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PHYSIOLOGICAL ECOLOGY - ORIGINAL RESEARCH

Sex-specific variation in brown-headed cowbird immunity following acute stress: a mechanistic approach

Loren Merrill · Frédéric Angelier · Adrian L. O'Loghlen · Stephen I. Rothstein · John C. Wingfield

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Abstract There is some discrepancy in the literature regarding whether acute stress is immunostimulatory or immunosuppressive. Studies of domesticated (laboratory and food) animals and humans typically indicate that acute stress is immunostimulatory, whereas studies of non-domesticated species document both immunostimulatory and immunosuppressive results. Few studies have examined the mechanisms responsible for changes in immune activity in species other than those classically used in laboratory research. We examined the effect of both acute stress and exogenous corticosterone (CORT) on the bactericidal capacity (BC) of blood plasma from captive, wild-caught brown-headed cowbirds (*Molothrus ater*) to determine if CORT is responsible for changes in levels of immune activity. We conducted "stress tests" in which we handled birds to elicit a stress response and then measured the birds' total CORT and BC at 30 or 90 min post-stressor. We also conducted non-invasive tests in which we administered exogenous CORT by injecting it into mealworms that were fed to the cowbirds remotely. Total, free, and bound CORT levels, corticosteroid binding globulins (CBGs), and BC at 7 or 90 min post-mealworm ingestion were measured. Both males and females exhibited significant increases in total CORT following handling stress and the administration of exogenous CORT. Experimental males and females also exhibited a significant increase in CBG capacity at 7 min post-mealworm ingestion compared to controls. Male cowbirds exhibited a significant decline in their BC following both handling stress and the administration of exogenous CORT whereas female cowbirds exhibited no decline under either condition. Female CBG levels were not different than those of males, suggesting that differences in BC could be due to differences between the sexes in the number of corticosteroid receptors which, along with CBGs, regulate the stress response. Female cowbirds may modulate their stress response as an adaptive life-history strategy for maximizing current reproduction.

Keywords Corticosterone · Bactericidal capacity · Corticosteroid binding globulin · Sex differences · Life-history strategy

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L. Merrill (✉) · A. L. O'Loghlen · S. I. Rothstein
Department of Ecology, Evolution and Marine Biology, University of California, Santa Barbara, Santa Barbara, CA 93106, USA
e-mail: lmerrill@lifesci.ucsb.edu

F. Angelier · J. C. Wingfield
Department of Neurobiology, Physiology and Behavior, University of California, Davis, CA 95616, USA

F. Angelier
Centre d'Etudes Biologiques de Chizé, CNRS, 79360 Villiers en Bois, France

Introduction

The vertebrate stress and immune responses are dynamic processes that, when activated, require significant coordination, and sometimes trade-offs, between different components of an organism's life history (Dhabhar 2009; Martin 2009). The magnitude of an organism's stress response and the strength/efficacy of its immune response depend on a combination of intrinsic and extrinsic factors (Lubach et al. 1995; Angelier et al. 2009; Mougeot et al. 2009; van de Crommenacker et al. 2010). Not surprisingly, stress and immune function are tightly linked (Biondi and

Zannino 1997; Maier and Watkins 1998), but our understanding of how and why remains tenuous, especially for non-laboratory and non-food animals (Dhabhar 2009; Martin 2009).

Much of the work examining the effects of stress on immunity in wild and domesticated animals has focused on moderate- to long-term stressors (hours–days/weeks), which typically result in suppression of the immune system (Dhabhar and McEwen 1997; Shini et al. 2010). In contrast, studies examining the effects of acute stress on immunity tend to find the opposite effect (Dhabhar and McEwen 1997; Sapolsky et al. 2000; Dhabhar 2000; reviewed in Martin 2009). In general, researchers have found that acute stress causes a rapid reduction in the number of circulating lymphocytes concurrent with a rapid increase in the number of circulating neutrophils (or heterophils in birds) (Apanius 1998; Shini et al. 2010; Tilgar et al. 2010). Neutrophils/heterophils act as sentinels, and an increase in the number of these cells in circulation may aid in immune surveillance capabilities (Shini et al. 2010). The lymphocytes are redistributed out of circulation to organs such as lymph nodes, the skin, and other "battle stations" where they are likely to encounter antigens, pathogens, and activated immune cells (Apanius 1998). If organisms are likely to sustain wounds during a stressful encounter with a rival or a predator, the redistribution of cell types may facilitate a rapid response (Dhabhar and McEwen 1997). Along these lines, acute stress has been documented to enhance skin immunity (Dhabhar and McEwen 1997).

However, the few studies that have examined the effects of acute stress on immunity in non-domesticated birds documented both positive (e.g., Buehler et al. 2008) and negative effects of stress on levels of immune activity (e.g., Davis 2005; Matson et al. 2006; Millet et al. 2007; Berzins et al. 2008; Kuhlman and Martin 2010). Matson et al. (2006) examined the effects of handling stress on the bactericidal capacity (BC) of both whole blood and plasma against *Escherichia coli* for five species of tropical birds and found that plasma was as effective as whole blood at killing *E. coli*, and that three of the species exhibited a significant decrease in BC, while the other two showed no change. Millet et al. (2007) also examined the BC of whole blood against *E. coli* and *Staphylococcus aureus* in wild birds and found that prolonged handling significantly decreased the BC against both. They also reported a significant negative correlation between the glucocorticoid (GC) hormone corticosterone (CORT) and BC against *E. coli*. Buehler et al. (2008) conducted a similar experiment using red knots (*Calidris canutus*) and examined the BC of whole blood against *E. coli* and *S. aureus* as well as antifungal capacity against *Candida albicans*. In the same study, these authors also measured circulating leukocyte numbers, complement, and natural antibody levels in the red knots. They found no effect of stress on the levels of monocytes, complement, and natural antibodies or on the killing of *E. coli*, but there was a positive effect on the killing of *S. aureus* and *C. albicans*. Based on these findings, Buehler et al. (2008) propose that the discrepancy between their results regarding the killing of *S. aureus* and *C. albicans* and those of Millet et al. (2007) lies in the fact that Millet et al. (2007) removed heterophils from the whole blood samples before assaying, whereas Buehler et al. (2008) did not. Janeway et al. (2004) found that heterophils are the most abundant phagocytic cells in birds and may therefore be responsible for most of the killing of *S. aureus* and *C. albicans*. Species-level differences in complement activity or activation of the stress response could explain the disparity between birds that exhibited a decrease in *E. coli* killing and those that showed no change following acute stress. Matson et al. (2006) documented such species-level differences in BC in response to handling stress and suggested that pace of life may determine the investment in BC—species with a slow pace of life tend to have stronger BC than those with a faster pace of life (Tieleman et al. 2005). In addition, differences between studies in the manner in which birds were captured (e.g., from an aviary with hand nets vs. in the wild with mist nets) and stressed (e.g., in a box vs. in a bag) could have potentially affected the results (Matson et al. 2006; Millet et al. 2007; Buehler et al. 2008).

