

# Experimentally elevated corticosterone does not affect bacteria killing ability of breeding female tree swallows (*Tachycineta bicolor*)

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## ABSTRACT

The immune system can be modulated when organisms are exposed to acute or chronic stressors. Glucocorticoids (GCs), the primary hormonal mediators of the physiological stress response, are suspected to play a crucial role in immune modulation. However, most evidence of stress-associated immunomodulation does not separate the effects of glucocorticoid-dependent pathways from those of glucocorticoid-independent mechanisms on immune function. In this study, we experimentally elevated circulating corticosterone, the main avian glucocorticoid, in free-living female tree swallows (*Tachycineta bicolor*) for one to two weeks to test its effects on immune modulation. Natural variation in bacteria killing ability (BKA), a measure of innate constitutive immunity, was predicted by the interaction between timing of breeding and corticosterone levels. However, experimental elevation of corticosterone had no effect on BKA. Therefore, even when BKA is correlated with natural variation in glucocorticoid levels, this relationship may not be causal. Experiments are necessary to uncover the causal mechanisms of immunomodulation and the consequences of acute and chronic stress on disease vulnerability. Findings in other species indicate that acute increases in GCs can suppress BKA; but our results support the hypothesis that this effect does not persist over longer timescales, during chronic elevations in GCs. Direct comparisons of the effects of acute vs. chronic elevation of GCs on BKA will be important for testing this hypothesis.

## 1. Introduction

Shifting resource allocation enables organisms to prioritize different physiological processes in response to different challenges. Endocrine signals coordinate regulation of these processes within complex physiological networks that control bodily functions (Cohen et al., 2012; Martin et al., 2011). One set of processes subject to endocrine regulation is the vertebrate immune system, which includes a dynamic set of responses that activate depending on the specific type of challenge that an animal is facing. Immune responses can be up- or down-regulated in response to challenges (de Assis et al., 2015; Love et al., 2017). Yet, while there is ample evidence supporting stress-related immunomodulation, the mechanisms behind this effect are unclear (Dietert et al., 1994; Koutsos and Klasing, 2013; Padgett and Glaser, 2003; Tort, 2011).

Depending on duration, stressors—defined here as unpredictable and/or uncontrollable stimuli (Romero et al., 2009)—can either enhance or suppress the immune response. Most commonly, acute stress

enhances immunity, while chronic stress suppresses it (Dhabhar, 2014; Dhabhar and McEwen, 1997; Titon et al., 2019). The presence and direction of these relationships can, however, differ depending on the type of immune response and its costs and benefits to organisms facing stressors. Upregulation of immunity following stressor exposure can be beneficial in preparing for potential injury or infection or recovering from exertion (Sapolsky et al., 2000). However, because immune investment can be expensive, and because enhanced immunity can lead to immunopathology (Dhabhar, 2008; Graham et al., 2005; Råberg et al., 1998), it may be beneficial to downregulate immune responses during chronic stress—or even in response to some acute challenges.

There is substantial evidence of immunosuppression in response to captivity in wild animals, which is often interpreted as a response to chronic stress. However, the relationship between captivity and the immune response is variable. Captivity can suppress different arms of the innate immune system like wound-healing mechanisms, the size of immune-related organs (Love et al., 2017), inflammation (Nazar and

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Marin, 2011), and bactericidal capacity (BKA; Matson et al., 2006; Titon et al., 2019). It can also reduce adaptive immune responses like antibody production (El-Lethey et al., 2003) and delayed hypersensitivity (Dhabhar and McEwen, 1997). Captivity has also been documented to increase adaptive processes like leukocyte proliferation (Begg et al., 2004). It is not clear, however, whether these results transfer to the kinds of stressors organisms encounter in the wild, because captivity is not an ecologically relevant condition. Thus, we know little about immunomodulation in response to ecologically relevant chronic stressors.

The varying effects of chronic stressors on immune activation may be because chronic stressors have inconsistent effects on glucocorticoid hormones (GCs; reviewed in Dickens and Romero, 2013). During the stress response GCs are secreted and regulated through the hypothalamic-pituitary-adrenal (HPA) axis (Romero and Butler, 2007; Selye, 1946). Elevated GC levels lead to changes in energy expenditure and metabolic rate that can promote responding to and recovering from challenges (Wingfield et al., 1998). When challenges are prolonged, GCs sometimes remain elevated (though this is not always the case; Dickens and Romero, 2013). With mounting evidence showing immunomodulation under chronic stress, and a vast body of knowledge on the role of GCs on individual immune pathways (Riccardi et al., 2002), the causal effects of GCs on the effectiveness of immune function in natural systems remains unclear. Experimental manipulations of GCs in the absence of other coordinated aspects of the stress response can help clarify how physiological components of the stress response can influence the immune system. They can also help uncover the lingering impacts of chronically elevated GC levels on the immune system due to past exposure to stressors. Testing the effects of GCs on constitutive innate immune mechanisms like bacterial killing ability (BKA) may be particularly illuminating because it can show how GCs may impact immune function in the presence of potential pathogens.

