Changes in corticosterone concentrations and behavior during *Mycoplasma gallisepticum* infection in house finches (*Haemorrhous mexicanus*)

Ashley C. Love^{a,1}, Sarah L. Foltz^a, James S. Adelman^{a,2}, Ignacio T. Moore^a, Dana M. Hawley^a

^aDepartment of Biological Sciences, Virginia Tech, Blacksburg, VA 24061, USA

¹ Present address: Department of Integrative Biology, Oklahoma State University, Stillwater, OK 74078, USA

² Present address: Department of Natural Resource Ecology and Management, Iowa State University, Ames, IA 50011, USA

Corresponding author:

Ashley C. Love

ashley.c.love@okstate.edu; 804-909-8868

501 Life Sciences West, Stillwater OK 74078

Abstract

Glucocorticoid stress hormones are important for energy mobilization as well as regulation of the immune system, and thus these hormones are particularly likely to both influence and respond to pathogen infection in vertebrates. In this study, we examined how the glucocorticoid stress response in house finches (*Haemorrhous mexicanus*) interacts with experimental infection of the naturally-occurring bacterial pathogen, *Mycoplasma gallisepticum* (MG). We also investigated whether infection-induced concentrations of corticosterone (CORT), the primary glucocorticoid in birds, were associated with the expression of sickness behavior, the lethargy typically observed in vertebrates early in infection. We found that experimental infection with MG resulted in significantly higher CORT levels on day 5 post-infection, but this effect appeared to be limited to female house finches only. Regardless of sex, infected individuals with greater disease severity had the highest CORT concentrations on day 5 post-infection. House finches exposed to MG exhibited behavioral changes, with infected birds having significantly lower activity levels than sham-inoculated individuals. However, CORT concentrations and the extent of sickness behaviors exhibited among infected birds were not associated. Finally, pre-infection CORT concentrations were associated with reduced inflammation and pathogen load in inoculated males, but not females. Our results suggest that the house finch glucocorticoid stress response may both influence and respond to MG infection in sex-specific ways, but because we had a relatively low sample size of males, future work should confirm these patterns. Finally, manipulative experiments should be performed to test whether the glucocorticoid stress response acts as a brake on the inflammatory response associated with MG infection in house finches.

Keywords: *Mycoplasma gallisepticum*, house finch, corticosterone, sickness behavior, acute phase response, inflammation

1. Introduction

Individuals vary considerably in their behavioral and hormonal responses to infectious agents, although the underlying causes of this variation are not always clear. When organisms are exposed to a chronic stressor, hormonal mediators of the stress response (e.g., glucocorticoids) can modify immune function (Sapolsky et al., 2000; Dhabhar, 2002; Dhabhar, 2009; Martin, 2009) and decrease disease resistance (Brown and Zwillig, 1994). Despite the often-suppressive effects of stress hormones on immune function, infections also incite the production of stress hormones (Webster et al., 2002; Webster and Sternberg, 2004; Weidenfeld et al., 1995; Turnbull and River, 1996; McEwen et al., 1997; Adamo, 2010). While this seems paradoxical, it is thought that glucocorticoid concentrations might rise following infection to help maintain homeostasis by minimizing overactive immune responses (Elenkov and Chrousos, 2006; Webster and Sternberg, 2004). Overall, given that glucocorticoids can both influence and respond to pathogen infection, examining changes in glucocorticoids over the course of experimental infection can help elucidate mechanisms underlying individual responses to infection.

Corticosterone (CORT), the main glucocorticoid in birds, is involved in the regulation of energy and is an integral part of the stress response. CORT enables animals to respond to changing energy demands by suppressing unnecessary physiological processes and redistributing energy to processes that promote immediate survival (Wingfield et al., 1998; Sapolsky et al., 2000;

