Chronically raised glucocorticoids reduce innate immune function in Belding's ground squirrels (*Urocitellus beldingi*) after an immune challenge

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ABSTRACT

The hypothalamic–pituitary–adrenal (HPA) axis releases glucocorticoids (GCs), or stress hormones, during the vertebrate stress response. GCs can both enhance and suppress the immune system depending on whether the experienced stressor is acute or chronic and what aspect of immune function is measured. More research is needed to fully understand how the immune system reacts to stressors. In this study, we examined the effects of chronically raised GCs on innate immune function in Belding's ground squirrels (*Urocitellus beldingi*). We measured immune function with a bacteria killing ability (BKA) assay, an integrative and functional assessment of an animal's ability to clear a bacterial infection. All studies to date have examined how acute stressors or repeated social stressors impact BKA. This study is the first to our knowledge to investigate how chronically raised GCs impact BKA both before and after an immune challenge. We noninvasively raised GCs in treatment squirrels for six days and then gave them, and a group of untreated (control) squirrels, an injection of lipopolysaccharide (LPS) to stimulate their innate immune system. Treatment squirrels exhibited lower BKA after, but not before, being challenged with LPS. These results suggest that experiencing chronic stress may not be detrimental to immune functioning until an individual is challenged with an infection.

1. Introduction

Wild animals are subjected to a myriad of natural stressors generated by their physical and social environment. Physical stressors can include predation threats, unpredictable weather or low food availability (Mateo, 2007). Social interactions, especially aggression or the threat of aggression, can also activate the stress response (Van Meter et al., 2009; Sapolsky et al., 1997). In response to a stressor, the hypothalamic–pituitary–adrenal (HPA) axis releases glucocorticoids (GCs), along with other hormones and neuropeptides, in order to protect the body and then bring it back to homeostasis. The stress response functions adaptively when an animal is trying to escape from a predator by increasing glucose uptake and cardiovascular tone and suppressing non-essential functions such as the reproductive, digestive and immune systems (Boonstra, 2013). However, repeated exposure to high GC levels can be detrimental to health, reproductive success and survival (Boonstra, 2005). The relationship between the stress response and the immune system has been the focus of much research over the past few decades, with a particular focus on GCs (Sapolsky et al., 2000; Webster Marketon and Glaser, 2008). Despite these many excellent studies on how GCs regulate immune function, we still do not fully understand the nature of this relationship, particularly in wild, non-model organisms.

Most of the studies on wild species have focused on acute stressors (minutes to hours), many times in relation to handling, a relevant study subject for researchers trapping animals in the wild. Handling stress may suppress, enhance or not affect immune function (Delehanty and Boonstra, 2009; Matson et al., 2006b; Merrill et al., 2012). In contrast, acute stressors in laboratory animals tend to enhance immune function, sometimes through the redistribution of immune cells from the blood stream to areas that are likely to first encounter an infection or injury, such as the skin, lymph nodes, gastrointestinal track and lungs (Bilbo et al., 2002; Dhabhar et al., 1995). These studies show that acute stressors affect immune function differently depending on when samples are collected and how immune function is measured (Dhabhar, 2009). For example, when molluscs are subjected to stress, their immune parameters decrease during the stressor and then increase above baseline between 30 and 120 min after the stressor has ceased (Malham et al., 2003). We still have much to learn about how and why acute stress affects immune function in such disparate ways (Adamo and Parsons, 2006; Matson et al., 2006b).

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Chronic stress can arise from repeated acute stressors or from a continuous stressor that lasts for days or months. Much of the work on chronic stressors and immune function has focused on laboratory animals (Chester et al., 2010; Dhabhar and McEwen, 1999). In contrast to acute stressors, chronic stressors tend to suppress immune function (Dhabhar and McEwen, 1997; Glaser et al., 2000; Nelson, 2005). However, responses to chronic stressors are not always suppressive. For example, when Siberian hamsters (*Phodopus sungorus*) are subjected to chronic social defeat over five days, they exhibit higher stress hormones but not consistently lower immune measures. In fact, defeated hamsters exhibit decreased anti-keyhole limpet hemocyanin (KLH) antibodies, enhanced bacteria killing ability and no difference in hemolytic complement activity compared to unstressed individuals (Chester et al., 2010). In one of the few studies on chronic stress in a wild species, we see similarly equivocal results. For example, when tree lizards (*Urosaurus ornatus*) are exposed to chronically high levels of GCs, they exhibit slower wound healing, a measure that involves multiple immune cells and proteins, than control lizards, but only when they are energetically compromised (French et al., 2007).

Additionally, an animal's infection status moderates measures of immune function. Many immune measures, such as anti-KLH antibodies and skin swelling responses to 2,4 dinitro-1-flourobenzene, rely on stimulating the immune system before measuring its response (Bilbo et al., 2002; Dhabhar, 2009). A study on chickens shows that bacteria killing ability (BKA) is enhanced in birds who were challenged with an immune system stimulant compared with birds that did not receive the stimulant (Millet et al., 2007). Altogether, these studies suggest that other factors, including an animal's energetic state and infection status, may play a role in how both acute and chronic stressors affect the immune system.