GC manipulations are known to have multiple effects on immune mechanisms that can shape innate immune responses like BKA (Baschant and Tuckermann, 2010; Riccardi et al., 2002). For example, mild or moderate stressors, along with GC elevation, can lead to heterophil mobilization which can influence bacterial inhibition and killing via immune activation and secretion of antimicrobial peptides (Genovese et al., 2013; Gross and Siegel, 1983; Maxwell, 1993; Maxwell and Robertson, 1998). Moreover, oral doses of GCs can directly impact the ability of heterophils to kill bacteria (Chuammitri et al., 2011). Meanwhile, synthetic GCs like dexamethasone suppress macrophage and monocyte activity in different tissues impacting phagocytosis, pro-inflammatory pathways and complement activity (Joyce et al., 1997; van de Garde et al., 2014; Walker, 1998).

Plasma-based BKAs, which are often used for wildlife studies, measure the effectiveness of antimicrobial peptides and complement activation of the membrane attack complex in the absence of leukocytes and other cell-mediated contributors to bacterial killing *in vivo*. *In vitro* studies have found that GCs affect gene expression of complement proteins in monocytes and macrophages (Lappin and Whaley, 1991; Lemercier et al., 1992; Walker, 1998), thus suggesting a mechanism that connects GCs and plasma BKA *in vivo*. However, while the expression of complement proteins can vary, it is unclear if these changes effectively impact bactericidal function in the absence of immune cells.

In wild systems, BKA is positively correlated with circulating corticosterone in red-winged blackbirds (*Agelaius phoeniceus*; Merrill et al., 2014), increases after acute restraint stress in slider turtles (*Trachemys scripta*; Terry et al., 2023), and increases with captivity-related chronic stress in house sparrows (*Passer domesticus*; Love et al., 2017). Very few studies have directly manipulated GC levels to test whether the effects of stress on BKA result from GC exposure. Those that have altered GCs directly have found patterns that sometimes differ from the relationships seen in studies that manipulate stressor exposure. In two species of birds, house sparrows and brown-headed blackbirds (*Molothrus ater*), an acute experimental elevation in corticosterone decreases BKA (Gao and

Deviche, 2019; Merrill et al., 2012). In contrast, a study in Cururu toads (*Rhinella icterica*) found that BKA does not change when GCs are artificially increased chronically, even though BKA decreases under chronic stress (de Assis et al., 2015). Thus, while the different findings of these studies may be due to differences between avian and amphibian immune systems, the results to date also suggest that acute and chronic exposure to elevated GCs may differ in their effects on BKA. In addition to duration and intensity of GC exposure, the physiological state of the organism—like reproductive state or body condition—may shape the impacts of GC elevation on BKA. All the previous studies of the effects of GCs on BKA tested their hypotheses with captive animals, which were thus already experiencing an artificial chronic stressor. Clarifying the role of chronic GC exposure in the modulation of BKA in free-living organisms can help identify the mechanisms that drive stress-induced immunomodulation.

In this study, we first examined the relationships between natural variation in GCs and plasma BKA, and then took an experimental approach to isolate the causal effects of GCs on BKA. We investigated the effects of elevated corticosterone, the main GC in birds, on BKA in free-living tree swallows (*Tachycineta bicolor*). Tree swallows are a useful system to study this question because they use nest boxes during the breeding season and can be captured multiple times per breeding attempt, allowing repeated sampling. This enabled us to test how endogenous circulating corticosterone affects BKA by comparing BKA before and after a corticosterone manipulation in a free-living bird. Tree swallows show signs of context-dependent variation in BKA (Chang van Oordt et al., 2022), as well as increased BKA when nesting in agricultural landscapes (Schmitt et al., 2017). Here we tested the hypothesis that corticosterone plays a causal role in regulating BKA. To test this hypothesis, we first investigated the correlation between BKA and baseline corticosterone levels in incubating tree swallows and then conducted an experimental manipulation of corticosterone to test its causal effects on BKA. For the latter, we artificially elevated circulating corticosterone using a non-invasive method in which incubating female tree swallows are dosed topically by putting corticosterone on a fake egg installed in their nest. In this species a series of five acute spikes in exogenous corticosterone can elevate endogenous corticosterone levels for days to weeks after the treatment has ended (Vitousek et al., 2018a), thus mimicking part of the physiological state of an organism experiencing a chronic stressor. Based on the relationship between chronic stressors and BKA shown in other studies we initially predicted that chronic GC elevation would suppress BKA; however, it is also possible that chronic GC elevation would have no effect on BKA, as seen in previous experiments in toads (de Assis et al., 2015; Titon et al., 2019).

