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Author for correspondence:

Laura A. Schoenle

e-mail: las86@cornell.edu

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Exogenous glucocorticoids amplify the costs of infection by reducing resistance and tolerance, but effects are mitigated by co-infection

Laura A. Schoenle^{1,2}, Ignacio T. Moore², Alana M. Dudek², Ellen B. Garcia³, Morgan Mays², Mark F. Haussmann⁴, Daniela Cimini³ and Frances Bonier^{2,5}

¹Office of Undergraduate Biology, Cornell University, Ithaca, NY, USA

²Department of Biological Sciences, and ³Department of Biological Sciences and Biocomplexity Institute, Virginia Tech, Blacksburg, VA, USA

⁴Department of Biology, Bucknell University, Lewisburg, PA, USA

⁵Biology Department, Queen's University, Kingston, Ontario, Canada

LAS, 0000-0002-9591-1194; MFH, 0000-0003-0021-0561; FB, 0000-0001-7245-0244

Individual variation in parasite defences, such as resistance and tolerance, can underlie heterogeneity in fitness and could influence disease transmission dynamics. Glucocorticoid hormone concentrations often change in response to fluctuating environmental conditions and mediate changes in immune function, resource allocation and tissue repair. Thus, changes in glucocorticoid hormone concentrations might mediate individual variation in investment in resistance versus tolerance. In this study, we experimentally increased glucocorticoid concentrations in red-winged blackbirds (*Agelaius phoeniceus*) that were naturally infected with haemosporidian parasites, and assessed changes in resistance and tolerance of infection. Glucocorticoid treatment increased burdens of *Plasmodium*, the parasite causing avian malaria, but only in the absence of co-infection with another Haemosporidian, *Haemoproteus*. Thus, glucocorticoids might reduce resistance to infection, but co-infection can mitigate the negative consequences of increased hormone concentrations. Glucocorticoid treatment also decreased tolerance of infection. We found no evidence that the inflammatory immune response or rate of red blood cell production underlie the effects of glucocorticoids on resistance and tolerance. Our findings suggest that exogenous glucocorticoids can increase the costs of haemosporidian infections by both increasing parasite numbers and reducing an individual's ability to cope with infection. These effects could scale up to impact populations of both host and parasite.

1. Introduction

Parasite infections are universal, but host responses to infection can vary widely across individuals, populations and species. When faced with infection, hosts can invest in two defence strategies: resistance and tolerance [1]. Resistance entails killing parasites and/or limiting their reproduction to reduce the total parasite burden. Tolerance involves minimizing the costs of infection for a given parasite burden by limiting or repairing damage. Importantly, tolerance allows a host to cope with infection without reducing the parasites' fitness [1,2]. Investment in resistance and/or tolerance could change parasite burdens and the costs of infection for the host, and as a result influence not only host fitness, but also parasite transmission [3,4].

Resistance and tolerance of infection can change when organisms encounter challenging environmental or social conditions, such as resource limitation or over-crowding [5–7]. Furthermore, glucocorticoid hormones, which are key mediators of responses to such challenges in vertebrates, also can directly

influence resistance and tolerance [8,9]. Experimental increases in glucocorticoids tend to reduce resistance, which is primarily attributed to immunosuppression caused by prolonged elevation of glucocorticoids [9,10]. Glucocorticoids influence immunity through diverse mechanisms, such as limiting inflammation through their influence on immune signalling pathways, regulating adaptive immunity via effects on lymphocyte activation and apoptosis, and influencing cytokine activity (reviewed in [11]). Glucocorticoid-driven decreases in resistance can cause elevations in parasite burden and/or the length of time organisms are infectious [8]. As a result, hosts with elevated glucocorticoids might be more likely to transmit infection, which ultimately could change disease transmission dynamics within a population [8,12].

The effects of glucocorticoids on tolerance are less clear. In an observational field study, we found that red-winged blackbirds (*Agelaius phoeniceus*) with naturally higher circulating concentrations of corticosterone, the primary avian glucocorticoid, were more tolerant of haemosporidian parasites, including avian malaria [13]. Birds with higher corticosterone maintained higher haematocrit for a given parasite burden than birds with lower corticosterone, suggesting corticosterone might increase tolerance. We found no evidence that corticosterone increased tolerance by elevating red blood cell production rates [13], but corticosterone could suppress pro-inflammatory Th1 activity and thereby reduce the number of cells destroyed by the host's immune system [11,14]. Experimental elevation of corticosterone in other host–parasite systems have been shown to decrease [8] or have no effect [15] on tolerance. However, to our knowledge, these laboratory-based experiments are not associated with similar field-based observational or experimental studies. Furthermore, these studies do not assess other factors that are likely to influence tolerance in free-living animals, which could result in differences between laboratory and field studies. For example, co-infection with multiple parasite species is common among wildlife and can alter the intensity of infections, the immune response and cost of infection [16–18]. Infection with one parasite species can stimulate an immune response that allows the host to better defend against other infections, or alternatively, one infection can increase host susceptibility to other infections through mechanisms such as immunosuppression [19]. Competition for host resources among parasite species or lineages might also alter parasite burdens within hosts such that the more competitive (and often more virulent) parasite will outnumber the less competitive parasite within the host [20]. Thus, co-infection could also impact the effects of glucocorticoids on resistance and tolerance.