In an attempt to better understand the relationship between acute stress and constitutive innate immunity and, in particular, the role that CORT plays on the BC of the blood, we conducted a series of tests in which we: (1) exposed brown-headed cowbirds (*Molothrus ater*; hereafter cowbird) to an acute stress event, or (2) experimentally manipulated CORT levels in the birds via the non-invasive administration of exogenous CORT independent of a stress event. In addition to documenting changes in BC and CORT, we also measured corticosteroid binding globulins (CBGs). Corticosteroid binding globulins are glycosylated globulins present in the plasma that bind steroid hormones, including CORT, with high affinity (Breuner and Orchinik 2002) and are important mediators of the stress response (Breuner et al. 2003, 2006; Lynn et al. 2003). Circulating CORT is therefore either in a bound or unbound ("free") state. In most species, the majority of CORT in circulation is bound to CBGs (Malisch and Breuner 2010) which act as carrier and storage molecules and possibly play a buffering role as CORT levels rise. Increases or decreases of CBG can allow for changes in free CORT levels under static total CORT conditions, and there is evidence that some animals do modulate free CORT levels in this manner (e.g., Lynn et al. 2003; reviewed in Malisch and Breuner 2010). There is also increasing evidence that CBG is involved in more than just buffering however. In mammals, CBG-

bound CORT can be transported to sites of inflammation and then cleaved from the CBG by serine proteases secreted by activated neutrophils, causing a localized increase in free CORT (Pemberton et al. 1988). This function allows elevated levels of free CORT to be targeted to specific tissues rather than requiring system-wide increases in free CORT which could trigger a suite of other physiological changes. In addition, CBG itself can bind to membrane receptors and activate intracellular second-messenger systems (Nakhla et al. 1988; Strelchyonok and Avvakumov 1991). It is still unclear which fraction of CORT is most biologically relevant, and as such it is important to examine both free and total CORT levels (Malisch and Breuner 2010).

For our study we used a group of captive, wild-caught cowbirds housed at an outdoor aviary. We then examined the effect of the different treatments on the birds' constitutive innate immune systems using the BC of blood plasma against *E. coli* as our measure of immune function. The bacteria-killing assay we use provides an easily quantified and interpreted measure of the bactericidal capacity of a bird's plasma—the higher the in vitro bacteria-killing in a bird, the better able that bird should be to clear a bacterial infection (Tieleman et al. 2005; Matson et al. 2006). Bacteria-killing in this assay appears to be primarily complement dependent, as inactivation of complement via heat treatment stops the killing of the *E. coli* (Matson et al. 2005; Merrill, unpublished data). Complement proteins are important to the first line of defenses as they can opsonize or directly lyse invading cells (Esser 1994). They can also target antigen to lymphoid organs and lower the threshold for B-cell activation (Ochsenbein and Zinkernagel 2000), thus linking innate immune responses to the acquired response. The multiple roles that complement plays in fighting an infection makes it a critical component of the immune system (Matson et al. 2006).

The results of studies examining complement activity, either directly or via the *E. coli* killing assay, suggest that complement is very sensitive to acute stress (but see Buehler et al. 2008). The direction of change is not uniform, however, as complement activity in the circulation was found to increase in people following acute stress (Burns et al. 2008) but decrease in birds (Matson et al. 2006; Millet et al. 2007). Additionally, Burns et al. (2008) documented sex-specific differences in which women exhibit greater increases in complement cascade reactivity than men during the post-stress recovery phase (30 and 60 min post-stressor). However, none of the studies examining the response of the BC of whole blood or plasma to acute stress in birds documented a sex-specific response (Matson et al. 2006; Millet et al. 2007; Buehler et al. 2008). We studied the effect of acute stress and exogenous CORT on the plasma BC of adult male and adult female cowbirds to determine if plasma BC against *E. coli* responds differently to handling stress versus ingestion of exogenous CORT and to determine if the sexes exhibit sex-specific responses. Based on the literature wherein the majority of bird species examined exhibited a decrease in *E. coli* killing following acute stress, and there was no reported effect of sex (Matson et al. 2006; Millet et al. 2007), we predicted that acute stress due to both handling and the non-invasive administering of exogenous CORT would negatively affect the plasma BC of both male and female cowbirds.

This is the first study to examine the effects of both short-term handling stress and experimentally elevated CORT via non-invasive, exogenous administering, on immune function in a non-domesticated species. This is also the first study to incorporate both CORT and CBG capacity in an examination of the effects of acute stress on immunity in a non-domesticated species, and the first study of CBG capacity in any cowbird species. By isolating the effects of CORT from those of stress on immunity, our aim was to gain a greater understanding of the role of CORT in mediating how stress affects immune function. This understanding is critical for the proper analysis and interpretation of the variability in stress and immune responses seen in vertebrate animals.

Materials and methods

Study species

Brown-headed cowbirds (*Molothrus ater*) are generalist brood parasites found throughout much of North America and are members of the family Icteridae. They are a primarily granivorous species, but will readily consume insects, especially during the breeding season (Ankney and Scott 1980). Cowbirds are gregarious and will assemble in large mixed-species flocks in the non-breeding season (Payne 1973). During the breeding season mated pairs will loosely defend home breeding ranges but typically congregate at communal feeding sites during the afternoon (Rothstein et al. 1984).

For this study, we used 28 wild-caught, captive cowbirds (16 male and 12 female) from a group of 31 birds housed at the University of California, Santa Barbara aviary. Birds were captured between June and August from three locations in California: the Sierra Nevada near Mammoth Lakes (37°38′N, 118°58′W) (2005), Santa Barbara (34°25′N, 119°41′W) (2006), and Filmore (34°23′N, 118°55′W) (2008). Birds were housed in single sex outdoor cages (approx. 6.0 × 1.2 × 2.7 m) with between three and five total males per cage and seven females per cage. Males were housed at slightly lower densities to keep agonistic

interactions down as they were more aggressive than females (L. Merrill, personal observation). Birds had ad libitum access to food (Mazuri's Small Bird Maintenance Kibble) and water. All birds were adults (>2 years of age) at the time of the experiments and had been in captivity for more than 9 months.

Experimental design

A series of stress tests were conducted in which we elicited a stress response in the birds by capturing and holding them in our hands for 5 min. We used this technique rather than placing them in a bag for 30 or 60 min to examine the effects of a stressor that may more accurately resemble an acute stressor in the wild. Tests were also conducted in which birds received exogenous CORT by eating mealworms that had been injected with the hormone. These tests were designed to assess the effects of CORT alone (i.e., without an associated stressor) on the immune system. The stress tests were run in June 2008, May 2009, and January 2010, and the non-invasive CORT experiments in June 2009 and January/February and July 2010. See Table 1 for details on sample size and dates for each test. We used the same individual birds for the different experiments, but initial sample sizes varied between treatments and tests based on the availability of isolation chambers. All procedures were approved for use by the University of California, Santa Barbara's Institutional Animal Care and Use Committee (IACUC protocol no. 185).