Angelier and Wingfield, 2013). While glucocorticoids may mediate energetic demands of immune responses, stress hormones also interact directly with components of the immune system, making these hormones a likely mechanism underlying variation in host responses to pathogens. One component of the immune system known to interact with stress hormones during infection is the acute phase response, an innate, non-specific defense against pathogens early in infection (Besedovsky, 1996). When an organism is challenged with a bacterial or viral pathogen, activated leukocytes release inflammatory cytokines that induce fever and sickness behaviors, increase other non-specific defenses such as the production of free-radicals, and often elevate glucocorticoid concentrations via activation of the hypothalamic-pituitary-adrenal (HPA) axis (Dantzer, 2004; Webster et al., 2002; Webster and Sternberg, 2004). It has been hypothesized that high concentrations of glucocorticoids may help prevent acute phase responses from progressing to harmful levels, acting as a brake on a costly innate immune component by, for example, suppressing inflammatory immune responses (Dantzer, 2004; Elenkov and Chrousos, 2006; Webster and Sternberg, 2004). This brake is important because inflammatory defenses are non-specific and can cause damage to host cells in addition to pathogen cells, resulting in immunopathology (Graham et al., 2005; Sears et al., 2011). Thus, CORT-mediated suppression of inflammation may be a crucial factor in limiting immunopathology during infection. Indeed, the inhibition of glucocorticoid release during experimental infections can result in increased disease severity and mortality, demonstrating that an intact HPA-axis and glucocorticoid release are crucial to surviving infection (Webster and Sternberg, 2004).

Behavioral responses to infection have also been linked to changes in CORT concentrations (Pezeshki et al., 1996; Propes and Johnson, 1997). Sickness behaviors, including the lethargy and

anorexia typically observed in vertebrates early in infection, are hypothesized to improve the ability of organisms to cope with infection by conserving energy needed for immune activation (Hart, 1988). However, sickness behaviors also come at significant costs (Adelman and Martin, 2009), which can include reduced caloric intake and lost reproductive opportunities (Lochmiller and Deerenberg, 2000; Konsman et al., 2002; Aubert et al., 1997). Although some studies suggest that a rise in glucocorticoids following exposure to a stressor may prolong sickness behavior during an acute phase response (Chijiwa et al., 2015), other studies indicate that glucocorticoids can suppress sickness behaviors through negative feedback on cytokine release (Goujon et al., 1995; Pezeshki et al., 1996; Propes and Johnson, 1997; Willette et al., 2007). The latter data support the hypothesis that glucocorticoids can act as a brake on the acute phase response by preventing severe behavioral disruptions caused by infection. Because glucocorticoids like CORT can have variable effects on sickness behavior, CORT may contribute to individual variation in behavioral as well as immunological responses to infection.

An excellent host-pathogen system for examining how CORT interacts with the acute phase response is the naturally-occurring infection of house finches (*Haemorrhous mexicanus*) with the bacterium *Mycoplasma gallisepticum* (MG). Since MG first appeared in free-living house finches in the mid-1990s, it has caused annual epidemics of Mycoplasmal conjunctivitis (Dhondt et al., 1998) and has been related to significant declines in eastern North American populations of house finches (Hochachka and Dhondt, 2000). Outbreaks of Mycoplasmal conjunctivitis are most common during winter and at the beginning of the breeding season when birds feed together in flocks (Altizer et al., 2004), periods generally associated with higher baseline CORT concentrations (Lindström et al., 2005). This study system is unique because the clinical signs of

115 this inflammatory disease can be visually and non-invasively scored. There are also potentially
116 high costs of inflammation associated with MG infection, which may underlie the increased
117 mortality rates in diseased birds (Faustino et al., 2004). These include the production of pro-
118 inflammatory cytokines, infiltration of lymphocytes and heterophils, and fever, all of which have
119 the potential to damage host tissue (Adelman et al., 2013b; Luttrell et al., 1998; Hawley et al.,
120 2012). The inflammatory eye lesions associated with Mycoplasmal conjunctivitis may also
121 impair vision, reducing the ability to evade predators or find food. Finally, infected house finches
122 also exhibit sickness behavior during infection (Kollias et al., 2004), decreasing activity levels to
123 points that could incur additional fitness costs.

124
125 While this host-pathogen system is well studied, little is known about the physiological factors
126 underlying individual variation in house finch responses to MG infection. Work on free-living
127 house finches found that birds with clinical signs of Mycoplasmal conjunctivitis had higher
128 stress-induced CORT concentrations than uninfected birds, but could not determine whether the
129 increased CORT concentrations were a cause or consequence of infection (Lindstrom et al.,
130 2005). Recent work by Adelman et al. (2015) on male house finches found that pre-infection
131 baseline CORT concentrations were negatively associated with the extent of conjunctival
132 swelling and sickness behavior during infection, but the detected association between CORT and
133 inflammation may have been confounded by population differences, making interpretation
134 difficult. Together these studies suggest that CORT concentrations may be linked to individual
135 variation in MG response in house finches. However, no studies to date have investigated how
136 CORT concentrations change throughout the course of experimental MG infection, or whether
137 post-infection CORT concentrations are linked to variation in individual responses to infection.