In our study, we examined how constitutive innate immune function in temporarily captive, wild-caught Belding's ground squirrels (*Urocitellus beldingi*) responded to chronically raised GCs, a proxy for experiencing chronic stress. Innate immune responses represent the first line of defense against pathogens and are non-specific to the nature of the invading microorganism. We measured immune function with a BKA assay. This assay measures the ability of plasma (or serum, if clotting factors have been removed from the plasma during blood collection) (Maton et al., 1993), proteins to kill *Escherichia coli* and represents an easily interpretable and functional immune measure; squirrels whose blood kills more bacteria *in vitro* should be able to better clear an *in vivo* bacterial infection (Matson et al., 2006b; Merchant et al., 2003). When using serum in this assay, as we did, complement proteins are primarily responsible for any bactericidal activity (Matson et al., 2006b). This method of measuring immune function is relatively new (first published in the ecology literature by Tieleman et al. (2005)). We know little about how BKA responds to chronically raised glucocorticoids. With the exception of Chester et al. (2010), all of the research specifically conducted on BKA and stress examines the effects of acute stress on BKA (Matson et al., 2006b; Merrill et al., 2012; Millet et al., 2007). Because different aspects of the immune system respond to stressors in different ways (Sapolsky et al., 2000), it is important for researchers studying BKA to know how this immune measure responds to both chronic and acute stressors, as well as how BKA responds to stressors during an immune challenge.

Belding's ground squirrels are an excellent species in which to explore questions regarding the stress response and immune system. Squirrels experience a multitude of stressors in the wild, including threat of predation, food shortages when they emerge from winter hibernation, and mate competition. Males suffer higher mortality that females, likely because they suffer wounds from male–male aggression during the mating season (Sherman, 1977; Sherman and Morton, 1984). Additionally, squirrels of both sexes exhibit higher glucocorticoids when faced with unpredictable predation threat and conspecific alarm calls (Mateo, 2007, 2010). We do not know, however, how *U. beldingi* immune systems respond to chronic stressors like predation threat or how well they can cope with infections from wounds when under chronic stress. Based on published work, we predicted that individuals subjected to chronically high GCs would exhibit lower BKA than control individuals (Dhabhar and McEwen, 1997). We also predicted that experiencing chronically high GCs would lower squirrels' ability to respond to an immune challenge (French et al., 2007; Glaser et al., 2000). This experimental design, with immune measures taken both after a chronic GC treatment and after an immune challenge, allowed us to explore the nuanced ways in which stressors, as modeled by GCs, impact immune function during an infection.

2. Material and methods

2.1. Study species

Belding's ground squirrels are ground-dwelling rodents that live in large colonies in the Sierra Nevada and Cascade mountains. They live primarily in alpine and sub-alpine meadows and hibernate for up to eight months per year (Jenkins and Eshelman, 1984). During their active summer season, roughly April–July, squirrels mate upon emerging from hibernation and females give birth to one litter about a month later (Sherman and Morton, 1984). Both terrestrial and aerial predators prey upon Belding's ground squirrels, including coyotes (*Canis latrans*), long-tailed weasels (*Mustela frenata*), pine martens (*Martus americana*), badgers (*Taxidea taxus*) and red-tailed hawks (*Buteo jamaicensis*) (Mateo, 2007). Overwinter mortality is high, with 36–39% of adults and 66–70% of juveniles disappearing from one active season to the next (Sherman and Morton, 1984).

2.2. Animals

Squirrels were live-trapped at Rock Creek Canyon, CA (2713 m) and brought to the animal lab at the Sierra Nevada Aquatic Research Lab (SNARL). At SNARL, each squirrel was individually housed in a plastic tub cage with a wire top (38 cm × 33 cm × 18 cm). We provided pine shavings and cotton squares for bedding material. We also provided each squirrel with four pieces of Mouse Diet 5015 (LabDiet, Richmond, IN, USA), sunflower seeds and water *ad libitum* each day. The animal lab was kept on a 13L:11D schedule and temperature was maintained at 10–18 °C with a combination of an air conditioner, fans and a furnace. After completing the experiment, squirrels were returned to their trapping site. This study was approved by the Institutional Animal Care and Use Committees at both the University of Chicago (protocol No. 71255) and the University of California Santa Barbara (protocol No. 5-03-532). Additionally, we had permission from the California Department of Fish and Game and the United States Forest Service to work with Belding's ground squirrels.

2.3. Fecal collection

We collected fecal samples by holding the animal over a clean bucket in the animal lab. If a squirrel did not defecate upon handling, it was placed in a clean trap inside a clean bucket until it defecated or until 30 min had past. Fecal glucocorticoid metabolites (FGMs) represent an integrated measure of circulating GCs over the past 12–24 h and handling does not impact FGMs until many hours after the handling event (Mateo and Cavigelli, 2005). Fecal samples were collected with clean tweezers and placed in polypropylene microcentrifuge tubes (Fisher Scientific, Hampton,

NH, USA). Tweezers were cleaned with 70% ethanol whereas buckets and traps were cleaned with Cidex Plus 28-Day Solution (Johnson & Johnson, New Brunswick, NJ, USA). All samples contaminated with urine were discarded. Samples were kept on ice until they could be transferred to a −20 °C freezer.

2.4. Blood collection

We collected blood from the saphenous vein. To collect blood, we removed each squirrel's tub cage from its shelf and grabbed the squirrel by hand. Squirrels were lightly anesthetized with isoflurane inhalant and the fur on the lower leg was smoothed with petroleum jelly to help visualize the saphenous vein. Veins were pricked with a sterile needle and blood was collected into a microcentrifuge tube. Samples were collected within 4.5 min of moving the animal's cage and represent baseline levels of circulating GCs (Mateo and Cavigelli, 2005). All samples were chilled for one hour before centrifuging. After being centrifuged, serum was frozen at −20 °C.