Table 1 Variables measured, date, and number of individuals run (*N*) for each test

Experiment[a]	Session[b]	Variable measured[c]	Date	*N*
Stress test 1	Males	BC at 90 min	15 May 2009	12
Stress test 1	Females	BC at 90 min	17 May 2009	12
Stress test 2	Males	Total CORT at 30 min	21 July 2008	12
Stress test 2	Females	Total CORT at 30 min	26 July 2008	10
Stress test 3	Males	BC at 90 min	22 January 2010	12
Stress test 3	Females	BC at 90 min	25 January 2010	12
CORT 1	Males (A)	Total CORT at 45 min	11 June 2009	12
CORT 1	Females (A)	Total CORT at 45 min	13 June 2009	10
CORT 1	Males (B)	Total CORT at 45 min	18 June 2009	12
CORT 1	Females (B)	Total CORT at 45 min	20 June 2009	10
CORT 2	Males (A)	BC at 90 min	29 January 2010	12
CORT 2	Females (A)	BC at 90 min	1 February 2010	10
CORT 2	Males (B)	BC at 90 min	5 February 2010	12
CORT 2	Females (B)	BC at 90 min	7 February 2010	10
CORT 3	Males (A)	Total, bound, free CORT; BC at 7 min	8 July 2010	4
CORT 3	Females (A)	Total, bound, free CORT; BC at 7 min	6 July 2010	8
CORT 3	Males (A)	Total, bound, free CORT; BC at 7 min	10 July 2010	8
CORT 3	Females (A)	Total, bound, free CORT; BC at 7 min	8 July 2010	4
CORT 3	Males (A)	Total, bound, free CORT; BC at 7 min	12 July 2010	4
CORT 3	Females (B)	Total, bound, free CORT; BC at 7 min	14 July 2010	8
CORT 3	Males (B)	Total, bound, free CORT; BC at 7 min	16 July 2010	4
CORT 3	Females (B)	Total, bound, free CORT; BC at 7 min	16 July 2010	4
CORT 3	Males (B)	Total, bound, free CORT; BC at 7 min	18 July 2010	8
CORT 3	Males (B)	Total, bound, free CORT; BC at 7 min	20 July 2010	4

[a] Experiments labeled Stress test are those in which birds were captured, bled, and handled for a total of 5 min to obtain baseline data. We ran three stress tests, each of which was divided into two "sessions"; one for males and one for females. Experiments labeled CORT are those in which an exogenous supply of corticosterone (CORT) was administered via mealworm without an associated stressor. We ran three CORT experiments, and for each session, half the birds received mealworms with CORT injected into them, and half received control mealworms

[b] The first time a group of birds was run for a given experiment, the session was labeled A. The second time that group was run, individuals that had received a mealworm with CORT received a control mealworm, and vice versa, and this session was labeled B. We ran a maximum of twelve birds per session except for the "CORT 3" experiment in which we ran a maximum of eight birds per session due to space limitations

[c] Variables were measured at approx. 7, 30, 45, or 90 min post-handling (Stress test), or post-mealworm ingestion (CORT). BC, Bactericidal capacity; total, bound, and free CORT refer to total circulating levels of CORT, the bound fraction of CORT in circulation, and the unbound (free) fraction of CORT in circulation, respectively

Stress tests

Birds were captured from the outdoor cages 48 h prior to the start of the experiment, and each bird was placed in a separate cage (46 × 27 × 27 cm). Preliminary studies documented a rapid (<3 h) return to baseline levels of CORT in cowbirds following handling stress (Merrill, unpublished data), and cowbirds adapt rapidly to captivity (Smith and Rothstein 2000); therefore, 48 h was deemed a conservative period of time to acclimate to changed housing. The cages were then transferred to individual sound attenuating isolation chambers (internal dimensions 53 × 28 × 30 cm). Each chamber was fitted with an external fan for air circulation and a 30-cm, 110 V fluorescent light controlled by a photocell switch so that the birds were maintained on the natural photoperiod. The birds continued to have ad lib access to Mazuri's Small Bird Maintenance Kibble and water while in the chambers.

On the second morning after being placed in the chamber, each bird was removed in turn from its cage and bled (see below) within 3 min of opening the chamber door to obtain a blood sample for "pre-stressor" baseline immunity data. The bird was then held so that the time in hand totaled 5 min, after which the subject was returned to its cage, which was placed back in the isolation chamber. Ninety minutes after being returned to its chamber the bird was again taken from its cage and re-bled as before to obtain a "post-stressor" blood sample. During the July 2008 stress tests we took blood samples to measure total CORT levels at baseline and 30 min post-stressor. Due to limitations in the number of females we could use during that period we only had post-stressor data at 30 min for four females and ten males. We failed to collect enough blood within 3 min from two females pre-stressor and one female post-stressor during the May 2009 test, so those samples were not used.

Non-invasive CORT experiment

Breuner et al.'s (1998) methods for experimentally increasing CORT levels in birds without an associated stress event were adopted to isolate the effects of CORT on the birds' BC. As in the stress tests, birds were transferred from the outdoor cages to individual cages, which were then placed in the isolation chambers for 48 h prior to the start of the experiment. The chambers were fitted with QuickCam Pro 4000 USB webcams (Logitech, Fremont, CA) that were attached to a Dell OptiPlex GX520 DT computer (Dell Corp, Round Rock, TX) running Windows XP (Microsoft, Redwood, WA) and using PBcam (J. Burt) video software. This arrangement allowed us to view the birds inside the chambers without disturbing them. Birds were given two mealworms (*Tenebrio molitor*) per day while in the chambers to ensure that the birds would recognize the mealworms as a food source and consume them during the experiment.

On the morning of the second day after the birds had been transferred to the chambers, we placed mealworms in the freezer for 10 min to decrease their mobility. We then injected mealworms with 20 μL of CORT (Sigma, St. Louis, MO) dissolved in dimethyl sulphoxide (DMSO) at a concentration of 0.5 mg CORT per 1 mL of DMSO, or pure DMSO for controls using a 27-gauge needle and 1-mL syringe. Any mealworms that "leaked" solution were discarded. Mealworms were injected immediately prior to the start of the experiment because they became hard and darkened 30–40 min after being injected, and the birds were less likely to eat them. A single mealworm was placed in a 100-mm, opaque petri dish that had a wire attached to its lid and double-sided adhesive tape on its bottom section to ensure it would not move when the cover was removed. The covered dish was then placed inside a bird's cage with the lid wire extending to the outside of the chamber. To do this, LM or an assistant slowly opened the chamber door, gently opened the cage door, and placed the dish inside the cage. This procedure took less than 30 s and often caused no change in the birds' behavior. The birds that did move around settled down as soon as the door was closed. We waited 25 min after closing the chamber door and then pulled gently on the wire to lift the lid and expose the mealworm. We used the webcams to determine when the mealworm was consumed by the test subject. For each session, half of the individuals received the control treatment (DMSO) and half received the experimental treatment (CORT). One week later, birds that had received DMSO initially were given CORT and vice versa. Individuals were given at least 1 week off between bleeding sessions to allow recovery from the stress of blood loss (Piersma et al. 2000).