We also evaluated whether the timing of breeding influences the relationship between corticosterone and BKA. Breeding early for tree swallows, as in many temperate birds, is a strong predictor of lifetime fitness (Winkler et al., 2020), and this relationship is likely to be driven by a combination of intrinsic and environmental factors (Shipley et al., 2020; Twining et al., 2018; Verhulst and Nilsson, 2008; Winkler et al., 2014). Breeding at different times may lead to differences in resource availability (Twining et al., 2018) and in the costs of immune investment (Chang van Oordt et al., 2022). Given that immune-based trade-offs can be facultative (French et al., 2007), the degree of GC-mediated immunomodulation may vary among individuals depending on their timing of breeding. Here, we predicted that late breeders would have a more negative relationship between corticosterone and BKA than earlier breeders.

## 2. Methods

### 2.1. Study site and reproductive monitoring

We studied female tree swallows breeding between May and July of 2020 at the Cornell University Experimental Ponds Facility in Ithaca, New York, U.S.A. (42.50° N, 76.45° W). The facility is composed of two

sites approximately 1 mile from each other, each of which has a series of experimental ponds built in an open field surrounded by woods. Combined, the study sites have ~385 nest boxes installed ~20 m from each other and set up in a grid. At this long-term study site, we monitor breeding in each box every two days and record the date of the start of laying ('lay date'), clutch completion date, hatching date, and fledge date. We also record clutch size, brood size and whether the nestlings fledge, are found dead, or are preyed upon.

## 2.2. Corticosterone-dosing experiment and blood sampling

Prior to the start of the experiment, 81 female tree swallows were captured between 06:00 a.m. and 10:00 a.m. on day 6–8 of incubation. Upon capture we collected a blood sample within the first three minutes of disturbance (hereafter called 'baseline'). The bird was then weighed, measured, and banded with an individual aluminum band if it did not already have one. For the purpose of other ongoing studies at our long-term study site that involve characterizing the acute glucocorticoid stress response (e.g., Vitousek et al., 2022), we collected two additional blood samples. The first of these was collected 30 min after the initial disturbance, and was immediately followed by an injection of dexamethasone, a synthetic GC, into the pectoral muscle, to measure the strength of negative feedback. We used a dose of 4.5  $\mu\text{L}$  per gram of body mass of dexamethasone sodium phosphate (2 mg  $\text{mL}^{-1}$ ), which was previously validated to stimulate maximal negative feedback of GC secretion (Zimmer et al., 2019). The final blood sample was taken 30 min after injection. In between samples, birds were restrained in a paper bag; after sampling, birds were released at the site of capture.

Following the initial capture and release, 65 females were included in a corticosterone-manipulation experiment: 33 females received a corticosterone-dosing treatment, and 32 females received a vehicle control treatment. We followed the protocol validated by Vitousek et al. (2018a) with some modifications. The treatment consisted of administering 60  $\mu\text{L}$  of 4 mg  $\text{mL}^{-1}$  corticosterone dissolved in dimethyl sulfoxide (DMSO) gel on a fake egg that had been tethered to the nest several days before the treatment was initiated. Corticosterone was applied once a day for 5 days during late incubation (days 8–12) at random times of day. Corticosterone dosing started 1–2 days after the first capture and continued daily for 5 days. The vehicle control treatment consisted of 60  $\mu\text{L}$  of DMSO only and was applied during the same time period. Females were in contact with the gel *via* their feather-free brood patch when incubating. This corticosterone-dosing treatment, which was originally designed to simulate the short-term changes in corticosterone that occur when an individual is exposed to an acute stressor, rapidly elevates circulating corticosterone within the range of natural variation of corticosterone in response to restraint. The exogenous corticosterone is rapidly metabolized and, in birds that experience this treatment once, corticosterone returns to baseline levels by 90 min after exposure (Vitousek et al., 2018a). However, birds that experience this protocol for five to six days in a row show a chronic upregulation in endogenous corticosterone secretion that persists for at least 9–11 days after treatment ends (Taff et al., 2018). Three days after eggs hatched (which was 4–6 days after the experimental treatment had concluded), we recaptured each experimental female tree swallow. The birds were again weighed and blood sampled using the sequence described above.

## 2.3. Sample processing and corticosterone quantification

After sample collection, blood samples were stored on ice until they were processed <4 h later. We spun blood samples for six minutes at 3500 rpm and extracted plasma using a Hamilton syringe. Plasma samples for each capture were then stored at  $-20^\circ\text{C}$  for the remainder of the field season, and then transferred to a freezer at  $-80^\circ\text{C}$  until corticosterone assays and bacteria killing assays were conducted.

