorticoid receptor antagonist, RU486, to investigate the role of the glucocorticoid receptor during stress-induced changes in natural antibody- and complement-mediated activity (Chapter Three). Finally, I investigated the sufficiency of CORT during stress-induced suppression of natural antibody- and complement-mediated immunity (Chapter Four). Additionally, I was able to assess the consistency of the relationship between stress and innate immune activity because I used similar experimental designs and captured breeding male House Sparrows from the same location in Phoenix, AZ for all experiments.

CHAPTER TWO

Corticosterone rapidly suppresses innate immune activity in the House Sparrow (*Passer domesticus*)

(Published in *Journal of Experimental Biology* 2016)

Abstract

Stress-induced effects on innate immune activity in wild birds have been difficult to predict. These difficulties may arise from the frequent assumptions that (a) the stress response influences different components of the immune response similarly, (b) stress-induced effects do not change over the course of the stress response, and (c) glucocorticoids are the primary regulators of stress-induced changes of immune activity. We tested the first two assumptions by measuring three components of innate immunity at two times during the stress response in captive adult male House Sparrows, *Passer domesticus*. Acute stress resulting from handling and restraint suppressed plasma lytic and microbicidal activity within 10 mins and reduced plasma agglutination ability within 120 mins. We tested the third assumption by measuring stress-induced effects in sparrows that were pharmacologically adrenalectomized by mitotane administration. Confirming the effectiveness of this treatment, mitotane-treated birds had lower pre-stress plasma CORT than control birds and showed no increase in plasma CORT during acute stress. The innate immune activity of mitotane-treated birds did not decrease during the stress response, but the pre-stress immune activity of these birds did not differ from that of vehicle-treated birds. These results suggest that elevated plasma CORT during

stress is primarily responsible for mediating stress-induced suppression of innate immune activity.

Introduction

In vertebrates, the stress response adjusts physiology and behavior (e.g., inhibit reproduction and mobilize glucose) to improve chances of surviving a stressor. Stress-related adjustments are orchestrated by a transient increase in catecholamine and glucocorticoid secretion (Sapolsky, 2000) and include a change in activity of the innate immune system, which serves as the most immediate line of defense against invading pathogens (Martin et al., 2008; Hasselquist and Nilsson, 2012). Stress-induced effects on innate immune activity have been investigated in free-living animals, but the direction of these effects remains difficult to predict. Stress-induced immunosuppression may occur because the animal lacks the resources that are necessary to sustain the activation and maintenance of multiple physiological systems (Martin et al., 2008; Moore and Hopkins, 2009; Nebel et al., 2012; Evans et al., 2015). Alternatively, animals may benefit from stress-induced immunoenhancement because exposure to stressors (e.g., predators and infectious agents) may increase the probability of injuries and infections (Dhabar, 2009; Martin, 2009). Finally, stress-induced effects on immune activity may change as a function of the duration of exposure to the stressor, such that immunoenhancement occurs initially but immunosuppression occurs during a prolonged stress response (Martin, 2009).

Empirical evidence of stress-induced effects on the immune system has been equally conflicting. Many studies on this topic have used free-ranging birds as models because the diversity of habitats used by birds and of life history characteristics among species may help identify broad patterns of stress-induced effects on the immune response (Hasselquist, 2007). Several avian species exhibit stress-induced immunosuppression such as reduced microbicidal (Matson et al., 2006; Merrill et al., 2012) and lysozyme activity (Zylberberg, 2015), decreased natural antibody and complement-mediated activity (Davies et al., 2016), and reduced cutaneous immune activity (Martin et al., 2005; Cyr et al., 2007). Stress, however, has also been observed to enhance acute phase protein activity in the Galápagos Flycatcher (*Myiarchus magnirostris*; Zylberberg, 2015) and phagocytic activity in Clay-colored Thrushes (*Turdus grayi*; Millet et al., 2007). These apparently inconsistent findings may result from most studies using one measure of immunity and/or sampling at one point during the stress response. In Red Knots (*Calidris canutus*), for example, the activation of the stress response enhances phagocytic ability and reduces plasma leukocyte concentration at different times during the stress response (Buehler et al., 2008). In such cases, stress-induced effects could not be properly characterized based on one measure of immunity and/or sampling at one time during the stress response.

Elevated plasma glucocorticoids inhibit immune activity in laboratory rodents and in clinical studies (Franchimont, 2004). These effects are mediated by the activation of glucocorticoid receptors (GR) and may involve both genomic and non-genomic mechanisms (Stahn and Buttgereit, 2008). In free-ranging birds, by contrast, it is unclear

whether corticosterone (CORT), the primary avian glucocorticoid (Schmidt et al., 2010), plays a role in the regulation of stress-induced effects on immunity and whether plasma CORT can directly alter immune activity (Davies et al., 2016). Most studies on this subject have been correlative and do not, therefore, establish a causal relationship between changes in plasma CORT and immune activity (Lindström et al., 2005; Matson et al., 2006; Cyr et al., 2007; Buehler et al., 2008; Zylberberg, 2015). Furthermore, demonstrating that elevated plasma glucocorticoids are the primary regulators of stress-induced changes in immune activity is complicated by the fact that the environment can also influence the development and the function of the immune system (Hasselquist and Nilsson, 2009). In addition, plasma catecholamines, which rapidly increase during stress, may also reduce immune activity (Brown-borg et al., 1991; Denno et al., 1994; Sapolsky, 2000; Martin, 2009). Most studies have used CORT administration or supplementation in food or drinking water to try and establish a causal relationship between this hormone and immune activity in free-living birds. This approach can, however, result in supraphysiological (Loiseau et al., 2008) or chronically elevated plasma glucocorticoids (Martin et al., 2005; Bourgeon and Raclot, 2006) and so does not necessarily demonstrate a role for transiently elevated plasma glucocorticoids as naturally experienced during acute stress.

Here we tested the hypothesis that activation of the stress response changes innate immune activity and that naturally elevated plasma CORT is the primary mediator of this change. We tested this hypothesis using male House Sparrows (*Passer domesticus*), a species whose stress response and immune systems are well characterized (Rich and

Romero, 2001; Martin et al., 2004; Martin et al., 2006; Kuhlman and Martin, 2010). We assessed the innate immune system by measuring the activity of natural antibodies and complement proteins, which regulate the ability to, respectively, recognize and agglutinate foreign antigens and kill foreign cells (Matson et al., 2005; French and Neuman-Lee, 2012). We measured stress-induced effects on innate immune activity at 10 minutes and at 120 minutes after the initiation of the stress response for two reasons. First, we aimed to discern whether these effects are controlled by genomic and/or non-genomic mechanisms based on the assumption that any change in immune activity within 10 minutes of the stress response initiation results from non-genomic mechanisms (Haller et al., 2008). However, it was difficult to predict if immune activity would be enhanced or suppressed as rapidly as 10 minutes after the activation of the stress response. Second, we expected that a prolonged stress response will suppress immune activity so as to limit the damage of the innate immune system on the body's tissues. To determine whether naturally elevated plasma CORT is primarily responsible for acute stress-induced changes in immune activity, we measured this activity in pharmacologically adrenalectomized birds (Breuner et al., 2000). If elevated plasma CORT plays an essential role in mediating stress-induced effects on immunity, we predicted that pharmacologically adrenalectomized birds will exhibit no change in immune activity during stress.

Methods

Study Animals

We captured 20 adult male House Sparrows with mist-nets and baited ground traps in late February 2015 in Phoenix, AZ (33.4° N, 111.6° W; 331 m a.s.l.). All birds had black beaks and thus, were in reproductive condition (Barfuss and Ellis, 1971). Birds were transported to Arizona State University's Animal Care Facilities, where they were randomly divided into two groups of 10 birds and housed in identical rooms (N=10 per room) under a long-day (13L:11D) photostimulatory photoperiod (Barfuss and Ellis, 1971). Birds were housed individually and were visually isolated from each other, and received ad libitum Mazuri Pellet Diet (PMI Nutrition International, Richmond, IN, USA) and water. All procedures were approved by the Arizona State University’s Institutional Animal Care and Use Committee and were conducted under Arizona Game and Fish Department scientific collecting permit SP719136.

Stress trials

We investigated 1) the stress-induced effects on innate immune activity at two times during the stress response and 2) whether pharmacological inhibition of CORT production influences these effects.

The experiment consisted in two identical stress trials such that, by the end of the experiment, each bird had experienced experimental stress twice, had received one mitotane and one control (vehicle) injection, and had yielded four blood samples (Fig. 2.1). For both trials, birds in one group (N=10; 10-min group) were exposed to experimental stress for 10 min and birds in the second group (N=10; 120-min group)

were exposed to experimental stress for 120 min. During the first trial, five birds in the 10-min group and five birds in the 120-min group (N=5) received mitotane treatment, and the other birds (five in each group) received the control (vehicle) treatment. During the second trial, which began 10 days after completion of the first stress trial, mitotane and control treatments were reversed. One bird was lost from each group over the course of the experiment.

All trials began at the same time of day (12 pm) to account for a potential daily rhythm of baseline (BL) plasma CORT (Rich and Romero, 2001). Each bird received one injection (mitotane solution or vehicle) two days prior to the beginning of the stress trial because mitotane administration to House Sparrows decreases plasma CORT within 36 hours of an injection (Breuner et al., 2000). On the day of the stress trial, we removed birds from their home cage, collected a baseline (BL) blood sample (220 µl) and induced a stress response by placing the bird in a breathable cloth bag and then placing the bag into the bird's cage. At the end of the restraint period, a second (stress-induced) blood sample was collected (220 µl). The volume of blood samples was determined by estimating the minimal volume that was necessary to measure plasma CORT and immune parameters (see below). All blood samples were obtained from the jugular vein using a heparinized microsyringe, and collected within 3 minutes of removing a bird from its cage and within 5 minutes of entering the room. Samples were immediately placed on ice and were centrifuged within hours of collection. Plasma was then separated and stored at -80oC until assayed.

Mitotane preparation and treatment

Mitotane (#25925, Sigma Aldrich, St. Louis, MO) was dissolved in peanut oil (90 mg/ml). This solution was stored at 4o C for up to five days before use and administered into the left pectoral muscle (100 µl, equal to 9 mg/bird per injection). Control injections consisted of 100 µl of peanut oil.

A mitotane injection at the same dose as used here to adult male House Sparrows reduced plasma CORT for up to 10 days (Breuner et al., 2000) and mitotane treatment inhibits CORT production in mammals (Chortis et al., 2013) and birds (Jonsson et al., 1994; DuRant et al., 2016). To counter potential problems of glucose mobilization resulting from low plasma CORT and to standardize the feeding regimen, we supplemented all birds, regardless of treatment, with fresh Nektar solution (13 g/100 ml 0.9% NaCl in water; Nekton, Germany) daily during the 10 days following injections of mitotane or vehicle.

Plasma corticosterone assay

We measured total plasma CORT with a validated commercial competitive enzyme-linked immunoassay according to the manufacturer's instructions (Enzo Life Sciences, Farmingdale, NY; Fokidis et al., 2009). House Sparrow plasma was diluted 15x in assay buffer containing steroid displacement reagent to dissociate the hormone from plasma binding proteins. Each assay plate included a standard curve and samples were assayed in duplicate. Samples were randomly assigned to assay plates but all four samples from the same individual were assayed on the same plate. The assay sensitivity was 4.90 pg/ml.

The average intra- and interassay coefficients of variation were 4.9% and 13.2%, respectively (N=2 plates).

Hemolysis–hemagglutination assay

We measured the activity of natural antibodies and complement following Matson et al.'s (2005) method. Samples were randomly assigned to 96-well plates, but all four samples from the same individual were assayed on the same plate. Plasma (40 µl) was added to the first column of each plate and then serially diluted in 0.9% phosphate buffered solution (PBS) until the 11th column. The 12th column contained only PBS and served as a negative control. We then added 20 µl of 0.5% whole sheep blood (#SB050, Hemostat Laboratories, Dixon, CA) to each well, covered the plates, and sealed them with Parafilm.

All plates were incubated at 37 °C, the incubation temperature that is effective for hemolysis–hemagglutination assay using House Sparrow plasma (Martin et al., 2006), for 90 min. Plates were then moved to room temperature and tilted at a 450 angle for 20 min. Plates were then scanned for agglutination at 600 dots per inch with a flat-bed scanner (ScanJet 3670; Hewlett-Packard Co, Palo Alto, CA). After scanning, plates were placed flat at room temperature for 70 min and then scanned again for lysis. Each row of wells was scored for agglutination and lysis by an individual without knowledge of the experimental treatment. High scores reflected high agglutination and lytic activity. All wells were scored in one session to maximize consistency.

Bacterial killing assays

To determine the microbicidal activity of plasma, we used French and Neuman-Lee's (2012) *ex vivo* bacterial killing assay (BKA) method with modifications. *Escherichia coli* (*E. coli*, ATCC NO. 8739) was reconstituted from a lyophilized pellet (Epower Assayed Microorganism Preparation; Microbiologics Inc., Saint Cloud, MN) in pre-warmed PBS to make a stock solution of 10^7 colony-forming units (CFU). For each BKA, we prepared a working solution of 10^5 CFU from the stock solution.