A total of three non-invasive CORT experiments were conducted with the cowbirds: one in June 2009 to determine total CORT levels at 45 min post-mealworm ingestion, one in January 2010 at 90 min post-mealworm to assess BC, and one in July 2010 at 7 min post-mealworm to measure BC, total CORT, CBG capacity, and free and bound CORT. We used the 7 min time frame for the non-invasive CORT experiment because Breuner et al. (1998) found circulating CORT levels peaked at this interval following the ingestion of CORT-injected mealworms. This peak occurs more rapidly than that from endogenous CORT production, which typically occurs 30–60 min after initiation of a stressor (Wingfield et al. 1998; Sapolsky et al. 2000). We followed the same procedure used in the stress test for blood collection as well as the storage of plasma (see below). In the June 2009 experiment, three experimental females and two experimental males as well

as two control females failed to eat their mealworms. Three of the remaining experimental samples and two of the remaining control samples were not run in the CORT assay due to space limitations. In the January experiment, two experimental males failed to eat their mealworms. For the July experiment, one control male and four experimental males failed to eat their mealworms. We ran CORT for every bird from which we had control and experimental samples and CBG for every bird from which we had sufficient plasma for both control and experimental samples.

Blood collection and storage

Blood was collected from the brachial vein using sterile 25-gauge needles to pierce the vein and 75-mm capillary tubes to draw up the blood. To decrease the chance of contamination, we swabbed the area around the brachial vein with 70% ETOH and allowed the alcohol to evaporate (approx. 10 s) before using the needle. To minimize any potential confounding effects of blood loss on CORT levels and BC, we collected only 75–100 μL of blood from each bird for the stress test and 100–150 μL of blood for the non-invasive CORT test. The blood was placed in the refrigerator until we were able to spin it down and pull off the plasma (within 3 h of collection). We aliquoted 10 μL of plasma into one 1.5-mL sterile microcentrifuge tube for use in the bacteria-killing assay, and the remainder was placed in another 1.5-mL sterile microcentrifuge tube for use in the CORT and CBG assays. All plasma samples were then frozen at −20°C until assayed (Morrison et al. 2009).

Bactericidal capacity

Circulating antimicrobial proteins, such as complement and acute phase proteins, provide an early line of defense against invading bacterial pathogens as part of the constitutive innate immune system. We used a strain of *E. coli* (ATCC no. 8739) for which the degree of killing is primarily dependent upon plasma proteins (Tieleman et al. 2005), and complement in particular (Matson et al. 2005; Merrill, unpublished data). Methods for the assay were derived from Millet et al. (2007) and Morrison et al. (2009). In brief, we added 5 μL of plasma to a combination of CO_2-independent media (Gibco, Invitrogen, Carlsbad, CA) plus 4 mM L-glutamine (90 μL) and bacterial broth (10 μL), incubated the solution for 20 min at 40°C, then pipetted out 50 μL in duplicate onto agar plates which were then incubated at 37°C overnight. The number of colonies was counted the following day and compared to control plates in which the bacterial broth and phosphate-buffered saline (PBS) were incubated together without any plasma. Killing capacity was determined by subtracting the mean number of colonies counted for a bird's two plates from the mean number of control colonies, and then dividing that by the control mean. We used an adjusted BC measure for the 7 min post-mealworm test due to the extreme variation in BC between weeks. Mean BC for the first sessions was significantly lower for both males and females, so we calculated mean killing values for each sex for each of the two sessions and subtracted that value from each individual's BC for that session.

Corticosterone assay

Plasma levels of total CORT were determined by a radioimmunoassay after Wingfield et al. (1992). Tritiated CORT (20 μL) was added to each sample and incubated overnight. Each sample was extracted using dichloromethane for 2 h before removal of the organic layer, dried using liquid nitrogen, and then re-suspended in PBS with gelatin. We assayed all samples in duplicate. The intra-assay coefficient of variation (CV) ranged from 2.7 to 12.5%, and inter-assay CV was 13.6%. Recoveries ranged from 68 to 100%.

Corticosteroid binding globulin assay

Corticosteroid binding globulin capacity was estimated in radioligand binding experiments using previously described methods (Orchinik et al. 2000; Breuner et al. 2003) that were modified and optimized for brown-headed cowbirds. Briefly, plasma was stripped of endogenous steroid with two volumes of dextran-coated charcoal solution (0.1% dextran, 1% Norit A charcoal in 50 mM TRIS). Samples were then centrifuged at 4°C for 10 min at 4,500 rpm. The supernatant was then diluted with 50 mM TRIS buffer in order to reach an optimized plasma dilution for the CBG assay of brown-headed cowbirds. Samples (100 μl) were then incubated in 100 μl of assay buffer with or without unlabeled CORT (100 μl of ^{3}H-CORT, specific activity 88 Ci/mM). Samples incubated with unlabeled CORT (1 μM) allowed us to measure non-specific binding. Free and bound ^{3}H-CORT were separated by rapid filtration of the samples over Whatman GF/B glass fiber filters (Whatman, Maidstone, Kent, UK) previously soaked for 1 h in ice-cold buffer containing 0.3% polyethylenimine. After filtration, the filters were rapidly rinsed with 9 mL ice-cold 25 mM TRIS (three rinses of 3 mL each) and then placed into liquid scintillation glass vials with 4.5 ml of scintillation liquid. Filter-bound radioactivity was quantified by standard liquid scintillation spectroscopy using a β-counter (LS 6500; Beckman Coulter, Fullerton, CA). All samples were assayed in triplicate.

The affinity of CBG for CORT was determined by a homologous competition following the method described

by Wingfield et al. (1984) with minor modifications. Plasma pool samples diluted in 50 mM TRIS were incubated with increasing quantities of unlabeled CORT (0.1–100 ng) and a constant amount (approx. 15,000 cpm) of ^{3}H-CORT for 2 h at 4°C. Separation of bound and free steroid was achieved by rapid vacuum filtration over GF/B glass fiber filters as described in the previous paragraph. It was then possible to calculate the specific binding (nM) and the total amount of CORT of each sample. A non-linear model (ligand-binding model) was fitted to the data of this homologous competition in order to obtain an estimate of the affinity of CBG for CORT in brown-headed cowbirds [model $F_{3,9} = 2686.96$, $P < 0.0001$, $R^2 = 0.9996$; estimate $K_d = 3.17 \pm 0.74$ (mean ± standard error)]. Homologous competition and saturation methods give very similar estimates for the affinity of CBG to CORT, and the homologous competition was preferred in this study because it has the advantage of using less radioactive reagents than the saturation method (Angelier and Wingfield, unpublished data).

Free CORT titers were estimated from total CORT concentrations and CBG binding parameters by using the equation of Barsano and Baumann (1989):

$$H_{free} = 0.5\{H_{total} - B_{max} - K_a^{-1} - [(B_{max} - H_{total} + K_a^{-1})^2 - 4(H_{total}(K_a^{-1}))]^{.5}\}$$

where H_{free} is free hormone, H_{total} is total hormone, B_{max} is total binding capacity of CBG, and K_a = dissociation constant^{-1} (K_d). Corticosterone values and CBG capacity are necessary to calculate free and bound CORT for an individual.

Statistical analyses

Stress tests

General linear models (GLMs) were used to compare differences in total CORT (2008) and BC (2009, 2010) between pre- and post-handling for each sex at each testing period. For the two GLMs examining the effect of handling on BC, we used treatment (pre/post) as a fixed factor and individual as a random factor, and we grouped the samples by sex to look at within-sex differences. To determine if the sexes differed in how their BC responded to handling stress, we combined the BC data from the two stress tests, incorporating individual as a random factor and treatment and sex as fixed factors, and the interaction between treatment and sex, and sex nested within date. We used two-tailed *t* tests to compare categories between seasons, e.g., "male pre-stressor in May" to "male pre-stressor in January." To test the effect of handling on circulating total CORT levels at 30 min post-handling, we used a model with treatment (pre/post) as a fixed factor and individual as a random factor, and we grouped the data by sex. To determine if total CORT levels differed between the sexes in response to stress, we used treatment and sex as fixed factors, individual as a random factor, and the interaction between sex and treatment.