We extracted steroids from plasma using a triple ethyl acetate extraction protocol and then measured corticosterone levels in duplicate

using a commercial EIA kit (DetectX Corticosterone, Arbor Assays: K014-H5) previously validated in tree swallows by Taff et al. (2019). We calculated the extraction efficiency of samples run on each plate by measuring corticosterone levels in six samples that had been spiked with 45  $\mu\text{L}$  of a corticosterone standard at 10 ng  $\text{mL}^{-1}$ . We obtained intra-assay variability by comparing measured concentrations from a pooled plasma sample run across each plate, and calculated intra-assay CV by averaging all within-sample covariances. The resulting extraction efficiency was 94.2 % with an intra-assay and inter-assay CV of 5.4 % and 11.5 %, respectively.

For the purposes of this study, we only use the corticosterone data from the baseline samples because they represent the natural state of an organism at the time of capture. The corticosterone data from the other two timepoints are not analyzed further in this study (but see Vitousek et al., 2022).

## 2.4. Bacteria killing assay

We measured the strength of one arm of the innate immune response using a bacteria killing assay (Matson et al., 2006; Millet et al., 2007) with blood plasma and *Escherichia coli* based on the protocol reported by French and Neuman-Lee (2012). Bacteria killing assays are performed *ex vivo*, thus reflecting standing immunity at the time of sample collection. We used frozen plasma samples that had been stored for at least four months at  $-80^\circ\text{C}$  (mean freezing time =  $165.1 \pm 6.91$  days). After sufficient plasma had been removed from each stored sample for corticosterone assays, we pooled all the remaining plasma from each individual's baseline, stress-induced and dex-induced plasma samples from each capture to gather enough material for the bacterial killing assay.

While sample storage can affect the accuracy of bacteria killing assays (Claunch et al., 2022; Jacobs and Fair, 2016), other studies have been able to uncover patterns using frozen samples stored for variable time periods (Downs et al., 2023; Merrill et al., 2012; Morrison et al., 2009; Schmitt et al., 2017). Among our samples, the variance in total freezing times is low relative to the total time frozen (CV = 4.19 % for all samples, CV = 2.46 % for correlational analysis, CV = 4.12 % for the experiment). Thus, since our samples received approximately the same storage treatment; we anticipated that no systematic biases were introduced by sample handling and storage. The decision to pool samples collected across the capture period was driven by the results of a pilot study that we conducted in 2019 comparing the BKA of fresh plasma taken at baseline and stress-induced timepoints: *i.e.*, within three minutes of capture, and 30 min after disturbance. Analyses showed that BKA did not differ between baseline and stress-induced samples taken from breeding female tree swallows (Appendix A). While over longer periods dexamethasone has been reported to impact the gene expression of complement proteins in monocytes and macrophages (Lappin and Whaley, 1991; Walker, 1998) and the expression of complement regulator proteins in different cells (Coulpier et al., 1995; Lemerrier et al., 1992; Muñoz-Cánoves et al., 1989), we do not expect these effects to manifest within 30 min of dexamethasone injection.

Prior to the assay, we suspended one pellet of bacteria (*Escherichia coli* ATCC® 8739™, Microbiology Power™ 0483E7) containing  $10^7$  CFUs in 40 mL of PBS, and then diluted 1 mL of this suspension in 9 mL of PBS to a final concentration of  $10^5$  CFU  $\text{mL}^{-1}$ . The stock and final suspension were kept and stored at  $4^\circ\text{C}$  for up to 24 h. We then diluted 5  $\mu\text{L}$  of blood plasma in 13  $\mu\text{L}$  of phosphate-buffered saline (PBS) in a 96 round-bottom microwell plate and added 6  $\mu\text{L}$  of the final bacterial suspension at  $10^5$  CFU  $\text{mL}^{-1}$ . For every two samples assayed, the plate included one blank control consisting of 24  $\mu\text{L}$  of PBS, and one positive control that consisted of 18  $\mu\text{L}$  of PBS with 6  $\mu\text{L}$  of the bacterial suspension. After administering the bacterial challenge to the sample dilutions, the plate was incubated at  $40^\circ\text{C}$  for 30 min and shaken at 300 rpm. When incubation was finished, we immediately added 125  $\mu\text{L}$  of cold Tryptic Soy Broth (TSB) to each well, and left it to incubate for 12 h

at 37 °C. Once incubation finished, the plate was shaken using an orbital shaker at 800 rpm for five minutes to resuspend bacteria that may have concentrated in the bottom of the wells. Finally, we measured absorbance at a wavelength of 300 nm using a BioTek Synergy HT Microplate Reader. When possible, we assayed up to three replicates of each pooled plasma sample.

