Infection with *Mycoplasma gallisepticum* Buffers the Effects of Acute Stress on Innate Immunity in House Finches

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ABSTRACT

When wild animals become infected, they still must cope with the rigors of daily life, and, thus, they still can be exposed to acute stressors. The suite of physiological responses to acute stress includes modifying the innate immune system, but infections can also cause similar changes. We examined the effects of an acute stressor (capture stress) on leukocyte abundance and bacteria-killing ability (BKA) in wild birds (house finches *Haemorhous mexicanus*) with and without a naturally occurring infection (*Mycoplasma gallisepticum*) to determine whether infection alters the typical immune response to stress. Birds were captured and bled within 3 min (baseline sample) and then held in paper bags for 2 h and bled again (stress sample). From blood smears made at both time points, we obtained estimates of total white blood cell (WBC) counts and relative numbers of each cell. We also measured BKA of plasma at both time points. In uninfected birds ($n = 26$), total WBC count decreased by 30% over time, while in infected birds ($n = 9$), it decreased by 6%. Relative numbers of heterophils did not change over time in uninfected birds but increased in infected birds. Combined with a reduction in lymphocyte numbers, this led to a threefold increase in heterophil-lymphocyte values in infected birds after the stressor, compared to a twofold increase in uninfected birds. There was a nonsignificant tendency for BKA to decline with stress in uninfected birds but not in diseased birds. Collectively, these results suggest that infections can buffer the negative effects of acute stress on innate immunity.

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Introduction

There is considerable evidence indicating that stress and infection are tightly linked within organisms and that one can give rise to the other. Infections stimulate the production of stress hormones and neurohormones (Lindström et al. 2005; Adamo 2010), and high levels of stress hormones over long periods ("chronic stress") can suppress immune function and increase susceptibility to parasite and pathogen infections (e.g., Cohen and Williamson 1991; Cohen et al. 1997; Owen et al. 2012). Conversely, acute (i.e., short-term) stress can lead to temporary enhancement of immune function (Dhabhar et al. 1996; Dhabhar and McEwen 1997; Dhabhar 2002), which could be adaptive for helping individuals cope with immediate short-term threats to survival. Specifically, acute stress leads to a temporary redistribution of circulating leukocytes, causing certain cell types (neutrophils in mammals and amphibians, heterophils in birds and reptiles) to increase in circulation in most species and other cells (lymphocytes and sometimes eosinophils) to migrate from blood to tissues (Dhabhar et al. 1994, 1995, 1996; Davis et al. 2008). Infections can cause similar changes to leukocyte populations (Aguirre et al. 1995; Davis et al. 2004), either because neutrophils and heterophils (phagocytic cells) are recruited to fight the pathogen or because infection-induced increases in stress hormones lead to reductions in lymphocytes (thereby causing relative increases in other cell types).

Acute stress can also modify noncellular components of the innate immune system. Recent studies investigating how acute capture stress affects the bacteria-killing ability (BKA) of blood plasma, a function mediated by serological immune components in the blood (e.g., complement, acute-phase proteins), reveal that effects vary across taxa and even within species. For instance, in a study of five tropical passerine species, capture stress caused reductions in plasma BKA in some species, while others showed no effect (Matson et al. 2006). Similarly, a study of brown-headed cowbirds (*Molothrus ater*) found that males showed a reduction in plasma BKA following acute stress while females did not (Merrill et al. 2012). Although few studies have examined the factors underlying these inter- and intraspecific differences in the immune response to acute stress, life history variation is thought to play a role (Matson et al. 2006; Merrill et al. 2012).

Animals that are infected must still face the daily rigors of survival, evading predators and finding food. In essence, they still could be exposed to acute stressors, even when their baseline stress levels are heightened by infection (Lindström et al. 2005; Adamo 2010). This leads to the question of how animals cope with the conflicting demands of both pressures. Specifically, if infections lead to activation of the innate immune sys-

tem (including leukocytes and BKA), how would the immune system respond if the animal then experienced an acute stressor? During an infection, it would be advantageous to maintain a high complement of immune cells in circulation. So, if acute stress normally leads to reductions in most leukocytes (Davis 2005; Davis and Maerz 2010; Cīrule et al. 2012), in animals fighting an infection this effect may be reduced. Similar patterns might be observed with BKA, which can be reduced during acute stress (Matson et al. 2006). To our knowledge, these ideas have never been examined specifically in birds or other vertebrates, that is, the combined effects of infection and acute stress on innate immunity.

