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The causative effects of corticosterone on innate immunity during the stress response in the House Sparrow, *Passer domesticus*

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ABSTRACT

Stress-induced inhibition of innate immune activity has been observed in a variety of wild birds and may increase chances of infection because this activity constitutes the first line of defense against pathogens. We previously reported that the transient elevation of plasma corticosterone (CORT; the primary avian glucocorticoid) that occurs during stress is necessary for stress-induced suppression of natural antibody-mediated, complement-mediated, and bactericidal activity. Here, we further investigated the regulatory role of CORT during this suppression. To this end, we treated House Sparrows (*Passer domesticus*) with mitotane to block endogenous CORT production, administered CORT at one of three doses (HI: 1.34 mg/kg; LO: 1.00 mg/kg; CON: vehicle), and assessed natural antibody-mediated, complement-mediated, and bactericidal activity during acute stress induced by handling and restraint. Mitotane administration eliminated the endogenous plasma CORT increase that normally takes place during stress, and corticosterone treatment increased plasma CORT to levels similar to those measured in intact birds during acute stress. As predicted, mitotane-treated birds receiving CON injections did not exhibit stress-induced suppression of complement-mediated and bactericidal activity, and CORT administration at both LO and HI doses restored this suppression. Contrary to expectations, mitotane-treated birds receiving CON injections demonstrated stress-induced suppression of natural antibody-mediated activity. Furthermore, CORT administration did not influence this parameter. These results suggest that stress inhibits innate immune activity through both CORT-dependent and CORT-independent mechanisms, but the contribution of these mechanisms can vary. This variation may result from effects of environmental factors, the identity and role of which warrant further research.

1. Introduction

The innate immune system serves as the first line of defense against pathogens and is composed of humoral constituents. These constituents consist of 1) natural antibodies, which 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); 2) the complement cascade pathways, which can lyse foreign cells through three mechanisms and can contribute to the induction of inflammation (Ochsenbein and Zinkernagel, 2000; Juul-Madsen et al., 2008; Unsworth, 2008); and 3) circulating antimicrobial peptides (Hellgren and Sheldon, 2011) and acute phase proteins (Chamanza et al., 1999; Zylberberg, 2015), which contribute to the lysing of foreign cells. All three constituents of the innate immune system have been identified in avian plasma. They act to rapidly contain and fight the earliest stages of infections as well as to recruit other components of the immune system to an infected site (Juul-Madsen et al., 2008). Therefore, susceptibility to infections may increase following inhibition of innate immune activity and identifying the factors that influence this activity may help predict when this inhibition will occur.

Acute stress is characterized by rapid activation of the hypothalamic-pituitaryadrenal axis, which leads to a swift (i.e., within minutes) and transient increase in plasma catecholamines and glucocorticoids (Sapolsky et al., 2000; Martin, 2009). This increase inhibits 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). We recently demonstrated that mitotane treatment, which blocks the production of glucocorticoids such as corticosterone (CORT,

Abbreviations: BL, baseline; CORT, corticosterone; CR, corticosteroid receptors; GR, glucocorticoid receptors; MR, mineralocorticoid receptors; BKA, bacterial killing assay

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the primary avian glucocorticoid; Breuner et al., 2000; DuRant et al., 2016; Deviche et al., 2017), prevents stress-induced suppression of humoral innate immune activities (Gao et al., 2016). These results imply that CORT is necessary for stress-induced suppression of innate immune activity. However, to confirm the critical role of CORT in the regulation of stress-induced inhibition of innate immune activity, one must demonstrate that treatment with CORT can restore stress-induced inhibition of innate immune activity when endogenous CORT is depleted.