Post-incubation absorbance was calculated as the difference between sample or positive absorbance and the mean absorbance of the blanks. Then, we calculated Bacteria Killing Ability (BKA) as one minus the ratio of sample absorbance to the mean positive control absorbance. Conceptually, BKA data should fall between zero, representing no killing ability, and one, representing complete inhibition of bacteria, though some studies report BKA values below zero (Tieleman et al., 2005). Because we had negative values, we prepared three alternative datasets. In the first, we left the values as is, including negative values and those above one. This approach is justified because negative values could be biologically meaningful, indicating more bacterial growth than inhibition during the challenge (Tieleman et al., 2005). In the second dataset we replaced all negative values with zero and values larger than one, with one ('zero-corrected'). This approach accounts for the fact that bacterial growth that occurs in the absence of bacterial inhibition is not caused by the same mechanisms as bacteria killing, and thus values outside of this range may not provide relevant information about bacterial killing ability. Finally, the third approach was to eliminate all instances where BKA was negative or above one because of the concerns described above and because the correction caused zero-inflation of the dataset ('zero-removed').

2.5. Statistical analyses

First, we assessed the association between natural variation in BKA and corticosterone levels in incubating tree swallows. For this analysis we used the data from all 81 females captured prior to the experiment. We constructed a linear regression model (LM) with standardized BKA as the outcome variable using the function *lm* in the program R (R Core Team, 2019). The LM included baseline corticosterone levels, lay date (as day of year), body mass, and the interaction of corticosterone and lay date, as predictors. All predictors were scaled to  $\mu = 0$  and  $\sigma = 1$ . The Breusch-Pagan test, using the function *bptest* from the package *lmtest* (Zeileis and Hothorn, 2002), indicated that the model's residual distribution was homoscedastic (BP = 4.2857, df = 4,  $p = 0.37$ ). We also include diagnostic plots for this model in Supplementary Fig. 1.

Next, we tested the effect of the experimental treatment on baseline corticosterone levels, and on BKA using the data at both sampling points (before and after treatment) from the 65 experimental females. For this analysis, we built two linear mixed models (LMMs) for BKA using the function *lmer* from the package *lme4* (Bates et al., 2015) and modified by the *lmerTest* package (Kuznetsova et al., 2017). Both LMMs were identical except for the outcome variables: one used corticosterone concentration as the outcome, and the other one used standardized BKA. Thus, we used lay date and the interaction between treatment group and experimental stage as fixed variables, and bird ID as a random variable in both models. Lay date was standardized in the BKA model only.

All BKA models, the correlational LM and the experimental LMM, were run three more times with the two data modifications: the zero-corrected dataset and the zero-removed. Both zero-corrected and zero-removed datasets were used to replicate the correlational LM and the experimental LMM, so the models were identical, but the dataset differed. Additionally, we ran a Generalized LMM using a beta zero-inflated data distribution model family with the zero-corrected dataset using the package *gamlss* (Rigby and Stasinopoulos, 2005). This last model used the unstandardized version of the BKA data to conform to the bounds of a beta distribution but used the same predictors as other models. For all regression models mentioned in this section, we report the results from the LM and LMMs with the full dataset only in the main text. However, we address the outcome of each of these models in the

Results section; and we report all model results in Appendix B of the supplementary materials.

3. Results

The correlational analysis showed that BKA was predicted by the interaction between baseline corticosterone concentration and timing of breeding, but the effect was small (Table 1). This association was retained in the models for the zero-corrected and zero-removed data (Supplementary Tables 1–3). The interaction showed that late breeders with higher corticosterone had lower BKA, while early breeders with higher corticosterone had higher BKA (Fig. 1).

When recaptured 4–6 days after treatments had concluded, baseline circulating corticosterone concentrations were elevated in previously corticosterone-dosed tree swallows (Table 2, Fig. 2A). However, we found no evidence that corticosterone dosing causally affected BKA (Table 3, Fig. 2B). The associations in the models that used the zero-corrected and zero-removed datasets were qualitatively similar to the main model (Supplementary Tables 4–6).

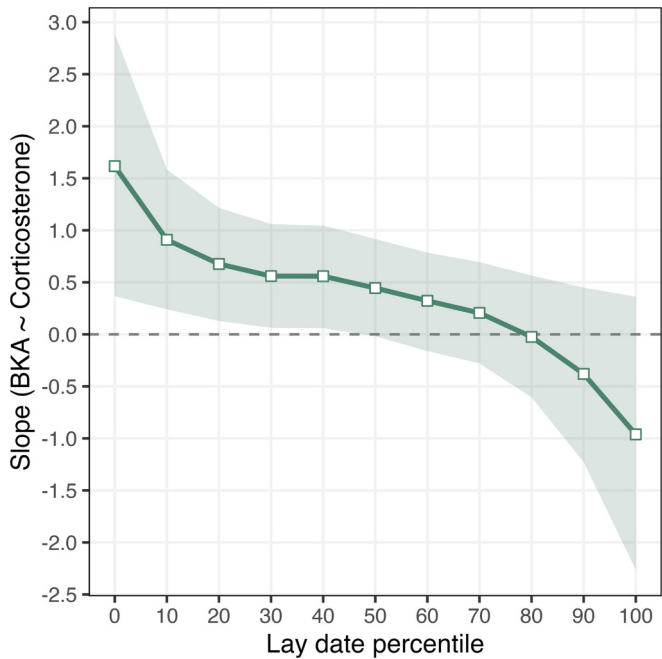
4. Discussion

We found a weak relationship between natural variation in corticosterone and BKA during incubation, but the nature of this relationship differed between early and late breeding birds. However, experimental elevation of circulating corticosterone did not causally affect BKA during the early nestling provisioning period. Our findings contribute to a growing body of evidence that relationships between GCs and immunity, and in particular between GCs and complement-dependent innate immunity, are complex and likely context-dependent. They also underscore that correlations between GCs and immune measures can result from processes other than GC mediation of immunity.