In this study, we examined the effects of an acute stressor (capture and handling) on leukocyte abundance and plasma BKA in wild birds with and without a naturally occurring infection to determine whether pathogen infection can alter the immune responses during stress. We studied house finches (*Haemorhous mexicanus*) that were naturally infected with the bacterial pathogen *Mycoplasma gallisepticum* (MG). This is a well-studied disease that is characterized by mild to severe unilateral or bilateral eyelid swelling, watery ocular discharge, and sometimes dried nasal exudate (Luttrell et al. 1998; fig. 1). Using the house finch–MG system, we tested the prediction that infection-induced enhancement of the innate immune response buffers the suppressive effects of acute stress on immunity. We expected that this buffering effect would be apparent for both cellular (leukocytes) and noncellular (plasma bacteria killing) innate immune responses.

Figure 1. Male house finch (*Haemorhous mexicanus*) with no visible clinical signs of *Mycoplasma gallisepticum* infection (*top*) and one with the typical signs of infection (*bottom*), including tissue swelling, ocular discharge, and feather loss around the eye. Both photos taken by A. K. Davis. A color version of this figure is available online.

Methods

Capturing and Handling Birds

House finches for this project were captured over a 2-mo period (May 10–July 6) during the summer of 2011 on the University of Georgia campus, in Athens, Georgia. A total of 35 house finches were captured. Birds were captured using mist nets set up around bird feeders, and the nets were set up such that we could visually monitor them for captures and birds could be immediately extracted (which is necessary for obtaining initial blood samples). Upon capture, we first obtained a blood sample via the brachial vein under the wing (Sheldon et al. 2008), which was used to make a standard blood smear. The remaining blood was transferred from a heparinized capillary tube to a microcentrifuge tube and was spun down to harvest plasma. Plasma was stored at -80°C until further processing. We ensured that the initial sample was taken within 3 min of the bird hitting the mist net (Romero and Romero 2002). After the initial sample was obtained, the bird was placed in a brown paper bag (with a hole in the top for ventilation) following Hill (2002) and left as such for 2 h. The paper bags containing birds were placed on a table away from the banding and bird-processing activities, and each bag was held fast by a wooden tray that held the bag upright and eliminated vibrations or disturbance. After that time, the bird was removed, and we obtained a second blood sample from the wing opposite the one from which the initial sample was taken. A second smear was made and plasma stored as described above. Throughout the project, we took care to ensure that all birds received the same postcapture treatment between the first and second blood-sampling times.

When all blood sampling was completed, we banded each bird with a numbered aluminum leg band, recorded its age (all were hatch-year birds in this study), and visually inspected its eyes for clinical signs of MG infection (fig. 1). Prior studies indicate a high degree of concordance between the visible signs of infection and the presence of the MG pathogen (Luttrell et al. 1998; Hartup et al. 2000; Sydenstricker et al. 2006). For the purposes of this study (and since sample size of infected birds did not permit additional categorization), we were concerned only with whether the bird was infected, as opposed to the severity of its infection. Of the 35 birds we captured, 9 (25.7%) showed visible signs of MG infection (conjunctivitis; fig. 1). Given that all birds were juvenile (hatched this year) in this study, we could be confident that the birds without conjunctivitis had not already contracted MG and cleared the infection. All birds were released at the site of capture following the 2-h protocol.

Leukocyte Counts

Dried blood smears were stained with a buffered Wright-Giemsa stain (Camco Quik Stain II) and then examined by one of us (M. Fratto) with a light microscope under high power (× 1,000). Each slide was scanned in a zigzag pattern (to sample multiple areas of the smear), and within each field of view, the number of all types of leukocytes (lymphocytes, heterophils, eosinophils, basophils, and monocytes; fig. 2) was counted. We counted 100 leukocytes for each slide, and from these data we calculated the relative numbers of each cell type. We also obtained an estimate of absolute numbers of all leukocytes (total white blood cell [WBC] count) and of each leukocyte type per 10,000 red blood cells based on the numbers of cells of each type per field of view (fields of view had approximately 400 red blood cells; A. K. Davis, unpublished data). Total WBC count was our index of cellular immunity for analyses. From the counts of individual cells, we determined the heterophil-lymphocyte (H-L) ratio for each bird, and this was used as an indicator of stress (Davis et al. 2008).