2.5. Cortisol assays

We extracted FGMs from fecal samples as described in Mateo and Cavigelli (2005). Thawed fecal samples were dried in a drying oven for 4–6 h, crushed and then weighed to 0.2 g. Glucocorticoids were extracted from weighed samples by adding 1.5 ml of 80% ethanol to the sample, vortexing and then centrifuging for 20 min. After centrifuging, the supernatant was poured off and frozen until assaying. Both extracted fecal samples and serum samples were assayed with commercial ^{125}I-cortisol Corticote® radioimmunoassay kits (MP Biomedicals, Irvine, CA, USA). Samples were assayed in duplicate. If the coefficient of variation (CV) between duplicates exceeded 20%, samples were reanalyzed. Each year, we created a high and a low control by pooling fecal extracts from 10 individuals with high and low binding. We ran both controls at the beginning and end of each assay. In 2010, the mean intra-assay and inter-assay CVs were 14.40% and 18.82%, respectively, for the low pool and 12.14% and 22.23% for the high pool. In 2011, the mean intra-assay and inter-assay CVs were 12.21% and 19.38%, respectively, for the low pool and 12.10% and 22.04% for the high pool. Fecal glucocorticoid metabolite concentrations were divided by the proportion of isotope recoveries, were corrected for dilution ratios and are expressed as nanograms per gram (ng/g) of dried feces. Serum corticoid concentrations (GCs) are expressed as nanograms per milliliter (ng/ml) of serum. Cortisol assays were performed in the Mateo Lab at the University of Chicago.

2.6. Bacteria killing ability assay

The BKA assay measures the capacity of serum proteins to kill *E. coli.* Our assay methods follow those of Chester et al. (2010) with a few exceptions. We used serum instead of plasma. We diluted serum samples in glutamine enriched CO_2-independent media (#18045, Gibco-Invitrogen, Carlsbad, CA, USA) to reach a final volume of 200 µl. To determine the optimal serum to media dilution for each year's samples, we serially diluted pooled samples (starting with a 1:5 dilution and ending with a 1:80 dilution). Using these dilutions, we determined which dilution exhibited 50% bacteria killing ability. We used a 1:10 dilution in 2010 and a 1:30 dilution in 2011. Although we used the same type of *E. coli* pellet (7×10^7 colony forming units (CFUs); EPower Microorganisms #0483E7, MicroBioLogics, St. Cloud, MN) in both years, the numbers of CFUs/ml working solution differed. Thus, we added 20 µl working solution to each diluted serum sample in 2010 and 3 µl working solution to each diluted serum sample in 2011 to obtain 100–200 CFUs of *E. coli* per sample. Positive control plates were

made with each assay and consisted of 200 µl media and 20 µl (in 2010) or 3 µl (in 2011) working solution. Negative control plates consisted of 200 µl media and 20 µl PBS. We incubated, vortexed and spread the serum bacteria cocktails onto agar plates following Chester et al. (2010). We assayed samples in triplicate in 2010 and in duplicate in 2011. After counting CFUs the following day, we averaged the number of colonies counted on a sample's plates. We conducted every part of the BKA assay (except incubation) under laminar flow conditions to prevent contamination during the assay. No negative control plates produced any CFUs. However, some samples resulted in negative bacteria killing ability. This occurs when more colonies grow on a sample plate than the positive control plate. Negative killing ability has been observed in other species and is ascribed to a combination of poor killing ability and high microbial division (Moore et al., 2011; Rubenstein et al., 2008; Tieleman et al., 2005). Bacteria killing ability is described as the percent of bacteria killed relative to the number of CFUS on the positive control plate. We used the formula below to calculate bacteria killing ability:

$$100^*(1 - \text{sample CFUs}/\text{positive control CFUs})$$

We assayed 2010 samples in the Demas Lab at Indiana University (Bloomington, IL) and 2011 samples in the Pritzker Lab at the Field Museum of Natural History (Chicago, IL). Some plates were contaminated with another bacterium species and were not included in analyses. We know that contamination occurred during blood collection because when we serially diluted samples, more dilute samples produced fewer contaminated bacteria CFUs. Other studies on BKA have also reported contamination (Beechler et al., 2012; Moore et al., 2011).

2.7. Experimental design

Squirrels were trapped upon emergence from hibernation in early May, so that females would be unmated and seasonal effects on glucocorticoid levels would be minimized (Nunes et al., 2006; Romero et al., 2008). Upon arrival in captivity, we dusted squirrels and their bedding with flea powder to eliminate ectoparasites and any effects they may have had on the study. Squirrels were allowed to acclimate to captivity for at least one week prior to the start of the study (Mateo and Cavigelli, 2005). We sampled 56 adult and yearling *U. beldingi* over two field seasons (N = 16 females and 16 males in 2010, N = 9 females and 15 males in 2011). We were not able to determine exact ages for the squirrels in the study because this population has not been part of a long-term study. However, we know that we only sampled adults and yearlings because juveniles had not yet been born this early in the active season.