Experimental manipulations of chronic stressors have found both positive and negative effects on BKA (Becker et al., 2019; Hopkins and DuRant, 2011; Love et al., 2017; Matson et al., 2006; Titon et al., 2018). Yet because chronic stressors can have variable effects on GCs (e.g., Dickens and Romero, 2013) it is difficult to determine whether these results stem from differences in the effects of chronic stressors on GCs or differences in the effects of GCs on BKA. The direct causal effects of GCs on BKA have rarely been investigated, but the available evidence suggests that these effects also vary. Our results, combined with those of previous studies, suggest that these differences may be explained by the duration of GC elevation. Experiments in two bird species show that short acute increases in corticosterone cause declines in BKA (Gao and Deviche, 2019; Merrill et al., 2012). However, one of these studies also found that injection with mitotane, an inhibitor of steroid hormone synthesis (including GCs), did not affect BKA two days later (Gao and Deviche, 2019). Thus, these data suggest that the causal effects of acute and chronic GC elevation may differ. Similarly, in *Rhinella ornata*, a toad, transdermal application of corticosterone for 13–20 days does not affect BKA (Titon et al., 2019). Our finding that chronically elevated corticosterone (for ~10 days) does not change BKA in provisioning tree swallows is thus consistent with the few previous studies that have manipulated corticosterone over longer timescales. Mechanistically,

**Table 1**  
Model summary for the linear model of BKA as a function of baseline corticosterone before the experiment using scaled predictors ( $\mu = 0$ ,  $\sigma = 1$ ). Table shows the coefficient estimates ( $\beta$ ), 95 % confidence interval (CI), and  $p$ -values of each predictor variable.

| Predictors                         | Estimates | CI             | p    |   |
|------------------------------------|-----------|----------------|------|---|
| (Intercept)                        | 0.1       | −0.13–0.32     | 0.40 |   |
| Baseline corticosterone (scaled)   | 0.37      | −0.10–0.84     | 0.12 |   |
| Lay Date (scaled)                  | 0.13      | −0.11–0.38     | 0.28 |   |
| Mass (scaled)                      | 0.06      | −0.20–0.32     | 0.65 |   |
| Baseline corticosterone × Lay Date | −0.5      | −0.97 to −0.03 | 0.04 | * |



**Fig. 1.** Predicted mean and 95 % confidence interval of the slope of BKA by circulating corticosterone (both scaled to  $\mu = 0$ ,  $\sigma = 1$ ) for every decile of lay date in the correlational regression model.