In this study, we experimentally increased circulating corticosterone concentrations in male red-winged blackbirds naturally infected with one to three genera of haemosporidian parasites: malaria (*Plasmodium*) and malaria-like parasites (*Haemoproteus* and *Leucocytozoon*). Avian haemosporidians are vector-transmitted parasites with complex life cycles, and are frequently used as a model system to evaluate the ecological and evolutionary consequences of infection [21]. Although observational studies report positive, negative and non-significant relationships between infection status or parasite burden and both health and fitness metrics, experimental treatments of chronic infections consistently demonstrate negative consequences of infection in terms of biomarkers (e.g. haematocrit, oxidative stress), survival and

reproductive success [22–25]. Here, we investigate if (i) corticosterone treatment affects resistance and tolerance to infection, (ii) effects of corticosterone on resistance and tolerance vary with co-infection status with multiple haemosporidian genera, and (iii) effects of corticosterone were mediated through mechanisms of immunosuppression and/or tissue repair. We predicted that exogenous corticosterone would increase parasite burden, indicating a reduction in resistance. Based on our findings in free-living birds, we also predicted that corticosterone would reduce the rate at which measures of host health decline as parasite burden increases, indicating an increase in tolerance [1,2]. Co-infection could modulate corticosterone's effects on resistance and/or tolerance to haemosporidians, and these effects could vary with genus of the infecting parasites. We also predicted that corticosterone would potentially increase tolerance and reduce resistance by suppressing immune function and/or increase tolerance by upregulating tissue repair.

2. Material and methods

(a) Study population

Red-winged blackbirds breeding in the marshes at the Queen's University Biological Station (QUBS; 44°34'02.3" N, 76°19'28.4" W) and in the surrounding Rideau Canal region of southeastern Ontario, Canada, have high haemosporidian infection prevalence. In the late 1980s to early 1990s, blood smear analyses revealed that 30–71% of birds in the QUBS population tested positive for the parasites [26–28]. In 2013–2015, PCR-based screening of breeding adults indicated that haemosporidian prevalence was $97.97 \pm 4.55\%$ (mean \pm s.e.) and $64.73 \pm .43\%$ (mean \pm s.e.) were infected with parasites from at least two genera of haemosporidians [13].

(b) Experimental design and data collection

In April–May 2015, we captured 89 adult males either using V-top Troyer traps baited with seeds and conspecific decoys or mist nets paired with conspecific song and call playback. We housed birds in groups of three in large outdoor flight aviaries (2.4 × 6.1 × 2.4 m, 30 aviaries in the complex; see the electronic supplementary material, Methods and Results, for detailed information about bird capture and husbandry).

We randomly assigned birds to one of three treatment groups, such that each aviary contained one bird from each treatment group, with the exception of one aviary containing only two birds. Treatment groups were low-dose corticosterone (0.1 mg implant, $n = 30$), high-dose corticosterone (0.5 mg implant, $n = 30$) and control (vehicle-only implant, $n = 29$) (Innovative Research of America, Sarasota, FL, USA). We sampled birds once pre-treatment, then immediately implanted the pellets subcutaneously on the birds' backs. We sampled each bird again 7, 14 and 21 days post-treatment. Corticosterone doses and sampling periods were selected based on a pilot study conducted in 2014 (methods and results for the pilot study are located in the electronic supplementary material). At each sampling period we collected 500 μ l of blood and measured body mass. We used blood samples to assess each individual's health, immune function, and tissue repair, and parasite presence and burdens. We evaluated host health using four metrics that can be impaired by haemosporidian infection: haematocrit [22], haemoglobin [22], body mass [29] and oxidative balance [30,31]. We also assessed an indicator of genomic stability, the percentage of red blood cells containing micronuclei [32,33]. Micronuclei are formed when chromosomes break or are not incorporated into

the nucleus during cell division [32]. Birds infected with haemosporidian parasites upregulate red blood cell production to compensate for the cells destroyed during infection [22], and this increase in cell production might result in higher error rates during cell division. We also assessed two immune metrics that can increase in response to haemosporidian infection: (i) nitric oxide, an inflammatory signalling molecule and anti-parasitic defence [34–36]; and (ii) PIT54 (avian analogue of haptoglobin), an acute-phase, anti-inflammatory protein and haemoglobin scavenger [37–39]. Because both haemosporidian parasites and the immune response to malarial parasites damage red blood cells [40], we assessed the level of red blood cell production as an indicator of tissue repair rate. Parasite presence/absence and burden were assessed pre-treatment and 7 days post-treatment, and all other measures were assessed at every sampling point.

After blood sample collection, we immediately measured haemoglobin in approximately 5 μ l of fresh blood using a haemoglobin meter (HemoCue HB 201+, HemoCue AB, Ängelholm, Sweden) and we created two replicate blood smears. We stained the blood smears with Giemsa stain and used the blood smears to assess haemosporidian parasite burdens (number of infected cells per approx. 10 000 red blood cells), and the level of red blood cell production (estimated with polychromasia, the relative number of young red blood cells which appear polychromatic, or intensely-stained with Giemsa). We centrifuged the remaining blood samples in capillary tubes and measured haematocrit using the average from two capillary tubes of blood. We then separated the plasma from the red blood cells and froze both at -20°C . We used the blood cells to assess the presence/absence of *Plasmodium*, *Haemoproteus* and *Leucocytozoon* via a PCR-restriction enzyme assay (modified from [41]). We used the plasma to measure corticosterone concentration via radioimmunoassay [42] and PIT54, nitric oxide, reactive oxygen species, and total antioxidant capacity via commercial colorimetric assay kits. See electronic supplementary material, Methods and Results, for details about blood sampling, blood processing and laboratory assays.

