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RESEARCH ARTICLE

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House sparrows prioritize skin repair over constitutive innate immunity during long-term chronic stress

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Abstract

The reactive scope model was created to address two major unanswered questions in stress physiology: how and when does the adaptive acute stress response turn into harmful chronic stress? Previous studies suggest that immunoenhancement should occur in reactive homeostasis (acute stress) and immunosuppression should occur in homeostatic overload (chronic stress). We used this dichotomy of immune function to further elucidate the transition from acute to chronic stress by treating house sparrows (*Passer domesticus*) with different intensities of chronic stress and then monitoring their immune function. By varying the number of stressors given per day and the length of chronic stress bouts over a period of 6 months, we produced four treatment groups: high, medium, and low stress, and captivity-only. We tracked immunity through the bacterial killing assay and monitored healing of a 4 mm skin biopsy punch. We hypothesized that higher-stress birds would repair their skin more slowly and have lower bacterial killing capacity. The opposite was true—high-stress birds initially repaired their skin fastest. Additionally, all birds dramatically reduced bacterial killing capacity after the biopsy and increased food-derived uric acid, suggesting increased energy acquisition and a shift in immune resources to a more immediate concern (healing). Once healing finished, only the high-stress birds were unable to recover circulating immune function, suggesting that the combination of high stress and an immune challenge pushed these birds into homeostatic overload. Prioritizing healing over other immunological processes might be the best defense for a bird in its natural habitat.

KEYWORDS
bacterial killing assay, chronic stress, house sparrow, immune function, reactive scope, uric acid

1 | INTRODUCTION

A major gap in the stress physiology field is how the adaptive acute stress response transitions into harmful chronic stress. The reactive scope model (Romero et al., 2009) was designed to elucidate this transition and suggests that pathology could arise due to an accumulation of damage caused by stress (wear-and-tear). Thus far, studies on chronic stress have primarily focused on the temporal aspect of stress, but a computational model of the reactive scope framework (Wright et al., in submission) suggested that an understudied aspect of stress that might aid in deciphering this transition is the intensity of stress. The experimental design of the present experiments was suggested by that computational model, and these experiments were part of a larger project created to manipulate the intensity of chronic stress, and thus the accumulation of damage, with the goal of investigating how chronic stress arises.

The reactive scope model (Romero et al., 2009) incorporates both physiological mediators and endpoints of the stress response.

Mediators are hormones, neurotransmitters, and cytokines that coordinate physiological adjustments (such as immune function) made during acute and chronic stress. These mediators exist in four ranges: predictive homeostasis (levels needed to respond to daily or seasonal changes), reactive homeostasis (levels needed to respond to an unpredictable or emergency change), homeostatic overload (levels in which the mediator itself begins to cause harm), and homeostatic failure (levels that are too low to sustain life). Immune function generally increases during acute stress and decreases with chronic stress (Dhabhar, 2014; Torres-Medina et al., 2018). This implies that each species would have a baseline level of immune function in the predictive homeostasis range, immunoenhancement in the reactive homeostasis range, and immunosuppression in the homeostatic overload and homeostatic failure ranges. The reactive scope model proposes that the transition from immunoenhancement to immunosuppression could happen in one of two ways: (1) if a single acute stressor was so strong that mediators were too high and thus caused damage to the immune system or (2) if a stressor lasted so long that damage accumulated and wear-and-tear occurred, thus gradually lowering immune function (Romero et al., 2009).

We assessed two components of immune function: circulating innate immunity (via an ex vivo bacterial killing assay) and biopsy healing ability. We used frozen plasma to measure bacterial killing ability and thus measured only the noncellular component of innate immune function (complement components, antimicrobial peptides, and natural antibodies) (Jennifer Terry, unpublished work; Field et al., 2022). The complement system cascade aids in lysing foreign cells and triggering inflammation (Juul-Madsen et al., 2014; Unsworth, 2008), antimicrobial peptides are necessary for limiting infections, and natural antibodies provide nonspecific recognition of bacterial molecules that may cause microbial infection and initiate an enzyme cascade that may lyse targeted cells (Matson et al., 2006). The in vivo measure of immunity used in this study, a biopsy healing assay, integrates many aspects of the immune system, including both cellular and noncellular components (Field et al., 2022). Elevated levels of corticosterone, a hormonal hallmark of the stress response, can slow the wound-healing process (DuRant, Arciniega, et al., 2016; Thomas & Woodley, 2015). We modified an established chronic stress protocol (Rich & Romero, 2005) to vary the intensity and duration of chronic stress to create four treatment groups of birds that should experience different "levels" of wear-and-tear. We hypothesized that the birds experiencing higher-intensity stress regimens would accumulate more wear-and-tear, thus exhibiting more symptoms of chronic stress, such as lower immune function and longer biopsy healing times.