Each year, half of the squirrels were placed in the treatment group and half of the squirrels were placed in the untreated, or control, group. In 2010, 16 males and 16 females were evenly divided between the treatment and control groups (treatment n = 8 males and 8 females; control n = 8 males and 8 females). Because we did not find any sex differences in 2010, we did not maintain an even sex ratio within treatment and control groups in 2011 (treatment n = 8 males and 4 females; control n = 7 males and 5 females). The treatment group had their cortisol levels raised noninvasively for six days (Days 1–6) to mimic a chronic stress response. We suspended hydrocortisone at a concentration of 0.09 mg/g bodyweight (Sigma, St. Louis, MO, USA) in sesame oil and added it to equal parts peanut butter and wheat germ to form a ball roughly 2 cm in diameter. Control animals were given peanut butter balls without hydrocortisone (Mateo, 2008). We distributed peanut butter balls each morning and made sure that every squirrel had eaten their ball each day. Most squirrels ate their peanut butter balls within 2 h.

In 2011, we collected pre-experiment weight measurements, fecal samples and blood samples before giving the first peanut butter ball on Day 1 of the experiment. In both years, we collected post-treatment weight measurements and fecal samples on Day 6 or 7 and a blood sample on Day 7 to assess the treatment's effect on weight, FGMs, serum GCs and BKA. Immediately after collecting these blood samples, we injected all squirrels intraperitoneally with 0.1 ml of 25 µg/kg lipopolysaccharide (LPS; from *E. coli*, phenol extracted; Serotype 0111:B4; Sigma, St. Louis, MO, USA) to induce a peripheral immune response. Two hours later, we collected another blood sample to assess treatment and immune-challenge effects on BKA.

2.8. Statistical analysis

Data analyses were completed using the lme4 (Bates et al., 2011), phia (De Rosario-Martinez, 2012), car (Fox and Weisberg, 2011) and base packages in R version 2.15.1 (Development Core Team, 2012). We examined all data for normality using Shapiro–Wilk tests. If a variable was not normally distributed, we attempted to transform it to improve normality. We used non-parametric statistics, parametric statistics and generalized linear mixed models (GLMMs) to analyze our data. If we used a GLMM, we included squirrel identity as a random effect to control for repeated measures on individuals. To better understand interaction results in GLMMs, we performed post hoc analyses on all of the pairwise interaction contrasts using the function testInteractions in the phia (De Rosario-Martinez, 2012) package of R. This method adjusts interaction *p*-values with a Holm-Bonferroni correction and removes influences from the model's main effects. These post hoc analyses allowed us to more accurately understand interactions between variables. We checked all model residual plots to ensure our models met assumptions. We looked for treatment, sampling period, sex and weight effects on FGMs, serum GCs and BKA. We pooled data from 2010 and 2011 because we did not find any difference between years in any of our response variables. Values are presented as mean ± SE unless otherwise noted. We used an alpha level of 0.05 as our threshold for significance.

To ensure that the treatment succeeded in raising GCs in a chronic fashion, we looked for treatment effects on FGMs. We also looked for sex and weight effects on FGMs to ensure that treatment effects were not caused by hidden variables. Fecal glucocorticoid metabolites were not normally distributed and transformations were unsuccessful. Thus, we used non-parametric statistics on these data. To avoid repeated measures on individuals, we looked for effects of weight, sex and treatment on FGMs using Spearman correlations and Wilcoxon rank–sum tests on pre-treatment samples separately from post-treatment samples. To determine whether there were differences in FGMs from pre- to post-treatment, we used paired Wilcoxon signed–rank tests on repeated measures for treatment squirrels separately from control squirrels.

We analyzed serum GCs to look for treatment effects and to ensure that our sampling method measured baseline GCs. If serum GCs are higher in treatment versus control squirrels, the peanut butter balls succeeded in raising circulating GCs for at least 12 h after consumption. We log transformed serum GCs and used a GLMM with a normal error distribution to analyze these data. Our model included three main effects: treatment, sampling day (pre- or post-treatment), and time to collect the blood sample as well as an interaction between treatment and sampling day. This model included data on 48 blood samples from 36 individuals. This sample size is lower than expected because we were not able to obtain blood from all individuals at all sampling points.

We were primarily interested in how chronically raised GCs affected innate immune function before and after an immune challenge. Bacteria killing ability was not normally distributed. As a proportion variable, a binomial model is most appropriate for analyzing BKA data (Warton and Hui, 2011). However, residuals from binomial models were poorly distributed. Thus, we logit transformed BKA and built a GLMM with a normal error distribution (Bolker et al., 2008; Warton and Hui, 2011). Residual plots were normally distributed indicating that this model best fit our data. The model results also align well with those of separate non-parametric tests. In order to use the logit transformation, we eliminated all negative and zero values from our dataset. Before transforming, we added 47 to each BKA sample so that our lowest value (-46% killing) was above zero. Our model included four main effects: treatment, sampling period (pre-treatment, post-treatment or post-LPS challenge), serum GCs and the BKA assay positive control plate CFUs as well as an interaction between treatment and sampling period. Because the BKA samples were assayed across multiple days, each assay had different positive control plate CFUs (107, 135, 230 CFUs). Although the variance in positive control plate was already included in each measure of BKA, the positive control plate CFUs still significantly affected BKA and was important to include in our model. Other studies have found that variance in positive control plate values also modulate sample BKA and have accounted for this variance in their analyses (Beechler et al., 2012; Townsend et al., 2010). The model for bacteria killing ability included data on 63 samples from 38 individuals. Again, our sample size is lower than expected because of difficulty obtaining blood samples.