(c) Statistical analyses

We performed all data analyses in R v. 3.3.2 [43]. Before conducting any analyses we transformed plasma corticosterone concentration values with a natural log function and parasite burdens as $\ln(\text{burden} + 1)$. Log transformations of corticosterone is common practice in field endocrinology because hormone concentrations are rarely normally distributed. Because parasite burdens within hosts are often overdispersed, parasite burden is frequently modelled using negative binomial or overdispersed Poisson error distributions. Neither of these distributions fitted our data as well as linear regression models with transformed parasite data (see electronic supplementary material, Analysis for resistance), and thus we used this transformation for our parasite data throughout our analyses. For all regression analyses, we initially summarized model results using Type III sums of squares using the R package ‘car’ [44]. However, if there were no significant interaction terms, we used Type II sums of squares, which is more statistically powerful in the absence of significant interactions [45,46]. However, if the switch to Type II sums of squares altered the statistical significance of interaction terms (the interaction terms change from non-significant to significant), we used Type III sums of squares, which is the more conservative approach. We compared the effects among levels within categorical factors (e.g. levels of corticosterone treatment) with pairwise contrasts adjusted with Tukey’s multiple comparisons using the R package ‘lsmeans’ [47]. All mixed models were run with the R package ‘lme4’ [48] and all graphs were created using the R package ‘ggplot2’ [49].

(i) Effectiveness of corticosterone treatment

We determined whether or not the implants effectively elevated corticosterone by performing a linear mixed effect model predicting change in corticosterone from pre-treatment. As predictor variables, we included individual identity as a random effect, and pre-treatment corticosterone, treatment, day of the experiment and the interaction between treatment and day as fixed effects.

(ii) Effect of glucocorticoids and co-infection on resistance

We assessed the effects of corticosterone on resistance and tolerance at 7 days post-implantation because (i) our pilot study indicated that parasite burden peaked at this day (electronic supplementary material, Pilot Study, figure S2) and (ii) this timing allowed us to avoid confusing corticosterone driven changes in parasite burden with the high burdens associated with the acute stage of a newly acquired infection, as it generally takes at least 8–15 days for a natural infection to reach the acute stage [39,50]. We assessed resistance as the change in parasite burden after treatment. Using a separate analysis for each parasite genus, we tested the effect of corticosterone treatment on change in parasite burden, including only individuals that were PCR-positive for the focal genus. We performed linear models including change in parasite burden as the response variable and corticosterone treatment, pre-treatment parasite burden, the presence/absence of each non-focal parasite genus, and the interactions between treatment and the presence/absence of each non-focal parasite genus as explanatory variables. Because we found an effect of the high-dose implant and co-infection status on *Plasmodium* burden (see Results below) we conducted two *post hoc* analyses aimed at determining if the effects of corticosterone on *Plasmodium* burden varied with co-infection status. We used an analysis of variance and Tukey’s multiple comparisons to assess how the change in *Plasmodium* burden varied with overall co-infection status within the high-dose treatment. We also tested if there was a relationship between co-infection status and *Plasmodium* burden pre-treatment using a simple linear regression with *Plasmodium* burden as the response variable and the presence/absence of both *Haemoproteus* and *Leucocytozoon*, and their interaction, as explanatory variables.

Because the *Haemoproteus* and *Leucocytozoon* parasites detected in blood cells can transmit infection to insect vectors, host resistance is directly linked to probability of disease transmission. However, *Plasmodium* has multiple parasite life stages within red blood cells, and only the gametocyte stage can infect vectors [40]. Therefore, to investigate specific effects of corticosterone on *Plasmodium* disease transmission, we also tested the effect of treatment on the change in *Plasmodium* gametocyte number. We measured gametocyte number rather than a ratio of gametocytes to other life stages because the raw numbers determine the likelihood of infecting a vector [51]. We performed a linear model analysis including change in gametocytes as the response variable and corticosterone treatment, pre-treatment parasite burden, the presence/absence of *Haemoproteus* and *Leucocytozoon*, and the interactions between treatment and the presence/absence of both *Haemoproteus* and *Leucocytozoon* as explanatory variables.

(iii) Effect of glucocorticoids and co-infection on tolerance

We measured tolerance as the slope of the relationship between parasite burden and proxies for host fitness or health among individuals within the same treatment group [2]. A sharper decline in fitness or health as parasite burden increases (a steeper slope) indicates lower tolerance. To test the effect of corticosterone on tolerance, we performed linear regressions predicting measures of health (haematocrit, haemoglobin, body mass, oxidative balance and micronuclei) including the following

predictor variables: treatment group, total haemosporidian parasite burden, and the interaction between treatment group and parasite burden. A significant interaction between corticosterone treatment and parasite burden indicates an effect of corticosterone on tolerance. Because we evaluated multiple, potentially non-independent metrics of tolerance, we adjusted the p -values for false discovery rate to account for multiple comparisons for analyses indicating significant corticosterone by parasite burden interaction terms [52,53]. We included all birds that were PCR-positive for haemosporidians and used total parasite burden across genera in the analyses because all three genera have the potential to influence health [23,54,55].