2 | METHODS

2.1 | Experimental design

Forty wild house sparrows (17M:23F) were caught with mist nets in Eastern Massachusetts in mid-June of 2021. Ages of the birds were not known, but all were either adults or older fledglings. Birds were doubly housed (M/F or F/F) in cages (45 cm × 37 cm × 33 cm), put on a light cycle of 12L:12D, and allowed to acclimate to captivity for at least 3 weeks in which they were not disturbed except for routine husbandry. Once the chronic stress period began, all chronic stress birds were housed together and the captivity-only group was housed in a different room. When certain groups needed more stressors/day, those groups were removed and the stressor was performed in a third room. When bouts for the low-stress or medium-stress groups ended, they were put into the captivity-only room, while chronic stress continued for the medium/high-stress groups.

Birds had ad libitum access to food (millet and sunflower seeds) and water during the acclimation period and for the duration of the experiment. The results and birds in this paper were part of a larger study and nonimmune measurements will be reported in future papers. Throughout the experiment, some birds died for unknown reasons. At any point in the experiment, if a bird dropped below 85% of its original weight or showed any signs of a health concern, the bird was removed from the chronic stress regimen until it recovered enough to continue. A plot of the sample sizes during each time point can be found in the supplemental materials (Supporting Information: Figure S1). The first two deaths occurred approximately 2.5 months after the birds were brought into captivity, so it is unlikely that birds were brought in with an acute disease, such as avian pox. Juveniles tend to acclimate less well to captivity, but it is unlikely this is the reason we lost birds, as any juveniles would have died much earlier if their death was because they were so young. Additionally, a cox survival analysis (Cox, 1972; Therneau, 2022) revealed that treatment group did not affect survival rate ($z = 0.26$, $p = 0.79$).

After acclimation, the birds were randomly separated into four experimental groups: captivity-only (5M:5F), low stress (3M:7F), medium stress (4M:6F), and high stress (5M:5F). An entirely stress-free control group was not possible because captivity is a background stressor for wild-caught birds. The captivity-only group experienced the stress of captivity, which involved routine husbandry and blood sampling for approximately 7.5 months and was as close to a control group as possible. We compensated for the lack of a true control group by employing a repeated-measures design for all metrics except the wound healing assay.

The other three groups were subjected to an established chronic stress protocol in which randomly selected psychological stressors were given multiple times/day at randomly chosen times (Cyr et al., 2007; Rich & Romero, 2005). The stressors included cage rolling (rolling the cage racks around the room), cage tapping (tapping the cages with a pen), playing a radio in the bird room, human voice in the bird room, restraint in a cloth bag, placing wind-up toys on the cage floor, running high-speed fans, and flashing colorful lights (always done in the dark period). All stressors were 30 min in duration.

The three applied chronic stress groups experienced six bouts of stress over a 6-month period. The three groups started with 2 weeks of chronic stress (of four stressors/day) followed by a 2-week break period. Each subsequent bout of stress for the low-stress group got

shorter by 2 days (and thus their breaks got longer by 2 days) and contained fewer stressors/day. Each subsequent bout of stress for the medium stress group remained at 2 weeks of four stressors/day and 2 weeks of breaks. Each subsequent bout of stress for the high-stress group got longer by 2 days (and thus their breaks got shorter by 2 days) and contained more stressors/day. Figure 1 contains a detailed timeline of this stress regimen and when samples were taken.

Animals were collected under a Massachusetts state collection permit and all experiments were performed according to the guidelines for use of wild birds in research (Fair et al., 2010) and approved by the Tufts University Institutional Animal Care and Use Committee.

2.2 | Bacterial killing assay

Before chronic stress began, at the end of every chronic stress bout, and 6 weeks after the experiment concluded (Figure 1), blood samples were taken to assess innate immunity via the bacterial killing assay. The bacterial killing assay tests the ability of plasma antibodies and proteins to combat a pathogen ex vivo. Birds were bled by puncturing the alar vein with a 26-guage needle and collecting approximately 90 µL of blood into a heparinized capillary tube. Samples were centrifuged at 1200*g* for 15 min and 10 µL of the extracted plasma was stored at −20°C for <10 days until used in the bacterial killing assay (Liebl & Martin, 2009). The remainder of the extracted plasma was used for another project. The bacterial killing assay (French & Neuman-Lee, 2012; Gormally et al., 2019; Liebl & Martin, 2009; Millet et al., 2007) was conducted in a 96-well plate and each sample was run in duplicate. Briefly, 4.5 µL of sample plasma was incubated at 37°C with 6 µL of diluted *Escherichia coli* (10^5 cells/mL; ATCC 8739) and 12.5 µL CO_2-Independent Media (ThermoFisher Cat. No. #18045088) + 4 mM L-glutamine (Gibco Cat. No. 25030-081) for 30 min. Negative controls contained only CO_2-Independent Media + L-Glutamine, positive controls contained CO_2-independent media + L-glutamine and *E. coli*. One hundred and twenty-five microliters of tryptic soy broth (Sigma-Aldrich Cat. No. 22092) was then added to every well and the plate absorbance was read at 300 nm on an M3 spectrophotometer to obtain a background reading. The absorbance was read again after a 12-h incubation period at 37°C. Bacterial killing capacity for each well was calculated as follows:

$$\%\text{Bacteria killed} = 100 \times \left(1 - \frac{\text{final absorbance of sample wells} - \text{background absorbance of sample wells}}{\text{final absorbance of pos. ctrl.} - \text{background absorbance of pos. ctrl.}}\right).$$