Bacteria-Killing Assay

We used a strain of *Escherichia coli* (ATCC 8739) for which killing is primarily mediated by complement proteins (Matson et al. 2006) to assay the BKA of house finch plasma. To implement the assays, we followed the protocol described in Matson et al. (2006) with minor modification. Specifically, we diluted 10 μL of plasma in 90 μL CO_2-independent media enriched with 4 mM L-glutamine, and to each dilution, we added 10 μL of bacteria suspension. Once bacteria were added

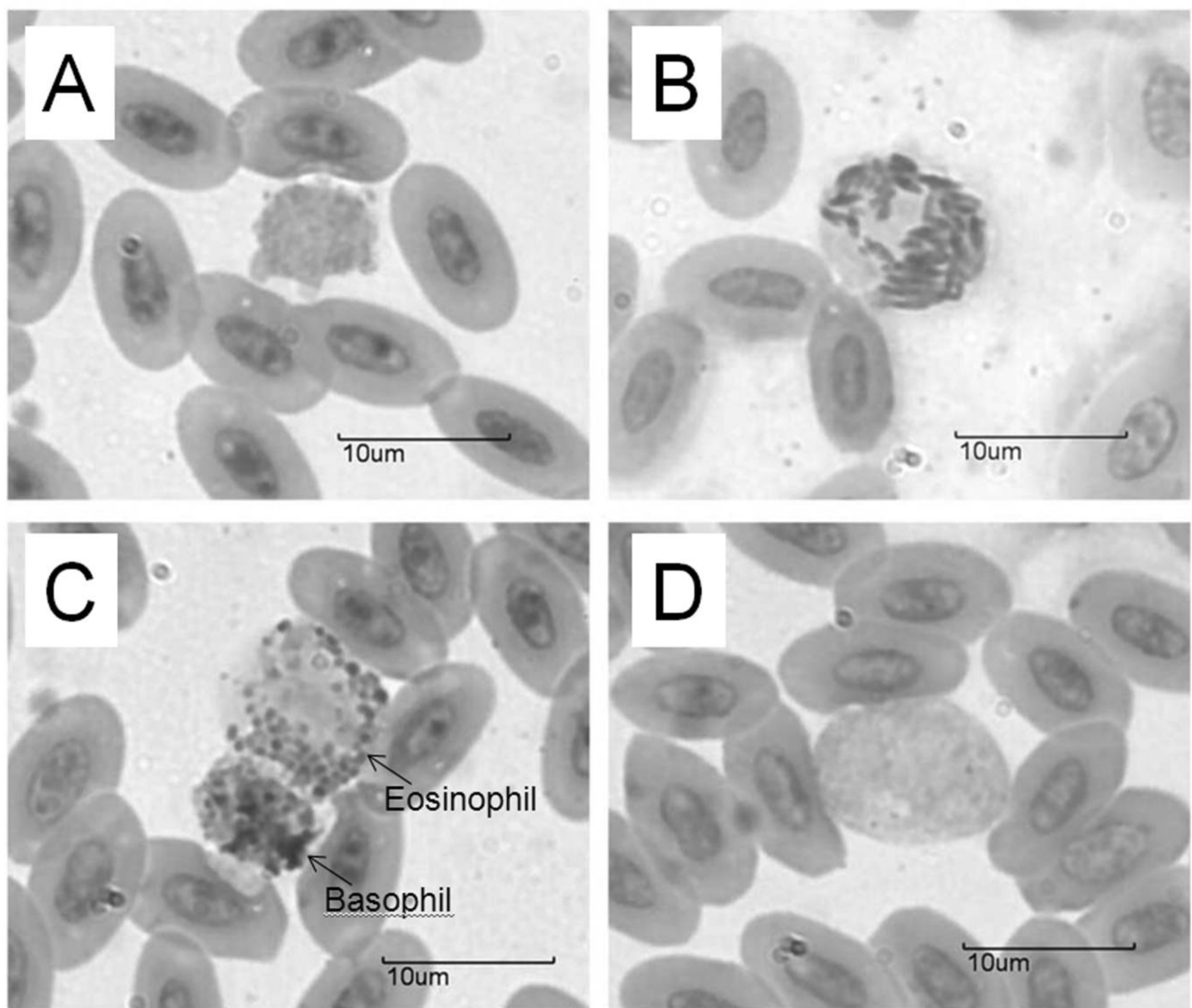

Figure 2. Photomicrographs of leukocytes from house finches. Cells shown are lymphocyte (*A*), heterophil (*B*), eosinophil and basophil (*C*), and monocyte (*D*). A color version of this figure is available online.

Table 1: Summary of leukocyte counts of house finches with and without mycoplasmal conjunctivitis and at both sample times

Disease status and sample time (min)	% Heterophils (SE)	% Lymphocytes (SE)	% Eosinophils (SE)	% Basophils (SE)	% Monocytes (SE)
Relative leukocyte counts:					
No conjunctivitis ($n = 26$):					
3	9.5 (1.4)	58.8 (3.1)	2.5 (.7)	29.1 (2.3)	.1 (.1)
120	11.8 (2.0)	38.8 (3.1)[b]	1.7 (.6)	47.4 (2.3)[b]	.3 (.1)
Conjunctivitis ($n = 9$)					
3	20.5 (3.3)	46.1 (4.5)	.7 (.3)	32.5 (3.7)	.2 (.1)
120	36.6 (5.1)[b]	29.8 (3.6)[b]	1.0 (.5)	32.3 (4.3)	.3 (.2)
Absolute estimates of leukocyte abundance:[a]					
No conjunctivitis ($n = 26$):					
3	2.2 (.4)	14.8 (1.6)	.5 (.1)	6.9 (.7)	.02 (.01)
120	1.3 (.2)[b]	4.5 (.5)[b]	.2 (.1)[c]	5.2 (.4)[c]	.02 (.01)
Conjunctivitis ($n = 9$)					
3	5.5 (1.0)	12.5 (1.9)	.2 (.1)	8.5 (1.0)	.1 (.04)
120	9.3 (1.7)[c]	6.5 (1.0)[c]	.3 (.2)	7.0 (1.0)	.1 (.04)

[a]Mean no. cells per 10,000 erythrocytes.
[b]Paired t-test comparing 3- and 120-min samples ($P < 0.001$).
[c]Means are not significant after Bonferroni correction.