We performed separate analyses to determine if co-infection was associated with tolerance or modulated any of the effects of corticosterone on tolerance. Only a subset of all possible combinations of co-infections were observed in our sample (electronic supplementary material, Methods and Results, table S3), and, as a result, we did not have the statistical power to include the presence/absence of each genus and their interactions in a regression model. Instead, we included infection status as a categorical variable where the levels were the combinations of infecting genera. We excluded birds with any infection status where $n < 5$ within any of the three treatment groups, which allowed us to test for effects of co-infection among three infection statuses: infection by *Plasmodium* only, by *Plasmodium* and *Haemoproteus*, and by *Plasmodium* and *Leucocytozoon*. The models testing the effect of co-infection and corticosterone treatment on tolerance included the measures of health as the response variables, and treatment, total haemosporidian parasite burden, infection status, and all two-way interactions as predictor variables. We did not perform this analysis for micronuclei because our sample was insufficient for a model with the predictors of interest.

(iv) The effects of corticosterone and co-infection on tissue repair and immune function

To investigate potential mechanisms for effects of corticosterone on resistance and tolerance, we assessed whether corticosterone treatment affected immune metrics and/or red blood cell production using linear mixed models. If we found any significant effects of corticosterone, we adjusted the p -values for false discovery rate to account for multiple comparisons [52,53]. We included individual identity as a random effect, and pre-treatment value of the response variable, day, treatment, change in parasite burden, and pairwise-interactions of day, treatment, and change in parasite burden as fixed effects. As in the analyses of tolerance, we tested for the additional effect of co-infection using a subset of the data including birds infected with *Plasmodium*, *Plasmodium* and *Haemoproteus*, or *Plasmodium* and *Leucocytozoon*, and added the following fixed effects to the model: co-infection status and the pairwise interactions between co-infection status and day, treatment, and parasite burden.

3. Results

(a) Effectiveness of corticosterone treatment

We measured glucocorticoid concentrations in 257 blood samples collected from 88 individuals. The high corticosterone treatment (0.5 mg) elevated plasma corticosterone concentrations throughout the experiment above both the control implant ($t = 2.58, p = 0.03$) and the low corticosterone implant (0.1 mg; $t = 3.18, p = 0.006$). At the times of sampling, the corticosterone concentrations of birds in the low dose treatment did not differ from birds receiving the sham implant ($t = -0.62, p = 0.81$) (electronic supplementary

material, Methods and Results, figure S3). Seven days after implantation, birds in the high-dose implant group had corticosterone concentrations of (mean \pm s.e.) 8.79 ± 1.89 ng ml⁻¹, which is similar to concentrations in free-living, breeding males, which we previously measured as 12.31 ± 1.86 ng ml⁻¹ [13]. Across all treatments, the change in corticosterone from the initial value decreased over time, indicating a waning effect of the implants or upregulated negative feedback and clearance mechanisms ($F = 14.66, p = 0.0002$).

(b) Effect of glucocorticoids and co-infection on resistance

The high-dose corticosterone treatment affected resistance, as indicated by an increased *Plasmodium* burden, but the effect depended on co-infection status. There were significant interactions between treatment and both *Haemoproteus* ($F = 16.74, p < 0.0001, n = 82$) and *Leucocytozoon* ($F = 4.15, p = 0.02, n = 82$) infection status, and the three-way interaction between treatment, *Haemoproteus*, and *Leucocytozoon* approached significance ($F = 2.93, p = 0.06, n = 82$). If birds were infected with *Plasmodium* but not *Haemoproteus*, the high-dose implant increased *Plasmodium* burdens more than the control implant (figure 1a,b), but the effect disappears in the presence of a *Haemoproteus* co-infection (figure 1c,d). The high-dose implant also increased *Plasmodium* burden more than the low-dose implant if a bird was only infected with *Plasmodium* (figure 1a), but not when co-infected with *Leucocytozoon* and/or *Haemoproteus* (figure 1b–d). In birds co-infected with *Plasmodium* and *Haemoproteus* (figure 1c), the low-dose implant increased *Plasmodium* burden more than the high-dose implant, but that effect is partially driven by a single bird with a large increase in parasite burden, and if that individual is removed from the analysis, the effect is no longer significant.

Within the high-dose treatment, change in parasite burden was associated with co-infection status ($F = 15.62, p < 0.0001, n = 28$). Birds infected with *Plasmodium* alone experienced greater increases in *Plasmodium* burden than those with co-infections, and none of the birds co-infected with *Haemoproteus* displayed increased *Plasmodium* burden (figure 2). The relationship between *Haemoproteus* co-infection and *Plasmodium* burden also existed pre-treatment. Pre-treatment, lower *Plasmodium* burdens were associated with *Haemoproteus* co-infection ($F = 54.9, p < 0.0001, n = 81$), but there was no relationship with *Leucocytozoon* co-infection ($F = 0.84, p = 0.36, n = 81$) or an interaction between *Haemoproteus* and *Leucocytozoon* ($F = 1.84, p = 0.18, n = 81$) (electronic supplementary material, Methods and Results, figure S4).