Non-invasive CORT

As with the stress test data, we used GLMs to compare the differences in BC, total, bound, and free CORT, and CBG capacity between treatments within each sex and to examine whether the sexes responded differently to the treatments. We also examined the effect of total, bound, and free CORT on the BC for the non-invasive CORT experiment at 7 min post-mealworm ingestion. To test the within-sex effect of non-invasive CORT treatment on BC in January 2010 and adjusted BC in July 2010, we used a GLM for each experiment with treatment (CORT/No CORT) and date as fixed factors and individual as a random factor, and we grouped the data by sex. To determine if the sexes differed in how their BC responded to non-invasive CORT, we combined the BC data from the two stress tests. To make the BC data comparable across the two datasets, we adjusted the BC values for the January test in the same way that we adjusted the July BC data (see above). The GLM was used with individual as a random factor, treatment, date, and sex as fixed factors, and the interaction between treatment and sex, and sex nested within date. To test the within-sex effect of treatment on circulating total CORT in June 2009, and the effect of treatment on total, bound, and free CORT, as well as CBG capacity (July 2010), we used GLMs with treatment (CORT/No CORT) and date as fixed factors, individual as a random factor, and we grouped the data by sex. To determine if total CORT (June 2009), total, bound, free CORT and CBG capacity (July 2010) differed between the sexes in response to administration of exogenous CORT without an associated stress event, we used GLMs with treatment, date, and sex as fixed factors, the interaction between sex and treatment, sex nested within date, and individual as a random factor.

We examined the effect of total CORT, free CORT, and CBG on BC for each sex/treatment separately using linear regression. We also examined correlations between total CORT, free CORT, and CBG for each sex/treatment separately using linear regression. Finally, to determine if the number of birds an individual was housed with affected the BC and/or total CORT of the birds, we ran a GLM for BC and total CORT (when available) for each experiment (total of 16 GLMs—eight for males and eight for females)

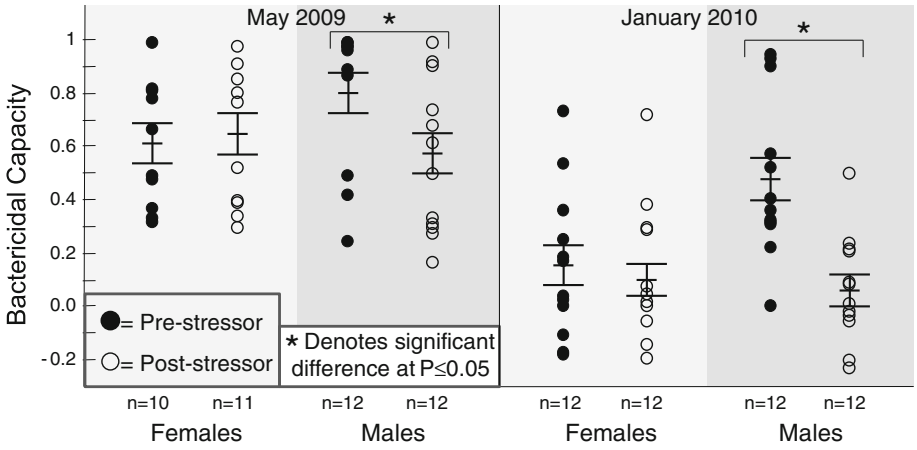


Fig. 1 Bactericidal capacity of the plasma in male and female cowbirds pre-stressor (*filled circles*) and 90 min post-stressor (*open circles*) in stress tests run in May 2009 and January 2010. Data are presented as the mean and standard error (SE) (*bars*) for each group

with treatment, date and "run" as fixed factors, and grouped the data by sex. "Run" refers to the outdoor cage the bird was housed in. We ran a sequential Bonferroni correction on the results of each GLM to determine significance of the results.

For all linear regressions and linear models the residuals were normally distributed, and for two-sample comparisons, the data were normally distributed, with the exception of "male pre-stressor in May". We used the Mann–Whitney U test for the comparison of "male pre-stressor in May" to "male pre-stressor in January". All analyses were performed in JMP 8.0 (SAS Institute, Cary, NC).

Results

Stress tests

Male cowbirds exhibited a significant decrease in BC from pre- to post-stressor in both the May ($F_{1,22} = 12.858$, $P = 0.004$) and January ($F_{1,23} = 64.79$, $P < 0.001$) experiments, whereas female BC did not change from pre- to post-stressor in either May ($F_{1,20} = 0.207$, $P = 0.659$) or January ($F_{1,23} = 0.389$, $P = 0.545$) (Fig. 1). There was a significant interaction effect of sex and treatment in the GLM incorporating BC data from both tests ($F_{1,62} = 15.276$, $P < 0.001$), showing that BC responded to handling stress in a significantly different manner between the sexes. BC was significantly higher in May than in January for each of the four categories (e.g., "male pre-stressor in May" compared to "male pre-stressor in January") ($P < 0.01$ for all comparisons) (Fig. 1). Both sexes exhibited significant increases in total CORT from pre- to 30 min post-stressor (males $F_{1,21} = 12.783$, $P = 0.002$; females $F_{1,15} = 20.28$, $P < 0.001$) (Fig. 2), but the interaction effect of sex and treatment was non-significant when we combined the sexes ($F_{1,29} = 1.39$, $P = 0.245$).

Non-invasive CORT

Total CORT levels did not differ between control and experimental birds at 45 min post-mealworm ingestion for either sex (males $F_{1,21} = 0.001$, $P = 0.907$; females $F_{1,8} = 0.911$, $P = 0.372$). At 90 min post-mealworm ingestion, experimental males exhibited significantly lower BC than control males ($F_{1,19} = 10.04$, $P = 0.018$), while females exhibited no difference in BC between the two groups ($F_{1,19} = 0.002$, $P = 0.966$) (Fig. 3). At 7 min post-mealworm ingestion, adjusted BC was significantly lower in experimental males than in control males ($F_{1,25} = 8.09$, $P = 0.014$), whereas there was no difference in adjusted

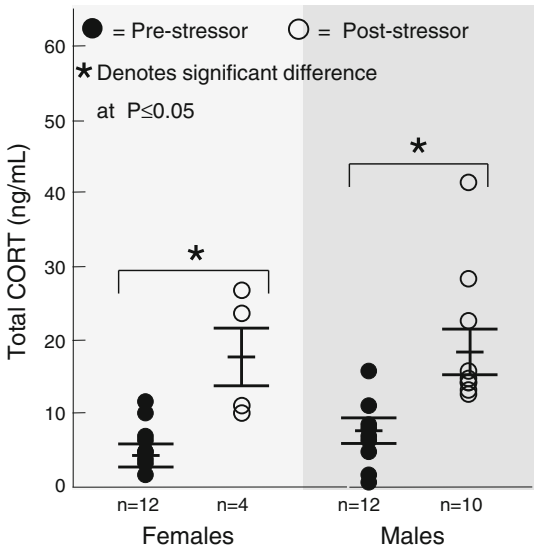


Fig. 2 Total corticosterone (*CORT*) for male and female cowbirds pre-stressor (*filled circles*) and 30 min post-stressor (*open circles*) for the July 2008 stress test. Data are presented as the mean and SE (*bars*) for each group