to plasma, samples were vortexed and incubated at 37°C for 30 min. After incubation, 50 μL of the plasma-bacteria mixture was plated in duplicate onto tryptic soy agar plates. On each day, three plates inoculated with 50 μL diluted bacteria served as positive controls, and three plates inoculated with 50 μL phosphate-buffered saline served as negative controls. All plates were incubated at 37°C, and the number of colony-forming units (CFUs) per plate was quantified after ~24 h. There was no bacterial growth on negative controls, so BKA per sample was calculated as $1 - (\text{mean CFU}_{\text{sample}}/\text{mean CFU}_{\text{positive controls}})$. We assayed BKA for 19 birds (8 infected, 11 uninfected) for which sufficient plasma sample was available for both time points (3 and 120 min). Plasma was stored at −80°C for 3–5 mo before processing.

Data Analyses

H-L ratios and total WBC counts were log transformed to approximate normal distributions prior to analyses. We then used repeated-measures ANOVAs to simultaneously examine the effect of sample time (i.e., 3 and 120 min) and MG infection on our three primary immune measures: total WBC, H-L ratio, and BKA. In all cases, time was the within-subjects factor, and infection was the between-groups factor, and the interaction effect of time × infection was also included in the models. Finally, we compared relative and absolute counts of individual leukocyte types between sample times (separately for infected and noninfected birds) using paired t-tests. Because multiple t-tests were performed here (five per disease group, reflecting one test for each cell type), significance for these comparisons was accepted if $\alpha < 0.01$ (Rice 1989). All analyses were performed using the Statistica 6.1 software package (Statsoft).