Corticosterone treatment increased the overall number of *Plasmodium* gametocytes. As with total *Plasmodium* burden, the effect of corticosterone treatment on the number of *Plasmodium* gametocytes depended on co-infection. The high-dose implants caused an increase in gametocytes relative to birds receiving both the control and low-dose implants in the absence of *Haemoproteus* (versus control, $t = 4.00, p = 0.0005, n = 28$; versus low, $t = 3.35, p = 0.004, n = 28$) and *Leucocytozoon* (versus control, $t = 2.92, p = 0.01, n = 28$; versus low, $t = 3.06, p = 0.009, n = 28$; additional details in the electronic supplementary material, Methods and Results).

Corticosterone treatment had no effect on resistance to *Haemoproteus* ($F = 1.95, p = 0.17, n = 23$) or *Leucocytozoon*

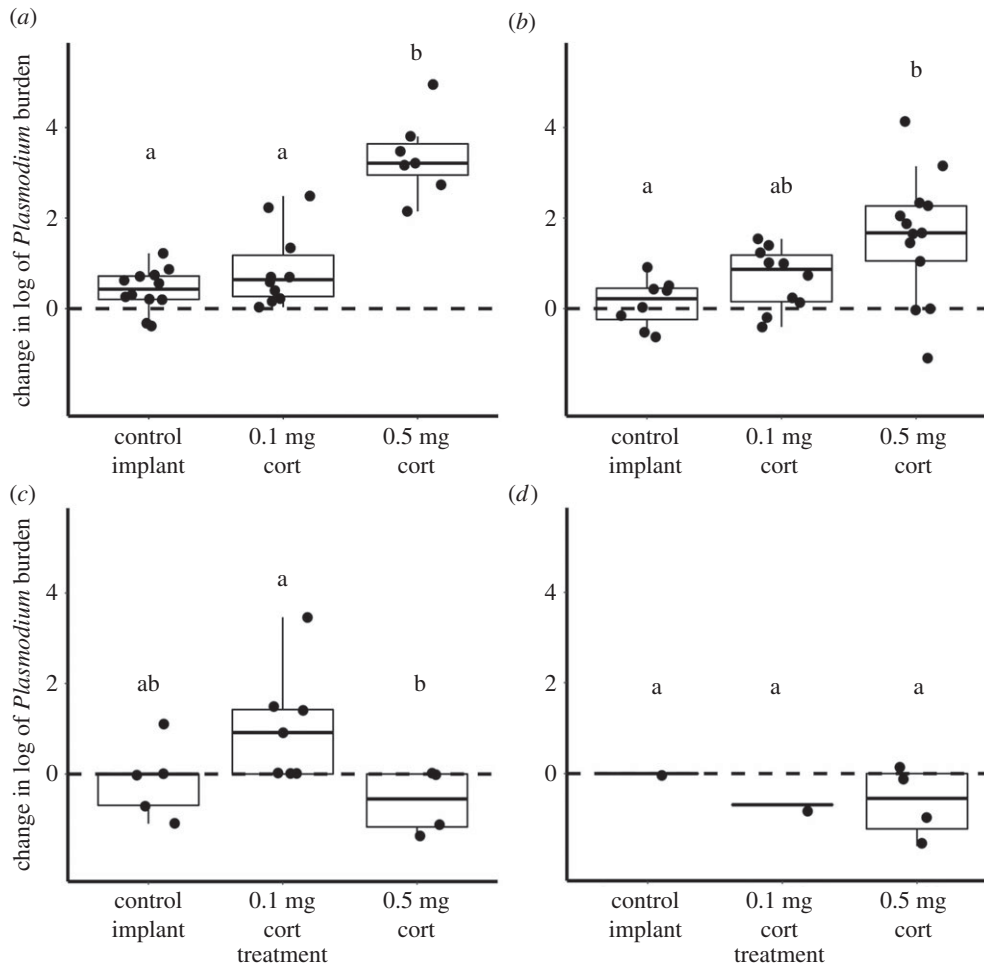


Figure 1. The effect of treatment on *Plasmodium* burden by co-infection status with (a) *Plasmodium* only, (b) *Plasmodium* and *Leucocytozoon*, (c) *Plasmodium* and *Haemoproteus*, and (d) *Plasmodium*, *Haemoproteus*, and *Leucocytozoon*. Letters (a, b) represent significant differences ($p < 0.05$) among treatments within the same panel. In the absence of *Haemoproteus*, (a,b), high-dose corticosterone causes an increase in *Plasmodium* burden, but the effect disappears with *Haemoproteus* co-infection (c,d).