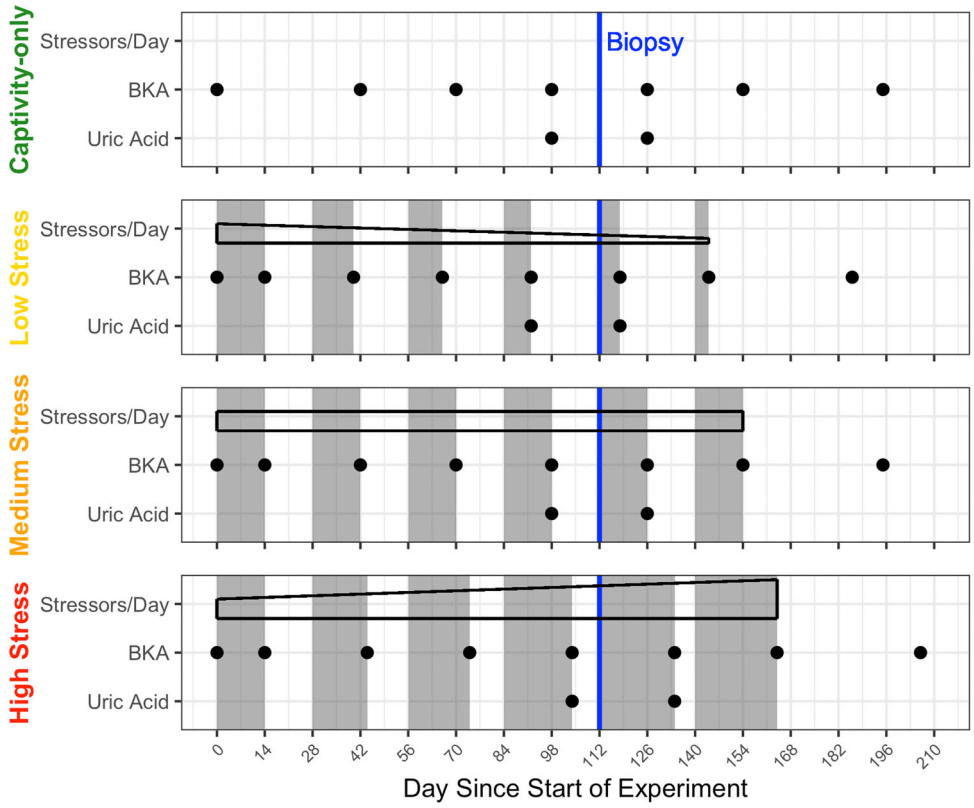

FIGURE 1 Timeline of chronic stress protocol and samples taken. Rows correspond to experimental group, with group names on the left side. Grey rectangles indicate periods of applied chronic stress. Black dots indicate a blood sample taken for a given metric. The right-most black dot was the recovery sample, which was taken 6 weeks after each group's last chronic stress bout concluded. Blue line indicates when biopsies were performed for the wound healing assay. BKA, bacterial killing assay.

Inter and intra assay variability, based on the positive controls, were 9.2% and 3.7%, respectively. Twenty-two samples did not have enough plasma to be run in duplicate so were run singly. Pairs of duplicates that had a CV above 20% were flagged and the "wrong" value (based on other values in that group/bout) were removed (29 out of 478 duplicates). Values that were under 0% (42 out of 250 samples) or over 100% (35 out of 250 samples) were assigned floor values of 0% or ceiling values of 100%, respectively.

2.3 | Healing assay

At the beginning of the fifth bout of applied chronic stress (Figure 1), we took superficial skin biopsies to assess wound healing capabilities (Carter et al., 2013; DuRant, Arciniega, et al., 2016; French et al., 2006). We performed the biopsies at this timepoint because we wanted to assess the cumulative effects of the differing stress regimens. The stress intensities would not have diverged enough had we taken biopsies earlier, and if we had taken them at the end of the 6 months, we may have impeded the birds' ability to recover. Birds were briefly anesthetized with isoflurane and administered a 4 mm biopsy to the skin of their right flank (between feather tracts) with a sterile, single-use biopsy punch (Miltex 33-34). Every day following the biopsy, images were taken of the wound and a ruler at level for scale. Using ImageJ, the circumference of the wounds in each image was traced and the area was determined. Wounds were considered fully healed when the margins were completely rejoined. Data from five birds was removed from the results. One bird died of unknown causes before healing was complete. Four birds had biopsies that expanded instead of healed during the extent of the daily monitoring (Supporting Information: Figure S2). The final sample sizes during the wound healing experiment were as follows: captivity-only (4M:4F), low stress (3M:6F), medium stress (3M:4F), high stress (3M:3F).