3. Results

3.1. Fecal glucocorticoid metabolites

We treated all post-treatment fecal and weight samples, whether collected on Day 6 or Day 7, as post-treatment samples because there was no difference in either variable based on collection day (FGMs: Wilcoxon rank–sum test: $W = 413$, $p = 0.1656$; weight: *t*-test: $t = -1.47$, $df = 65$, $p = 0.146$). Fecal glucocorticoid metabolites did not differ between treatment and control squirrels prior to treatment (Wilcoxon rank–sum test: $W = 57$, $p = 0.608$; Fig. 1a). Treatment squirrels, however, had higher FGMs than control squirrels post-treatment (Wilcoxon rank–sum test: $W = 519$, $p = 0.0022$; Fig. 1a). A repeated measures analysis showed that control squirrels' FGMs did not change from pre- to post-treatment (paired Wilcoxon signed–rank test: $V = 22$, $p = 0.625$; Fig. 1b). A repeated measures analysis on treatment squirrels showed that their FGMs were higher post-treatment compared to pre-treatment (paired Wilcoxon signed–rank test: $V = 0$, $p = 0.00098$) (Fig. 1b). Sample sizes were lower in the paired Wilcoxon signed–rank tests than in the Wilcoxon rank–sum tests because of missing data. These differences in FGM values were not driven by sex (Wilcoxon rank–sum tests: pre-treatment samples: $W = 76$, $p = 0.325$, females = 261.9 ± 35.33 ng/g, $n = 8$; males = 219.7 ± 11.89 ng/g, $n = 15$; post-treatment samples: $W = 420$, $p = 0.183$, females = 1156 ± 216.2 ng/g, $n = 23$; males = 875.9 ± 160.3 ng/g, $n = 30$). Pre-treatment weight did differ between treatment (212.2 ± 12.3 g) and control (215.1 ± 9.18 g) squirrels (*t*-test: $t = -0.1901$, $df = 22$, $p = 0.851$) and did not influence FGMs (Spearman correlation: $S = 1986$, $\rho = 0.019$, $p = 0.932$, $n = 23$). However, during the post-treatment period, individuals with lower weight had higher FGMs (Spearman correlation: $S = 19,089$, $\rho = -0.345$, $p = 0.022$, $n = 44$). Both treatment (Spearman correlation: $S = 2773$, $\rho = -0.371$, $p = 0.082$, $n = 23$) and control (Spearman correlation: $S = 2283$, $\rho = -0.483$, $p = 0.027$, $n = 21$) squirrels exhibited lower FGMs with higher body weights post-treatment, although this relationship was only a trend in treatment squirrels. There were no post-treatment weight differences between control (219.4 ± 6.62 g) and treatment squirrels (228.9 ± 6.92 g) (*t*-test: $t = 0.996$,
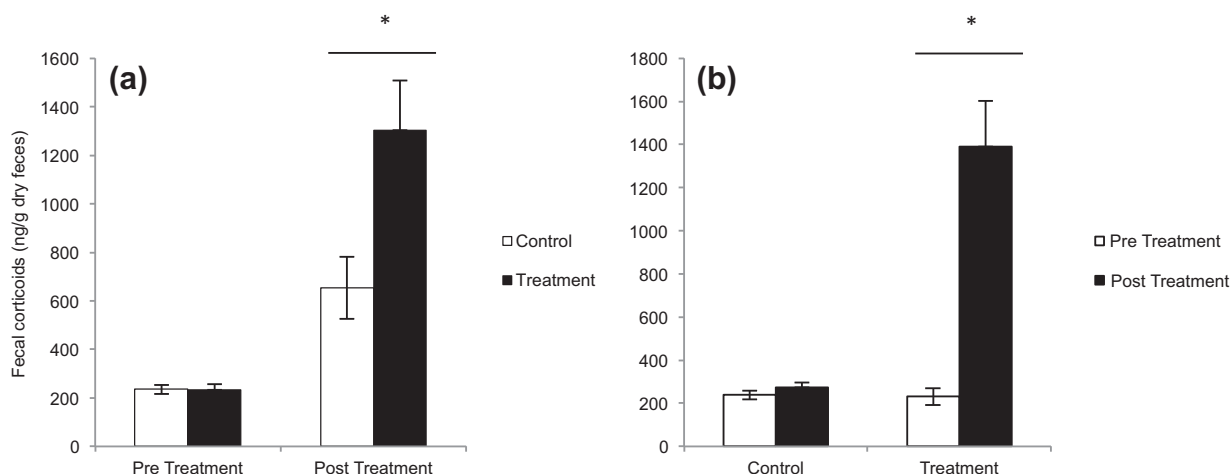
Fig. 1. Mean + SE fecal glucocorticoid metabolites (ng/g dry feces) for control and treatment squirrels at both pre-treatment (Day 1 before beginning treatment) and post-treatment (Day 6 or 7, after six days of treatment). (a) data from separate Wilcoxon rank sum test on FGM differences between control and treatment squirrels at pre- and post-treatment sampling (control group pre-treatment n = 11, treatment group pre-treatment n = 12, control group post-treatment n = 25, treatment group post-treatment n = 28). (b) data from separate paired Wilcoxon signed-rank tests on FGM differences within control and treatment squirrels at pre- and post-treatment sampling (control group n = 10, treatment group n = 11). Lines over columns indicate significant differences between groups. *indicates $p < 0.05$.

df = 42, p = 0.33). In fact, all squirrels, except for one in the treatment group, gained weight from pre- to post-treatment (Pearson correlation: t = −4.71, df = 15, p = 0.0003, pre-treatment = 220.6 ± 9.21 g, post-treatment = 245.3 ± 6.43 g, mean weight change from pre- to post-treatment = 24.75 ± 5.26 g). Treatment and control squirrels gained 15.7% and 10.8%, respectively, of their original body weight over the course of the experiment.