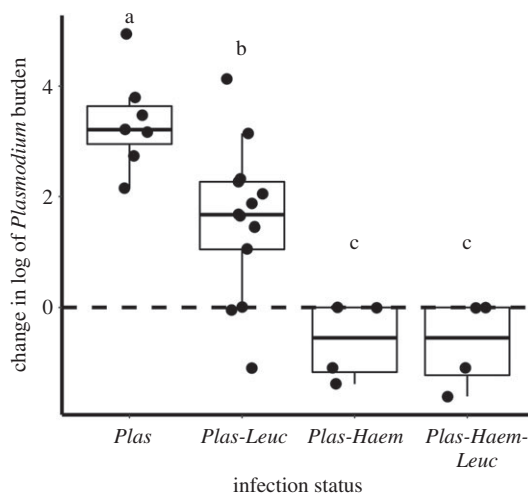


Figure 2. The relationship between co-infection status and change in parasite burden in burdens receiving the high-dose corticosterone treatment. Letters (a, b, c) represent significant differences ($p < 0.05$) among co-infection statuses. Co-infection with either or both *Haemoproteus* and *Leucocytozoon* limits the effect of treatment on *Plasmodium* burden.

($F = 0.92$, $p = 0.41$, $n = 37$), as measured by change in burdens of these parasites. Because fewer individuals were infected with *Haemoproteus* and *Leucocytozoon* than with *Plasmodium*, and there were limited sample sizes of some co-infection

statuses in our study (electronic supplementary material, Methods and Results, table S3), we could not test for an effect of co-infection on change in burdens of these genera.

(c) Effects of glucocorticoids and co-infection on tolerance

Corticosterone treatment reduced tolerance to infection with haemosporidian parasites as estimated through body mass (interaction between treatment and parasite burden; $F = 5.98$, $p = 0.004$, $n = 87$, false discovery rate adjusted $p = 0.02$). Body mass of birds in the high-dose group declined more steeply as parasite burden increased compared with birds in the low-dose group ($t = -3.35$, $p = 0.004$, $n = 87$) (figure 3a). However, the slope of the mass–parasite burden relationship did not differ between the high-dose and control groups ($t = -1.60$, $p = 0.25$, $n = 87$) or the low-dose and control groups ($t = 0.62$, $p = 0.81$, $n = 87$; figure 3a). The range of parasite burden in the control group (2–174 parasites per 10 000 red blood cells) is much smaller than the range in the low-dose (1–2143 parasites per 10 000 red blood cells) or high-dose (0–4346 parasites per 10 000 red blood cells) groups, and thus we might not have been able to detect a difference between the high-dose and control groups, if it were present. However, when we performed the same analysis combining the control and low-dose groups, which had similar

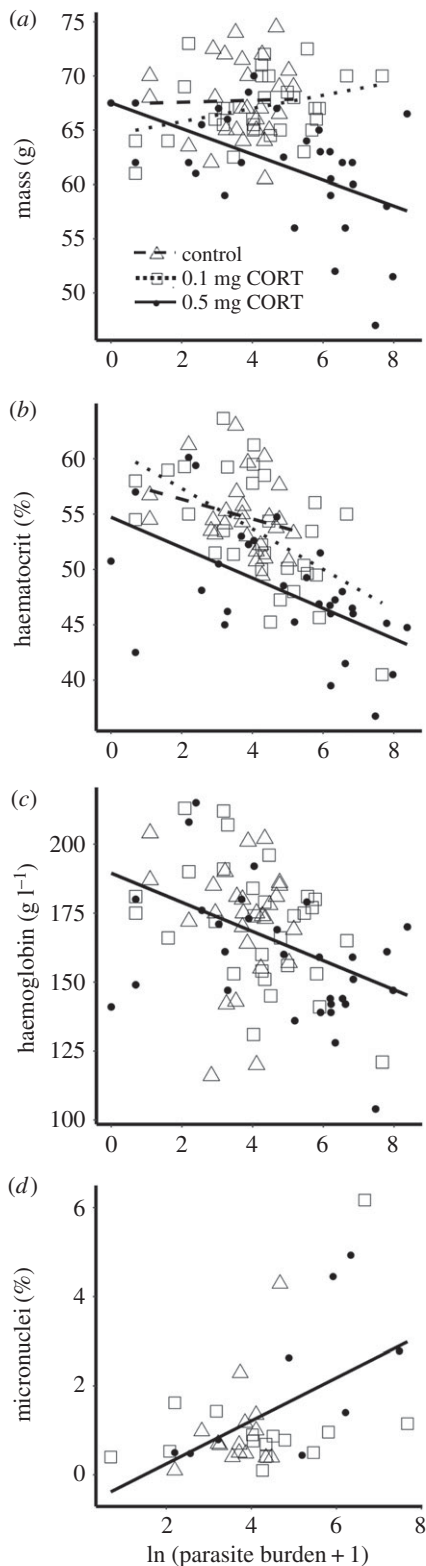


Figure 3. The effect of corticosterone treatment on tolerance to infection estimated using (a) body mass, (b) haematocrit and (c) haemoglobin. Open triangles and dashed line represent birds in the control group, open squares and dotted line represent the low-dose group, and solid circles and solid line represent the high-dose group. (a) Among birds implanted with the high dose, body mass was lower in birds with higher parasite burdens, and the slope was significantly different from the low-dose group or the low and control groups combined, indicating lower tolerance. (b) Birds in the high corticosterone group had lower mean haematocrit than birds in the other treatment groups. There was no difference in the slope of the relationship between haematocrit and parasite burden among treatment groups, indicating no difference in tolerance. (c) There was no difference in mean haemoglobin concentrations or the slope of the haemoglobin–parasite burden relationship among treatment groups.

corticosterone concentrations at the time of sampling, birds in the high-dose group demonstrated a steeper decline in body mass with increasing parasite burden than the other groups combined ($t = -3.35$, $p = 0.0012$, $n = 87$).