In this study, we hypothesized that baseline CORT concentrations vary during the course of MG infection and are linked to individual variation in the acute phase response. Specifically, we predicted that 1) CORT concentrations would increase early in infection and 2) CORT concentrations during infection would be positively correlated with the severity of conjunctival inflammation and sickness behaviors. We also predicted that, consistent with prior work, pre-infection CORT concentrations would negatively correlate with the extent of eye inflammation. Ultimately, this study aimed to enhance our understanding of the endocrine mechanisms that underlie variation in host immune responses and shed light on how CORT-immune interactions could impact disease dynamics in an ecologically relevant host-pathogen system.

2. Methods

2.1. Bird Capture and Housing

Thirty-four house finches (24 female, 10 male) were captured using cage traps in Auburn, Alabama, in September 2010, then transported to the Virginia Polytechnic Institute and State University campus in Blacksburg, Virginia. All birds were housed individually in cages on a 12 L: 12 D light cycle. Cages had two wooden perches, a water dish, and a cylindrical feeder in which birds were fed an *ad libitum* pelleted diet (Roudybush Maintenance Diet). Birds were housed in four rooms and treatments (MG infection or control) were equally distributed among rooms. On each rack, three cages could be stacked vertically and individual racks only housed birds belonging to the same treatment in order to prevent cross-contamination of pathogen. Sheets were placed on the sides of all racks to limit visual contact with birds housed on other racks. Housing and research protocols were approved by the Virginia Tech Institutional Animal Care and Use Committee.

Six months prior to this study, all birds had been used in a prior experiment (Hawley et al., 2013) as either controls (inoculated with media alone) or were inoculated with a low virulence isolate of MG (CA2006, CA2008, or CA2009; Hawley et al., 2013). We thus included infection history (previously exposed to MG or not) as a categorical covariate in all initial analyses. No birds in this study had clinical signs of Mycoplasmal conjunctivitis or detectable pathogen load prior to experimental inoculation.

2.2. Experimental Timeline

To assess changes in CORT and behavior during MG infection, we measured both variables prior to, and at multiple time points following, inoculation with either MG or media alone (sham control). To allow collection of CORT within three minutes of capture, we staggered inoculation dates by splitting birds into three sampling groups. Treatments were approximately equally distributed across the three sampling groups, and sampling groups were equally distributed across the four captive rooms. For each group, seven days prior to inoculation we collected baseline measurements for mass, baseline plasma CORT concentrations, pathogen load, and eye score (Fig. 1; detailed methods below). Video recordings were taken four days prior to inoculation to assess baseline behavior. Birds were inoculated with MG or control media at day 0. Blood samples were taken for baseline CORT at 6 and 24 hours (day 1) post-infection and on days 5 and 14 post-infection. Blood samples for CORT were also taken 21 days post-infection but were stored improperly and were therefore excluded from all analyses. On days 1, 5, 14, and 21 post-infection, mass and eye scores were recorded, and conjunctival swabs were taken to quantify pathogen load. Videos of behavior were taken post-infection on days 2, 6, and 22. These

time periods were chosen to correspond temporally with CORT samples (Fig. 1) but a one-day lag was used between CORT and video sampling to ensure that recorded behaviors were unbiased by human handling. All data (baseline and post-infection) were collected between July 6 and Aug 6 2011.

2.3. *Experimental Inoculation*

Birds were inoculated bilaterally in their palpebral conjunctiva with either 0.025 mL Frey's media alone (sham controls; n=15), or with 0.025 mL of a NC2006 isolate of *Mycoplasma gallisepticum* (n=19). The specific MG isolate (NC2006) used in this study was obtained from a free-living male house finch from North Carolina in September 2006 (stock ID 2006.080-5-4P; D. H. Ley, North Carolina State University, College of Veterinary Medicine, Raleigh, NC). This particular isolate was chosen because it is more virulent relative to other house finch MG isolates (Hawley et al., 2013), ensuring successful infection in the subset of birds previously infected with a less virulent isolate of MG. Prior to inoculation, inoculum was stored at -80°C but was thawed immediately before experimental inoculation. All individuals in the MG treatment showed clinical signs of Mycoplasmal conjunctivitis and had detectable loads of MG following inoculation. Pathogen load and eye lesion scores were used to assess individual variation in infection and disease, respectively.