We conducted BKAs using 96-well plates and plasma that had not been previously thawed. Each plate included negative (24 µl PBS) and positive controls (18 µl PBS and 6 µl 10^5 E. coli CFU). For each sample, we added 7 µl of plasma to 11 µl of PBS and 6 µl of bacteria working solution and assayed each sample in duplicate. Samples were randomly assigned to plates, but all four samples from the same individual were assayed on the same plate. We added 125 µl of Tryptic Soy Broth (15 g broth/500 ml nanopure water; #T8907 Sigma-Aldrich, St. Louis, MO, USA) to all wells and obtained a background reading (300 nm) with a microplate reader (Multiskan GO, Thermo Scientific, Waltham, MA, USA.) All plates were then incubated at 37 ˚C for 12 hours and then read again.

To evaluate the capacity of the plasma to kill *E. coli*, we first subtracted the background reading from the 12 hour reading. We then averaged the positive controls and the duplicates for each sample and calculated the percentage of bacteria killed in each well as described by French and Neuman-Lee (2012).

Statistics

We analyzed all data sets using a three-way mixed design analysis of variance (ANOVA) to examine the effects of the duration of experimental stress (10 min or 120 min), treatment (mitotane or control), and stress (BL or stress-induced) on plasma CORT, agglutination scores, lysis scores, and bacterial killing capacity. All data were first tested for normality with the Shapiro-Wilk test. Data sets that could not be normalized by log transformation were ranked before ANOVA (Conover and Iman, 1981). When significant main effects and/or interactions were detected, we used simple main effects to compare specific groups. We compared BL plasma CORT during the first and second trials using a paired Student's t -test to test the potential effect of captivity-associated stress. All statistical analyses were performed with SPSS Statistics 21 (IBM Corporation, New York, NY) and GraphPad Prism 6 (GraphPad Software, Inc., San Diego, CA). The statistical significance level of all tests was set to $P=0.05$.

Results

Effects of mitotane treatment, restraint, and captivity on plasma CORT

Baseline and stress-induced plasma CORT were lower in mitotane-treated than in control birds ($F_{1, 16}=44.524$; $P<0.001$ and $F_{1, 16}=129.319$; $P<0.001$, respectively; Fig. 2.2). The duration of restraint did not affect the strength of the stress response ($F_{1, 16}=1.881$; $P=0.189$; Fig. 2.2). However, mitotane treatment suppressed the stress response: stress-induced plasma CORT was higher than BL plasma CORT in vehicle-treated ($F_{1, 16}=77.314$; $P<0.001$) but not in mitotane-treated birds ($F_{1, 16}=2.248$; $P=0.153$; Fig. 2.2).

Baseline CORT did not differ during the first and second stress trials (t=-0.029; d.f.= 17; P=0.977).

Effects of mitotane treatment and restraint on innate immune measures

Bacterial killing capacity changed during the stress response in vehicle-treated birds ($F_{1, 16}$=11.959; P=0.003; Fig. 2) but not in those receiving mitotane ($F_{1, 16}$=4.267; P=0.055; Fig. 2). The duration of experimental stress did not influence how bacterial killing capacity changed during the stress response ($F_{1, 16}$=0.023; P=0.882). We also found no evidence for an effect of mitotane treatment on BL bacterial killing capacity ($F_{1, 16}$<0.001; P=0.986).

Agglutination scores decreased during the stress response in birds that experienced experimental stress for 120 min ($F_{1, 16}$=14.754; P=0.001) but not 10 min ($F_{1, 16}$=0.192; P=0.667; Fig. 3). However, this decrease took place only in birds receiving the vehicle injection (vehicle: $F_{1, 16}$=40.210; P< 0.001; mitotane: $F_{1, 16}$=0.031; P=0.864). There was no effect of treatment on BL agglutination scores ($F_{1, 16}$=1.584; P=0.226).

Lysis scores decreased during the stress response in birds receiving the control ($F_{1, 16}$=5.508; P=0.032) but not the mitotane ($F_{1, 16}$=0.650; P=0.432; Fig. 3) treatment. The duration of experimental stress did not influence the change of lysis scores during the stress response ($F_{1, 16}$=1.122; P=0.066). Furthermore, mitotane- and vehicle-treated birds had similar BL lysis scores ($F_{1, 16}$=1.567; P=0.229).

Discussion

We found in male House Sparrows that acute stress is associated with suppression of the innate immune system activity. The onset of this suppression was rapid and the effects were persistent, as shown by the fact that complement-mediated lysis and bacteria killing ability decreased within 10 min of stress exposure and remained low for the next 120 min of this exposure. However, the onset of agglutination, which is mediated by natural antibodies, began between 10 and 120 min of restraint. These results are consistent with the hypothesis that prolonged activation of the stress response inhibits innate immune activity but do not support the hypothesis that the effects of acute stress on innate immunity change over the duration of the stress response. Furthermore, the results suggest the involvement of both non-genomic and genomic mechanisms. We also found that pharmacological adrenalectomy induced by mitotane administration eliminated the immunosuppressive effects of stress, suggesting that elevated plasma CORT during stress plays an essential role in mediating these effects. To our knowledge, this is the first study demonstrating the necessity of elevating plasma CORT to induce immunosuppression in free-living birds. The findings also indicate that mitotane is an effective agent to investigate relationships between plasma CORT and immune activity.

Stress-induced suppression of innate immune constituents

The effects of acute stress on the innate immune system can be difficult to predict, and investigations of stress-induced effects on innate immune activity in free-living birds have yielded conflicting results. Overall, our results show stress-induced inhibition of

innate immune activity and are consistent with previous findings in other free-living birds (Martin et al., 2005; Matson et al., 2006; Cyr et al., 2007; Merrill et al., 2012; Zylberberg, 2015; Davies et al., 2016). They also indicate that the inhibition of complement-mediated activity occurs with a shorter latency than the inhibition of natural antibody-mediated activity. Two other studies have observed differing latencies when investigating the effects of stress on immune activity but contrary to our findings, these studies showed immunoenhancing effects of stress. In the Small Ground Finch, stress elevated natural antibody-mediated activity at a faster rate than complement-mediated activity (Zylberberg, 2015). In Red Knots, stress increased phagocytic activity against *S. aureus* faster than against *C. albicans*, and furthermore, did not alter microbicidal activity against E. coli (Buehler et al., 2008). Together, these results highlight the importance of measuring immune parameters at various times during the stress response and contribute to accounting for disparities between results from avian studies examining the relationship between stress and the immune system. We proposed two explanations for our finding that stress did not result in immunoenhancement. First, this observation may indicate a fixed allocation of resources between reproduction, stress, and immunity (Moore and Hopkins, 2009). Supporting this hypothesis, male House Sparrows in reproductive condition, such as those in this study, mount weaker cutaneous immune responses than molting, non-breeding males even when receiving food *ad libitum* (Greenman et al. 2005; Lee et al., 2006). Second, stress-induced immunosuppression may reflect a transient re-direction of limited resources in preparation for energetically expensive behavior such as flight. For example, innate immune activity is reduced in

European Starlings, *Sturnus vulgaris*, immediately after prolonged flight but partially recovers within 48 hours (Nebel et al., 2012). One such limited resource may be protein availability, as indicated by the fact that low protein stores are associated with reduced activity of constitutive immunity in fasted Mallards, *Anas plathyrhynchos* (Bourgeon et al., 2010), and in Red Knots during migration (Buehler et al., 2010).

The involvement of non-genomic mechanisms in the observed effects of stress on immunity is suggested by the finding that complement-mediated lytic and microbicidal activity decreased within 10 min. To our knowledge, only one other study has investigated rapid stress-induced effects on immune activity in free-living birds. In this study and contrary to the present work, stress for 15 min enhanced agglutination activity (Zylberberg, 2015). These conflicting results may arise from the differing pathogen environment of temperate and tropical birds (Buehler et al., 2008), although stress-induced effects on immune activity may vary even within tropical avian species (Zylberberg, 2015). Despite the disparity, these findings are consistent with the hypothesis that that stress alters immune activity through non-genomic mechanisms. One such mechanism may consist in a decrease in constitutive immunity following loss of blood during collection of the baseline sample. We consider this possibility to be unlikely because we did not observe a rapid decrease in agglutination activity. Furthermore, Buehler et al. (2008) found no effect of the number of blood sampling within a short time frame on constitutive immunity. Alternatively, acute stress may stimulate the movement of complement proteins from the blood to other tissues. Mammalian studies have demonstrated stress-induced immunoredistribution of leukocytes (Dhabar, 2009).

Leukocytes are also redistributed from the blood into other tissues during long-term captivity in the House Sparrow (Kuhlman and Martin, 2010). We are not aware of studies demonstrating this phenomenon in response to restraint, but our results do not exclude this possibility.

In laboratory rodents, an increase in plasma glucocorticoid during stress is a primary inhibitor of immune activity (Sapolsky, 2000). Most studies on this subject in free-living species have been correlational and so there is limited support for a causal relationship between elevated glucocorticoids during stress and immunosuppression (Berger et al., 2005; Linstrom et al., 2005; Matson et al., 2006; French et al, 2010; Kuhlman and Martin, 2010; Hopkins and Durant, 2011). By manipulating endogenous plasma CORT while controlling for the presence of the stressful stimulus, our results suggest a causal relationship between elevated plasma CORT during stress and the suppression of innate immunity. A negative, causal relationship between these two factors has been also observed in male Brown-headed Cowbirds (*Molothrus ater*) that were treated with CORT and then restrained for 90 minutes (Merrill et al., 2012). Our results are also consistent with studies simulating stress by experimentally elevating plasma CORT but not involving exposure to a stressor such as restraint (Martin et al., 2005; Loiseau et al., 2008; Shini et al., 2008). Our findings do, however, not reveal whether elevated plasma CORT directly reduces innate immunity or exerts this effect through other mediators. In Abert's Towhees (*Melozone aberti*), the stress-induced suppression of agglutination and lysis scores were not correlated with stress-induced plasma CORT levels (Davies et al., 2016), suggesting that plasma CORT acts indirectly on the innate immune system. With the

present data, these observations suggest that stress-induced suppression of innate immune activity is at least indirectly caused by elevated plasma glucocorticoids rather than result from exposure to a stressor per se.

We found that the decrease of BL plasma CORT resulting from mitotane administration was not associated with suppression of innate immune activity prior to restraint. Baseline plasma CORT is thought to facilitate the energetic demands to fuel innate immune activity, although both positive (Merrill et al., 2014) and negative (Zylberberg, 2015) correlations have been found in free-living birds between BL CORT levels and innate immune activity. Our results, while not supporting this hypothesis, suggest mechanisms that may be involved in the observed effects. In House Sparrows, low BL plasma CORT acts on cells by binding to high affinity mineralocorticoid receptors (MR) whereas high plasma CORT, such as during stress, acts by binding to high affinity MR as well as low affinity glucocorticoid receptors (GR; Lattin et al., 2011). Accordingly, stress-induced immunosuppression may be mediated primarily through GR. This hypothesis would explain the lack of relationship between BL plasma CORT and innate immune activity, but warrants further research. Indeed, we are not aware of studies investigating the role of GRs during stress-induced changes in immune activity in adult free-ranging birds. Research on this subject and using specific GR antagonists may help clarify this relationship.

Summary

Our results suggest that elevated plasma CORT is a primary component of the stress response that inhibits natural antibody and complement mediated immune activity. The rapid stress-induced suppression of complement-mediated activity is especially intriguing given the sparse data on stress-induced immune effects within a non-genomic time frame in free-living birds. Our results also highlight the necessity to measure immunological parameters at various times during the stress response, as it appears that the activation of the stress response does not act on all constituents of the innate immune system with the same latency. Our findings do not reveal the involvement of any specific CORT receptor type or whether plasma CORT acts directly or indirectly to suppress the activity and/or concentration of complements and natural antibodies in the plasma. However, given that the reduction of BL plasma CORT resulting from mitotane treatment was not associated with suppression of innate immune activity prior to restraint, our results suggest that CORT acts through GRs rather than MRs to reduce innate immune activity. Furthermore, the rapid onset and persistence of stress-induced immunosuppression implies a role for both non-genomic and genomic pathways in causing the observed immune effects of stress.

Fig. 2.1. Experimental design to assess effect of restraint and mitotane treatment on immune activity. Adult male House Sparrows, *Passer domesticus*, were divided into two groups (N=10): 10 minutes and 120 minutes. Each bird was first treated with either mitotane or vehicle solution (A). Two days later, each bird was exposed to a stress trial (B). We collected a baseline blood sample, restrained the bird for the duration of the bird's group, and collected a stress-induced blood sample. Each bird then rested for ten days, received the opposite treatment (A), and was exposed to a second stress trial (B).