Results

Results from the examination of house finch blood smears for leukocytes are shown in table 1. The strongest and most consistent effect of our capture and holding protocol was on the abundance of circulating lymphocytes; for birds with and without conjunctivitis, there was a significant decrease in both relative and absolute lymphocyte numbers between the 3-min sample and the 120-min sample. For heterophils, absolute counts decreased over time in birds without conjunctivitis, but there was a nonsignificant increase over time in birds with the disease. Absolute counts of eosinophils and basophils tended to decrease over time in birds without conjunctivitis, but these differences were not significant after Bonferroni correction of P values (table 1).

Analysis of total WBC abundance (number of cells per 10,000 erythrocytes) showed an effect of both infection and sample time (table 2). WBC counts were higher in birds with MG than in uninfected birds and declined significantly over time between the 3-min sample and the 120-min sample (fig. 3*A*). A significant interaction effect pointed to a difference in the response of infected and noninfected birds to capture stress; in birds without conjunctivitis, total WBC count decreased by approximately 30% over time, while in infected birds it decreased by only 6% (fig. 3*A*).

H-L ratios were significantly higher in house finches with conjunctivitis than in those without and were higher in the 120-min sample than in the 3-min sample (table 2; fig. 3*B*). However, the magnitude of the effect of time depended on infection status (i.e., significant time × infection interaction; table 2). At 120 min, H-L ratios of infected birds more than

Table 2: Summary of repeated-measures tests examining each of the innate immune measures of house finches at 3- and 120-min time periods (within-subjects factor = time) and the presence or absence of mycoplasmal conjunctivitis (between-groups factor = infection)

Dependent and predictor	*F*	df	*P*	Mean square
Total white blood cell count:				
Infection	11.011	1	.0022	.516
Error		33		.047
Time	20.481	1	.0001	.504
Time × infection	7.566	1	.0096	.186
Error		33		.025
Heterophil-lymphocyte ratio:				
Infection	15.075	1	.0005	.361
Error		33		.024
Time	22.422	1	.0000	.259
Time × infection	4.858	1	.0346	.056
Error		33		.012
Bacteria-killing ability:				
Infection	.958	1	.3415	1,001.099
Error		17		1,045.232
Time	.725	1	.4062	194.130
Time × infection	2.240	1	.1528	599.386
Error		17		267.592

doubled, whereas the change in H-L ratio for uninfected birds was less pronounced (fig. 3*B*).

Finally, there were no significant effects of infection, sample time, or their interaction on BKA, a measure of humoral innate immunity (table 2). However, there was a nonsignificant tendency for BKA to be higher in house finches with conjunctivitis than in those without, as well as a tendency for BKA to decrease over time in uninfected birds but not in infected birds (fig. 3*C*).

Discussion

The collective results from this study show how the innate immune system response to acute stress differs between infected and noninfected birds and specifically how infection counterbalances the negative effects of acute stress on immunity. The typical response to acute stress in this and a large variety of other species is a reduction in total leukocytes (Davis 2005; Noda et al. 2006; Buehler et al. 2008; Seddon and Klukowski 2012), and this is what we observed in uninfected house finches when stressed (a 30% decline in WBC abundance; fig. 3*A*). However, when birds were infected with MG, the decline in WBC abundance was much less (a 6% decline), and we interpret this as the demands of the infection (for leukocytes to remain in circulation) outweighing the trafficking or redistribution of cells that normally occurs with acute stress (Dhabhar et al. 1996). Keeping a strong complement of immune cells mobilized in circulation would be advantageous for combating infection. Moreover, while the effect was not significant, we saw a similar pattern with the bacteria-killing assay (fig. 3*C*), in that the suppression of bacteria-killing response that sometimes occurs after stress (e.g., Matson et al. 2006) seemed to be counterbalanced by the demands of infection.