3.2. Serum glucocorticoids

Sex and weight did not affect GCs according to non-parametric statistics on GC data separated into sampling days (pre-treatment, post-treatment) and GLMMs with sex or weight as the only main effect in the model or as an interaction in a single model. Thus, we did not include these terms in the model. The interaction between treatment and pre-versus post-treatment period was nearly significant (generalized linear mixed model: t = −2.037, p = 0.066; Fig. 2, Table 1). A post hoc analysis indicated that this interaction
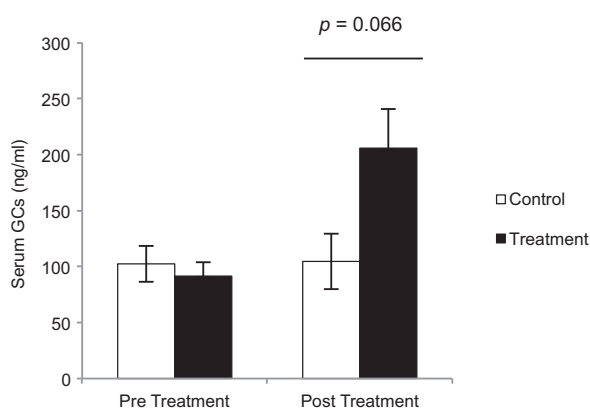

Fig. 2. Mean + SE serum GCs (ng/ml) for control and treatment squirrels at pre-treatment (Day 1 before beginning treatment) and post-treatment (Day 7, after six days of treatment). Control group pre-treatment n = 9, treatment group pre-treatment n = 7, control group post-treatment n = 16, treatment group pre-treatment n = 16. Lines over columns indicate differences between groups. The interaction between Treatment and Sampling Day was significant (p = 0.042) in a post hoc analysis that removed the influence of the main effects from the p value of the interaction term.

was significant after removing the influence of the main effects, showing that treatment squirrels had higher GCs post-treatment than control squirrels (interaction contrast with Holm-Bonferroni correction: χ^2 = 0.738, p = 0.042). The time it took to collect the blood sample did not affect GCs (generalized linear mixed model: t = 1.143, p = 0.278; Table 1).

3.3. Bacteria killing ability

Sex and weight did not affect BKA according to non-parametric statistics on BKA data separated into sampling periods (pre-treatment, post-treatment, post-LPS challenge) and GLMMs with sex or weight as the only main effect in the model or as an interaction in a single model. Thus, we did not include these terms in the model. There was a significant interaction between treatment and sampling period for post-treatment and post-LPS challenge samples (generalized linear mixed model: t = 2.758, p = 0.011; Table 2). A post hoc analysis confirmed this result (interaction contrast with Holm-Bonferroni correction: χ^2 = 7.61, p = 0.017). Control squirrels had higher BKA after the LPS challenge than treatment squirrels (Fig. 3, Table 2). The interaction between treatment and sampling period for pre-treatment and post-treatment samples was not significant (generalized linear mixed model: t = 1.164, p = 0.256; Table 2), indicating that treatment alone did not suppress BKA. A post hoc analysis confirmed these interaction results and also demonstrated that there was no difference between control and treatment squirrels' BKA when comparing pre-treatment and post-LPS samples (interaction contrast with Holm-Bonferroni correction: χ^2 = 1.244, p = 0.489). Higher circulating GCs corresponded to lower BKA (generalized linear mixed model: t = −3.06, p = 0.005; Fig. 4, Table 2), indicating that GCS suppressed immune function as measured by BKA. Additionally, higher positive control plate CFUs corresponded to higher BKA (generalized linear mixed model: t = 2.68, p = 0.013; Table 2).

4. Discussion

This study is, to our knowledge, the first to examine the effects of chronically elevated glucocorticoids on bacteria killing ability after an immune challenge. This immune measure is gaining popularity in field ecology studies because of its functional interpretability and ease of use in many species. Bacteria killing ability,

Table 1
Results from a generalized linear mixed model with serum glucocorticoids as the dependent variable and squirrel identity as a random effect (n = 48 samples from 36 squirrels).

Independent variables	Estimate	SE	t	p
Intercept	3.911	1.040	3.760	0.003[**]
Treatment (Control)	−0.6754	0.2200	−3.070	0.011[*]
Sampling Day (Pre-Treatment)	−0.6411	0.2723	−2.355	0.038[*]
Time to Collect Blood	0.0051	0.0045	1.143	0.278
Treatment (Control)*Sampling Day (Post-Treatment)	0.7380	0.3623	−2.037	0.066[†]

[*] Indicates p < 0.05.
[**] Indicates p < 0.01.
[†] Indicates p < 0.10.

Table 2
Results from a generalized linear mixed model with bacteria killing ability as the dependent variable and squirrel identity as a random effect (n = 63 samples from 38 squirrels).