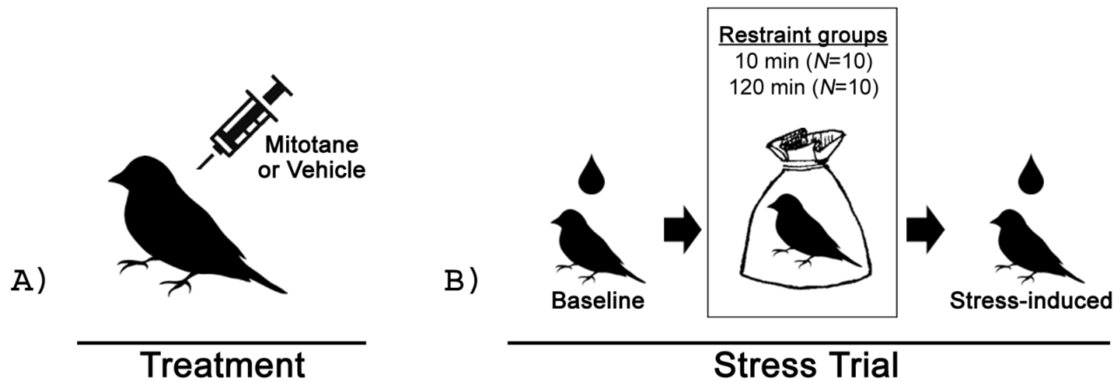


Fig. 2.2. Baseline and stress-induced levels of plasma CORT (left) and bacterial killing capacity (right) of mitotane-treated and vehicle-treated House Sparrows. Birds (N=9 per group) treated with mitotane or vehicle were restrained for either 120 minutes (Mitotane-120 and Control-120, respectively) or 10 minutes (Mitotane-10 and Control-10, respectively). Letters indicate significant differences ($P < 0.05$) between groups and data are shown as means ± s.e.m.

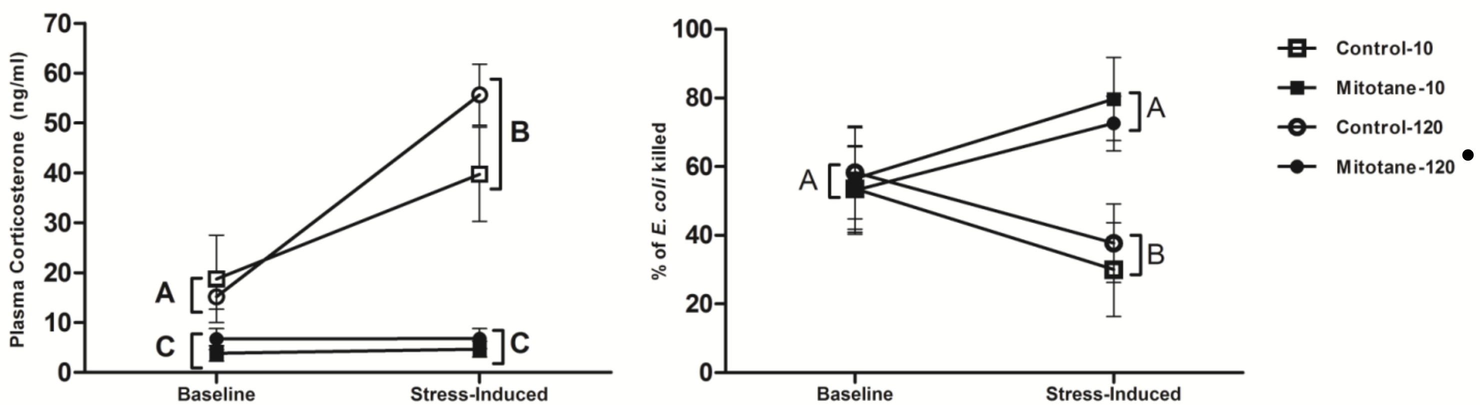
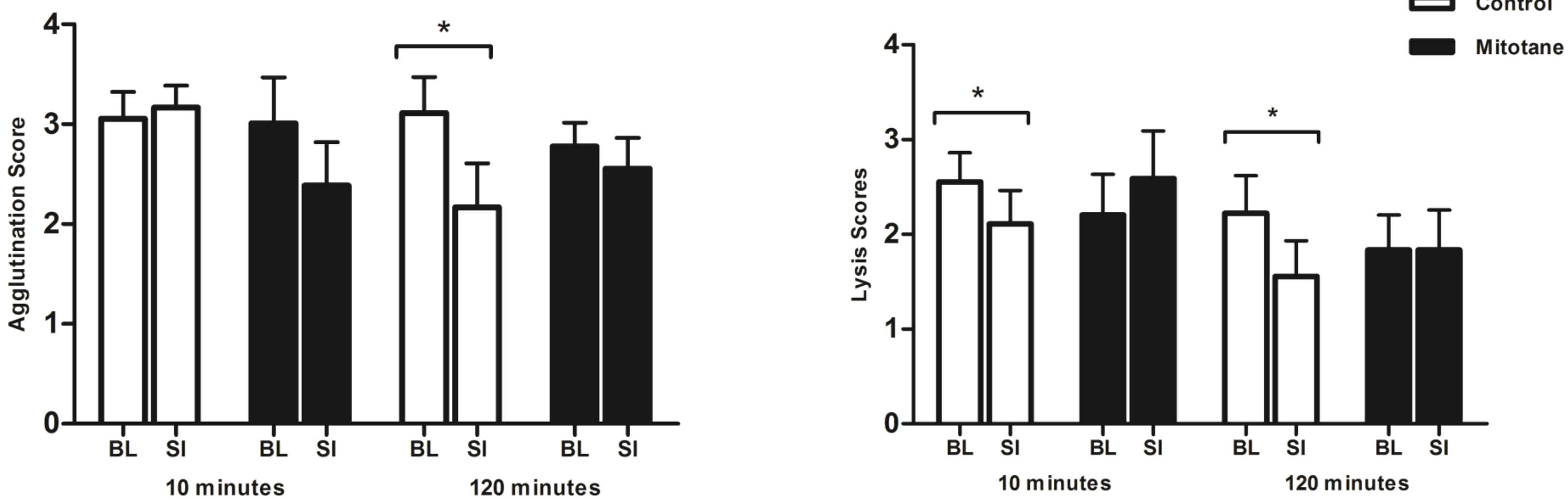


Fig. 2.3. Baseline and stress-induced levels of agglutination (left) and lytic scores (right) of mitotane-treated and vehicle-treated House Sparrows. Birds (N=9 per group) treated with mitotane or vehicle were restrained for either 120 minutes (Mitotane-120 and Control-120, respectively) or 10 minutes (Mitotane-10 and Control-10, respectively). Asterisks indicate significant differences (P< 0.05) between groups and data are shown as means ± s.e.m.



CHAPTER THREE

The glucocorticoid receptor selectively mediates stress-induced suppression of innate immunity in the House Sparrow, *Passer domesticus*

Abstract

Stress-induced inhibition of innate immune activity is widespread in free-ranging birds, but the mechanisms that are responsible for this inhibition are poorly understood. I previously demonstrated that an increase in plasma corticosterone (CORT), the primary avian glucocorticoid, is necessary for the inhibition of natural antibody- and complement-mediated activities to occur during stress. Here I investigated the role of cytosolic glucocorticoid receptors in stress-induced inhibition of natural antibody and complement-mediated activities within non-genomic (< 10 min) and genomic (< 120 min) time frames in male House Sparrows, *Passer domesticus*. Treatment with the selective glucocorticoid receptor antagonist mifepristone (RU486) attenuated stress-induced suppression of natural antibody-mediated activity within 10 min and 120 min of experimental stress. By contrast, this treatment did not influence stress-induced suppression of complement-mediated activity. These results suggest that stress-induced elevated plasma CORT inhibits natural antibody-mediated activity, but not complement-mediated activity, by activating cytosolic glucocorticoid receptors and that both non-genomic and genomic mechanisms underlie this activation. Additional research is needed to identify the receptors that regulate inhibitory effects of elevated plasma CORT on complement-mediated activity.

Introduction

The vertebrate innate immune system serves as the first line of defense against pathogens and includes two humoral components, natural antibodies and the complement system. Natural antibodies are found in non-immunized animals (Avrameas, 1991) and help reduce susceptibility to infections (Congdon et al., 1969; Longenecker et al., 1969; Ochsenbein and Zinkernagel, 2000; Parmentier et al., 2004; Davison et al., 2008). Components of the complement system can lyse foreign cells and induce inflammation through a catalytic complement cascade which culminates in the formation of a membrane attack complex that attaches to the target cell's plasma membrane (Juul-Madsen et al., 2008; Unsworth, 2008). This cascade can be activated through the natural antibody-dependent classic pathway and/or through the natural antibody-independent alternative and/or lectin pathways (Ochsenbein and Zinkernagel, 2000; Nauta et al., 2004; Juul-Madsen et al., 2008). All three pathways have been identified in birds (Davison et al., 2008).

Acute stress often stimulates the secretion of glucocorticoids (corticosterone [CORT] in birds; Romero et al., 2006) within minutes and can be immunosuppressive (Martin et al., 2004; Matson et al., 2006; Merrill et al., 2012; Zylberberg, 2015; Gao et al., 2016). There is evidence that elevated plasma glucocorticoids are responsible for this inhibition (Martin et al., 2004; Bourgeon and Raclot, 2006; Loiseau et al., 2008; Gao et al., 2016), but the mechanism and, in particular, the receptor type(s) that is(are) involved is(are)

unclear. Corticosterone can bind to three types of receptors. At low plasma levels, such as in non-stressed animals, CORT primarily binds to high affinity cytosolic mineralocorticoid receptors (MR; Breuner and Orchinik, 2009; Groeneweg et al., 2012). By contrast, at high plasma levels, such as during stress, CORT also activates low affinity cytosolic glucocorticoid receptors (GR). Activation of MR and GR is thought to result in genomic changes, the expression of which requires at least 30 minutes (Buttgereit and Scheffold, 2002). In addition, CORT can bind to membrane-bound GR (Moore and Orchinik, 1994; Breuner and Orchinik, 2009) to induce non-genomic changes. These changes can be rapid, being observed within 15 minutes of receptor activation (Haller et al., 2008).

I recently found that acute stress inhibits natural antibody- and complement-mediated activity in the House Sparrow, *Passer domesticus*, and that elevated plasma CORT during stress may be the primary mediator of this inhibition (Gao et al., 2016). Both stress-induced inhibitory effects occurred 120 minutes after the onset of the stress response. Furthermore, stress-induced inhibition of complement-mediated activity could occur within 10 minutes of the onset of the stress response. I also found that administration of the CORT synthesis inhibitor mitotane (Breuner et al., 2000; Chortis et al., 2012) reduces pre-stress plasma CORT but not pre-stress immune activity. Together, these results suggest the involvement of non-genomic mechanisms (Breuner et al., 1998; Haller et al., 2008) and imply that effects of stress on immunity depend on activation of GR and not MR.

Here, I investigated the role of the cytosolic GR in rapid (< 10 min) and prolonged (< 120 min) stress-induced suppression of natural antibody and complement-mediated immunity by using the glucocorticoid receptor antagonist RU486 (mifepristone; Baulieu 1991). In birds, RU486 antagonizes cytosolic GR (Landys et al., 2004; Breuner and Orchinik, 2009; Lattin et al., 2013), has a low affinity for membrane-bound GR (Breuner and Orchinik, 2009), and does not bind to MR (Lattin et al., 2011). Unlike in mammals, RU486 does not antagonize avian progesterone receptors (Groyer et al., 1985). Effects of RU486 treatment in birds, therefore, likely result from antagonism of cytosolic GR (Koch et al., 2002; Landys et al., 2004a, b). Accordingly, I predicted that this treatment would mitigate stress-induced inhibition of both natural antibody and complement-mediated activity after 120 minutes. I also predicted that RU486 treatment would not mitigate rapid stress-induced effects on complement-mediated activity because these effects have been previously reported to result from activation of a non-genomic mechanism and, therefore, presumably from activation of membrane-bound GR. Identification of the type of CORT receptor that mediates stress-induced immunosuppression may help predict when innate immune activity is most sensitive to stress because the density of cytosolic GR and MR varies as a function of the life history stage (Breuner and Orchinik, 2001; Lattin and Romero, 2013; Lattin et al., 2013).

Methods

Study species and housing

Twenty adult male House Sparrows were captured with mist nets and ground traps in early May 2015 in Phoenix, AZ (33.4° N, 111.6° W; 331 m.a.s.l.). The reproductive condition of all birds was determined based on their beak color (Barfuss and Ellis, 1971). I transported birds to Arizona State University's Animal Care Facilities and randomly assigned them to one of two groups (N=10) that were housed in identical rooms. Birds were housed individually, visually isolated from other birds, and exposed to 13L:11D (lights on at 9 a.m.). They were caught at least one week prior to experimental procedures began and received ad libitum water and Mazuri Pellet Diet (PMI Nutrition International, Richmond, IN, USA). All procedures were approved by the Arizona State University's Institutional Animal Care and Use Committee and were conducted under Arizona Game and Fish Department scientific collecting permit SP719136.

Stress trials

Each sparrow experienced two consecutive stress trials (see below): Once after receiving RU486 and once after receiving the control (vehicle) treatment. During both trials, ten birds experienced experimental stress for 10 minutes (10 mins group) whereas the other ten birds experienced experimental stress for 120 minutes (120 mins group). These durations were chosen as to discern between solely non-genomic (10 mins) and both non-genomic and genomic (120 mins) effects of stress.

For the first trial, five birds in the 10 minutes group and five birds in the 120 minutes group received a RU486 injection (see below), and the remaining sparrows received a vehicle injection. During the second trial, I reversed the RU486 and vehicle treatments so

that each sparrow served as its own control. The two trials were separated by a period of 14 days to allow the effects of RU486 injection to dissipate (Földesi et al., 1996; Sitruk-Ware and Spitz, 2003). Consistent with previous studies on passerines, treatments were administered the day preceding a stress trial (Landys et al., 2004a, b).