To this end, we examined the effect of acute stress on innate immune activity in House Sparrows (*Passer domesticus*) treated with mitotane and then injected with CORT to induce stress levels of the steroid. Mitotane treatment and exogenous CORT injections allowed us to investigate the role of CORT independent from other factors on innate immune activity during the stress response. We predicted that, consistent with previous results, mitotane treatment would mitigate stress-induced suppression of all measures of innate immune activity. We also expected that, if CORT is critical to mediating this suppression, administration of the hormone to mitotane-treated birds would restore the inhibitory effect of stress on innate immunity. These predictions were based on studies demonstrating that mitotane treatment mitigates stress-induced suppression of innate immunity in House Sparrows (Gao et al., 2016) and that CORT administration inhibits immune activity in wild birds (Martin et al., 2005; Loiseau et al., 2008; Shini et al., 2008; Merrill et al., 2012). Our results strongly suggest that elevated plasma CORT during stress plays an essential role in stress-induced suppression of complement-mediated and bactericidal activity, but, surprisingly, does not affect natural antibody-mediated immunity. This finding differs from those in Gao et al. (2016) despite both studies using reproductively-ready male House Sparrows of similar body masses and obtained from the same population.

2. Materials and methods

2.1. Study species and housing

Thirty adult male House Sparrows were captured with mist-nets and ground traps in late March 2016 in Phoenix, Arizona, USA (33.4° N, 111.6° W; 331 m.a.s.L.). We confirmed the reproductive condition of all birds by their black beaks (Barfuss and Ellis, 1971). Upon capture, we measured the body mass of all birds as in Gao et al. (2016). We then transported birds to Arizona State University's Animal Care Facilities and housed them in two identical rooms. Sparrows were housed individually, visually isolated from each other, 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. Three birds died during this adjustment period.

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.

2.2. Experimental design

We randomly divided birds into three groups (n = 9 per group): high CORT (HI), low CORT (LO), and controls (CON). The experiment consisted of two stress trials as previously described (Gao et al., 2016; Fig. 1). For each bird, 12 days separated the two trials because the effects of one mitotane injection on plasma CORT persisted for 10 days in House Sparrows (Breuner et al., 2000). 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. Experimental stress was induced by restraint following Remage-Healey and Romero (2001) and Loiseau et al. (2008).

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 suppresses plasma CORT within 36 h 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), restrained the bird in a breathable cloth bag for one hour, and collected a second (stress-induced) blood sample before returning the bird to its home cage. Blood was collected from the jugular vein with a heparinized microsyringe and was obtained within 3 min of catching the bird from its cage, and within 5 min of entering the room. Because of this time constraint, only two birds from each room were tested per day and each stress trial was conducted over the course of seven consecutive days. The order of sampling was randomized for the first stress trial but due to the 10-day effect of a single mitotane injection, the order was retained for the second stress trial. The volume of blood collected (220 µl per sample; 440 µl per bird during each trial) was based on the minimal amount of plasma needed to measure plasma CORT and assess immune parameters (see below), and is not expected to influence immune measures (Buehler et al., 2008; Gao et al., 2016). Blood samples were held on ice before being centrifuged within four hours of collection. Plasma was harvested and stored at −80 °C until assayed.

2.3. Mitotane solution and treatment

Mitotane inhibits CORT production in mammals (Maher et al., 1992; Martz and Straw, 1977) and birds (Jonsson et al., 1994) by associating with P-450 cytochromes to inhibit the adrenocortical synthesis of steroid hormones (Young et al, 1973). Importantly, 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; DuRant et al., 2016; Gao et al., 2016). We are not aware of any direct CORT-independent effects of mitotane on immune activity.

We prepared and administered mitotane 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). 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, we 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.

2.4. Corticosterone solutions and treatment

In a pilot study, an injection of 1.34 mg/kg 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, we 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 at 4 °C before use.