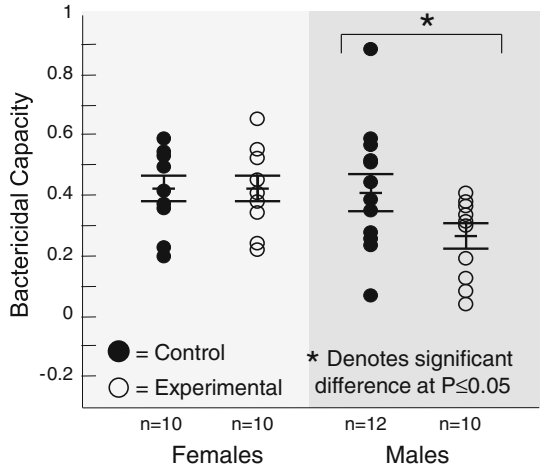


Fig. 3 Bactericidal capacity of the plasma in male and female cowbirds at 90 min post-mealworm ingestion for the non-invasive CORT experiment. Birds were fed either control mealworms (*filled circles*) or mealworms injected with CORT (experimental; *open circles*) and plasma was collected 90 min following ingestion of the mealworm. Data are presented as the mean and SE (*bars*) for each group

BC for the two female groups ($F_{1,23} = 0.394$, $P = 0.544$) (Fig. 4). There was a significant interaction effect of sex and treatment in the GLM incorporating BC data from both tests ($F_{1,90} = 5.275$, $P = 0.025$).

Total CORT at 7 min post-mealworm ingestion was significantly higher in experimental birds for both sexes (males $F_{1,21} = 10.089$, $P = 0.011$; females $F_{1,23} = 5.68$, $P = 0.038$) (Fig. 5a), and there was no difference in total CORT between the sexes for either treatment (No CORT $F_{1,22} = 1.475$, $P = 0.239$; CORT $F_{1,22} = 0.443$, $P = 0.513$). CBG capacity was not significantly different in experimental males and females compared to control

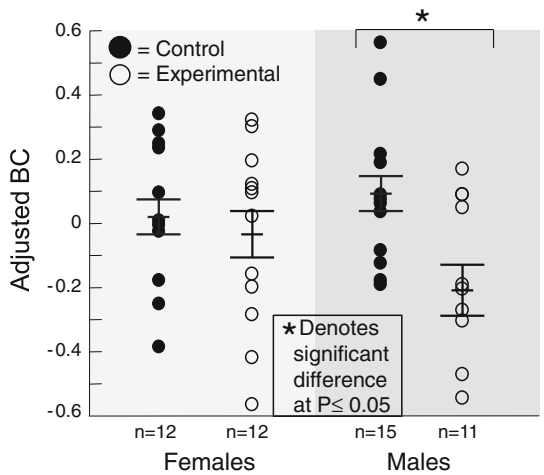


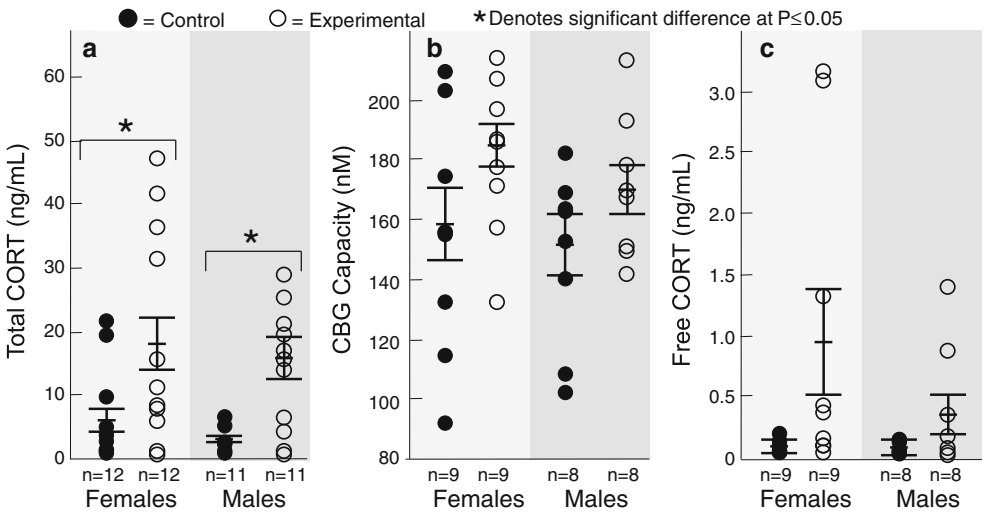
Fig. 4 Adjusted bactericidal capacity (*BC*) of the plasma for male and female cowbirds at 7 min post-mealworm ingestion for the non-invasive corticosterone experiment. Data are presented as the mean and SE (*bars*) for each group

birds (males $F_{1,15} = 4.923$, $P = 0.068$; females $F_{1,17} = 3{\cdot}51$, $P = 0.103$) (Fig. 5b). When the sexes were combined, however, CBG capacity in experimental birds was significantly higher than that in control birds ($F_{1,33} = 5.44$, $P = 0.026$). The capacity of CBGs did not differ between the sexes for either treatment (No CORT $F_{1,16} = 0.112$, $P = 0.743$; CORT $F_{1,16} = 0.669$, $P = 0.427$). Free CORT was not significantly different in experimental birds compared to controls for both sexes (males $F_{1,15} = 2.415$, $P = 0.171$; females $F_{1,17} = 4.07$, $P = 0.083$) (Fig. 5c), but when the sexes were combined, free CORT was significantly higher in experimental birds than in control birds ($F_{1,33} = 6.964$, $P = 0.013$).

There was a non-significant negative correlation between total CORT and BC for experimental males in the 7 min post-mealworm experiment ($N = 11$, $R^2 = 0.317$, $P = 0.072$) but no correlation for control males ($N = 11$, $R^2 = 0.110$, $P = 0.319$) or either female treatment (No CORT $N = 12$, $R^2 = 0.000$, $P = 0.998$; CORT $N = 12$, $R^2 = 0.006$, $P = 0.809$). There was no correlation between BC and free CORT for either sex in the control treatments (males $N = 8$, $R^2 = 0.085$, $P = 0.483$; females $N = 9$, $R^2 = 0.015$, $P = 0.754$), nor for experimental males ($N = 8$, $R^2 = 0.197$, $P = 0.270$), but there was a nearly significant negative correlation for experimental females ($N = 9$, $R^2 = 0.437$, $P = 0.052$).