I began all stress trials at 12 p.m. to account for a potential daily rhythm of baseline (BL) plasma CORT (Rich and Romero, 2001). I first collected a BL blood sample (220 μl) within 3 minutes of removing a bird from its cage and within 5 minutes of entering the room, and then induced a stress response by restraining the bird in a breathable cloth bag and placing the bag into the bird's cage for 10 minutes or 120 minutes. At the end of the restraint period, a second stress-induced blood sample was collected (220 μl). Accordingly, four blood samples were collected from each sparrow during the course of the experiment. Samples were obtained from the jugular vein with a heparinized microsyringe and then immediately placed on ice until centrifuged within hours of collection. Plasma was then separated and stored at -80 °C until assayed.

RU486 preparation

I dissolved RU486 (Sigma Aldrich, St. Louis, MO, #M8046, purity > 98%) in peanut oil via sonication. The solution was stored at 4 °C for up to five days and administered into the left pectoral muscle (1.35 mg in 100 μl of peanut oil; 50 mg/kg; Landys et al., 2004a, b). Control injections consisted of the same volume of peanut oil.

Corticosterone assays

I measured total plasma CORT with a validated commercial competitive enzyme-linked immunoassay according to the manufacturer's instructions (Enzo Life Sciences, Farmingdale, NY; Fokidis et al., 2009). I diluted House Sparrow plasma 15x in assay buffer containing steroid displacement reagent to release the hormone from plasma binding proteins. Each sample was assayed in duplicate and each assay plate included a standard curve. All four samples from the same bird were assayed on the same plate and individuals were randomly assigned to assay plates. The assay sensitivity was 3.90 pg/ml. The average intra- and interassay coefficients of variation were 4.53% and 6.42%, respectively (n = 3 plates).

Measurements of immune activity

I used a hemolysis-hemagglutination assay to assess activity levels of natural antibodies and complement. I used methods previously described (Matson et al., 2005; Gao et al., 2016) and tested samples against 20 µl of 0.5% sheep red blood cell (SRBC; #SB050, Hemostat Laboratories, Dixon, CA). All four samples from a same bird were assigned to the same plate, but individuals were randomly assigned to plates. I diluted plasma (40 µl) in 0.9% phosphate buffered solution (PBS). All plates were scanned at 600 dots per inch with a flat-bed scanner (ScanJet 3670; Hewlett-Packard Co, Palo Alto, CA). Each row of wells was scored for agglutination and lysis by an individual (S.G.) without knowledge of the experimental treatment. High scores reflected high agglutination and lytic activity. All wells were scored in one session to maximize consistency.

I used French and Neuman-Lee’s (2012) *ex vivo* bacterial killing assay (BKA) method with modifications described in Gao et al (2016) to determine the complement-mediated microbicidal activity of plasma against *Escherichia coli* (*E. coli*, ATCC NO. 8739). *Escherichia coli* was reconstituted from a lyophilized pellet (Epower Assayed Microorganism Preparation; Microbiologics Inc., Saint Cloud, MN) in pre-warmed PBS to make a stock solution of 10^7 colony-forming units (CFU). I prepared a working solution of 10^5 CFU from the stock solution for each BKA. All plates were incubated for 12 hours at 37 ˚C and all plate readings were obtained with a microplate reader (Multiskan GO, Thermo Scientific, Waltham, MA, USA). All samples were assayed in duplicate. I calculated the percentage of bacteria killed for each sample as described by French and Neuman-Lee (2012).

Statistics

I used three-way mixed model analyses of variance (ANOVA) to determine the effects of experimental stress duration (10 or 120 minutes of restraint), treatment (RU486 or control), and stress (BL or stress-induced) on plasma CORT, agglutination scores, lysis scores, and bacterial killing capacity. All data sets were first analyzed for normality (Shapiro-Wilk test). I normalized lysis scores through logarithmic transformation. I ranked plasma CORT, agglutination scores, and bacterial killing percentage data sets before analysis because other transformations failed to normalize these sets (Conover and Iman, 1981). When the ANOVA revealed statistically significant interactions between main effects, I used simple effects to test for significant differences between specific groups (Gao et al., 2016). I used paired Student’s t-tests with a Bonferroni adjustment to

assess any potential effect of the duration of captivity on the four dependent variables. All statistical analyses were performed with SPSS Statistics 21 (IBM Corporation, New York, NY) and GraphPad Prism 6 (GraphPad Software, Inc., San Diego, CA). The statistical significance level of the three-way mixed model ANOVA results was set to P= 0.05 and the statistical significant level of the Student's t-tests were set to P=0.0125 (*i.e.,* 0.05/4 independent tests).

Results

Effects of RU486 treatment and of restraint on plasma CORT

Plasma CORT increased during restraint and neither RU486 treatment nor the duration of restraint affected the magnitude of this increase (Table 3.1; Figure 3.1). Baseline plasma CORT did not differ whether birds received RU486 or only vehicle ($F_{1, 18}$=1.615; P=0.220; Fig 3.1). The duration of captivity did not affect BL plasma CORT (t=0.225; d.f.= 19; P=0.801).

Effects of RU486 treatment and restraint on innate immunity

All birds, irrespective of RU486 or control treatment, had similar BL agglutination scores ($F_{1, 18}$=0.606; P=0.446). However, the type of treatment influenced agglutination scores during stress (treatment x stress interaction: P=0.001; Table 3.1 and Fig 3.2): When compared to BL agglutination scores, restraint reduced agglutination scores in vehicle-treated sparrows (Table 3.1; Fig 3.2) but not in RU486-receiving sparrows (Table 3.1; Fig

3.2). The duration of restraint did not influence agglutination scores (Table 3.1; Fig 3.2). The duration of captivity did not affect BL agglutination scores (t=1.205; d.f.= 19; P=0.243).

All birds, irrespective of treatment, had similar BL killing percentages ($F_{1, 18}$=0.310; P=0.585; Fig 3.1). The percentages of killed bacteria decreased during restraint (Table 3.1; Fig 3.1). Neither the type of treatment nor the duration of restraint (Table 3.1; Fig 3.1) influenced bacterial killing ability. Baseline killing percentages were lower during the first than the second stress trial (first trial: mean=66.62% ± s.e.m. 5.42; second trial: 83.91% ± 4.25; t=-3.871; d.f.= 19; P=0.001).

Restraint reduced lysis scores (Table 3.1; Fig 3.1), but neither the type of treatment (Table 3.1; Fig 3.1) nor the duration of restraint (Table 3.1; Fig 3.1) affected these scores. The type of treatment did not affect BL lysis scores ($F_{1, 18}$=0.813; P=0.379). The duration of captivity did not affect BL lysis scores (t=-1.175; d.f.= 19; p=0.254).

Discussion

I investigated the role of cytosolic GR in acute stress-induced immunosuppression in the adult male House Sparrow. Consistent with a previous study (Gao et al., 2016), acute stress inhibited natural antibody- and complement-mediated immunity. This suppression was observed within 10 minutes and persisted for 120 minutes of stress. These results suggest either the involvement of both non-genomic and genomic mechanisms or the

involvement of non-genomic mechanisms resulting in effects that persist for at least 120 minutes. Treatment with RU486 mitigated stress-induced inhibition of natural-antibody mediated activity but not complement-mediated activity. Given the evidence in birds that RU486 selectively antagonizes cytosolic GR, these results suggest that activation of these receptors is primarily responsible for mediating effects of elevated CORT on natural-antibody mediated activity, but not complement-mediated activity.

Stress-induced suppression of innate immune activity and the role of the glucocorticoid receptor

Stress-induced inhibition of natural antibody- and complement-mediated activity has been observed in House Sparrows (Gao et al., 2016) and other birds (Martin et al., 2004; Matson et al., 2006; Merrill et al., 2012; Zylberberg, 2015; Davies et al., 2016), but the underlying mechanisms are unclear. The present results suggest that stress-induced inhibition of natural antibody-mediated activity is primarily regulated by activation of cytosolic GR and this inhibition involves non-genomic mechanisms. Non-genomic actions mediated by cytosolic GR have been reported (Croxtall et al., 2000) and may depend on actions of proteins that associate with unbound cytosolic GR (Buttgereit and Scheffold, 2002).

The mechanism by which GR activation reduces circulating natural antibody-mediated activity may involve the degradation of these antibodies and/or their redistribution from plasma to other tissues. Stress-induced immunoredistribution of leukocytes from the plasma to the skin has been described in mammals (Viswanathan and Dhabhar, 2005) and

House Sparrows (Kuhlman and Martin, 2010), and in mammals CORT is necessary for this redistribution to occur (Viswanathan et al., 2005). I am not aware of any study assessing stress-induced immunoredistribution of natural antibodies and/or CD5+ B1 cells, the primary producers of natural antibodies (Ochsenbein and Zinkernagel, 2000). However, increased filtration of natural antibodies through the spleen and skin may, respectively, help improve pathogen detection and clearance (John, 1994; Ochsenbein and Zinkernagel, 2000), and cytosolic GR have been identified in the spleen and skin of House Sparrows (Lattin et al., 2011; Lattin et al., 2013).

Besides providing evidence supporting the hypothesis that CORT inhibits natural antibody-mediated activity through cytosolic GR, the present results suggest that CORT inhibits complement-mediated activity through a different mechanism. This difference implies that the hemolytic and microbicidal activities in my immune assays are primarily regulated by antibody-independent complement pathways. Indeed, the natural antibody-independent alternative pathway is involved in the lysis of *E. coli* (Betz and Isliker, 1981). However, the primary complement pathway involved in the lysis of SRBCs in avian plasma is not known (see below). High glucocorticoid levels can inhibit the activation of complement pathways and complement-mediated lysis (Imai, 1981; Schumer et al., 1974; Dauchel et al., 1990) and this inhibition may serve an anti-inflammatory function (Dauchel et al., 1990; Nauta et al., 2004). It is, however, unclear as to how elevated plasma CORT inhibits complement-mediated activity in the male House Sparrow. Previous studies suggested that MR are not involved in this inhibition (Gao et al., 2016). Elevated plasma CORT may, therefore, influence the complement system by binding to

membrane-bound GR. These receptors have been proposed to mediate CORT-induced inhibition of macrophage activity in mice (Long et al., 2005) and may mediate the inhibitory effects of glucocorticoids on human neutrophil degranulation (Liu et al., 2005).

Effect of captivity on innate immune activity

Baseline microbicidal activity, which is primarily mediated by complement activity, increased between the first and second trials. This increase may reflect a change in complement-mediated activity given that the complement system mediates inflammation (Unsworth, 2008) and an increase in inflammation during captivity has been reported in House Sparrows (Martin et al., 2011). However, the precise mechanism underlying this increase is unclear because I did not observe a concurrent increase in lytic capacity, which is also mediated by the complement system. As previously explained, my results suggest that the antibody-independent alternative pathway primarily mediates microbicidal activity against *E. coli*. To my knowledge, the complement pathway that mediates the in vitro lysis of SRBC is not identified. Furthermore, in other taxa this process is mediated by the classic pathway (mammalian plasma: May et al., 1972; fish plasma: Nonaka et al., 1981) as well as the alternative pathway (reptilian plasma: Merchant et al., 2005). The classic pathway may primarily control the lysis of SRBC, but this mechanism does not explain my observation that BL agglutination scores did not also increase as a function of the duration of captivity. My results, therefore, only indicate that the complement-mediated mechanism that regulates the lysis of *E. coli* differs from the mechanism that mediates the lysis of SRBC.

Variation in stress-induced suppression of innate immune activity

I previously observed stress-induced inhibition of natural antibody-mediated activity in male House Sparrows, but contrary to the present results, this inhibition did not occur within 10 minutes of restraint (Gao et al., 2016). The House Sparrow breeding season includes a pre-nesting phase and three breeding stages (Hegner and Wingfield, 1986), and pairs can produce up to three clutches per season (Lendvai et al., 2007). Birds used by Gao et al. (2016) were captured at the beginning of their breeding season whereas birds in the present study were captured in the middle of this season and were, therefore, presumably at a more advanced reproductive stage. The innate immune activity of wild birds can vary between (Pap et al., 2010; Hegemann et al., 2012; Merrill et al 2015) and within (Pap et al., 2010) life history stages. The above difference between studies may, therefore, reflect the fact that the activity of natural antibodies, but not complement, varies as a function of the reproductive stage. The mechanisms that account for this variation are, however, speculative. These mechanisms may relate to the fact that natural antibodies and antigen-antibody complexes are filtered through the avian spleen (John, 1994), and changes in the density of splenic cytosolic GR occur during the breeding season (Lattin et al., 2013).

Conclusions

My previous research found that an elevation in plasma CORT during stress is necessary for stress-induced inhibition of natural antibody and complement-mediated activities, and indicated that this inhibition does not involve cytosolic MR activation (Gao et al., 2016).

Consistent with this conclusion, the present study suggests that elevated CORT activates cytosolic GR to inhibit natural antibody activity, but further research is needed to identify the underlying mechanism. I also found no evidence that cytosolic GR mediate effects of elevated CORT on complement-mediated activity. The mechanisms that underlie these effects remain, therefore, speculative. Microbicidal and lytic results, however, do imply the involvement of natural antibody-independent complement pathways in the lysing of foreign cells and pathogens in the male House Sparrow.

Overall, the present results reveal that multiple mechanisms regulate effects of acute stress on innate immune activity. Studies investigating relationships between stress hormones and innate immunity should, therefore, not assume that a single mechanism mediates the effects of these hormones on various aspects of immunocompetence.