2.4 | Uric acid

Before and after the wound healing assay, the antioxidant, uric acid, was quantified from blood samples. The birds' food is a mixture of millet and sunflower seeds, however, we have observed that they preferentially eat the sunflower seeds, which are very high in uric acid (Hafez et al., 2017). We can thus use blood uric acid levels as a proxy for sunflower seed consumption and approximate energy intake.

Birds were fasted overnight and blood samples for uric acid quantification were taken 1 h after food addition. Immediately after blood sampling, approximately 2 µL of blood was used with the UASure Blood Uric Acid Meter (Cat. No. U3003) to quantify uric acid levels (Beattie et al., 2022; Malisch et al., 2018; Morales et al., 2020; Stoot et al., 2014). In instances where the value was higher than the detection limit of the device, a ceiling value of 500 mg/dL was assigned. Seven out of 69 samples exceeded the uric acid test.

2.5 | Statistics

All statistical analyses were run in R version 4.0.3. For each step of the following statistical analyses, we checked for the homogeneity of variances using Levene's Test and by visually inspecting the residual plots for each model. No data transformation was required.

To analyze bacterial killing capacity, we ran a linear mixed effect model ("lmer" function, lme4 package; Bates et al., 2015) using group and bout as fixed effects and bird identity as a random effect. Then, a Type III analysis of variance (ANOVA) ("Anova" function, car package; Fox & Weisberg, 2011) was run to test if the model was significant. Because the interaction between group and bout was significant, we split the groups and ran four separate linear mixed effect models with bout as the fixed effect and bird identity as the random effect. A standard ANOVA was run to compare the recovery samples (those taken 6 weeks after the experiment concluded). Data from the medium birds were excluded from the recovery timepoint statistics, because only three birds remained.

We ran a similar linear mixed-effect model analysis for the wound healing data, however, the fixed effects were group and day-since-biopsy and the random effect was bird identity. We also used a survival analysis (Cox, 1972) to analyze the "survival" of the wounds against group and using bird as a random effect ("coxme" function, coxme package [Therneau, 2022]). Within the terms of a survival analysis, a fully healed wound was considered to be a "death" and there was no censored data by the end of the experiment (all birds' wounds healed). To analyze the total length of time it took for a full wound recovery, we ran an ANOVA across the four groups.

For uric acid and bacterial killing capacity, we analyzed data from before and after the biopsies. To do this we used a linear mixed-effect model with group and time (before/after biopsy) as fixed effects and bird identity as a random effect. In the case of bacterial killing capacity (isolating the samples before and after the biopsies), the interaction between group and time was not significant, so it was taken out and the model was rerun.

3 | RESULTS

3.1 | Bacterial killing capacity

Bacterial killing capacity initially increased with chronic stress (Figure 2a; main effect of bout, $F_{6,170} = 3.71$, $p < 0.01$) but sharply declined immediately after biopsies were taken. The magnitude of these changes depended on treatment group (group:bout interaction, $F_{17,171} = 2.64$, $p < 0.001$). The bacterial killing capacity of the captivity-only ($F_{5,40} = 3.20$, $p = 0.02$), medium stress ($F_{6,37} = 3.16$, $p = 0.01$), and high stress ($F_{6,47} = 6.16$, $p < 0.001$) groups decreased after the biopsy, however the killing capacity of the low-stress group did not ($F_{6,46} = 1.26$, $p = 0.29$). After the biopsies were healed, the captivity-only, low, and medium-stress groups regained their bacterial killing capacity, but the high-stress group's ability to fight a pathogen collapsed.