Corticosterone treatment had no effect on tolerance when estimated using haematocrit, haemoglobin or micronuclei data. Although both corticosterone treatment ($F = 11.78$, $p < 0.0001$, $n = 87$) and parasite burden ($F = 31.40$, $p < 0.0001$, $n = 87$) were associated with haematocrit, the slope of the relationship between haematocrit and parasite burden was similar across corticosterone treatments ($F = 0.59$, $p = 0.55$, $n = 87$; figure 3b). Haemoglobin declined with increasing parasite burden ($F = 13.19$, $p = 0.0005$, $n = 87$), but did not differ with treatment ($F = 1.40$, $p = 0.25$, $n = 87$) or the interaction of treatment and parasite burden ($F = 0.37$, $p = 0.69$, $n = 87$; figure 3c). Micronuclei increased with increasing parasite burden ($F = 10.11$, $p = 0.0032$, $n = 39$), indicating that higher infection intensities are associated with greater chromosomal damage, but micronuclei were unrelated to corticosterone treatment ($F = 0.69$, $p = 0.51$, $n = 39$) and the interaction of treatment and parasite burden ($F = 0.73$, $p = 0.49$, $n = 39$; figure 3d). Oxidative balance was not associated with parasite burden ($F = 0.46$, $p = 0.50$, $n = 84$), and there was no interaction of treatment and parasite burden ($F = 0.40$, $p = 0.96$, $n = 84$).

We assessed whether or not co-infection was correlated with tolerance using a subset of the full dataset, including individuals with the most common combinations of infecting genera (more than five birds per group): *Plasmodium* only, *Plasmodium* and *Haemoproteus*, and *Plasmodium* and *Leucocytozoon*. Within this subset, the interaction between co-infection status and parasite burden was not significant in any of the models predicting costs of infection (all $p > 0.07$), suggesting that co-infection does not influence tolerance.

(d) The effects of corticosterone and co-infection on tissue repair and immune function

Corticosterone treatment did not change rates of red blood cell production (Wald $\chi^2 = 2.01$, $p = 0.37$, 240 observations of 81 individuals). However, red blood cell production was correlated with change in parasite burden, but the effect varied across days of the study (Wald $\chi^2 = 3.62$, $p \leq 0.0001$, 240 observations of 81 individuals). Co-infection status had no effect on red blood cell production (Wald $\chi^2 = 0.29$, $p = 0.86$, 210 observations of 71 individuals).

Corticosterone treatment did not change birds' plasma haptoglobin (Wald $\chi^2 = 1.20$, $p = 0.55$, 237 observations of 82 individuals) or nitric oxide concentrations (Wald $\chi^2 = 0.14$, $p = 0.93$, 139 observations of 56 individuals). Neither immune measure was associated with change in parasite burden (haptoglobin, Wald $\chi^2 = 0.10$, $p = 0.75$, 237 observations of 82 individuals; nitric oxide, Wald $\chi^2 = 0.08$, $p = 0.78$, 139 observations of 56 individuals). Co-infection was not associated with either immune measure (haptoglobin, Wald $\chi^2 = 0.29$, $p = 0.86$, 210 observations of 73 individuals; nitric oxide, Wald $\chi^2 = 0.98$, $p = 0.61$, 123 observations of 50 individuals).

4. Discussion

Our results suggest that treatment with exogenous glucocorticoid hormones can amplify the costs of haemosporidian infection for a host by reducing both resistance and tolerance

to infection. In addition, we found evidence indicating that exogenous glucocorticoids might increase the risk of disease transmission. The higher of two biologically conservative exogenous corticosterone doses caused an increase in avian malaria (*Plasmodium*) burden and in the total number of gametocytes, the malaria life stage responsible for transmission to the insect vector. Interestingly, we only found this effect of exogenous hormone on malaria burden in the absence of co-infection with a related parasite, *Haemoproteus*. Corticosterone treatment also reduced infection tolerance such that birds in the high-dose treatment experienced steeper declines in body mass with increasing parasite burden. We found no evidence supporting an immunosuppressive role for either corticosterone or co-infection, suggesting that suppression of the inflammatory response might not underlie glucocorticoids' effects on resistance and tolerance and/or other components of immunity might be more important for responses to malaria infection in this species. Our findings suggest that individual variation in both glucocorticoid concentration and co-infection status can influence parasite dynamics within hosts and, as a result, might also alter transmission dynamics in a population.

Our findings are similar to those of other studies also reporting that increases in corticosterone can cause increases in parasite burden [9,56]. Additionally, we find that corticosterone treatment increases the total number of malaria gametocytes. Glucocorticoids' effects on parasite burden have often been interpreted as the result of immunosuppression (e.g. [9,11]); however, rather than reducing host resistance, the treatment might have directly caused the parasites to increase their rate of replication within the host. Haemosporidian parasites can respond to subtle changes in their hosts, including detecting and responding to the host's response to a mosquito bite [57], and *in vitro* studies of malaria and related protozoans have shown that glucocorticoids can increase parasite replication in the absence of a host immune system [58–60]. Therefore, we cannot determine if changes in parasite burden were caused by responses to corticosterone by the host or the parasites, or both. In addition, elevated glucocorticoids can increase host attractiveness to mosquito vectors [61], and increasing the total number of gametocytes could further improve transmission success [51].