Table 3.1. Results of three-way mixed model analyses of variance used to determine the effects of restraint (stress) and its duration (10 or 120 minutes), and that of RU486 or vehicle treatment (treatment) on plasma corticosterone, bacterial killing capacities (BKA), sheep red blood cell agglutination scores, and sheep red blood cell lysis scores in captive male House Sparrows. The Treatment x Restraint interaction is not shown due to physiological irrelevance as treatments were administered in a random order. Bolded text indicates significant differences (P < 0.05) between groups).

	Plasma Corticosterone	BKA	Agglutination Scores	Lysis Scores
Treatment	$F_{1,18} = 2.599$ $p = 0.12$	$F_{1,18} = 2.477$ $p = 0.13$	$F_{1,18} = 2.392$ $p = 0.14$	$F_{1,18} = 0.586$ $p = 0.45$
Stress	**$F_{1,18} = 158.293$ $p < 0.001$**	**$F_{1,18} = 39.396$ $p < 0.001$**	**$F_{1,18} = 16.073$ $p < 0.001$**	**$F_{1,18} = 9.661$ $p < 0.001$**
Restraint duration	$F_{1,18} = 0.197$ $p = 0.66$	$F_{1,18} = 0.170$ $p = 0.69$	$F_{1,18} = 0.125$ $p = 0.73$	$F_{1,18} = 0.609$ $p = 0.45$
Stress x Restraint duration	$F_{1,18} = 1.751$ $p = 0.20$	$F_{1,18} = 0.644$ $p = 0.43$	$F_{1,18} = 3.160$ $p = 0.09$	$F_{1,18} = 3.444$ $p = 0.08$
Treatment x Stress	$F_{1,18} = 0.003$ $p = 0.96$	$F_{1,18} = 2.584$ $p = 0.13$	**$F_{1,18} = 17.862$ $p < 0.001$**	$F_{1,18} = 0.119$ $p = 0.73$
Treatment x Stress x Restraint duration	$F_{1,18} = 1.142$ $p = 0.30$	$F_{1,18} = 0.490$ $p = 0.49$	$F_{1,18} = 1.000$ $p = 0.33$	$F_{1,18} = 1.102$ $p = 0.31$

Fig. 3.1. Stress-induced (SI) plasma CORT levels were higher than baseline (BL) levels, and SI bacterial killing capacity and lysis scores were lower than their respective BL values in adult male House Sparrows. Because the effects of both treatment and duration of restraint were not statistically significant, baseline and SI data from treatment and restraint groups were pooled together to best represent the effect of stress on the dependent variables. An asterisk indicates a statistically significant difference (P< 0.05) from the BL score and data are shown as means ± s.e.m.

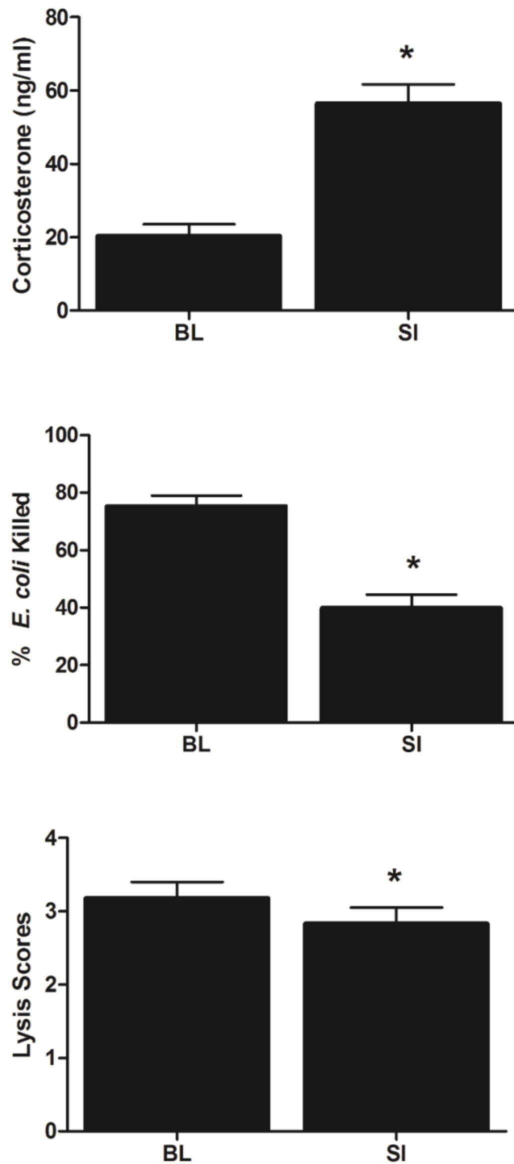
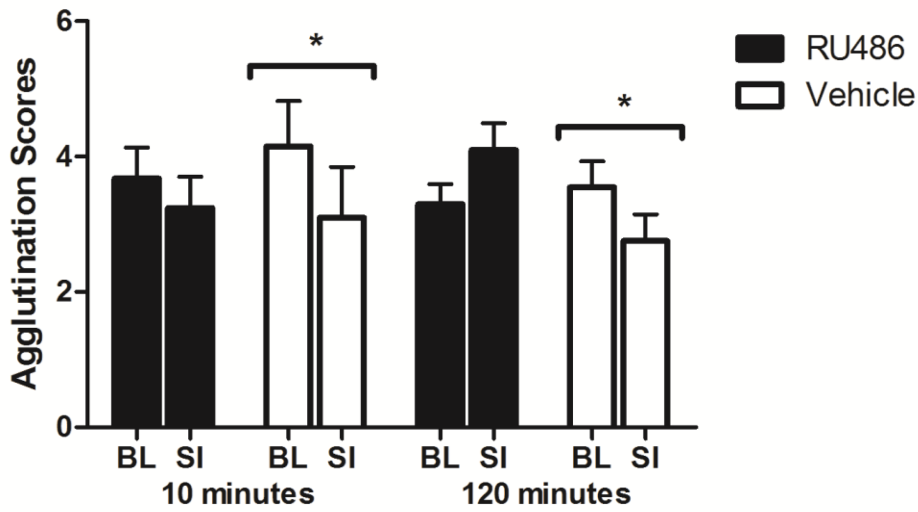


Fig. 3.2. Vehicle-treated, but not RU486-treated birds, showed lower (SI) agglutination scores when compared to baseline (BL) scores in adult male House Sparrows. Effect of stress, treatment (vehicle or RU486; n =10 per group), and duration of restraint (10 or 120 minutes) on red blood cell agglutination scores (means + s.e.m.) are shown. Asterisks indicate significant differences ($P < 0.05$) between groups and data are shown as means ± s.e.m.



CHAPTER FOUR

Sufficiency of corticosterone in stress-induced inhibition of innate immunity in male House Sparrows, *Passer domesticus*

Abstract

Stress-induced inhibition of innate immune activity has been observed in a variety of wild birds, and may increase chances of infection as innate immune activity serves as the first line of defense against pathogens. I have previously reported that the transient elevation of plasma corticosterone (CORT; the primary avian glucocorticoid) which occurs during stress is necessary for stress-induced suppression of both natural antibody- and complement-mediated activity. The goal of the present study, therefore, was to determine the sufficiency of CORT for these two inhibitory relationships. To investigate this, I first experimentally suppressed endogenous CORT production in House Sparrows (*Passer domesticus*) using the CORT secretion blocker mitotane. I then administered these birds exogenous CORT at one of three doses (HI: 1.34 mg/kg; LO: 1.00 mg/kg; CON: vehicle) and assessed their natural antibody- and complement-mediated activity during stress. Mitotane administration suppressed plasma CORT such that stress-induced elevation of plasma CORT was not observed. Exogenous CORT treatment increased plasma CORT to high levels within the species' physiological range. As expected, mitotane-treated birds receiving the CON injections did not exhibit stress-induced suppression of complement-mediated activity. Furthermore, administration of CORT at the low dose restored this suppression, indicating that CORT suffices to inhibit

complement activity during stress. Contrary to expectations, however, mitotane-treated birds receiving CON injections demonstrated stress-induced suppression of natural antibody-mediated activity. Furthermore, CORT administration did not influence this parameter. These present results, which compared to those in the previous study, suggest inter-annual variation in natural antibody-mediated activity during stress. This variation may be a result of changes in the pathogen environment or food availability, but additional studies are needed to test these hypotheses.

Introduction

The innate immune system serves as the first line of defense against pathogens. Two important humoral components of the innate immune system are natural antibodies and the complement system. Natural antibodies are found in non-immunized animals (Avrameas, 1991) and reduce susceptibility to infections by detecting pathogens (Congdon et al., 1969; Longenecker et al., 1969; Ochsenbein and Zinkernagel, 2000; Parmentier et al., 2004; Davison et al., 2008). Complement cascade pathways can lyse foreign cells, induce inflammation, and recruit other components of the immune system (Ochsenbein and Zinkernagel, 2000; Juul-Madsen et al., 2008; Unsworth, 2008). Constituents of the innate immune system act to rapidly contain and fight the earliest stages of infections, and to recruit other components of the immune system to an infected site (Juul-Madsen et al., 2008). Inhibition of innate immune activity may, therefore, increase susceptibility to infections. Elucidating the mechanisms which regulate innate

immune activity may help to predict when these inhibitions will occur. Such elucidation is especially important in urban birds, such as House Sparrows (*Passer domesticus*), because these birds are exposed to higher prevalence, abundance, and transmission of pathogens than their rural counterparts (Bradley et al., 2007; Giraudeau et al., 2014; Gil and Brumm, 2013; Galbraith et al. 2016).

Stress is characterized by a swift and transient increase in plasma catecholamines and glucocorticoids (Martin, 2009), and can inhibit innate immune activity in wild birds (Martin et al., 2004; Matson et al., 2006; Cyr et al., 2007; Merrill et al., 2012; Zylberberg, 2015; Gao et al., 2016). I recently demonstrated that mitotane treatment, which blocks the production of endogenous glucocorticoids such as corticosterone (CORT, the primary avian glucocorticoid; Breuner et al., 2000; Chortis et al., 2013), prevents stress-induced suppression of natural antibody- and complement-mediated activity (Gao et al., 2016). These results imply that CORT is necessary, but do not demonstrate that it is sufficient, for stress-induced suppression of innate immune activity. Demonstration of sufficiency would confirm CORT to be the primary regulator of stress-induced suppression of natural antibody- and complement-mediated activity.

To assess the sufficiency of CORT in stress-induced suppression of innate immune activity, I tested the effect of experimental stress on natural antibody- and complement-mediated immunity in birds treated with mitotane and injected with different doses of exogenous CORT. I predicted that mitotane treatment would mitigate stress-induced suppression of natural antibody- and complement-mediated activity. I also predicted CORT to be sufficient for stress-induced suppression of innate immunity and that CORT

administration would restore the inhibitory effect of stress on innate immunity in mitotane-treated birds. These predictions were based on studies demonstrating that mitotane treatment mitigated stress-induced suppression of innate immunity in House Sparrows (Gao et al., 2016) and that CORT administration inhibited immune activity in wild birds (Martin et al., 2005; Loiseau et al., 2008; Shini et al., 2008; Merrill et al., 2012).

Methods

Study Species and Housing

Thirty adult male House Sparrows were captured with mist-nets and ground traps in late March, 2016 in Phoenix, AZ (33.4° N, 111.6° W; 331 m.a.s.l.). I confirmed the reproductive condition of all birds by their black beaks (Barfuss and Ellis, 1971). All birds were transported to Arizona State University's Animal Care Facilities and were housed in two identical rooms. Sparrows were housed individually, visually isolated from each other, and exposed to 13L:11D (lights on at 9 am), and received *ad libitum* water and Mazuri Pellet Diet (PMI Nutrition International, Richmond, IN, USA). All birds were housed for at least one week prior to experimental procedures. All procedures were approved by the Arizona State University’s Institutional Animal Care and Use Committee and were conducted under Arizona Game and Fish Department scientific collecting permit SP751901.

Experimental Design

I randomly divided birds into three groups (n=9 per group): high CORT (HI), low CORT (LO), and controls (CON). One bird in each group died during the experiment. The experiment consisted of two stress trials as described in Gao et al. (2016). At least ten days separated the two stress trials. During the first stress trial, HI and CON birds received mitotane treatment (see below for details) whereas LO birds received vehicle treatment. These treatments were reversed during the second stress trial so that by the end of the experiment, each bird had been treated once with mitotane and once with vehicle, and thereby served as its own control. For each stress trial, experimental stress was induced by restraint. Doses and the duration of experimental stress were determined from previous avian studies (Remage-Healey and Romero, 2005; Loiseau et al., 2008) and from a pilot study as to ensure elevated but physiologically relevant levels of "stress-induced" plasma CORT.

All stress trials began at the same time (11 am) to account for potential diurnal variation in baseline (BL) plasma CORT (Rich and Romero, 2001). Mitotane was known to suppress endogenous plasma CORT levels within 36 hours of administration in House Sparrows (Breuner et al., 2000). Birds, therefore, received either mitotane or vehicle treatment two days before a stress trial. At the start of each trial, we collected a BL blood sample from each bird, gave an injection of CORT (HI or LO dose) or of control solution (CON dose; see below), restrained the bird in a breathable cloth bag for 1 hour, and collected a second (stress-induced) blood sample before returning the bird to its home cage. Blood samples were obtained from the jugular vein with a heparinized

microsynringe and were collected within 3 minutes of catching the bird from its cage and within 5 minutes of entering the room. The volume of blood collected (220 µl per sample; 440 µl per bird during each trial) should not influence immune measures (Buehler et al., 2008; Gao et al., 2016) and was based on the minimal amount of plasma needed to measure plasma CORT and assess immune parameters (see below). All blood samples were held on ice before being centrifuged within four hours of collection. Plasma was harvested and stored at -80 °C until assayed.