Independent variables	Estimate	SE	t	p
Intercept	1.679	1.028	1.634	0.115
Treatment (Control)	−1.173	0.4164	−2.818	0.01[**]
Post-LPS challenge vs Post-Treatment	−0.6947	0.3923	−1.771	0.089[†]
Pre-Treatment vs Post-Treatment	−0.5958	0.5107	−1.167	0.255
log Serum GCs	−0.5905	0.1929	−3.060	0.005[**]
Positive Control Plate CFUs	0.008	0.003	2.680	0.013[*]
Treatment (Control)*Sampling Period (Post-LPS Challenge)	1.440	0.522	2.758	0.011[*]
Treatment (Control)*Sampling Period (Pre-Treatment)	0.7404	0.6361	1.164	0.256

[*] Indicates p < 0.05.
[**] Indicates p < 0.01.
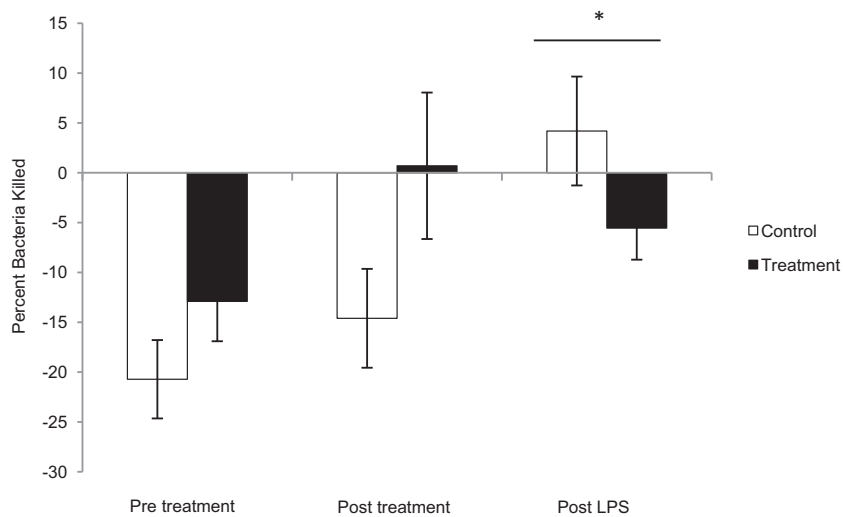[†] Indicates p < 0.10.

Fig. 3. Mean + SE percent bacteria killed in control and treatment squirrels at pre-treatment (Day 1 before beginning treatment), post-treatment (Day 7, after receiving six days of treatment) and post-LPS challenge (Day 7, two hours after receiving an injection of LPS to stimulate the immune system) sampling periods. Control group pre-treatment n = 9, treatment group pre-treatment n = 5, control group post-treatment n = 12, treatment group post-treatment n = 11, control group post-LPS challenge n = 14, treatment group post-LPS challenge n = 12. Lines over columns indicate significant differences. *indicates p < 0.05.

however, is subject to modulation by the HPA axis, as are all aspects of the immune system. Because BKA has only been used in ecological studies since 2005, little work has been done on how it responds to stressors. The work that has been done has focused on acute or repeated social stressors (Chester et al., 2010; Hopkins and Durant, 2011; Matson et al., 2006b; Merrill et al., 2012; Millet et al., 2007). In this study, we exposed Belding's ground squirrels to chronically raised glucocorticoids for six days via hydrocortisone-laced peanut butter balls. This non-invasive technique allowed us to raise glucocorticoid levels without subjecting squirrels to physical or social stressors. This increase in GCs likely mimics the GC surge experienced during a natural stress response (Merrill et al., 2012). After the treatment period, we stimulated the squirrels' immune systems with lipopolysaccharide. We found that raising GC

levels, through exogenous treatment with hydrocortisone, caused treatment squirrels to have lower bacteria killing ability compared to untreated (control) squirrels after receiving LPS (Fig. 3, Table 2). However, the chronic GC treatment did not lower BKA in the absence of an immune challenge. Thus, one of two predictions for the study were met.

Our results indicate that the treatment succeeded in raising treatment squirrels' GC levels as measured by both fecal glucocorticoid metabolites and serum glucocorticoids (Figs. 1 and 2, Table 1). Repeated measures analyses of FGMs and serum GCs showed that treatment squirrels exhibited higher GCs during post-treatment sampling compared to pre-treatment sampling (Figs. 1 and 2, Table 1). Although the interaction between treatment and sampling day in the serum GC model was a trend, a post
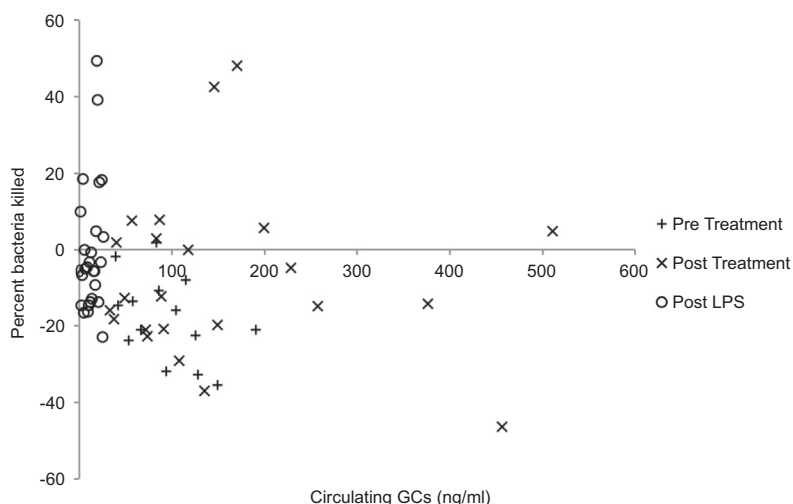
Fig. 4. Relationship between serum GCs (ng/ml) and percent bacteria killed (n = 63 blood samples from 38 squirrels). Samples are from all three sampling periods (pre treatment, post treatment and post LPS challenge) and from both control group and treatment group squirrels.