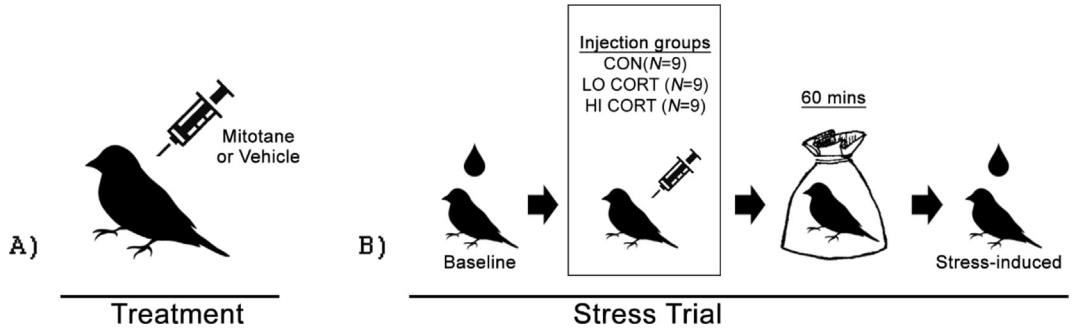

Fig. 1. Experimental design of the stress trials. Twenty-seven House Sparrows were divided into three groups (N = 9 each), in which each received either control (CON), low (LO), or high (HI) doses of corticosterone treatment. A) Each bird was administered either mitotane or vehicle solution prior to the stress trial. B) We collected a baseline blood sample (blood drop), injected the bird with the CORT dose of the bird's group, restrained all birds in a breathable cloth bag for 60 min, and collected a stress-induced blood sample (blood drop). Each bird then rested for at least 10 days, received the opposite treatment (A), and underwent a second stress trial (B) with the CORT dose of the bird's group.

2.5. Corticosterone assays

We 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 included all four samples from the same bird. Prior to the assay, plasma was diluted 15× 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).

2.6. Measurements of immune activity

A hemolysis-hemagglutination assay was conducted to measure complement-mediated and natural antibody-mediated activity, respectively. Our protocol is identical to previously reported methods (Gao et al., 2016; Gao and Deviche, 2018) and similar to that used by Matson et al. (2005). Briefly, samples were serially diluted with 0.9% phosphate buffered solution (PBS), and 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). Wells were scored by an individual without knowledge of the experimental treatments and all plates were scored in one sitting to ensure consistency. High scores indicated high agglutination and lytic activities.

A bacterial killing assay (BKA) was conducted to assess bactericidal activity against *Escherichia coli* (*E. coli*; ATCC NO. 8739). Non-cellular bactericidal activity is primarily mediated through antimicrobial peptides, acute phase proteins, and complement (Juul-Madsen et al., 2008). We used a protocol that was developed by French and Neuman-Lee (2012) and slightly modified for House Sparrow plasma (Gao et al., 2016). House Sparrow plasma was obtained after cellular components were removed through centrifugation, and thus retained all non-cellular components. 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, we prepared a working solution of 10^5 CFU from the stock solution. For each well, we 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, 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. All plates were incubated at 37 °C for 12 h. All readings were obtained with a microplate reader (Multiskan GO, Thermo Scientific, Waltham, MA, USA). We averaged the positive controls and the triplicates for each sample and calculated the percentage of bacteria killed, which represented the bactericidal activity, for each plasma sample as described by French and Neuman-Lee (2012) and Gao et al. (2016).

2.7. Statistics

We performed statistical analyses 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). To account for the potential influence of captivity duration on measurements, we used paired Student's t-tests with Bonferroni adjustments to compare BL hormone and immune measures from stress trials 1 and 2. Because we collected four blood samples from each bird over the duration of the two stress trials, we then used a three-way repeated-measures analysis of variance (ANOVA) to determine 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 or to P = 0.0125 when a Bonferroni adjustment was used.

3. Results

3.1. 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 1). Baseline and stress-induced plasma CORT did not differ in mitotane-treated birds receiving a CON injection (Fig. 2), but differed in birds belonging to the other groups (Table 2). Corticosterone administration increased stress-induced plasma CORT in a dose-related manner (HI > LO > CON) in both mitotane- and vehicle-treated birds (Table 3; Fig. 2; Supplemental Fig. 1). Overall, mitotane-treated birds had lower BL plasma CORT than vehicle-treated birds (Table 4; Supplemental Fig. 1). Duration of captivity did not influence BL plasma CORT ($t_{26} = -1.202$; P = 0.240).