2.4. *Pathogen Load*

To collect conjunctival swab samples, sterile cotton swabs were dipped in a tryptose phosphate broth and used to swab each conjunctiva (one swab per conjunctiva). After gently twirling the swab in each conjunctival sac for 5 sec, the swab was swirled and wrung out in a microcentrifuge

207 tube containing 300 μ L sterile tryptose phosphate. Swabs were then removed from the broth and
208 discarded. Samples were frozen at -20°C. Qiagen DNeasy 96 Blood and Tissue kits (Qiagen,
209 Valencia CA) were used to extract genomic DNA from our samples. Pathogen load was
210 measured using quantitative PCR (qPCR) to target the *mgc2* gene of MG as described in Grodio
211 et al. (2008) and Adelman et al. (2013b).

213 *2.5. Eye Lesion Scoring*

214 Eyes were scored for the severity of conjunctival inflammation on a 0-3 scale (as per Hawley et
215 al., 2011) each time conjunctival swabs were collected. Eye scores of 0 show no sign of
216 swelling. Scores of 1 display minor swelling around the eye, while a score of 2 shows moderate
217 swelling and eversion of the conjunctiva tissue. An eye score of 3 is given when the eye is
218 almost or completely swollen shut. Eye scores for both eyes were summed into a total eye score
219 within each time point, so that all scores ranged from 0 to 6. This total eye score was used as a
220 metric of disease severity at each sampling point.

222 *2.6. Corticosterone concentrations*

223 Samples were collected for baseline CORT concentrations within three minutes of entering the
224 room where birds were housed (an accepted time period before baseline CORT concentrations
225 begin to rise in response to stress initiation; Romero and Romero, 2002). Approximately 60 μ L of
226 blood was collected from the wing vein in heparinized micro-hematocrit capillary tubes and
227 immediately placed on ice. Within 1 h of sampling, hematocrit tubes were centrifuged for 5 min
228 to separate blood plasma from erythrocytes. Plasma samples were stored at -80°C until CORT
229 could be extracted from plasma and assayed via radioimmunoassay (RIA). Average plasma

volume was 28.4 μ l (range: 10.5 - 40 μ l). Briefly, CORT was extracted from plasma using distilled dichloromethane. Plasma CORT concentrations were measured in duplicate following standard radioimmunoassay techniques (Moore et al., 2002) and CORT concentrations were corrected for individual extraction efficiency. The assay detection limit was ~0.94 ng/ml with an intra-assay variation of 8.15%. All samples were obtained within three minutes of entering the room with the exception of two samples (one control male, one infected female) at the 24 hr time point collected at 3:30 and 4:00 minutes, respectively. Both samples were well within the range of the other 24 hour samples (control sample was below 75% quartile; infected sample below the median) and were included in the analyses as their removal did not change the results in any way. Additionally, we were unable to collect a sample for CORT for one infected female at the 24 hr time point; thus, this bird was excluded from CORT analyses at that time point.

2.7. Sickness Behavior

All birds were video recorded for one hour between 800-1330 EDT on days that behavioral data were collected (Fig. 1). Videos were analyzed by watching three different five-minute increments (min 15-20, min 30-35, min 45-50) of each hour-long video. These time periods were chosen to give a buffer period for the initial disturbance of setting up cameras and to best represent the entire recording period. The type and number of each of four behaviors (perching, eating, walking, flying) that occurred were recorded individually during the observed portions. All videos were watched by the same individual (ACL) to avoid inconsistencies in scoring. Videos were watched while blind to treatment. Video data was collected for all individuals at every time point with the exception of one control bird at day 22 post-infection from which we were unable to collect behavioral data.

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2.8. Statistical Analyses

We used principal component analysis in JMP 10.0 (SAS Institute, Cary, NC) to collapse our non-independent behavioral variables into one axis, PC1, which explained almost 60% of the observed variation in behavior (Table 1). This principal component, which positively loaded with eating, flying, and walking, and negatively loaded with perching, was essentially a measure of overall activity. We log10 transformed PC1 and CORT concentrations to meet the assumptions of parametric statistics. All further statistical analyses (detailed below) were conducted in SAS 9.3 (SAS Institute, Cary, NC).