A more detailed examination of WBC patterns in response to capture stress highlights some key findings. Focusing on individual leukocyte types, we found that stress was associated with a decline in four out of five cell types in uninfected birds, whereas in MG-infected birds only one cell type (lymphocytes) decreased in number following stress. Specifically, the estimated lymphocyte numbers in uninfected birds declined by 70% after the acute-stress treatment (table 1), but in MG-infected birds this decline was 48%. Meanwhile, estimated heterophil numbers increased following stress in infected birds, which is in contrast to the decrease seen in uninfected birds (table 1). This pattern makes sense given the role of this cell in the immune system; studies of domestic chickens indicate that heterophils, being phagocytic in nature, are important in the host response to MG infection (Branton et al. 1997). A previous survey of leukocyte profiles in MG-infected and uninfected house finches also found that infected birds had greater numbers of heterophils (Davis et al. 2004). These results support the idea that capture stress had an overall weaker suppressive effect on immune cells of infected finches compared to uninfected finches.

It has been suggested that stress-induced redistribution of WBCs is an adaptive response that allows for the enhancement of immunity in other tissues (Dhabhar et al. 1996; Dhabhar 2009). This redistribution is what results in the characteristic rise in H-L ratios in the blood within hours of a stressful event. In support of this hypothesis, redistribution of blood WBCs in response to acute stress has been linked to enhanced immunity in the skin, where they can be quickly mobilized in response

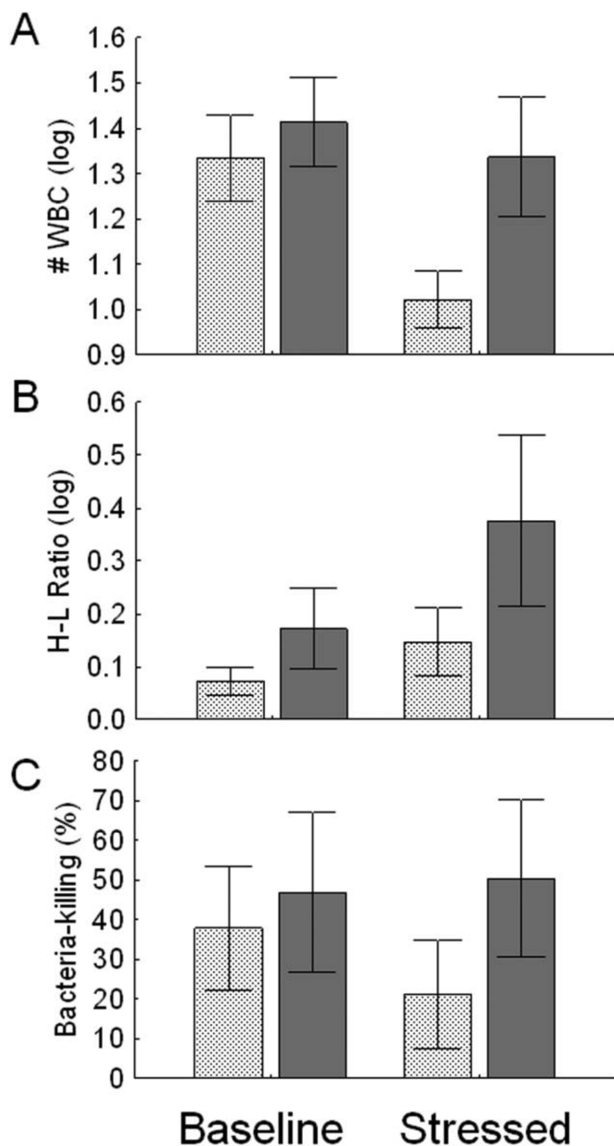

Figure 3. Average white blood cell (WBC) counts (*A*), heterophil-lymphocyte (H-L) ratios (*B*), and bacteria-killing ability (BKA; *C*) of uninfected (stippled bars) and *Mycoplasma gallisepticum*–infected (dark gray bars) house finches at baseline and stressed intervals (3 and 120 min after capture, respectively). Sample sizes for *A* and *B* were 26 uninfected and 9 infected birds, respectively. For *C* there were samples from 11 uninfected and 8 infected birds. See Methods for descriptions of each parameter. Whiskers over bars represent 95% confidence intervals. The analysis of BKA did not show a significant effect of time or infection (see table 2).

to wounding or entry of pathogens (reviewed in Dhabhar 2009). If redistribution serves the purpose of enhancing immunity where needed, the increase in heterophils we saw in MG-infected birds may reflect an altered redistribution pattern, where in the face of infection immunity is enhanced in the most relevant compartment. The functional significance of the reduction in circulating lymphocyte numbers following stress is less clear. Their drop in abundance is a result of migration to extravascular spaces and, to a lesser extent, apoptosis of cells (Dhabhar et al. 1995). That they are shunted from the peripheral blood supply into tissues during stress would suggest that mobility of these cells is not a priority during this phase.