3.2. 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 bactericidal activity (Table 1). Baseline and stress-induced bactericidal activity did not differ in

Table 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 (bactericidal activity), lysis scores, and agglutination scores in male House Sparrows. The statistical significance level of all tests was set to $p = 0.05$, and significant effects/interactions were bolded. Significant three-way interactions were observed for the three independent variables and post-hoc simple effect tests were further conducted (see Tables 2–4).

	Corticosterone	Bactericidal activity	Lysis Scores	Agglutination Scores
Treatment	$F_{1,24} = 55.49$ **$p < 0.01$**	$F_{1,24} = 1.18$ $p = 0.29$	$F_{1,24} = 7.21$ **$p = 0.01$**	$F_{1,24} = 16.32$ **$p < 0.01$**
Stress	$F_{1,24} = 309.51$ **$p < 0.01$**	$F_{1,24} = 342.44$ **$p < 0.01$**	$F_{1,24} = 33.23$ **$p < 0.01$**	$F_{1,24} = 35.66$ **$p < 0.01$**
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 × 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 × Injection	$F_{2,24} = 26.00$ **$p < 0.01$**	$F_{2,24} = 19.79$ **$p < 0.01$**	$F_{2,24} = 0.43$ $p = 0.66$	$F_{2,24} = 1.11$ $p = 0.35$
Treatment × 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 × Stress × Injection	$F_{2,24} = 11.25$ **$p < 0.01$**	$F_{2,24} = 18.85$ **$p < 0.01$**	$F_{2,24} = 3.76$ **$p = 0.04$**	$F_{2,24} = 3.08$ $p = 0.06$

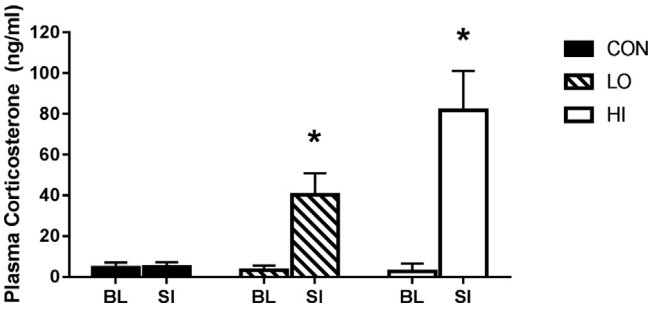

Fig. 2. Baseline (BL) and stress-induced (SI) plasma CORT in mitotane-treated birds after CORT injection. Corticosterone was injected at three doses (CON, LO, HI). Asterisks mark a significantly different SI plasma corticosterone concentration relative to BL. The statistical significance level of all tests was set to p = 0.05 and error bars show s.e.m.

Table 2
Differences between BL and stress-induced (SI) plasma corticosterone, percent of bacteria killed (bactericidal 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 $p = 0.05$, and significant effects were bolded.

Injection	Treatment	Corticosterone BL vs SI	Bactericidal 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	$F_{1,24} = 47.97$ **$p < 0.01$**	$F_{1,24} = 80.62$ **$p < 0.01$**	$F_{1,24} = 11.78$ **$p < 0.01$**
LO	Mitotane	$F_{1,24} = 79.12$ **$p < 0.01$**	$F_{1,24} = 115.02$ **$p < 0.01$**	$F_{1,24} = 13.91$ **$p < 0.01$**
	Vehicle	$F_{1,24} = 65.01$ **$p < 0.01$**	$F_{1,24} = 92.64$ **$p < 0.01$**	$F_{1,24} = 5.17$ **$p = 0.03$**
HI	Mitotane	$F_{1,24} = 165.50$ **$p < 0.01$**	$F_{1,24} = 93.06$ **$p < 0.01$**	$F_{1,24} = 17.56$ **$p < 0.01$**
	Vehicle	$F_{1,24} = 126.12$ **$p < 0.01$**	$F_{1,24} = 129.75$ **$p < 0.01$**	$F_{1,24} = 7.48$ **$p = 0.01$**