Free CORT was positively correlated with total CORT for both treatments in males (No CORT $N = 8$, $R^2 = 0.985$, $P < 0.001$; CORT $N = 8$, $R^2 = 0.878$, $P < 0.001$) and females (No CORT $N = 9$, $R^2 = 0.863$, $P < 0.001$; CORT $N = 9$, $R^2 = 0.903$, $P < 0.001$) (Fig. 6). There was a non-significant interaction effect of "Sex" by "total CORT" in experimental birds as free CORT tended to increase more rapidly in females than in males ($F_{1,16} = 3.939$, $P = 0.069$). CBGs were also significantly positively correlated with total CORT in control birds (males $N = 8$, $R^2 = 0.670$, $P = 0.013$; females $N = 9$, $R^2 = 0.450$, $P = 0.048$), but not in experimental birds (males $N = 8$, $R^2 = 0.027$, $P = 0.695$; females $N = 9$, $R^2 = 0.062$, $P = 0.519$) (Fig. 6).

To determine if there was an effect of handling or bleeding the birds on subsequent experimental sessions, we compared total CORT levels for the first batch of control birds to those of the second batch of control birds in the 2010 non-invasive CORT experiment (Table 1 experiment "CORT 3", controls from session 1A vs. controls from 1B). There did not appear to be an effect of repeated handling or bleeding as there was no difference between the CORT levels of control birds that were bled first and those of control birds that were bled second (control birds bled first $N = 11$, mean CORT = 5.618 ng/mL; control birds bled second $N = 12$, mean CORT = 4.112 mL; two-tailed t test $P = 0.529$). In addition, we found no difference



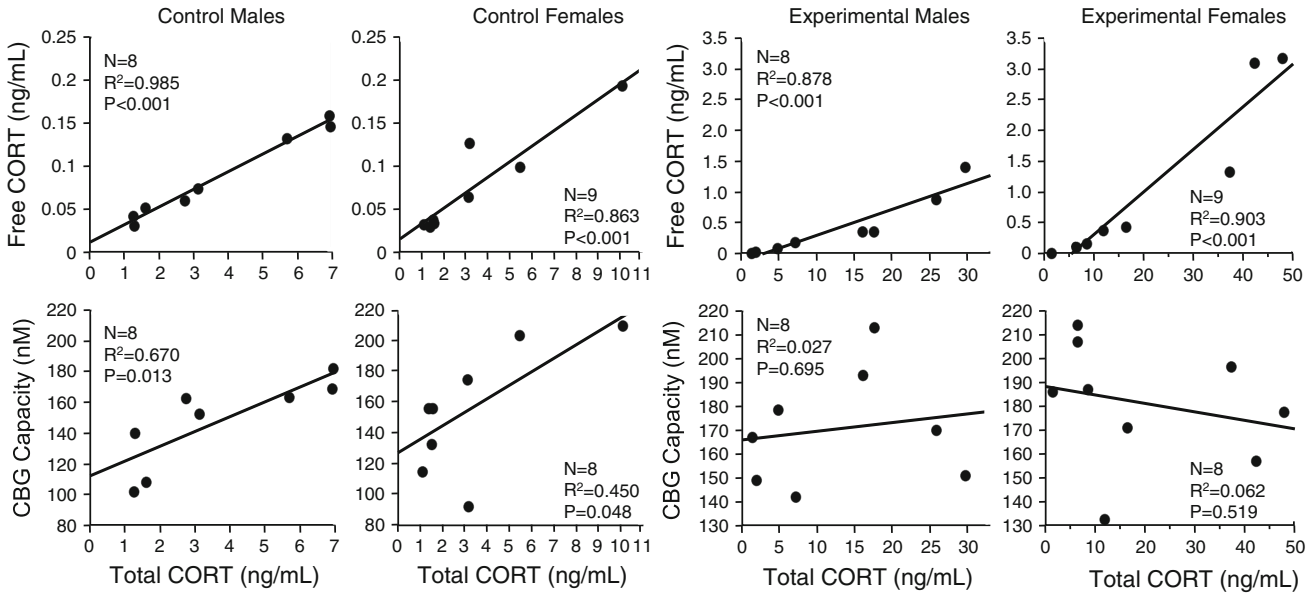


Fig. 5 Total CORT (**a**), corticosteroid binding globulin (*CBG*) capacity (**b**), and free CORT (**c**) for control (*filled circles*) and experimental (*open circles*) male and female cowbirds at 7 min post-mealworm ingestion. Data are presented as the mean and SE (*bars*) for each group

Fig. 6 Free CORT and CBG capacity by total CORT for control and experimental male and female cowbirds at 7 min post-mealworm ingestion

between CORT levels in control birds from June 2009 to July 2010 (Table 1 experiments "CORT 1" and "CORT 3") and baseline CORT in wild-caught cowbirds during the 2006–2009 breeding seasons (control birds $N = 40$, mean CORT = 5.262 ng/mL; wild caught birds $N = 100$, mean CORT = 4.601 ng/mL; two-tailed t test $P = 0.474$).

We attempted to minimize the effect of housing arrangement on the experiments by placing birds in the sound chambers for 48 h prior to experimentation, and we did not find an effect of run on any experiment. As noted in the "Materials and methods", males and females differed in the number of cage-mates they lived with, but there were no differences between the BC or total CORT of males and females in the "pre-stressor" or control treatments after sequential Bonferroni correction.

Discussion

This study is the first to examine the effects of both handling stress and non-invasive exogenous CORT on immune activity, as well as CBG capacity as part of the dynamic relationship between acute stress and innate immune function, in a wild-caught vertebrate. We demonstrated that the cowbird's complement-mediated BC responds to an exogenous supply of CORT in the same manner as it responds to capture and handling stress. This result suggests that CORT is responsible for the decline in BC in male cowbirds and that female cowbirds are in some manner modulating the effects of CORT.

By measuring CORT levels during the stress test we were able to determine that female cowbirds do not block

the production of GCs as a way to suppress a stress response. If they did, we would not have documented a significant increase in CORT from pre- to post-stressor (Fig. 2). The release of CORT is tightly regulated by feedback loops initiated in the hypothalamus (Sapolsky 1992), and the magnitude of the stress response is determined by the amount of CORT released, the concentration of CBGs, and the number of corticosteroid receptors (Breuner and Orchinik 2002). There are two types of intracellular, ligand-activated transcription factors: mineralocorticoid receptors (MR), which have high affinity for CORT, and glucocorticoid receptors (GR), which have a much lower affinity for CORT (Breuner and Orchinik 2001). Corticosterone also binds to specific, non-genomic receptors in the plasma membranes of cells and other tissues (Breuner and Orchinik 2001). We were unable to measure receptors, but we did measure CBG capacity to determine if female cowbirds could be mitigating the effects of stress via elevated levels of plasma CBG. If both males and females exhibit an increase in total CORT following exposure to a stressor, but females produce more CBG, the latter could be buffered against the physiological changes that males experience due to differences in levels of free CORT. Our data from the non-invasive CORT experiment do not support this scenario in cowbirds, however, as females had comparable or higher levels of free CORT compared to males in the experimental treatment (Fig. 5c). To say with more certainty that there are no differences between the sexes in CBG capacity we would want to have multiple sampling intervals. It may be that CBGs spike immediately post-stressor in cowbirds, or that they peak later than we sampled. Some studies examining changes in CBG capacity following stress have documented changes in CBGs within 10 min of the onset of a stressor (e.g., Bassett and Henry 1988; Tinnikov 1999; Breuner et al. 2006), whereas others did not find changes until 60 min or even days following the stressor or administration of exogenous CORT (e.g., Breuner et al. 2006; Almasi et al. 2009). Some of the disparity in these results is a consequence of species-level differences in CBG production (e.g., Breuner et al. 2006), but the method used for elevating CORT levels (e.g., implants, handling stress, foot shocks, ingestion) may also play a role. The rapid (7 min) change in CBG and BC (in males) following CORT ingestion in our study is potentially a result of CORT acting via non-genomic mechanisms, such as corticosteroid membrane receptors (Breuner et al. 1998). When CORT binds to an intracellular GR, the receptor regulates gene expression, leading to changes in protein levels and/or cellular activity on the order of 30–60 min following hormone treatment (Wehling 1995). The binding of CORT to membrane receptors is thought to initiate rapid changes in cellular activity, and the involvement of membrane receptors rather than intracellular receptors may allow organisms to time behavioral and physiological responses to an acute stressor more appropriately (Breuner et al. 1998).