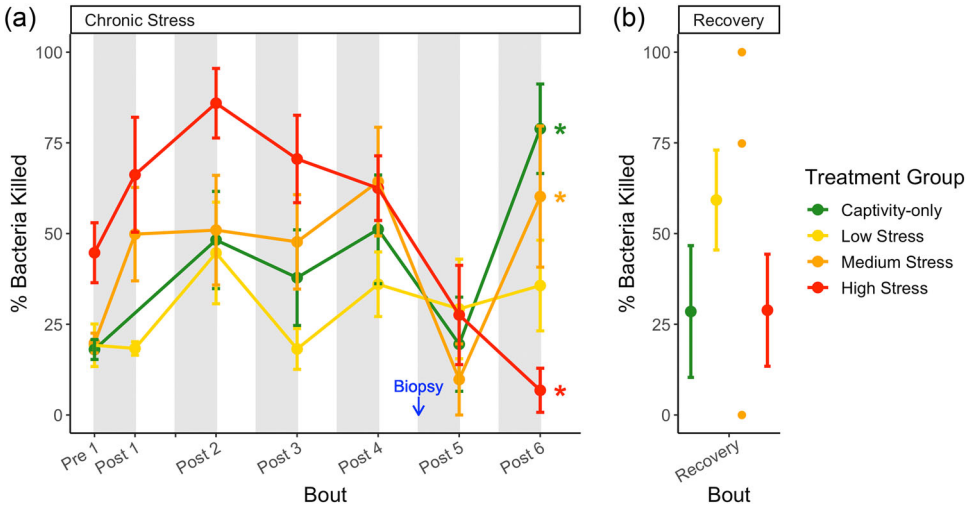

FIGURE 2 Changes in innate immunity over 6 months of chronic stress and after 6 weeks of recovery. Intensity of chronic stress affected bacterial killing capacity (a), but the effect did not last through the recovery period (b). A small, superficial skin biopsy (indicated by the arrow) preceded a dip in killing capacity. Lower % bacteria killed means lower innate immunity. Asterisks in panel A indicate groups that had a significant effect of bout number. Data from the medium stress group was excluded for the recovery timepoint statistics because only three birds remained, but data is graphed (as individual points) for completeness. Grey regions indicate times of applied chronic stress for the low, medium, and high-stress groups. Note that the number of days of each grey and white region differ depending on group, as they experienced differing durations of chronic stress (see Figure 1). Graphed values represent mean ± SE.

Six weeks after the conclusion of the chronic stress experiment, there was no difference in killing capacity between the captivity-only, low-stress, medium-stress, and high-stress treatment groups (Figure 2b, $F_3 = 1.01$, $p = 0.41$). We also reran the recovery statistics without the medium group, since they only had three birds remaining at that timepoint. Without the medium group in the model, the result was the same ($F_2 = 1.36$, $p = 0.28$).

3.2 | Healing assay

There was a significant interaction between treatment group and time to heal (Figure 3a, $F_{3,454} = 5.24$, $p < 0.005$). The survival analysis indicated that treatment group significantly affected healing ($z = 2$, $p = 0.04$). Biopsy healing in the low-stress (hazard ratio [HR] = 2.48, $p = 0.63$) and medium-stress (HR = 0.47, $p = 0.71$) groups was not different than the captivity-only group. Healing in the high-stress group (HR = 291.83, $p < 0.01$) was significantly different than the captivity-only group (Figure 3c). Although the high-stress birds showed significant differences in their healing profiles and healed their wounds ~4.7 days faster than the other three groups, the results of the ANOVA on the number of days to fully heal the biopsy revealed a nonsignificant trend (Figure 3b; $F_3 = 2.32$, $p = 0.09$).

3.3 | Physiological comparisons before and after biopsies

There was a significant decrease in bacterial killing capacity from before the biopsies were taken to while the birds are healing their wounds (Figure 4a; effect of time; $F_{1,34} = 11.5$, $p < 0.005$) that is the same across all four treatment groups (effect of treatment group; $F_{3,31} = 0.40$, $p = 0.75$).

All treatment groups increased plasma uric acid during wound healing (Figure 4b; comparing before to after biopsies $F_{1,29} = 16.50$, $p < 0.001$). There was a difference between the groups ($F_{3,46} = 4.99$, $p < 0.005$), but the interaction was not significant ($F_{3,29} = 1.70$, $p = 0.19$), indicating that, despite different initial uric acid levels, every group had a similar increase after the biopsy.

4 | DISCUSSION

In this study, we aimed to track innate immunity over a long-term chronic stress series as well as test wound healing ability. We did so in four groups of house sparrows: captivity-only, low stress, medium stress, and high stress. We manipulated the length of stress bouts and the number of stressors/day to force different levels of wear-and-tear. The reactive scope model (Romero et al., 2009) posits that wear-and-tear will cause an animal to be less likely to cope with chronic stress, so we predicted that our high-stress birds would have higher levels of wear-and-tear, and thus earlier (or more) symptoms of chronic stress. This study thus provides unique insight into the impact of long-term, repeated chronic stress and wear-and-tear on immune function.

4.1 | Prebiopsy bacterial killing capacity

Previous review papers documented an overall pattern where immune function increases with acute stress and decreases with