2.8.1 Treatment differences

To examine how CORT concentrations, sickness behaviors, and mass changed over time with treatment, we built three linear mixed models (PROC MIXED), each of which included treatment (control or infected), infection timing (days post-inoculation), sex, mass (CORT and behavior models only), and all pairwise interactions as predictors. Because individuals were repeatedly sampled over time, bird identity was included as a random effect in all models. We considered infection timing as a categorical variable (pre-infection, day 0, 1, 5, 14, 22) because pathogen load and eye score do not change linearly with infection (Hawley et al., 2010). Because we found a significant interaction between sex and treatment on corticosterone concentrations (see below), we also ran separate linear mixed models for each sex (CORT model only) in order to determine how infection timing varied by sex. For all mixed models, we used the Satterthwaite approximation to calculate denominator degrees of freedom because this approximation is more robust for unbalanced designs.

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277 *2.8.2 Individual level variation within the infected treatment*

278 To determine which factors predicted variation in CORT concentrations or activity levels during
279 infection, we limited the analyses to birds in the infected treatment only (n=19). Because CORT
280 and activity responses were only significantly different from controls on day 5 (see below), we
281 examined day 5 post-infection. Our predictor variables for both CORT and activity levels (PC1)
282 were sex, pathogen load, eye score, mass, and all pairwise interactions. For the behavior analysis,
283 we also included CORT concentrations as a predictor variable to test whether CORT
284 concentrations measured on day 5 correlated with activity levels on day 6. Because each
285 individual was only included once in these analyses, we used General Linear Models (PROC
286 GLM).

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288 *2.8.3 Effect of pre-infection corticosterone on overall disease responses*

289 We examined whether pre-infection CORT concentrations predicted individual variation in
290 responses to MG within the infected treatment only (n=19). Because we wanted to capture
291 whether pre-infection CORT concentrations were associated with overall susceptibility to
292 infection or disease among individuals, we generated two measures – infection severity (overall
293 pathogen load) and disease severity (overall eye score) – by summing pathogen loads and eye
294 scores across all sampling dates post-infection for each individual. Because each individual was
295 only included once in these analyses, we used General Linear Models (PROC GLM). Our
296 predictor variables were pre-infection CORT, sex, and their pairwise interaction, and the
297 response variables for the two independent models were overall disease severity (sum of eye
298 scores) or overall pathogen load.

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300 For all analyses, we removed non-significant pairwise interactions from the final model.
301 Infection history (exposed to MG in a previous experiment) was initially included as a covariate
302 in all analyses, but because it was never significant, it was removed from the final models. We
303 present F-tests using Type III Sums of Squares for all analyses. For significant categorical
304 variables, post-hoc t-tests were conducted on all pairs of least square means in SAS using a
305 Tukey-Kramer adjustment for multiple comparisons. Final models and parameter estimates for
306 all models are presented in Supplementary Material.

307

308 **3. Results**

309 *3.1. Corticosterone*

310 *3.1.1. Treatment differences*

311 Experimental infection with MG influenced baseline plasma CORT concentrations, but
312 the extent of this effect varied across the sampling intervals (infection timing*treatment: $F_{4,108}=$
313 5.01; $p=0.001$; Table S1). Plasma CORT concentrations of MG-infected birds were similar to
314 that of control birds prior to ($t=-1.67$; Adj $p=0.81$) and immediately following infection (6 hours:
315 $t=0.86$, Adj $p=0.98$; 24 hours; $t=0.74$; Adj $p=0.99$), but were significantly higher than control
316 birds on day 5 ($t=-3.61$; Adj $p=0.016$) post-infection (Fig. 2). By day 14 post-infection, CORT
317 concentrations in infected birds had begun to decline and were no longer significantly different
318 from control birds ($t=-1.77$; Adj $p=0.75$). Because of the time dependence of treatment effects on
319 corticosterone concentrations, the main effect of treatment was not significant (treatment:
320 $F_{1,32}=0.60$; $p=0.44$). Individual mass significantly predicted CORT concentrations, with birds of

higher body mass having lower CORT concentrations (mass: $F_{1,49.6} = 6.09$; $p = 0.017$), regardless of treatment or infection timing.