If malaria-infected birds were co-infected with *Haemoproteus*, the higher-dose treatment did not increase malaria parasite burden or the number of gametocytes. Similarly, another study of co-infection demonstrated that the presence of one avian haemosporidian species can reduce the probability of infection with another species [62]. Competitive interactions among parasites can yield negative relationships between parasite infection intensities [63]. For example, competition for shared resources, like blood cells or haemoglobin in the case of haemosporidians, could result in higher burdens of the more competitive parasite [64]. Alternatively, infection with a parasite could result in cross immunity, in which a host has increased resistance to a different parasite [65,66]. However, our study found no support for differences in immune function with co-infection using the immune metrics we measured. Regardless of the mechanism, co-infections with multiple haemosporidian parasites could contribute to parasite dynamics within bird populations.

In our previous field study of red-winged blackbirds, higher endogenous plasma corticosterone concentrations were associated with maintaining higher haematocrit for a

given parasite burden [13]. In the present study, exogenous corticosterone treatment had no effect on tolerance when measured using haematocrit or haemoglobin and instead reduced tolerance as estimated with body mass. Our results also differ from a study of zebra finches (*Taeniopygia guttata*), where corticosterone manipulation had no effect on tolerance of West Nile infection when estimated with body mass, although there was a tendency for the birds receiving a high-dose implant to have lower tolerance in terms of flight ability [8]. Energy regulation is a central role of glucocorticoids [67], and corticosterone-driven changes in metabolism coupled with the challenge of parasite infection might lead to the decline in tolerance as estimated with body mass. As a measure of energy regulation, change in body mass could reflect the cumulative energetic costs of infection, such as the resources required to upregulate some immune defences or repair damaged tissue. Thus, we might be better able to detect changes in this more integrative measure of tolerance than with those metrics that reflect more limited costs of infection, such as haematocrit or micronuclei, which are indicative of red blood cell damage. In addition, the discrepancy between our observational and experimental studies could be the result of an unmeasured physiological factor driving the observational correlation.

Dissimilarities between natural and captive environments, or the effects of manipulated, exogenous hormone concentrations and endogenous hormone concentrations, could contribute to the contrasting outcomes observed here and in the prior field study [13]. Physiological responses to stimuli can vary in captive and wild settings [68,69]. In our captive study, birds are freed from food limitation and the energetic and physical demands of breeding, but experience a different physical and social environment as well as more frequent encounters (visual and physical contact) with humans, which could be perceived as increased predation risk. Any of these differences could alter hypothalamic–pituitary–adrenal (HPA) axis activity at various levels (e.g. receptors, hormone concentrations, negative feedback) or change various components of the immune response [69,70]. Further, exogenous corticosterone manipulations might not always effectively simulate the effects of individual variation in hormones. Even if hormone implants increase corticosterone within a natural physiological range, as they did in our study, they do not match the pulsatile, cyclic release of endogenous hormones [70,71]. As a result, experimental elevations of corticosterone could affect the HPA axis differently than endogenous corticosterone concentrations even if plasma levels of the hormone appear similar (e.g. binding to different receptor types or altering negative feedback), which could alter the downstream effects of corticosterone. In addition, individuals' regulation of hormone concentrations has been shaped by selection; any hormone manipulation that shifts individuals away from endogenous levels could have negative effects, such as the reduction in tolerance seen here. Moving forward, investigators need to address whether factors underlying variation in glucocorticoids, including life-history strategy or the physical and social environment, are also associated with changes in resistance and tolerance to parasites.

In summary, experimentally augmented glucocorticoid concentrations and co-infection status can contribute to among individual differences in the two key host defence strategies, resistance and tolerance. To our knowledge, this is the first study to demonstrate a significant glucocorticoid-driven

change in parasite burden that varies with co-infection status. Elevated glucocorticoids had twofold costs: birds suffered reduced resistance, as indicated by higher parasite burdens and associated reductions in haemoglobin and haematocrit, and reduced tolerance, reflected in higher costs of infection, in terms of reduced body mass. The increase in *Plasmodium* burden could also increase malaria transmission rates, suggesting a mechanism by which individual glucocorticoid levels could link to population-level processes. Thus, through its effects on an individual's endocrine physiology, environmental variation could lead to variation in host–parasite dynamics at the individual and population level.

Ethics. All procedures were approved by the Virginia Tech Institutional Animal Care and Use Committee (13-030 BIOL) and the Queen's University Animal Care Committee (2013-027).

Data accessibility. The datasets supporting this article have been uploaded as part of the supplementary material and are available from the Dryad Digital Repository: <https://doi.org/10.5061/dryad.g9n8g17> [72].

Authors' contributions. L.A.S., I.T.M. and F.B. designed the experiment. L.A.S. and A.M.D. conducted the experiment. L.A.S., M.F.H., I.T.M., E.B.G., M.M., A.M.D. and D.C. contributed to laboratory assays. L.A.S. performed statistical analyses and drafted the manuscript. All authors edited and approved the manuscript.

Competing interests. We declare we have no competing interests.

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