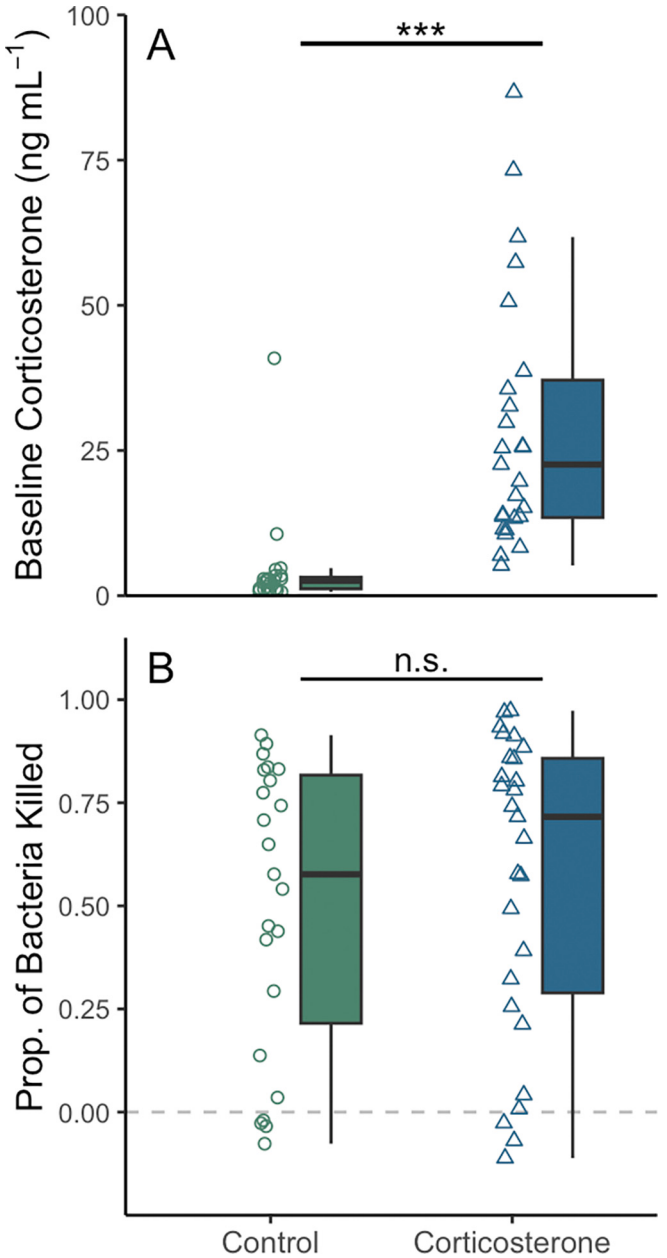
**Table 2**

Model summaries for the linear mixed regression model of baseline corticosterone concentrations. Individual ID was treated as a random variable, and stage refers to the experimental time point (before or after treatment application). The table shows the coefficient estimates ( $\beta$ ), 95 % confidence interval (CI), and p-values.

| Predictors                        | Estimates | CI               | p          |
|-----------------------------------|-----------|------------------|------------|
| (Intercept)                       | 50.22     | −43.20–143.64    | 0.289      |
| Treatment [cort]                  | 27.1      | 19.12–35.08      | <0.001 *** |
| Stage [before]                    | −2.26     | −10.07–5.55      | 0.567      |
| Lay Date (day of year)            | −0.33     | −0.99–0.34       | 0.331      |
| Treatment [cort] × Stage [before] | −25.54    | −36.36 to −14.73 | <0.001 *** |

chronic GC elevation may cause declines in immune sensitivity to GCs, possibly through shifts in GC receptor density in cells involved with the immune response. Although, decreases in BKA after acute GC elevation seen in other studies may also be due to fast catecholamine release during acute stress—specifically restraint during handling—considering that immune activation can be fast (Gao et al., 2017; Sapolsky et al., 2000). Studies like ours, that use free-living animals and that manipulate GCs via non-invasive methods, allow the effects of chronic GC elevation to be separated from GC-independent physiological responses, and from the effects of other physiological mediators of the stress response (e.g., catecholamines) on immune function. Studies of wild animals in captivity, which is often perceived as a stressor, are often unable to separate these effects. Thus, future studies that combine acute and chronic GC manipulations with manipulations of stressor exposure in free-living systems will be important for testing the hypothesis that immune sensitivity to GCs differs based on the duration of GC elevation, as well as the role of catecholamines in immunomodulation.

It is also possible that the effects of GCs on BKA and other immune parameters differ based on the degree of increase in GCs. Most studies to date have increased circulating GCs within the range of response to a major acute stressor (where substantial binding at glucocorticoid receptors is expected). Smaller increases in GCs (that bind predominantly at mineralocorticoid receptors which have a higher affinity for GCs)



**Fig. 2.** Boxplots of baseline circulating corticosterone and BKA four to six days after treatment ended in the experimental study. **A**, post-treatment baseline corticosterone in control and corticosterone-dosed female tree swallows. Baseline corticosterone was significantly higher in corticosterone-dosed females than in control females. **B**, boxplots of post-treatment BKA in individuals that received the control or corticosterone treatment. There was no statistically significant difference in BKA between control and corticosterone-dosed females.

**Table 3**

Model summaries for the linear mixed regression model of standardized BKA after treatment. The table shows the coefficient estimates ( $\beta$ ) for each predictor, 95 % confidence interval (CI), and p-values. Lay date was standardized.

| Predictors                        | Estimates | CI         | p    |
|-----------------------------------|-----------|------------|------|
| (Intercept)                       | −0.07     | −0.48–0.34 | 0.74 |
| Treatment [cort]                  | 0.15      | −0.41–0.72 | 0.59 |
| Stage [before]                    | 0.27      | −0.26–0.81 | 0.31 |
| Lay Date (scaled)                 | 0.11      | −0.09–0.32 | 0.28 |
| Treatment [cort] × stage [before] | −0.37     | −1.11–0.36 | 0.32 |

could have very different effects on immune function. In humans and house mice, mineralocorticoid receptors are expressed in many immune cell types, further suggesting that different GC levels may have differing effects on immune function (Grafte-Faure et al., 1999; Herrada et al., 2009; Rickard et al., 2007; Rocha et al., 2002). For example, heterophil mobilization differs when exposed to mild or severe stressors: mild stressors lead to heterophilia, but more severe conditions cause heteropenia in chickens (Gross and Siegel, 1983; Maxwell and Robertson, 1998). Additionally, the relationships between GCs and BKA could differ between life history stages as has been shown in other taxa (French and Moore, 2008; Ruoss et al., 2019).