Mitotane preparation and treatment

Mitotane inhibits CORT production in mammals (Chortis et al., 2013) and birds (Jonsson et al., 1994), and one mitotane injection can reduce plasma CORT for 10 days in adult male House Sparrows (Breuner et al., 2000). Furthermore, mitotane treatment has been used to investigate the effects of glucocorticoids on immune activity (Gabaglia et al., 2007; Gao et al., 2016). I am not aware of any direct, CORT-independent effects of mitotane on immune activity

Mitotane was prepared and administered as described in Gao et al. (2016) and Breuner et al., (2000). Each bird received an injection of mitotane (#25925, Sigma Aldrich, St. Louis, MO) dissolved in peanut oil into the left pectoral muscle (100 µl, equal to 9 mg/bird per injection). Vehicle injections consisted of the same volume of peanut oil. To protect against potential negative effects of low plasma CORT on glucose mobilization and to standardize feeding regimens, I supplemented all birds, regardless of treatment, with

fresh Nektar solution (13 g/100 ml 0.9% NaCl in water; Nekton, Germany) for the ten days following mitotane or vehicle administration.

Corticosterone preparation and injection

In a pilot study, an injection of 1.34 mg CORT to House Sparrows elevated plasma CORT to approximately 80 ng/ml one hour post-injection. This concentration is within the range of that measured during stress in intact birds (Breuner et al., 2000; Romero et al., 2006; Gao et al., 2016). Therefore, I prepared CORT solutions at three concentrations: HI (0.35 mg/ml), LO (0.25 mg/ml) and CON (peanut oil) and injected 100 µl of these solutions (corresponding to 1.34 mg/kg [HI], 1 mg/kg [LO], and 0 mg/kg [CON]) into the right pectoral muscle. Corticosterone (#C2505, Sigma Aldrich, St. Louis, MO) was dissolved by sonication and solutions were stored for up to 10 days before use.

Corticosterone assays

I measured total plasma CORT using a validated commercial competitive enzyme-linked immunoassay and according to the manufacturer's instructions (Enzo Life Sciences, Farmingdale, NY; Gao et al., 2016). Samples were assayed in duplicate and each assay plate contained all four samples from the same bird. Prior to the assay, plasma was diluted 15x with assay buffer containing steroid displacement reagent to dissociate the hormone from plasma binding proteins. The assay sensitivity was 7.75 pg/ml. The average intra- and interassay coefficients of variation were 3.36% and 9.85%, respectively (n=3 plates).

Measurements of immune activity

A hemolysis-hemagglutination assay was conducted to measure complement and natural antibody-mediated activity, respectively. My protocol is identical to previously reported methods (Gao et al., 2016) and similar to Matson et al. (2005). Briefly, samples were serially diluted with 0.9% phosphate buffered solution (PBS), and were then incubated at 37°C with 20 µl of 0.5% whole sheep blood (#SB050, Hemostat Laboratories, Dixon, CA). Plates were scanned first for agglutination and then for lysis at 600 dots per inch with a flat-bed scanner (ScanJet 3670; Hewlett-Packard Co, Palo Alto, CA). Each row of wells was scored by an individual without knowledge of the experimental treatments, and all plates were scored in one sitting to ensure consistent scoring. High scores indicated high agglutination and lytic activities.

A bacterial killing assay (BKA) was conducted to assess complement-mediated microbicidal activity against *Escherichia coli* (*E. coli*). I used a protocol developed from French and Neuman-Lee (2012) and slightly modified for House Sparrow plasma (Gao et al., 2016). Briefly, a stock solution of 10^7 colony-forming units (CFU) *E. coli* in PBS was prepared from a lyophilized pellet (Epower Assayed Microorganism Preparation; Microbiologics Inc., Saint Cloud, MN). For each BKA, I prepared a working solution of 10^5 CFU from the stock solution. For each sample, I mixed 7 µl of plasma with 11 µl of PBS and added 6 µl of bacteria working solution. Each sample was assayed in triplicate and all four samples from the same bird were assayed on the same 96-well plate. Prior to incubation, I added 125 µl of Tryptic Soy Broth (15 g broth/500 ml nanopure water; #T8907 Sigma-Aldrich, St. Louis, MO, USA) to all wells. All plates were incubated at 37

˚C for 12 hours. All readings were obtained with a microplate reader (Multiskan GO, Thermo Scientific, Waltham, MA, USA). I averaged the positive controls and the duplicates for each sample and calculated the percentage of bacteria killed in for each plasma sample as described by French and Neuman-Lee (2012) and Gao et al. (2016).

Statistics

Statistical analyses were performed with SPSS Statistics 21 (IBM Corporation, New York, NY) and GraphPad Prism 6 (GraphPad Software, Inc., San Diego, CA). All data sets were first tested for normality with the Shapiro-Wilk test. Data sets that were not normalized by log transformation were ranked prior to additional statistical tests (Conover and Iman, 1981). I analyzed the effect of the duration of captivity on BL plasma CORT and on BL immune measures with paired Student's t-tests with Bonferroni adjustments. I then used a three-way repeated-measures analyses of variance (ANOVA) design to investigate the effects of treatment (mitotane or vehicle), CORT administration (CON, LO, or HI dose), and experimental stress (BL or stress-induced samples) on plasma CORT, agglutination scores, lysis scores, and microbicidal activity. When statistically significant interactions between independent variables were detected, we used simple effects to compare specific groups. The statistical significance level of all tests was set to P=0.05 and set to P=0.0125 with a Bonferroni adjustment.

Results

Effects of mitotane treatment, CORT injection, and experimental stress on plasma CORT

A significant 3-way interaction between treatment, injection, and experimental stress was detected for plasma CORT (Table 4.1). Overall, mitotane-treated birds had lower BL plasma CORT than vehicle-treated birds (Table 4.3; Fig 4.1). Baseline and stress-induced plasma CORT levels did not differ in mitotane-treated birds receiving a CON injection, but differed in birds belonging to the other groups (Table 4.2). Corticosterone administration increased plasma CORT in a dose-related manner (HI > LO > CON) in both mitotane- and vehicle-treated birds (Table 4.4; Fig 4.1). Duration of captivity did not influence BL plasma CORT levels (t_{26}=-1.202; P=0.240).

Effects of mitotane treatment, CORT injection, and experimental stress on innate immunity

A significant 3-way interaction between treatment, injection, and experimental stress was detected for microbicidal capacity, as reflected in BKA values (Table 4.1). Baseline and stress-induced microbicidal capacity did not differ in mitotane-treated birds given a CON injection, but differed in birds belonging to the other groups (Table 4.2; Fig 4.2). Among mitotane-treated birds, stress-induced microbicidal capacity was lower in birds administered a CON injection than in birds receiving a LO or HI injection, and was similar between birds administered LO and HI injections (Table 4.4; Fig 4.2). Duration of captivity did not significantly affect BL bacterial killing percentages (t_{26}=-1.690; P=0.103).

A significant 3-way interaction between treatment, injection, and experimental stress was detected for lysis scores (Table 4.1). Stress-induced lysis scores were similar in birds from all groups (Table 4.4). However, BL and stress-induced lysis scores did not differ in mitotane-treated birds administered CON injections but were different in birds from all other groups (Table 4.2; Fig 4.2). In birds administered CON injections, stress-induced lysis scores differed between mitotane-treated and vehicle-treated birds (Table 4.3; Fig 4.2). However, for birds administered LO or HI doses, stress-induced lysis scores were similar between mitotane-treated and vehicle-treated birds (Table 4.3; Fig 4.2). Duration of captivity did not significantly affect BL lysis scores (t_{26}=0.222; P=0.826).

Treatment and experimental stress affected agglutination scores (Table 4.1). Mitotane-treated birds demonstrated higher BL and stress-induced agglutination scores when compared to respective scores in vehicle-treated birds (Fig 4.3). Experimental stress significantly reduced agglutination scores in birds from all groups (Fig 4.3). Injection did not have a significant effect (Table 4.1). Duration of captivity did not significantly affect BL agglutination scores (t_{26}=0.404; P=0.689).

Discussion

I investigated the role of CORT in stress-induced inhibition of innate immunity by assessing the hormone's sufficiency and by characterizing the relationship between elevated plasma CORT and innate immunity. I used mitotane treatment to inhibit CORT production and then administered exogenous CORT at two different doses (LO, HI) to

elevate hormone levels. These levels were within the physiological range after injection of the lower CORT dose, and slightly above physiological range in response to injection of the higher CORT dose (Romero et al., 2006). Consistent with previous results, I observed stress-induced inhibition of innate immunity after one hour of experimental stress and mitotane treatment mitigated stress-induced inhibition of complement-mediated activity (Gao et al., 2016). Injection of CORT at the lower dose restored stress-induced suppression of complement-mediated activity in mitotane-treated birds, which demonstrated the sufficiency of CORT. The magnitude of suppression of lysis scores did not increase with increasing levels of stress-induced plasma CORT, suggesting that birds defended a baseline level of complement-mediated activity. Stress-induced inhibition of natural antibody-mediated activity was observed in vehicle-treated birds and, unexpectedly, also in mitotane-treated birds. Furthermore, overall, natural antibody-mediated activity was stronger in mitotane-treated birds and exogenous CORT did not have any effect.

Overall, when compared to Gao et al (2016), my results show that the inhibitory relationship between elevated CORT and complement-mediated activity is consistent while the relationship between elevated CORT and natural antibody-mediated activity can vary inter-annually. The predictable, inhibitory relationship between elevated CORT and complement-mediated activity may reflect the anti-inflammatory role of glucocorticoids as complement pathways contribute to inflammation. Inter-annual variation in the regulation of natural antibody-mediated immunity suggests that multiple mechanisms regulate natural antibody-mediated immunity during stress.

Corticosterone is sufficient for stress-induced inhibition of complement-mediated activity

Stress inhibits complement-mediated immune activity in several wild bird species (Matson et al., 2006; Davies et al., 2016) including House Sparrows (Gao et al., 2016). The present study, along with my previous work (Gao et al., 2016), demonstrates that CORT is necessary as well as sufficient for stress-induced suppression of complement-mediated activity. These results are consistent with studies showing that CORT treatment can inhibit complement-mediated in Brown-headed Cowbirds (*Molothrus ater*, Merrill et al., 2012) and cutaneous immune activity in adult and nestling House Sparrows (Martin et al., 2005), and that it can reduce the weight of immune organs in chickens (*Gallus gallus domesticus*, Shini et al., 2008). I also note in the present study that both LO and HI doses of CORT exerted similar effects on complement-mediated activity despite the fact that HI doses elevated plasma CORT to higher levels than LO doses. In other words, administration of the LO CORT dose sufficed to exert a maximum inhibitory effect on complement-mediated activity. The complement system is essential for the clearance of pathogens and the recruitment of additional constituents from both innate and adaptive immune systems (Ochsenbein and Zinkernagel, 2000). Thus, birds may defend a minimum level of complement-mediated activity irrespective of their plasma CORT levels.

The mechanism by which CORT inhibits complement-mediated activity is unclear. High glucocorticoid levels can disrupt complement cascade pathways (Imai, 1981; Schumer et al., 1974; Dauchel et al., 1990). In birds, low (*i.e.,* at baseline) plasma CORT levels act through cytosolic mineralocorticoid receptors (MR) whereas elevated plasma CORT, as

is the case during stress, induced effects by activating cytosolic glucocorticoid receptors (GR) or membrane-bound GR (Moore and Orchinik, 1994; Breuner and Orchinik, 2009; Groeneweg et al., 2012). Here I observed that mitotane treatment decreased BL plasma CORT but not BL lysis scores or microbicidal capacity. Thus, and consistent with previous results (Gao et al., 2016), inhibitory actions of CORT on complement-mediated activity may not involve MR. However, a previous study with the selective GR antagonist RU486 has indicated that CORT does not inhibit complement-mediated activity through the activation of cytosolic GR (Chapter Three). I conclude that CORT may inhibit complement-mediated activity by binding to membrane-bound GR. It is noted in mammals that this receptor type may mediate inhibitory effects of glucocorticoids on immune activity, such as macrophage activity (Long et al., 2005) and neutrophil degranulation (Liu et al., 2005).

When compared to Gao et al (2016), my results demonstrate that the inhibitory relationship between elevated CORT and complement-mediated activity during the stress response is consistent. This predictable relationship may be attributed to the anti-inflammatory role of glucocorticoids (Laue et al., 1988; Baschant and Tuckermann, 2010) as activated complement pathways contribute to the inflammatory response (Unsworth, 2008). My results, therefore, suggest that it is necessary to suppress the inflammatory response during stress.