Unexpectedly, the effect of infection on CORT concentrations also differed between the sexes (Fig. 3; treatment*sex: $F_{1,28} = 10.16$; $p = 0.0035$): infected females had significantly higher CORT concentrations than control females ($t = -3.48$; Adj $p = 0.0085$) but there was no effect of MG infection on male CORT concentrations ($t = 1.41$, Adj $p = 0.22$). The main effect of sex ($F_{1,28.1} = 0.39$; $p = 0.54$) was not significant. Separate mixed models for females and males indicated that only female house finches showed a significant increase in CORT concentrations with treatment (treatment $F_{1,29.8} = 11.1$; $p = 0.0024$; Table S2), and this response varied with time post-infection (infection timing $F_{4,71.1} = 12.4$, $p < 0.0001$; infection timing*treatment: $F_{4,72.1} = 4.87$; $p = 0.0016$): post-hoc tests revealed that infected females had higher CORT concentrations than controls on days 5 (Fig. 3A; $t = -5.28$; Adj $p < 0.0001$) and 14 post-infection ($t = -3.45$; Adj $p = 0.03$). In contrast, male house finches did not show significant effects of treatment ($F_{1,8.17} = 2.91$; $p = 0.13$), infection timing ($F_{4,29} = 2.52$, $p = 0.06$), or their interaction (infection timing*treatment: $F_{29} = 0.89$; $p = 0.48$) on CORT responses.

3.1.2. Individual-level variation in corticosterone within the infection treatment

Disease severity (total eye score) on day 5 significantly predicted variation in CORT concentrations in response to MG infection (Fig. 4; Table S3; $F = 7.51$, $p = 0.016$; Model $R^2 = 0.54$). Consistent with the sex * treatment effect detected earlier, plasma CORT concentrations for infected females were higher than for infected males on day 5 (sex: $F = 4.46$, $p = 0.053$) but this result was not quite statistically significant. Pathogen load ($F = 0.92$, $p = 0.35$) and mass ($F = 3.77$, $p = 0.07$) did not significantly predict CORT concentrations within the infected treatment.

3.1.3. Effect of pre-infection corticosterone on overall disease responses

The interaction between pre-infection CORT concentrations and sex predicted overall disease severity (Fig. 5; $F_{3,15}=11.8$; $p=0.0036$; Model $R^2=0.45$) and overall pathogen load ($F_{3,15}=6.55$; $p=0.02$; Model $R^2=0.34$) across the course of infection (Table S4). Within infected males ($n=6$), higher pre-infection CORT concentrations were associated with lower overall disease severity ($\beta=-20.86 \pm 5.45$; $t=-3.83$, $p=0.018$) and pathogen load ($\beta=-19.6 \pm 6.65$; $t=-2.95$, $p=0.042$). In contrast, the parameter estimates for females ($n=19$) were positive but not significantly different from zero for both the relationship between pre-infection CORT and overall eye score ($\beta=7.53 \pm 3.93$; $t=1.92$, $p=0.082$) and pre-infection CORT and overall pathogen load ($\beta=9.53 \pm 5.55$; $t=1.74$, $p=0.11$). Corticosterone concentrations and sex as main effects did not predict overall pathogen load (both $F<2.97$, all $P<0.11$) but sex as a main effect significantly predicted overall eye score ($F_{3,15}=11.8$; $p=0.0036$).

3.2. Sickness behaviors

3.2.1 Treatment differences

Treatment ($F_{1,40.8}=14.7$, $p=0.0004$), infection timing ($F_{3,79.7}=14.0$, $p<0.0001$), and their pairwise interaction ($F_{3,80.9}=3.02$, $p=0.034$) were all significant predictors of activity levels (PC1; Table S1). Activity levels of MG-infected birds were similar to that of control birds prior to ($t=0.27$; Adj $p=1.0$) and immediately following infection ($t=2.06$; Adj $p=0.45$), but were significantly lower than control birds on day 6 ($t=-4.20$; Adj $p=0.0017$) post-infection (Fig. 6). By day 22 post-infection, activity levels in infected birds had begun to increase and were no longer significantly different from control birds ($t=2.19$; Adj $p=0.37$).

3.2.2 Individual-level variation in sickness behavior within the infection treatment

None of our predictor variables (sex, mass, total eye, or pathogen load) significantly explained individual variation in activity levels within the infected treatment on day 5 (all $F < 2.95$, all $p \geq 0.11$; Model $R^2 = 0.27$).