Our correlational results suggest context-dependence in the relationship between corticosterone and BKA. We found that the relationship between natural variation in BKA and circulating corticosterone depended on timing of breeding: late breeders with higher corticosterone had lower BKA but this relationship was not present in earlier breeders, although the effect sizes were small. Context-dependence is consistent with facultative resource-based trade-offs seen in other studies in which individuals with more resources can simultaneously invest in reproduction and immunity, or in immunity and mounting a strong stress response, whereas those with fewer resources face an investment trade-off (Ardia, 2005; French et al., 2007; Titon et al., 2018). It is also consistent with our previous finding that immune-based trade-offs vary with timing of breeding in tree swallows (Chang van Oordt et al., 2022). Small differences in resource availability between early and late breeders may drive these differences in the association between corticosterone and BKA.

Many studies also show that inherent underlying phenotypic variation is associated with lay dates in temperate breeding birds, including tree swallows; higher quality birds breed earlier and have elevated reproductive success (Verhulst and Nilsson, 2008; Winkler et al., 2020). Thus, differences in individual quality may also result in varying trade-offs where only low quality (late breeding) phenotypes suppress BKA with higher corticosterone (Fig. 1). The correlational analysis of BKA and corticosterone shows a decline of the slope of BKA by baseline corticosterone as the breeding season increases, and that the association becomes negative after May 24th (Supplementary Fig. 2)—which is 2–3 weeks after tree swallows usually start laying eggs in our population (see Winkler et al., 2020). Interestingly, in our data only the latest ~20 % of breeding birds showed negative associations between BKA and corticosterone levels before the treatment (Fig. 1).

It is also possible that early and late breeders differ in their corticosterone levels and immune responses, but that the covariation between these measures does not reflect a trade-off between investment in stress responses and investment in immunity. Regardless of whether there is a trade-off between investment in immunity and stress responses, differing relationships between corticosterone and BKA may also stem from differences in the cause of GC elevation in early and late breeders. In early breeding tree swallows—high quality birds that tend to be in good condition—GCs may be more likely to rise to facilitate increased reproductive investment (Bonier et al., 2009; Vitousek et al., 2018b). Meanwhile, in late breeders, which tend to be lower quality birds, elevated GCs may be a signal that birds are failing to cope effectively with environmental challenges. It is also important to note that while we excluded data from nests that we knew were the second attempts of birds at our study site, some of the apparently late breeding birds in our dataset were likely birds that initiated nests first in a different location, but when those nests failed they subsequently moved to one of our study sites. Thus, the latest breeding birds in our dataset include two distinct groups of individuals that are breeding late for different reasons, and thus may invest differently in reproduction and face different reproductive trade-offs. Furthermore, the relationships found in this study do not fully represent GC-mediated immunomodulation of all arms of the immune system. For example, immune mechanisms with higher risk of immunopathology or resource requirements may be more responsive to elevated GCs than BKA (e.g., inflammatory

responses: Martin et al., 2005), which uses few resources and thus may be able to be maintained during chronic stress without significant costs to the organism (Iseri and Klasing, 2013). In accordance with this hypothesis, inflammatory assays and hemagglutination assays often show immunosuppression during GC elevation (acute: Gao et al., 2017; chronic: Martin et al., 2005).

## 5. Conclusions

We found that the association between corticosterone and BKA differs between early- and late-breeding birds, but that chronically elevated GCs do not causally affect BKA. These results highlight that the correlational patterns between stress and immunity do not necessarily indicate a causal relationship between GCs and the immune system, and thus, experimental manipulations are necessary to assess causality. We suggest that these patterns, together with findings from other studies where acute GC manipulation can decrease BKA in birds, indicate that the duration of GC exposure may be a key factor in determining its effects on complement-dependent innate immunity. Further research is necessary to test this hypothesis, as well as to understand the roles of the degree of GC elevation and life history stage in the immunomodulatory effects of GCs. Finally, it is likely that different arms of the immune system respond differently to chronically elevated GCs. BKA, a relatively inexpensive type of defense, may be maintained under chronically elevated GCs as a baseline protective mechanism against pathogens while other more energy-demanding immune defenses are down-regulated, potentially mitigating the fitness costs of full immunosuppression.

## CRedit authorship contribution statement

**David A. Chang van Oordt:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Visualization, Writing – original draft, Writing – review & editing. **Conor C. Taff:** Data curation, Formal analysis, Investigation, Methodology, Project administration, Writing – review & editing. **Monique A. Pipkin:** Methodology, Writing – review & editing. **Thomas A. Ryan:** Methodology, Writing – review & editing. **Maren N. Vitousek:** Conceptualization, Funding acquisition, Investigation, Project administration, Supervision, Writing – review & editing.

## Data availability

Data has been archived and made available on Figshare: <https://doi.org/10.6084/m9.figshare.25119104.v2>.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.6084/m9.figshare.25119104.v2>.

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