Inter-annual variation in natural antibody-mediated activity during stress

The present results suggest that elevated plasma CORT either does not mediate or is not the primary mediator of stress-induced inhibition of natural antibody-mediated immunity. This finding is unexpected because it is inconsistent with previous results (Gao et al., 2016), and because a previous study has demonstrated that elevated plasma CORT can inhibit natural antibody-mediated activity through the activation of the cytosolic GR (Chapter Three). The mechanisms which underlie stress-induced effects on natural antibody-mediated activity in the birds of my present study are unclear. CORT-independent mechanisms which inhibit innate immune activity have been implicated in Abert's Towhees (*Melozone aberti*; Davies et al., 2016), and may involve catecholamines. Elevated catecholamine levels have been reported to inhibit immune activity in immature chickens (Denno et al., 1994), and norepinephrine can reduce cutaneous immunity through the activation of the beta-adrenergic receptor in chickens (Brown-borg et al., 1991).

When combined with data previously reported (Gao et al., 2016), I demonstrate inter-annual variation in the regulation of natural antibody-mediated activity during stress in House Sparrows. Inter-annual variation in innate immune activity has been observed in wild birds but the causes of this variation are poorly understood. This variation may be associated with fluctuations in food availability (Horrocks et al., 2012; Pigeon et al., 2013) and with changes in the pathogen environment (Pigeon et al., 2013). Furthermore, the relationship between plasma CORT and immune activity may vary as a function of the pathogen environment (Martin et al., 2005) and with the energetic status of the bird (Bourgeon et al., 2010). However, in skylarks (*Alauda arvensis*), innate immune activity

can vary independently of body mass across years (Hegemann et al., 2012), which suggests that the effects of food availability and/or energetic status on innate immunity may differ between species. The mechanisms which underlie these inter-annual variations are unknown, but may involve changes in GR and MR densities. My present results, which show that low plasma CORT levels may exert a mild inhibition on natural antibody-mediated immunity, suggest the involvement of the MR, not GR, but previous studies have shown the involvement of the GR, not MR (Gao et al., 2016; Chapter Three). Seasonal variation in GR and MR densities have been reported in House Sparrow tissues (Lattin et al., 2015), including splenic tissue (Lattin et al., 2013), but it is not known if receptor densities can vary among years as well. Additional research is needed to specifically investigate the causes of and the mechanisms underlying inter-annual variation in innate immune activity during the stress response in free-living birds.

Summary

I investigated the role of CORT during stress-induced suppression of complement- and natural antibody-mediated activity in House Sparrows. Mitotane treatment mitigated stress-induced suppression of complement-mediated activity, and an injection of exogenous CORT successfully restored this inhibitory relationship. Interestingly, the magnitude of complement-mediated inhibition did not increase with increasingly higher doses of exogenous CORT. My results, therefore, demonstrate that CORT is sufficient for stress-induced suppression of complement-mediated activity, but birds may defend a

minimal level of complement-mediated activity during stress. Because my lowest dose of exogenous CORT in mitotane-treated birds elevated plasma CORT to stress-induced plasma CORT levels in intact birds, additional studies are needed to investigate whether plasma CORT inhibits complement-mediated immunity in a dose-dependent manner.

My results unexpectedly demonstrated that stress-induced inhibition of natural antibody-mediated immunity occurs independently of plasma CORT during stress, which is inconsistent with Gao et al (2016). Inter-annual variation in innate immune activity has been reported in wild birds, but the causes and mechanisms which underlie this variation are unknown. Further studies are needed to elucidate the mechanisms which mediate the interactions between the stress and immune systems as these pathways may identify valuable targets for the fields of conservation and veterinary sciences.

Table 4.1. Results from a three-way repeated measures ANOVA assessing the effects of treatment (mitotane and vehicle), injection (HI, LO, or CON), and experimental stress (BL or stress-induced) on plasma corticosterone levels, percent of bacteria killed (microbicidal activity), lysis scores, and agglutination scores in male House Sparrows. The statistical significance level of all tests was set to P=0.05. Significant three-way interactions were observed for three dependent variables and post-hoc simple effect tests were further conducted (see Tables 4.2, 4.3, 4.4).

	Corticosterone (ng/ml)	Microbicidal activity	Lysis Scores	Agglutination Scores
Treatment	$F_{1,24}$=55.49 $p < 0.00$	$F_{1,24}$=1.18 $p = 0.29$	$F_{1,24}$=7.21 $p = 0.01$	$F_{1,24}$=16.32 **$p < 0.00$**
Stress	$F_{1,24}$=309.51 $p < 0.00$	$F_{1,24}$=342.44 $p < 0.00$	$F_{1,24}$=33.23 $p < 0.00$	$F_{1,24}$=35.66 **$p < 0.00$**
Injection	$F_{2,24}$=6.22 $p < 0.01$	$F_{2,24}$=2.45 $p = 0.17$	$F_{2,24}$=0.19 $p = 0.83$	$F_{2,24}$=0.46 $p = 0.64$
Treatment x Injection	$F_{2,24}$=2.35 $p = 0.12$	$F_{2,24}$=6.23 $p = 0.01$	$F_{2,24}$=0.32 $p = 0.73$	$F_{2,24}$=0.27 $p = 0.77$
Stress x Injection	$F_{2,24}$=26.00 $p < 0.00$	$F_{2,24}$=19.79 $p < 0.00$	$F_{2,24}$=0.43 $p = 0.66$	$F_{2,24}$=1.11 $p = 0.35$
Treatment x Stress	$F_{2,24}$=9.67 $p = 0.01$	$F_{2,24}$=9.28 $p = 0.01$	$F_{2,24}$=3.89 $p = 0.06$	$F_{2,24}$=0.07 $p = 0.79$
Treatment x Stress x Injection	$F_{2,24}$=11.25 **$p < 0.00$**	$F_{2,24}$=18.85 **$p < 0.00$**	$F_{2,24}$=3.76 **$p = 0.04$**	$F_{2,24}$=3.08 $p = 0.06$

Table 4.2. Differences between BL and stress-induced (SI) plasma corticosterone, percent of bacteria killed (microbicidal activity), and lysis scores are assessed within treatment groups (Mitotane vs Vehicle) at the level of each exogenous corticosterone injection (CON, LO, HI). Values represented below were generated from post-hoc simple effects which compared specific groups. The statistical significance level of all tests was set to P=0.05.

Injection	Treatment	Corticosterone (ng/ml) BL vs SI	Microbicidal activity BL vs SI	Lysis scores BL vs SI
CON	Mitotane	$F_{1,24}=0.25$ $p = 0.622$	$F_{1,24}=0.03$ $P = 0.86$	$F_{1,24}=0.06$ $p = 0.80$
	Vehicle	$F_{1,24}=47.97$ **$p < 0.00$**	$F_{1,24}=80.62$ **$p < 0.00$**	$F_{1,24}=11.78$ **$p < 0.00$**
LO	Mitotane	$F_{1,24}=79.12$ **$p < 0.00$**	$F_{1,24}=115.02$ **$p < 0.00$**	$F_{1,24}=13.91$ **$p < 0.00$**
	Vehicle	$F_{1,24}=65.01$ **$p < 0.00$**	$F_{1,24}=92.64$ **$p < 0.00$**	$F_{1,24}=5.17$ **$p = 0.03$**
HI	Mitotane	$F_{1,24}=165.50$ **$p < 0.00$**	$F_{1,24}=93.06$ **$p < 0.00$**	$F_{1,24}=17.56$ **$p < 0.00$**
	Vehicle	$F_{1,24}=126.12$ **$p < 0.00$**	$F_{1,24}=129.75$ **$p < 0.00$**	$F_{1,24}=7.48$ **$p = 0.01$**

Table 4.3. Differences in plasma corticosterone, percent of bacteria killed (microbicidal activity), and lysis scores for mitotane-treated or vehicle-treated birds are examined at each level of experimental stress (BL vs stress-induced [SI]) for each corticosterone injection (CON, LO, HI). Values represented below were generated from post-hoc simple effects which compared specific groups. The statistical significance level of all tests was set to P=0.05.

Injection	**Stress**	**CORT ng/ml Mitotane vs Vehicle**	**Microbicidal activity Mitotane vs Vehicle**	**Lysis scores Mitotane vs Vehicle**
CON	BL	$F_{1,24}$=1.25 $p = 0.27$	$F_{1,24}$=0.03 $p = 0.87$	$F_{1,24}$=0.65 $p = 0.43$
	SI	$F_{1,24}$=159.76 **$p < 0.00$**	$F_{1,24}$=52.81 **$p < 0.00$**	$F_{1,24}$=13.25 **$p < 0.00$**
LO	BL	$F_{1,24}$=6.11 **$p = 0.02$**	$F_{1,24}$=1.35 $P = 0.26$	$F_{1,24}$=1.23 $p = 0.28$
	SI	$F_{1,24}$=24.00 **$p < 0.00$**	$F_{1,24}$=0.10 $p = 0.76$	$F_{1,24}$=1.71 $p = 0.20$
HI	BL	$F_{1,24}$=4.21 **$p = 0.05$**	$F_{1,24}$=2.55 $p = 0.12$	$F_{1,24}$=0.94 $p = 0.34$
	SI	$F_{1,24}$=11.68 **$p < 0.00$**	$F_{1,24}$=3.39 $p = 0.08$	$F_{1,24}$=1.63 $p = 0.21$

Table 4.4. Differences in plasma corticosterone, percent of bacteria killed (microbicidal activity), and lysis scores among different corticosterone injection doses (CON, LO, HI) for mitotane- and vehicle-treated birds at BL and stress-induced (SI) levels. Values represented below were generated from post-hoc simple effects which compared specific groups. The statistical significance (Sig) level of all tests was set to P=0.05.

Stress	Treatment	Injection Dose vs Dose		Corticosterone (ng/ml) F	Sig	Microbicidal activity F	Sig	Lysis scores F	Sig
BL	Mitotane	CON	LO	0.832	.34	0.818	.25	0.020	.86
		CON	HI		.24		.89		.98
		LO	HI		.82		.31		.88
	Vehicle	CON	LO	0.184	.90	2.313	.99	0.102	.66
		CON	HI		.66		.08		.86
		LOW	HI		.57		.08		.79
SI	Mitotane	CON	LO	79.814	**.00**	27.892	**.00**	1.399	.16
		CON	HI		**.00**		**.00**		.16
		LO	HI		**.00**		.62		.99
	Vehicle	CON	LO	16.263	**.05**	0.421	.56	0.030	.84
		CON	HI		**.00**		.76		.83
		LO	HI		**.00**		.38		.99

Figure 4.1. Baseline (BL) and stress-induced (SI) plasma CORT levels in mitotane- and vehicle-treated birds which were administered exogenous CORT. Exogenous CORT was injected at three doses (CON, LO, HI). Asterisks mark significant differences between BL and SI plasma CORT levels, and how that BL and SI plasma CORT different in all groups except for mitotane-treated birds which were administered a CON dose. Among vehicle-treated birds, each exogenous CORT dose elevated SI plasma CORT to a significantly different level, which is marked by different capital letters. Among mitotane-treated birds, each exogenous CORT dose elevated SI plasma CORT to a significantly different level, which is marked by different lowercase letters. Among birds which have received the same dose of exogenous CORT, stress-induced levels were significantly higher in vehicle-treated birds than in mitotane-treated birds and this effect is indicated by the difference in the capitalization of the same letter. The statistical significance level of all tests was set to P=0.05.

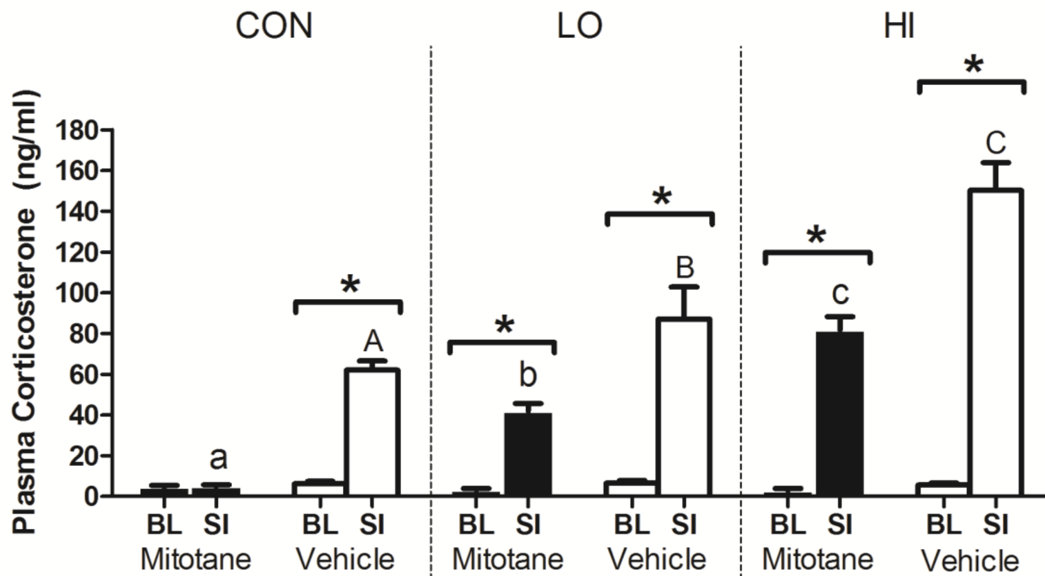


Figure 4.2. Mitotane treatment mitigated stress-induced suppression of microbicidal activity (*top*) and lysis scores (*bottom*) and administration of exogenous CORT restored this interaction. Exogenous CORT was injected at three doses (CON, LO, HI). Asterisks mark significant differences between BL and SI microbicidal activity (*top*) and lysis scores (*bottom*). Baseline (BL) and stress-induced (SI) microbicidal activity and lysis scores were similar in mitotane-treated birds administered CON doses, but SI microbicidal activity and lysis scores were lower than their respective BL values for all other groups. The statistical significance level of all tests was set to P=0.05.