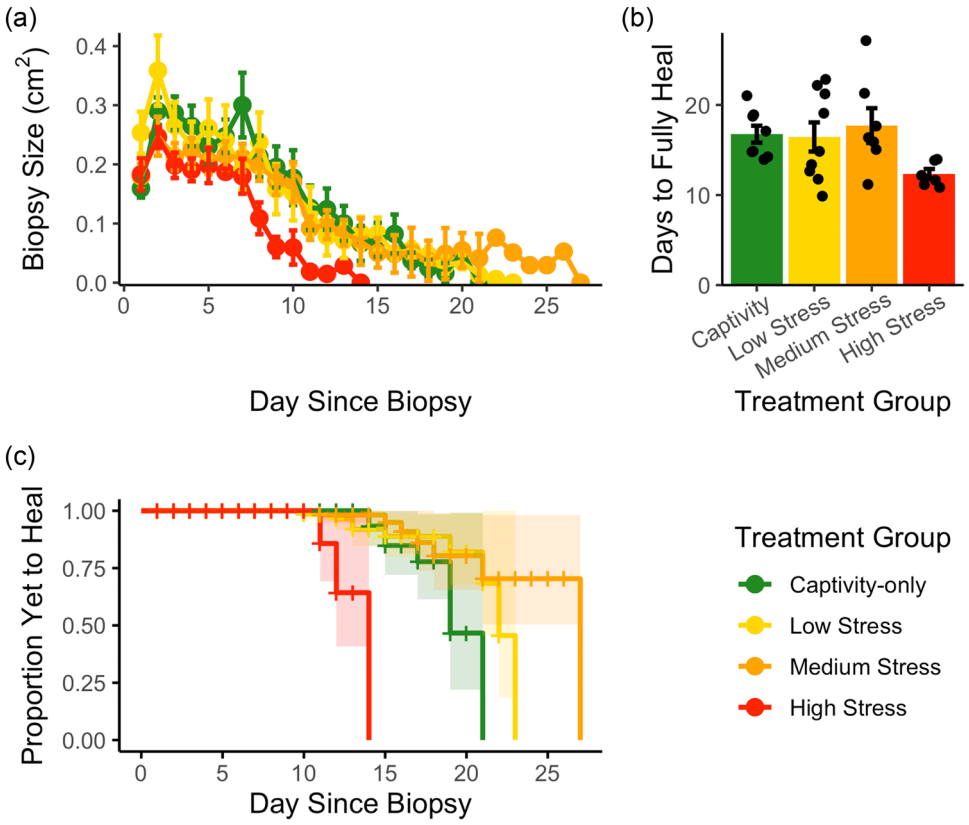

FIGURE 3 Intensity of chronic stress affects wound healing rates. Graphs show the change in wound size throughout the healing process (a), total amount of time until wounds are fully healed (b), and survival curves for wounds (c). Biopsies were taken at the start of the 5th bout of stress. The high-stress group exhibited a faster rate of healing, however, the total days to wounds being fully healed was not significant at an α level of 0.05. Graphed values represent mean ± SE.

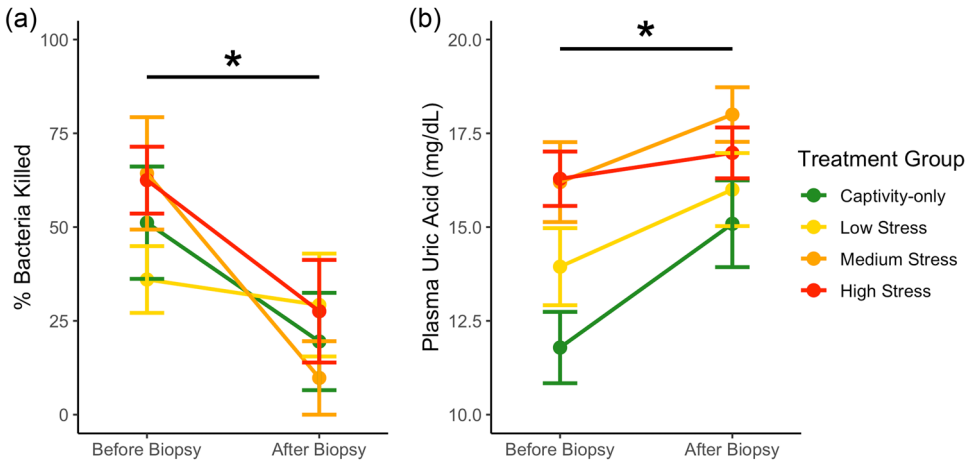

FIGURE 4 Physiological changes before and after biopsies. Innate immunity decreased while birds healed wounds (a), while plasma uric acid (a proxy for food consumption) increased (b). Graphed values represent mean ± SE. *Significant difference of $p < 0.001$ over time.

chronic stress (Dhabhar, 2014; Martin, 2009), however, our noncellular immune function data are contrary to this pattern. In the first two bouts of chronic stress, bacterial killing capacity increased by about 25% in all treatment groups (Figure 2a, "Pre 1" to "Post 2"). This increase in killing capacity is contrary to other work in house sparrows (Gormally et al., 2018, 2019; Martin et al., 2012), lizards (McCormick et al., 2015), and snakes (Neuman-Lee et al., 2015), but is in line with one study in house sparrows that showed increased bacterial killing capacity with captivity stress (Love et al., 2017). However, it is not clear why bacterial killing capacity also increased in the captivity-only group that was not exposed to experimental chronic stress. We propose that introduction to captivity of

wild-caught birds also acted as a chronic stressor, albeit milder than the additional chronic stress protocol, which is why we present this group as "captivity-only" rather than "no stress."