mitotane-treated birds given a CON injection, but differed in birds belonging to the other groups (Table 2; Fig. 3A; Supplemental Fig. 2). Among mitotane-treated birds, stress-induced bactericidal activity was higher 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 3; Fig. 3A). 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 1). Baseline and stress-induced lysis scores did not differ in mitotane-treated birds administered CON injections but differed in birds from all other groups (Table 2; Fig. 3B; Supplemental Fig. 2). In mitotane-treated birds administered LO or HI injections, stress-induced lysis scores were significantly lower when compared to BL scores (Table 2; Fig. 3B). Duration of captivity did not significantly affect BL lysis scores ($t_{26} = 0.222$; P = 0.826).

Only treatment and experimental stress affected agglutination scores while injection type did not have a significant effect (Table 1). Overall, regardless of the mitotane/vehicle treatment or injection dose, agglutination scores significantly decreased after experimental stress (Fig. 3C; Supplemental Fig. 3). Interestingly, both BL and stress-induced agglutination scores were higher in mitotane-treated birds than in vehicle-treated birds regardless of injection (Supplemental Fig. 3). Duration of captivity did not affect BL agglutination scores ($t_{26} = 0.404$; P = 0.689).

4. Discussion

We investigated the role of CORT in stress-induced inhibition of innate immunity by characterizing the causative relationship between elevated plasma CORT and innate immunity. For this, we used an experimental protocol that allowed us to study the role of CORT on innate immunity independent of other components of the stress response. Specifically, we administered mitotane to inhibit endogenous CORT production at baseline and during stress, and then treated mitotane-administered birds with CORT at two doses (LO or HI) in order to restore plasma CORT levels. Mitotane-treated birds had induced CORT levels that were within physiological range after injection of the LO CORT dose, and slightly above this range after injection of the HI CORT dose (Romero et al., 2006). Consistent with previous results, we observed stress-induced inhibition of innate immune activity after one hour of experimental stress, and mitotane treatment attenuated stress-induced inhibition of complement-mediated and bactericidal activity (Gao et al., 2016). Importantly, injection of CORT at the low dose restored stress-induced suppression of complement-mediated and bactericidal activity in mitotane-treated birds, thus demonstrating an essential role for elevated plasma CORT in suppressing these components of innate immune activity. The magnitude of suppression of lysis scores and percentage of bacteria killed did not increase with increasing levels

Table 3
Differences in plasma corticosterone, percent of bacteria killed (bactericidal 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 level of all tests was set to $p = 0.05$, and significant effects were bolded.

Stress	Treatment	Injection Dose vs Dose		Corticosterone		Bactericidal Activity		Lysis Scores	
				$F_{2,24}$	P	$F_{2,24}$	P	$F_{2,24}$	P
BL	Mitotane	CON	LO	0.83	0.34	0.82	0.25	0.02	0.86
		CON	HI		0.24		0.89		0.98
		LO	HI		0.82		0.31		0.88
	Vehicle	CON	LO	0.18	0.90	2.31	0.99	0.10	0.66
		CON	HI		0.66		0.08		0.86
		LOW	HI		0.57		0.08		0.79
SI	Mitotane	CON	LO	79.81	**< 0.01**	27.89	**< 0.01**	1.40	0.16
		CON	HI		**< 0.01**		**< 0.01**		0.16
		LO	HI		**< 0.01**		0.62		0.99
	Vehicle	CON	LO	16.26	**0.05**	0.42	0.56	0.03	0.84
		CON	HI		**< 0.01**		0.76		0.83
		LO	HI		**< 0.01**		0.38		0.99

Table 4
Differences in plasma corticosterone, percent of bacteria killed (bactericidal 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 $p = 0.05$, and significant effects were bolded.