The distinct H-L patterns we observed among infected and uninfected birds are in line with prior work on this disease using a different stress assay, levels of plasma corticosterone; Lindström et al. (2005) showed how MG-infected house finches that were acutely stressed from capture had corticosterone levels that were five to six times higher than unstressed birds without the disease. Meanwhile, acute stress caused only a threefold increase in corticosterone in uninfected birds. In light of evidence suggesting that corticosterone is a key mediator of immune enhancement during acute stress (Dhabhar 2009) and findings from Lindström et al. (2005), corticosterone may be an important modulator of the shift in WBC redistribution patterns we noted among MG-infected birds in our study.

More generally, results from this study also provide insight for interpreting field and lab experiments involving H-L ratios as indicators of stress (reviewed in Davis et al. 2008). As expected, we found that H-L ratios increased in both uninfected and MG-infected birds in response to a capture and holding protocol (capture plus 2 h holding period), and this suggests that our protocol reliably induced stress and that this stress was effectively measured with this hematological index. For the field or lab researcher, it is important to note that H-L ratios approximately doubled as a result of this stressor (in uninfected birds). Knowing the magnitude of change in H-L ratios after acute stress would be very useful for interpreting leukocyte profiles of birds. It is also important to know that H-L ratios tripled in infected birds after acute stress. The more pronounced response in MG-infected birds was largely driven by an increase in heterophils at the 120-min sampling point (table 1). For the researcher trying to interpret patterns from H-L ratio data, these results emphasize that individuals harboring undetected infections (as opposed to the easily observed signs of MG) could show especially large H-L ratios if the study involves stress treatments.

Finally, while our BKA results reveal a trend that is consistent with the idea that stress-induced suppression of BKA may be moderated in MG-infected birds, we also are aware that there was no significant difference between baseline and stress BKA responses for either infected or uninfected birds. There are a few potential explanations for this result. First, we had sufficient plasma to test bacteria-killing responses in only 8 infected and 11 uninfected birds; thus, we may not have had sufficient power to detect differences due to the stress treatment (power to detect a difference at 120 min between infected and uninfected birds given the observed effect size was ~30%). The lag time between sample collection and implementing the BKA assays (range = 110–155 d) may have further compounded this issue if the time delay resulted in decays in the killing response that minimized differences between treatment groups. Rapid declines in *E. coli* killing ability of plasma can occur with increasing storage time (Liebl and Martin 2009); however, high rates of killing (>95%) have been reported in at least one study with storage times

extending beyond 1 yr (Rubenstein et al. 2008). In our study, killing rates were still fairly robust, ranging from 3% to 85%. Importantly, the nonsignificant negative effect of stress on BKA that we observed in uninfected birds may simply reflect weak and/or variable effects of acute stress on this component of innate immune function, as has been reported in other studies (Matson et al. 2006; Merrill et al. 2012). Future work will be needed to fully understand associations between acute stress, humoral immunity, and MG infection in this study system.

In summary, the collective results of this study highlight how infections can modify the normal physiological response to acute stress in animals in ways that may be adaptive. Infection appears to override the stress-induced redistribution of leukocytes, thereby ensuring that a strong complement of cells remains in circulation. Infections also stimulate the release or production of important phagocytic cells (heterophils) above the levels normally seen during stress. In future work, it will be interesting to determine whether the heterophils present during stress are functionally equivalent to those produced during infections. Moreover, it would also be helpful to know whether there are any morphological characteristics that could be used to differentiate heterophils associated with stress versus those that increase with infection (Latimer et al. 1988). Addressing these and related questions will help expand our knowledge of the conflicting demands of stress and infection on the innate immune system.

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