Stress-related immunosuppression is primarily mediated by glucocorticoids and the glucocorticoid receptor (Gao & Deviche, 2018; Gao et al., 2017), but glucocorticoid changes do not always occur during chronic stress (Dickens & Romero, 2013). Corticosterone data from this project (data in prep.) did not show a concurrent decrease, so the increase in bacterial killing capacity must have occurred through another mechanism.

Within the lens of the reactive scope model, it appears as though the birds in the present study were able to maintain immune function in the reactive homeostasis range far longer than we would have expected. While an increase in immune function during acute stress is typically thought of as adaptive, a prolonged increase in killing capacity on the order of months is not necessarily a benefit to the animal and may itself cause wear-and-tear (Romero et al., 2009). If the innate immune system is unnecessarily upregulated for too long, there is a potential for host cells and tissues to be the target of nonspecific immune attack. This side effect of increased innate immunity could eventually lead to pathology (Graham et al., 2005; Love et al., 2017). Another study in house sparrows (Martin et al., 2011) showed a hyperinflammation response with captivity stress and concluded that chronic stress might, more-broadly, cause immune dysregulation, not simply immunosuppression. The changes in killing capacity before the biopsy (Figure 2a) might be consistent with these ideas.

Treatment groups did respond differently throughout the chronic stress series, with the changes in bacterial killing capacity being slightly more extreme in the high-stress group, moderate changes in the captivity-only and medium-stress groups, and no change in the low-stress group. These results suggest that there is a "dose effect" with innate immunity and chronic stress even though none of the groups entered into homeostatic overload initially.

4.2 | Healing

At the start of the fifth bout of stress, we administered a superficial wound to every bird and monitored healing until completion. In the middle of the healing process, the high-stress group appeared to be healing fastest (Figure 3a), though the total number of days to fully heal was not significantly different from the other groups (Figure 3b). The avian wound healing process consists of three distinct phases: the inflammatory phase, the collagen phase, and the maturation phase (Ritzman, 2004). The inflammatory phase occurs within the first few days after wounding, with the first 12 h consisting of vasoconstriction, vasodilation, the invasion of polymorphonuclear leukocytes and monocytes around the margins of the wound, and active phagocytosis of necrotic cellular tissue and debris (MacLeod & Mansbridge, 2015; Ritzman, 2004). The collagen phase occurs over approximately the next 2 weeks when new capillaries are formed and begin to invade the wound area and new epithelial cells begin spreading from the margins of the wound across the surface. The final maturation phase occurs over weeks to months when the number of fibroblasts decrease and thicker collagen replaces weaker collagen (Ritzman, 2004). In our study, it seems as though a high-stress treatment affected healing in the collagen phase, but not enough to cause a significant difference in the total time to heal.

The birds in our study took approximately 2 days longer to fully heal their wounds than birds in similar studies in house sparrows (DuRant, Arciniega, et al., 2016; Love et al., 2017) and European starlings (DuRant, de Bruijn, et al., 2016). This difference may be a result of the long-term chronic stress these birds were exposed to. However, it is not clear that these 2 extra days represents a biologically relevant time period because evidence suggests that wound healing ability is robust to perturbations. Despite slight differences in the speed of healing, there is no difference in total time to heal with chronic stress (this study; DuRant, de Bruijn, et al., 2016), molt (DuRant, de Bruijn, et al., 2016), corticosterone injection, or when corticosterone secretion is blocked (DuRant, Arciniega, et al., 2016). Only one other study showed a significant increase in healing while house sparrows were experiencing the stress of acclimation to captivity (Love et al., 2017). The lack of consensus on the interaction of stress and wound healing is not unique to birds; wounds were larger mid-way through the healing process in salamanders (with corticosterone patches, but not chronic stress; Thomas & Woodley, 2015), snakes (with chronic stress; Neuman-Lee et al., 2015), and lizards (with chronic stress; French et al., 2006), though the time to fully heal was either not reported (salamanders and snakes) or not significant (lizards).

The dramatic decrease in bacterial killing capacity in the "Post 5" timepoint (Figure 2a) was likely due to the active wound healing. It takes birds about 14 days to heal a 4 mm biopsy (this study; DuRant, Arciniega, et al., 2016; DuRant, de Bruijn, et al., 2016; Love et al., 2017), which means the samples for the bacterial killing assay at the "Post 5" timepoint were taken while the birds were healing their wounds or shortly thereafter. This decrease is significant when compared to the time point immediately before the biopsies (Figure 4a). This result could potentially indicate a shift in immune resources from circulating innate immunity to prioritize wound healing, as there was no difference across groups in the total time to heal wounds (Figure 3b).