Injection	Stress	Corticosterone Mitotane vs Vehicle	Bactericidal 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	$F_{1,24} = 159.76$ **$p < 0.01$**	$F_{1,24} = 52.81$ **$p < 0.01$**	$F_{1,24} = 13.25$ **$p < 0.01$**
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	$F_{1,24} = 24.00$ **$p < 0.01$**	$F_{1,24} = 0.10$ $p = 0.76$	$F_{1,24} = 1.71$ $p = 0.20$
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	$F_{1,24} = 11.68$ **$p < 0.01$**	$F_{1,24} = 3.39$ $p = 0.08$	$F_{1,24} = 1.63$ $p = 0.21$

of stress-induced plasma CORT, suggesting that the LO dose sufficed to elicit maximal suppression and that birds defended a baseline level of complement-mediated and bactericidal activity. We also found that all birds, regardless of mitotane treatment, exhibited stress-induced inhibition of natural antibody-mediated activity, this suggesting that plasma CORT does not have an essential role in regulating natural antibody-mediated activity during stress. This differs from results reported by Gao et al. (2016), despite the fact that House Sparrows in both studies had similar body masses and came from the same population. Interestingly, our present study reveals that natural antibody-mediated activity was stronger in mitotane-treated than vehicle-treated birds, suggesting CORT to be mildly suppressive on natural antibody-mediated immunity, regardless of stress.

4.1. Elevated corticosterone plays an essential role in stress-induced inhibition of complement-mediated and bactericidal activity

Stress inhibits complement-mediated and bactericidal 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 our previous work (Gao et al., 2016; Gao and Deviche, 2018), demonstrates that CORT plays an essential role in this suppression, as CORT administration to mitotane-treated birds sufficed to restore this relationship. This conclusion is consistent with the results of studies showing that CORT treatment can inhibit complement-mediated (Brown-headed Cowbird, *Molothrus ater*, Merrill et al., 2012) and bactericidal activity (Crimson-backed Tanager, *Ramphocelus dimidiatus*, Matson et al., 2006; Clay-colored Thrush, *Turdus grayi*, Millet et al., 2007). Treatment with the LO and HI doses of CORT exerted similar effects on complement-mediated and bactericidal activity, suggesting that the LO CORT dose was able to exert a maximum inhibitory effect on these two components of innate immune activity. The complement system, acute phase proteins, and antimicrobial peptides are essential for the clearance of pathogens, contributing to the inflammatory response and the recruitment of additional constituents from both the innate and adaptive immune systems (Ochsenbein and Zinkernagel, 2000; Juul-Madsen et al., 2008). Our results, therefore, suggest that birds defend a minimum level of complement-mediated and antibacterial activity irrespective of their plasma CORT levels.

The mechanism by which CORT inhibits complement-mediated and bactericidal activity is speculative. High glucocorticoid levels can disrupt complement cascade pathways (Imai et al., 1981; Schumer et al., 1974; Dauchel et al., 1990). In birds, low (i.e., at baseline) plasma CORT acts through cytosolic mineralocorticoid receptors (MR) whereas elevated plasma CORT, as is the case during stress, induces effects by activating cytosolic glucocorticoid receptors (GR) or membrane-bound corticosteroid receptors (CR; Moore and Orchinik, 1994; Breuner and Orchinik, 2009; Groeneweg et al., 2012). Here we observed that mitotane treatment decreased BL plasma CORT but not BL lysis scores or bactericidal activity. Thus, and consistent with previous results (Gao et al., 2016), inhibitory actions of CORT on complement-mediated activity may not involve MR. Furthermore, a recent study using the selective GR antagonist mifepristone demonstrated that CORT does not inhibit complement-mediated or bactericidal activity through the activation of cytosolic GR (Gao and Deviche, 2018). Accordingly, this inhibition may result either from CORT binding to the membrane-bound CR or to circulating CORT binding globulin (Strel'chyonok and Avvakumov, 1991), or from the steroid directly interacting with the plasma membrane (Dindia et al., 2012).