3.3 Mass

3.3.1 Treatment differences

The interaction between treatment and infection timing was the only factor that significantly predicted body mass in our study ($F_{5,136} = 3.75$, $p = 0.0032$; Table S1). In general, mass levels increased slightly for infected birds over time, as has been detected in prior work (Adelman et al., 2013a). However, no post-hoc tests between control and infected birds were statistically significant (Fig. S1; all $|t| \leq 1.11$, all Adj $p \geq 0.99$). Main effects of treatment ($F_{1,31.5} = 0.05$, $p = 0.82$), sex ($F_{1,30.9} = 0.25$, $p = 0.62$), and infection timing ($F_{5,136} = 1.92$, $p = 0.095$) on mass were all not significant.

4. Discussion

Here we examined how baseline CORT concentrations in house finches both predict and respond to experimental infection with a naturally-occurring bacterial pathogen. Consistent with our predictions, CORT concentrations significantly increased following experimental infection with MG, but this increase was not yet detectable at 24 hours post-inoculation. Due to the inability to repeatedly blood-sample small birds at finer temporal intervals, we were unable to pinpoint in this study when plasma CORT concentrations began to increase between 24 hours and 5 days

post-infection. However, our results (Fig. 2) suggest that the house finch CORT response to MG infection is not a transient, immediate effect (*i.e.*, hours to days), but is relatively prolonged (*i.e.*, days to weeks), as has been documented in mice (Dunn et al., 1989) and tadpoles (Warne et al., 2011) in response to experimental viral infections.

Unexpectedly, CORT responses to MG infection appeared to differ by sex in our study, with only females showing a significant effect of MG treatment on CORT concentrations (Fig. 3A), while CORT concentrations were similar for control and infected males (Fig. 3B). Because we had a significantly smaller sample of males (n=10) than females (n=24) in our study, the lack of treatment effect in males may be due to low statistical power alone, and thus the detected interaction between sex and treatment should be interpreted with caution. Although further confirmation of sex-specific responses is needed, the interaction between sex and infection treatment on CORT responses does not appear to be driven by body mass in our study: mass did not differ between male and female house finches, either alone or in interaction with infection treatment. Future work with larger and more balanced sample sizes should confirm whether CORT responses to infection are, in fact, sex-specific in house finches, and if so, should investigate potential mechanisms driving these sex differences.

In addition to examining changes in CORT across treatments, we also examined whether individual-level variation in responses to infection predicted variation in CORT concentrations on day 5 post-infection, which was the day of peak eye score (Fig. S2) and pathogen load (Fig. S3). We found that within the infected treatment, individuals with greater conjunctival swelling (total eye score) had the highest baseline CORT concentrations (Fig. 4). This relationship implies

that baseline CORT concentrations and inflammatory responses to MG are linked, but our data do not allow us to determine causality. Evidence that glucocorticoids can enhance pro-inflammatory cytokine activity in other vertebrates (Haddad et al., 2002; Viswanathan et al., 2005; Dhabhar, 2009; Frank et al., 2010) indicates that increases in CORT could be stimulating inflammatory responses that would lead to increased conjunctival inflammation. Conversely, the pro-inflammatory response associated with severe swelling around the eye could trigger increases in CORT in order to ensure that inflammation does not progress to pathological levels (Elenkov and Chrousos, 2006; Webster and Sternberg, 2004; Dantzer, 2004). This could be especially important in maintaining immune homeostasis in house finches infected with MG, as MG infection is characterized by local and systemic inflammatory responses (Adelman et al., 2013b; Luttrell et al., 1998; Hawley et al., 2012), all of which can cause damage to host tissue. Manipulative experiments that augment or block CORT would help clarify whether the observed rise in CORT during MG infection suppresses inflammatory immune responses or pathology associated with MG infection.

We also looked for potential associations between CORT and sickness behaviors during infection. As has been detected previously (Kollias et al., 2004; Bouwman and Hawley, 2010; Adelman et al., 2013b), infected birds responded to experimental MG infection with significantly lower activity levels (Fig. 6). While CORT and sickness behavior both peaked at day 5 post-infection, individual variation in CORT concentrations at this time point did not predict variation in activity levels within the infected treatment. It is still possible that CORT and sickness behaviors are linked in MG-infected house finches, but we failed to capture the relevant timing of this link by sampling behavior and CORT concentrations on different days (Fig. 1). Indeed,

the contradictory evidence on the interactions between glucocorticoids and sickness behavior in the current literature indicate that timing is central to capturing the relationship between these two endpoints (Goujon et al., 1995; Pezeshki et al., 1996; Dantzer, 2004; Willette et al., 2007; Chijiwa et al., 2015). Because CORT coordinates energy balance and redistribution within an organism and sickness behaviors incur costs that require both the expenditure and redirection of metabolic resources (Lochmiller and Deerenberg, 2000; Konsman et al., 2002), more work should be done to investigate the relationship between CORT and behavioral responses to MG infection.