Further support for immune trade-offs comes from a study in lizards that showed prioritization of wound healing ability over stress-induced bacterial killing ability (Neuman-Lee & French, 2014). Our study and the lizard study both show a decrease in circulating immunity during the time of wound healing. Corticosterone is known to inhibit immune function during chronic stress (Dhabhar, 2014; Martin, 2009), but there was not a concurrent increase in corticosterone (data in prep.) with the decrease in bacterial killing. Alternatively, immune components could have shifted from circulation in the bloodstream (where it is measurable by the BKA) to the site of the biopsy to promote healing (Dhabhar et al., 2012). A similar study on house sparrows also suggests a trade-off between circulating innate immunity and wound healing, albeit in the opposite

manner. Love et al. (2017) reported an increase in wound healing time (implying lower immune function), but an increase in bacterial killing ability (implying higher immune function) with captivity stress. While it is unclear why circulating immunity was prioritized in that study, but wound healing was prioritized in our study and Neuman-Lee and French (2014), it is further evidence that wound healing and circulating innate immunity may not be upregulated at the same time.

To further investigate the idea that birds may be resource limited, we analyzed plasma uric acid levels just after a meal. Four potential mechanisms have been suggested to be mediators of immune trade-offs: energetic costs, nutrient costs, autoimmunity, and oxidative stress (Archie, 2013; Hasselquist & Nilsson, 2012). Uric acid might give insight into both the nutrient cost mechanism and the oxidative stress mechanism. Because the birds in our study ate a high-uric-acid diet (Hafez et al., 2017), uric acid can be used as a proxy for sunflower seed consumption (Beattie et al., 2022). Uric acid significantly increased after the biopsies (Figure 4b), potentially indicating that the birds ate more to cope with the increased resources needed to heal their wounds. This is consistent with food-restricted rats healing wounds more slowly than nonfood-restricted rats (Hunt et al., 2012). Conversely, food restriction did not have an effect on wound healing in gartersnakes (Neuman-Lee et al., 2015), but the physiology of an infrequently-feeding gartersnake may be fundamentally different than a constantly-foraging house sparrow.

Additionally, uric acid has been proposed to be the dominant antioxidant in birds (Stinefelt et al., 2005). The increase in uric acid seen during biopsy healing in this study (Figure 4b) could have been in response to an increase in free radicals during an immune challenge. While we cannot disentangle information on food consumption from antioxidant status with our data, these two mechanisms of immune trade-off may not need to be mutually exclusive. The energetic cost trade-off mechanism also has support through a study in which nonmolting, chronically stressed birds decreased energy expenditure after wounding (DuRant, de Bruijn, et al., 2016). Regardless of the mechanism, these immune trade-offs may exist to help an animal avoid entering homeostatic overload.

Prioritizing wound healing over circulating innate immunity makes sense in the context of a bird in its natural habitat. A visible wound might be an unwanted and damaging signal to a conspecific or predator (Sapolsky et al., 2000) as well as an open avenue for pathogens and parasites to enter the body and cause infection (Rojas et al., 2002), so prioritizing healing over other physiological processes might be the best defense. The longer an animal shows a visible wound, the longer it might be subject to differential treatment from conspecifics, the fewer mating opportunities it might have, and the higher likelihood it might be predated upon or be susceptible to deadly infection.

4.3 | Postbiopsy bacterial killing capacity—Evidence for homeostatic overload

The decrease in bacterial killing capacity after the biopsies (Figure 2a) is reversed in the "Post 6" timepoint, in all groups except for the high-stress group. The birds in this group only received a 3-day break between the fifth and sixth bouts of stress (Figure 1), meaning that the birds experienced 6 weeks of almost constant chronic stress. The reduction of killing capacity to almost 0% could be an indication of homeostatic overload (Romero et al., 2009) in the high-stress group. The birds may have been able to cope with the stress regimen in the first four bouts of the experiment, but when they were faced with a wound healing challenge on top of chronic psychological stress, the effects of wear-and-tear manifested, and their systems failed to cope. The 6-week recovery period (Figure 2b) may have been enough time to bring the high-stress group back into the range of immune function as the other three groups, though it was not quite as high as their initial timepoint.

5 | CONCLUSIONS

House sparrows subjected to different intensities of a long-term chronic stress regimen showed differences in innate immunity, manifested as an increase in bacterial killing capacity. This prolonged increase within the reactive homeostasis range of reactive scope might lead to wear-and-tear, dependent on chronic stress intensity. Bacterial killing capacity rapidly decreased with the administration of a superficial biopsy, potentially representing a shift of immune resources to a more immediate concern: the wound. A concurrent elevation in uric acid suggests birds also ate more high-quality food to cope with the increased energy demands of healing a wound. These data suggest that when a wound is present, other physiological demands are downregulated to prioritize healing, perhaps to avoid appearing weakened to predators or conspecifics. Once healing concluded, all groups except the high-stress group were able to recover their circulating innate immunity, indicating that the high-stress group entered homeostatic overload.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request. The data and code are available upon request.

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