Along with our previous results (Gao et al., 2016; Gao and Deviche, 2018), the present findings demonstrate a consistent inhibitory relationship between elevated CORT and complement-mediated and bactericidal activity during stress. This relationship may be attributed to the anti-inflammatory role of glucocorticoids (Laue et al., 1988; Baschant and Tuckermann, 2010) because activated complement pathways, acute phase proteins, and antimicrobial peptides all contribute to the inflammatory response (Unsworth, 2008; Juul-Madsen et al., 2008). Thus, stress may be obligatorily associated with

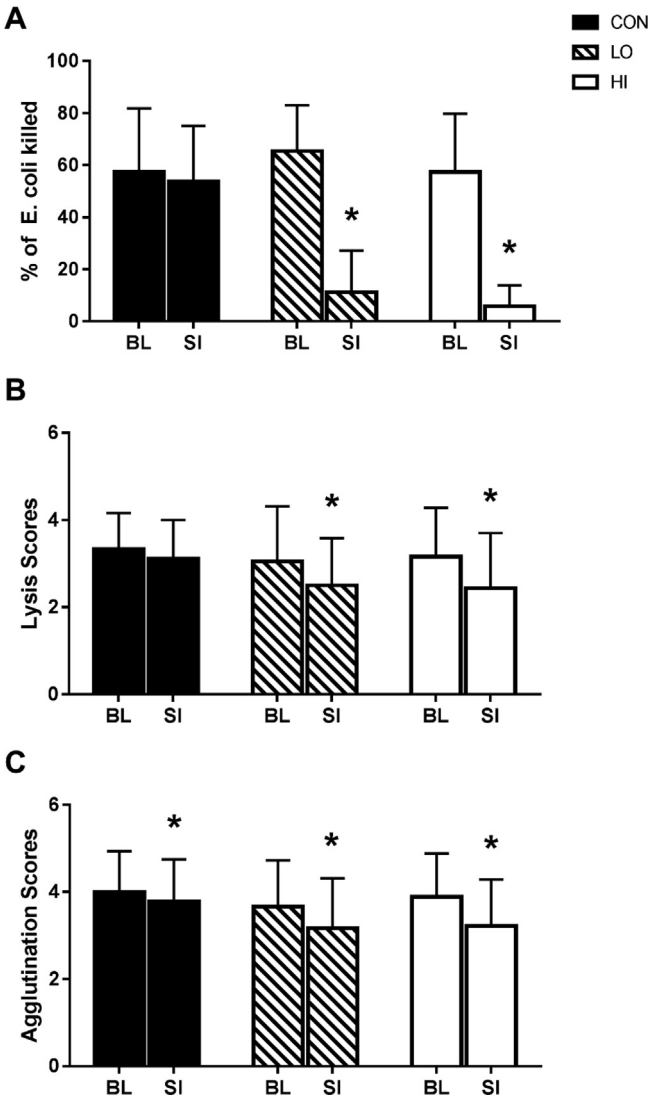

Fig. 3. The effect of corticosterone (CORT) injections on three measures of immune activity in mitotane-treated birds. Corticosterone was injected at three doses (CON, LO, HI). Mitotane-treated birds injected with vehicle (CON) showed mitigated stress-induced suppression of bactericidal activity (A) and lysis scores (B), but not agglutination scores (C). Both LO and HI CORT doses restored stress-induced suppression of bactericidal activity (A) and lysis scores (B). Experimental stress induced suppression of agglutination scores occurred regardless of CORT treatment. The statistical significance level of all tests was set to $p = 0.05$ and error bars show s.e.m.

suppression of the inflammatory response.