Total CORT and free CORT were tightly linked in control and experimental birds for both sexes, whereas CBG was significantly correlated with total CORT for control birds only (Fig. 6). The change in the relationship between total CORT and CBG in the two treatments, coupled with the fact that free CORT and total CORT levels were so tightly linked, suggests either that the birds actively facilitated increases of free CORT via low levels of CBG production, or that CBG production could not keep up with the rapid increase in total CORT in the 7-min-long period we studied. We would need to examine CBG levels at multiple time points post-CORT ingestion to answer this question. Many studies of CBG following a stress response document no change or a decrease in CBG (e.g., Tinnikov 1999; Breuner et al. 2006; Almasi et al. 2009), which is thought to allow for a more robust stress response as more CORT is free to bind to GR ["free hormone hypothesis" (Mendel 1989)]. However, we found a significant increase in CBG, as did Bassett and Henry (1988) 15 min after the initiation of a stressor in rats and Boonstra and Singleton (1993) 30 min after the injection of adrenocorticotropic hormone in hares. The reason for the discrepancy between studies is unclear, but it may be a product of differences in how animals were stressed (e.g., restraint, foot shock, CORT implant), or differences in the interval between the initiation of the stressor and blood sampling. This is an area of research that needs more investigation.

Because female cowbirds did not have higher CBG levels than males, we suspect that the difference in immune response between sexes may be due to differences in the number of CORT receptors (Endres et al. 1979) or hormonal binding capacity of the receptors (Elaković et al. 2010). If female cowbirds have fewer receptors or a lower binding affinity than males, then comparable (or even elevated) levels of free CORT would result in a lower responsiveness to CORT than males. Sex-specific differences in receptor levels or binding capacity have not been well studied, but Endres et al. (1979) found that female rats had fewer GR than males in the cytosols of the thymus and liver, while Elaković et al. (2010) found that the GR of female rats had a higher binding capacity than those of males. These two studies provide some indication that there are multiple ways in which animals can modulate their response to changes in CORT levels and that the sexes may respond differently to stressful stimuli.

The ultimate cause for the difference in immune response may lie in the cowbird's reproductive strategy. Female cowbirds, which are thought to lay between 20 and 40 eggs over one breeding season (Scott and Ankney

1983), lay their eggs in the nests of other species which subsequently raise the parasitic chick as if it were their own. Female cowbirds may therefore have adopted a breeding strategy focused on current reproduction at the expense of self-maintenance; females have significantly lower annual survivorship (Woolfenden et al. 2001; Ortega and Ortega 2009) and have reduced levels of immunocompetence in nature compared to males during the breeding season (Merrill, unpublished data). One of the major consequences of a stress response is a reduction in, or complete shut-down of, reproductive physiology (Sapolsky 1992; Wingfield and Sapolsky 2003). If female cowbirds are investing heavily in current reproduction, it may not be adaptive to interrupt egg production and they may have therefore evolved ways of tamping down the effects of a stress response. A consequence of this would be no change in levels of immune activity. Alternatively, females may be avoiding CORT-induced increases in metabolic activity. Egg production is thought to be energetically expensive (Monaghan et al. 1995; Ward 1996), and an increase in metabolic activity results in an increase in reactive oxygen species (ROS) which can cause damage to tissues, cells, and DNA (Beckman and Ames 1998; Apel and Hirt 2004). The high egg production of female cowbirds could result in sustained elevated levels of ROS, and mounting a full stress response on top of the heavy reproductive expenditure could result in damaging levels of ROS. We did not find a difference between summer (May 2009–July 2010) and winter (January/February 2010) in how stress and CORT affect BC, but there would be no change between seasons if female cowbirds were not plastic in their ability to modulate receptor numbers. We did document a significant difference in BC between seasons in which both sexes exhibited greater killing in May compared to January. This variation could be due to lower ambient temperatures in January drawing resources away from immunity towards thermal regulation (Nelson and Demas 1996).

The biological significance of the changes in male cowbird plasma BC is unknown, especially since it is unclear whether the decline in plasma BC is a result of the redistribution, or degradation, of complement proteins. Few studies have examined changes in immunity so rapidly after initiation of a stressor, but Gelling et al. (2009) found a significant negative effect of handling stress on respiratory burst capacity within 5 min in small mammals, and Burns et al. (2008) found a significant increase in complement activity in humans within 30 min of the onset of stress. If the change in BC is a form of immunoredistribution, the rapid deployment to "battle stations" would be important in helping to staunch the growth of invading microbes. Conversely, if the change in BC is a result of complement degrading, this may be an adaptive response to avoid increased free radical damage or other forms of immunopathology (Dhabhar 2009). Complement activation has been linked to inflammatory disorders in people, and psychological stress has been shown to exacerbate the problem (Burns et al. 2008). In this study, we did not examine BC for more than 90 min following CORT ingestion or handling, but we do have some preliminary data indicating that BC levels return to baseline 2–3 h following handling stress (Merrill, unpublished data). This relatively ephemeral change in BC may be very important in fighting off pathogens or avoiding immunopathology, but will require further investigation. Once the functional basis for the change in BC is determined, we can begin to understand the consequences of, and reasons why, female cowbirds do not exhibit a change in BC.

Of particular relevance to our study is work conducted by Berzins et al. (2008) examining the effects of handling stress on the PHA swelling response in zebra finches (*Taeniopygia guttata*). They found that an increase in handling time resulted in a decreased swelling response in males, but that it had no effect on the swelling response of females. Zebra finches are opportunistic breeders (Serventy 1971) and capable of high levels of egg production (Willie et al. 2010). Wingfield and Sapolsky (2003) discuss examples in which avoiding a stress response may be adaptive, particularly with respect to maintaining reproductive condition. Female cowbirds and zebra finches may face similar constraints to each other and are likely candidates to exhibit adaptive suppression of the stress response. Caution needs to be exercised when extrapolating results from animals in captivity to large-scale adaptive strategies, but these experiments provide first-line evidence for sex-specific differences in modulation of the stress response in the brood–parasitic cowbird. More integrated approaches that examine how organisms respond to stress are needed to push our understanding of why and how individuals respond the way they do.

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