Finally, we examined whether CORT concentrations prior to infection were predictive of individual variation in disease outcome, as has been detected in prior work on male house finches (Adelman et al., 2015). Intriguingly, we found that pre-infection CORT concentrations interacted with sex in our study: male house finches with higher pre-infection CORT concentrations showed lower overall disease severity and pathogen load in response to MG infection, whereas female pre-infection CORT concentrations did not significantly predict individual variation in either metric. Our results are consistent with those of Adelman et al. (2015), who found that higher pre-infection baseline CORT concentrations were predictive of lower disease severity among male house finches. It is intriguing that we did not detect a significant relationship between pre-infection CORT and disease response within female house finches, despite having a larger sample size for this group. Furthermore, although not significant, the parameter estimates for females were in the opposite direction of that detected for males, with higher pre-infection CORT concentrations associated with higher disease severity and pathogen load. Adelman et al. (2015) only included male house finches (n=22), and thus our

study is the only one to date to examine how pre-infection CORT concentrations predict host responses to MG in female versus male house finches. However, further study is needed with larger, and more balanced sample sizes, to examine the potential for sex-specific influences of pre-infection CORT concentrations on disease responses to MG.

5. Conclusions

By investigating relationships between CORT concentrations and MG infection in house finches, this study demonstrates the importance of examining CORT-infection interactions at multiple time points. Overall, our results suggest that the house finch glucocorticoid stress response may both influence and respond to MG infection, potentially in sex-specific ways. Moreover, the fact that CORT concentrations are linked to conjunctival inflammation during MG infection could have important implications for MG transmission. Adelman et al. (2013a) found that individuals with greater conjunctival pathology (eye score) deposited higher relative amounts of the MG bacterium onto bird feeders (one of the main modes of transmission for MG). This possibility highlights how characterizing individual variation in CORT-immune interactions could help us understand population-level processes, such as pathogen transmission, in some systems.

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Figure Captions

Fig. 1. Experimental timeline for infected and control birds. (B* - Samples were collected for this time point but were stored improperly and therefore excluded from all analyses).

Fig. 2. Effects of *Mycoplasma gallisepticum* treatment on corticosterone concentrations over time. The bars denote the average corticosterone concentrations ($\pm$ SE) of birds in the control (n=15) and infected (n=19) treatments. *P<0.05.

Fig. 3. Effects of *Mycoplasma gallisepticum* treatment on corticosterone concentrations over time by sex. (A) Average corticosterone concentrations of female birds in the control (n=11) and infected (n=13) treatments. (B) Average corticosterone concentrations of male birds in the control (n=4) and infected (n=6) treatments. Bars represent means ($\pm$ SE) of CORT concentrations. ***P<0.001, *P<0.05.

Fig. 4. Corticosterone concentrations predict the extent of disease severity (measured as total eye score) in response to *Mycoplasma gallisepticum* at day 5 post-infection (n=19).

636

637 **Fig. 5.** Pre-infection corticosterone concentrations predict overall disease severity (the sum of all
638 total eye scores across the post-infection sampling points) in males (black squares, solid line,
639 $n=6$) but not females (gray circles, dashed line, $n=13$). Corticosterone concentrations are $\log_{10}$
640 transformed (as in the statistical analysis) to improve visualization of the interaction.

641

642 **Fig. 6.** Effects of *Mycoplasma gallisepticum* treatment on activity levels (measured as the first
643 principal component of multiple behaviors) over time. The bars denote the average activity levels
644 ($\pm$ SE) of birds in the control ($n=15$) and infected ($n=19$) treatments. ** $P<0.01$.

Inoculation with MG or control media

Days Post-Infection:

-7 -4 0 6 hr 1 2 5 6 14 21 22

B Blood sampling
for CORT

P Pathogen Load
and Eye Score

V Video recording
for behavior

M Mass

A. Females

B. Males

Table 1. Principle component loadings for the four behavioral variables used to assess sickness behavior. PC1 explained 56.9% of the variation in recorded behaviors.

Variable	PC1
Perching	-0.97
Eating	0.67
Flying	0.73
Walking	0.56