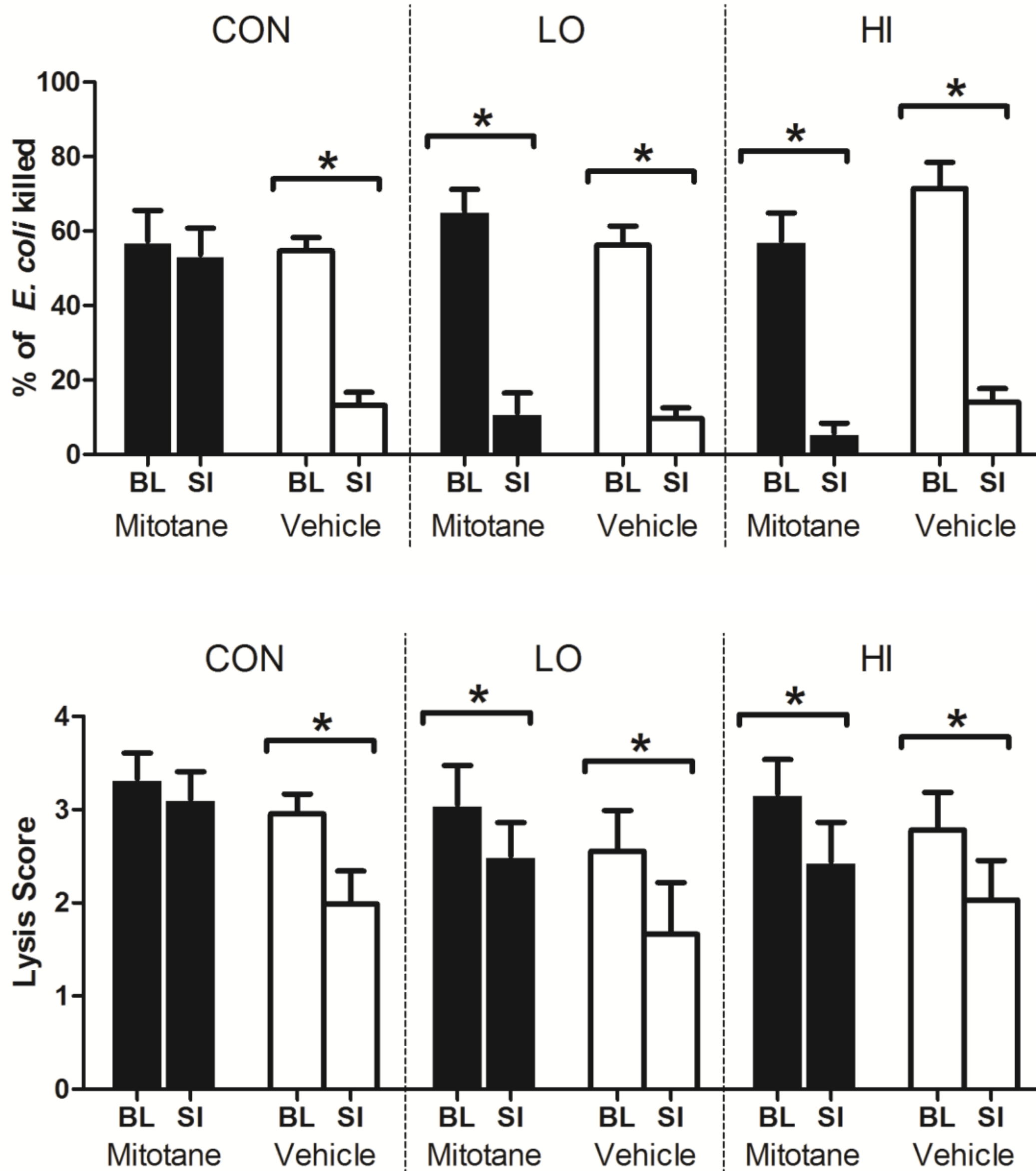
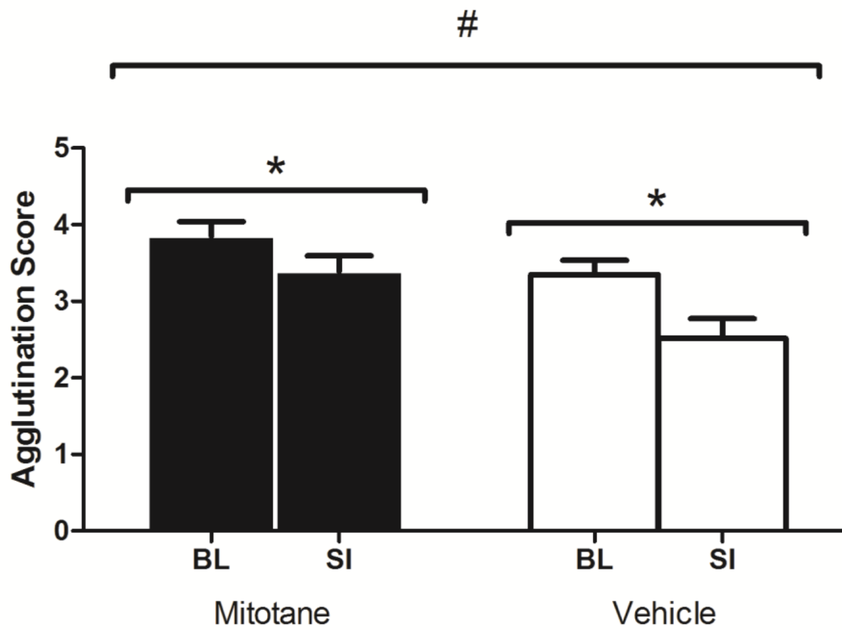


Figure 4.3. Stress-induced (SI) agglutination scores were significantly lower than baseline (BL) agglutination scores in both mitotane- and vehicle-treated birds, and agglutination scores were greater in mitotane-treated birds when compared to vehicle-treated birds. A significant difference between BL and SI agglutination scores is marked with a * while the significant difference of agglutination scores between mitotane- and vehicle-treated birds is marked with #. The statistical significance level of all tests was set to P=0.05.



CHAPTER FIVE

Conclusions and Future Directions

Stress-induced effects on innate immune activity have been observed in wild birds, but the nature of these results has been conflicting. Some studies have reported stress-induced immunosuppression (Martin et al., 2005; Matson et al., 2006; Merrill et al., 2012; Zylberberg, 2015; Davies et al., 2016), while other have observed stress-induced immunoenhancement (Millet et al., 2007; Zylberberg, 2015). My dissertation demonstrates that stress inhibits innate immune activity in adult male House Sparrows, and that these effects can be induced within a rapid, non-genomic time frame. However, the mechanisms which mediate the interaction between stress and complement-mediated activity differ from those that mediate stress-induced effects on natural antibody-mediated activity.

The inhibitory relationship between stress and complement-mediated activity is consistent, and corticosterone (CORT) is both necessary and sufficient for this interaction. Stress-induced elevation of plasma CORT suppresses complement-mediated activity either through non-genomic mechanisms which induce effects that persist for at least 120 mins, or through both non-genomic and genomic mechanisms. The magnitude of this inhibition, however, does not increase with increasingly high levels of plasma CORT, which suggests that a minimum level of complement-mediated activity is defended. Interestingly, my dissertation indicates that CORT activates neither cytosolic mineralocorticoid receptors (MR) nor glucocorticoid receptors (GR) to inhibit

complement-mediated immunity, which implies that this interaction may be mediated through membrane-bound GR. Involvement of membrane-bound GR during GC-dependent inhibition of immune activity has been implied (Liu et al., 2005; Long et al., 2005), but difficult to prove without the development of an effective antagonist for this receptor. The consistent, inhibitory relationship between elevated plasma CORT levels and complement-mediated activity may reflect the role of glucocorticoids in the cessation of the inflammatory response (Imai et al., 1981; Baschant and Tuckermann, 2010). Activated complement pathways are necessary for the elimination of foreign cells through lysis (Ochsenbein and Zinkernagel, 2000) but also contribute to the inflammatory response (Unsworth, 2008). The predictable, suppressive relationship between elevated plasma CORT and complement-mediated activity during stress suggests that a robust inflammatory response may be maladaptive during the stress response.

When compared to the role of CORT in stress-induced inhibition of complement-mediated activity, the role of CORT in stress-induced inhibition of natural antibody-mediated activity is more puzzling and inconsistent. Elevated, stress-induced levels of plasma CORT can suppress natural antibody-mediated through the activation of the cytosolic GR. However, between years, CORT may vary between acting as primary, necessary mediator of this interaction to a partial mediator of this interaction. This inconsistency may result from the possibility that both CORT-dependent and CORT-independent mechanisms underlie the interaction between stress and natural antibody-mediated immunity. It is not clear as to how or why these mechanisms would vary between years, but changes in the pathogen environment (Martin et al., 2005; Pigeon et

al., 2013; Maina, 2015) and food availability (Bourgeon et al., 2010; Horrocks et al., 2012; Pigeon et al., 2013) can affect regulation of innate immunity. Furthermore, this redundancy suggests that it is crucial to suppress natural antibody-mediated activity during stress. Natural antibodies, which are produced from B1 cells, tag and quarantine "non-self" elements so that other components of the innate immune system, such as complement proteins and macrophages, can eliminate those foreign elements. In other words, natural antibodies are needed to help initiate the immune response against infections. The redundancy of mechanisms which underlie the inhibitory relationship between stress and natural antibody-mediated activity suggests that there is something maladaptive about activating immune activity during the stress response. The cause of this maladaptation is unclear. Activation of the immune response requires protein and increases oxidative damage, and stress-induced suppression of natural antibody-mediated immunity may reflect nutritional constraints and/or a method of limiting tissue damage. Additional studies are needed to explore the factors which influence the relationship between glucocorticoids and natural antibody-mediated immunity during stress in wild birds.

Studies which elucidate the mechanisms that regulate immune activity under stressful circumstances and/or in response to disturbances are needed in birds so that effective programs can be developed to target the mediators of this relationship. For example, there is great interest in exploring methods of improving the health of chickens against common poultry diseases such as Newcastle disease virus as to increase food production (Dawkins, 2017, Rehman et al., 2017; Sharma et al., 2017). Such elucidation is especially

important in studies using urban birds because pathogen transmission, abundance, and prevalence can increase around anthropogenic food sources such as bird feeders and waste disposal sites (Bradley et al., 2007; Giraudeau et al., 2014; Gil and Brumm, 2013; Galbraith et al., 2016). Furthermore, global warming and climate change may alter patterns of pathogen transmission and prevalence (Liao et al., 2017), and birds are excellent hosts for zoonotic pathogens (Komar et al., 2003; Del Amo, 2014; Borges et al., 2017). For example, House Sparrows are competent, amplifying hosts for both the L1 and L2 strains of West Nile Virus (Del Amo, 2014; Nemeth et al., 2009), capable of transmitting the virus to mosquitos during viremia or to other animals when the bird is consumed (Nemeth et al., 2009). Decreased immunocompetence may increase host competence in birds (Jankowski et al., 2008), and thus increase the persistence and transmission of pathogens.

Additional research, however, is needed to clarify the interaction between stress and immune activity in wild birds. Research aimed that improving avian health under stressful conditions, such as in poultry sciences or captive breeding programs, should consider identifying the mechanisms which underlie this interaction. Such identification will provide targets for developing treatments that can mitigate stress-induced inhibition of immune activity. Research aimed at improving or predicting avian health under different environmental contexts, such as in the fields of urban ecology and conservation, should consider investigating the environmental variables which change the regulation of avian immune activity.

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APPENDIX A

COAUTHOR APPROVAL

All coauthors of published chapters granted permission to use those chapters in this dissertation.

APPENDIX B

APPROVAL DOCUMENTATION FROM UNIVERSITY INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE

Institutional Animal Care and Use Committee (IACUC)
Office of Research Integrity and Assurance
Arizona State University
660 South Mill Avenue, Suite 315
Tempe, Arizona 85287-6111
Phone: (480) 965-4387 *FAX:* (480) 965-7772

Animal Protocol Review

ASU Protocol Number:	**15-1414R Amendment #5**
Protocol Title:	**Environmental control of reproduction and immunity**
Principal Investigator:	**Pierre Deviche**
Date of Action:	**2/25/2016**

The animal protocol review was considered by the Committee and the following decisions were made:

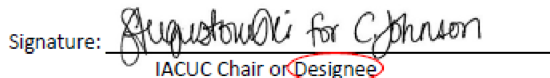
The amendment was approved by Full Committee Review to add additional procedures, 36 birds and Emerald Byakeddy and Piper Benson as additional personnel to the protocol.

If you have not already done so, documentation of Level III Training (i.e., procedure-specific training) will need to be provided to the IACUC office before participants can perform procedures independently. For more information on Level III requirements see https://researchintegrity.asu.edu/training/animals/levelthree.

Total # of Animals:	**151**	
Species:	**Birds**	**Pain Level: C-131; D-20**

Protocol Approval Period: **1/22/2015 – 1/21/2018**

Sponsor:	**N/A**
ASU Proposal/Award #:	**N/A**
Title:	**N/A**

Signature:  Date: 3/9/2016

IACUC Chair or Designee

Cc: IACUC Office
IACUC Chair