4.2. Variation in natural antibody-mediated activity during stress

The present results show that stress inhibits natural antibody-mediated immunity, but suggest that the transient elevation of plasma CORT during the stress response either does not mediate or is not the primary mediator of this inhibition. These results are partly consistent with other observations. For example, CORT-independent mechanisms may mediate effects of stress on innate immune activity in the Abert's Towhee (*Melozone aberti*; Davies et al., 2016). These mechanisms are unidentified and may involve catecholamines, which can inhibit immune activity in immature chickens (Denno et al., 1994). Consistent with this observation, norepinephrine can reduce cutaneous immunity through the activation of β-adrenergic receptors in chickens (Brown-Borg et al., 1991).

By contrast, our recent results in House Sparrows suggest that elevated plasma CORT can inhibit natural antibody-mediated activity through GR activation (Gao and Deviche, 2018). Differences between the present study and Gao and Deviche's investigation are probably not due to methodological inconsistencies because both studies used the same species, were performed in the same laboratory, and used the same equipment and similar experimental protocols. It is conceivable that differences between studies result from natural fluctuations in food availability (Horrocks et al., 2012; Pigeon et al., 2013) or the energetic condition of the experimental birds (Bourgeon et al., 2010). Indeed, natural antibody-mediated activity serves as the first step of the humoral immune response (Boes, 2000), the activation of which is highly energetically costly in birds (Hasselquist and Nilsson 2012). However, in skylarks (*Alauda arvensis*), innate immune activity can vary independently of body mass across years (Hegemann et al., 2012). Furthermore, the body mass of sparrows in the present study did not differ from that of birds used by Gao et al. (2016). The relationship between plasma CORT and immune activity may also vary as a function of the composition of environmental pathogen populations (Martin et al., 2005; Maina, 2015). Additional studies are warranted to identify factors that account for intraspecific variations in innate immune activity such as observed here.

The mechanisms that underlie variation of the effect of stress on natural antibody-mediated activity are also unknown. These mechanisms may involve changes in GR and MR densities. Interestingly, the present results, showing that low plasma CORT may exert a mild inhibition of natural antibody-mediated immunity, suggest the involvement of MR rather than GR in this inhibition, but previous studies led to the opposite conclusion (Gao et al., 2016; Gao and Deviche, 2018). House Sparrow GR and MR tissue concentrations vary seasonally (Lattin and Romero, 2015), but it is not known if these densities also vary between years. If so, additional research is also needed to identify the origin of and the mechanisms underlying variation between years in innate immune activity during stress.

4.3. Conclusions

We investigated the role of CORT during stress-induced suppression of complement- and natural antibody-mediated as well as bactericidal activity in House Sparrows. Administration of CORT restored stress-induced suppression of complement-mediated and bactericidal activity in mitotane-treated birds. Furthermore, the magnitude of this suppression did not increase with increasing doses of CORT. These results demonstrate that CORT plays a primary role in stress-induced suppression of complement-mediated and bactericidal activity, and that birds may defend a minimal level of complement-mediated and bactericidal activity during stress.

On the other hand, our results indicate that stress-induced inhibition of natural antibody-mediated immunity during stress can occur independently of plasma CORT. This conclusion differs from that reached in Gao et al. (2016) and Gao and Deviche (2018). Variation in innate immune activity has been reported in wild birds, but the causes and mechanisms that underlie this variation in the present study are unknown. Further studies are warranted to elucidate the mechanisms that mediate interactions between the stress and immune systems as these pathways may identify valuable targets for the fields of conservation and veterinary sciences.

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Declaration of interests

The authors declare no competing interests.

Author Contributions

S.G. and P.D. designed the experiments and drafted the manuscript. S.G. administered all of the treatments and collected blood samples with some help from P.D. S.G. performed the corticosterone and immune assays. S.G. performed all the statistical analyses.

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Appendix A. Supplementary data

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