hoc analysis that removed the influence of the model's main effects from this interaction showed that this relationship was, in fact, significant. Because the treatment succeeded in raising serum GCs, we know that the exogenous GCs crossed the gut epithelium barrier and entered circulation (Breuner et al., 1998) instead of merely passing through the gut and being excreted in squirrels' feces and urine. These treatment effects were not due to underlying physiological or sex differences between the control and treatment groups. Control and treatment squirrels did not differ in FGMs, GCs or weight prior to treatment. Additionally, the blood samples measured baseline, not stress induced, serum GCs because the time it took to collect a blood sample did not affect GCs (Table 1). We have seven years of data on FGMs in wild *U. beldingi*. The FGM values observed in treatment squirrels are higher than the average values observed in wild *U. beldingi* but are within the observed range (Mateo, unpublished data). Thus, the results from this study should apply to the ways in which wild *U. beldingi* immune systems respond to chronic stressors.

To date, no other studies have examined the effect of chronically raised glucocorticoids on bacteria killing ability both before and after an immune challenge. However, we can compare each part of our BKA results with previous studies. In the only other study dealing with chronic stress and BKA, Chester et al. (2010) found that Siberian hamsters (*Phodopus sungorus*) subjected to chronic social defeat exhibit enhanced BKA over control animals. These results contrast with our own results, where individuals who experienced chronically high GCs did not exhibit a change in post-treatment BKA compared to control individuals. Social stressors, however, may modulate immune function differently than physical or environmental stressors or chronically high GCs. In studies on acute stressors and BKA, immune function may be enhanced, suppressed or unaffected by raised glucocorticoids. In birds and rodents, for example, acute stressors tend to suppress or not affect BKA (Matson et al., 2006b; Merrill et al., 2012; Millet et al., 2007; Previtali et al., 2012). In eastern hellbenders (*Cryptobranchus alleganiensus*), however, acute stressors enhance BKA (Hopkins and Durant, 2011). In our study, we found a significant negative relationship between circulating GCs and BKA, which parallels the relationship between FGMs and BKA found in *U. beldingi* by Dosmann, Brooks and Mateo (in review). This linear relationship between GCs and BKA exists even in the absence of a treatment difference in post-treatment BKA levels.

Both control and treatment squirrels exhibited higher BKA after the LPS challenge compared to pre-treatment levels. These results align well with the theory that immune challenges should enhance immune function (Bilbo et al., 2002; Bison et al., 2009; Hasselquist et al., 1999; Millet et al., 2007; Owen and Moore, 2008; Zysling et al., 2009). Treatment squirrels, however, exhibited lower BKA than control squirrels after being challenged with LPS, suggesting that experiencing chronic stressors reduces bacteria killing ability during an infection (Fig. 3, Table 2). Alternatively, treatment squirrels' serum proteins may have already been redistributed into surrounding tissues during the immune challenge (Adamo, 2004). White blood cells are known to redistribute into skin, lymph nodes, spleen, bone marrow and lungs during stress responses (Bilbo et al., 2002; Dhabhar and McEwen, 1997; Martin et al., 2006) and it is possible that other immune components do this as well.

It is worth discussing why roughly 70% of the BKA values observed in this study were negative. This percentage of negative values is larger than that observed in other studies. However, many studies do observe negative killing values (Ezenwa et al., 2012; Merrill et al., 2012, 2013; Moore et al., 2011; Rubenstein et al., 2008; Sparkman and Palacios, 2009; Stambaugh et al., 2011; Tieleman et al., 2005). Negative killing occurs when more bacteria grows on a sample plate than the positive control plate. This can occur because bacteria, and *E. coli* in particular, have a rapid generation time, on the order of 30–90 min (Plank and Harvey, 1979). Thus, during the 30 min incubation of serum, L-glutamine enriched CO_2 independent media and *E. coli*, it is expected that if the serum proteins do not kill all of the added bacteria that this bacteria will duplicate. This mixture is an even more nutrient rich environment than that of the positive control because the added serum has additional nutrients in it. Thus, even if a sample exhibits negative killing it is likely that it killed some proportion of the added bacteria and its value is still relevant to understanding the immune function of that individual animal. Even though we optimized this assay so that individual samples would kill, on average, 50% of the added bacteria, this optimization was completed with a subset of the overall pool of samples. In the future, we will complete a dilution response with all of our samples. This tactic should increase the killing ability in the overall study.

Our results suggest that the observed variation in the relationship between GCs and immune function within and between species may be due to infection status (Berzins et al., 2008;

Delehanty and Boonstra, 2009). Treatment squirrels exhibited suppressed BKA after, but not before, being challenged with an immune stimulant. Thus, experiencing chronic stressors may not be detrimental to animals unless they experience an immune challenge in the form of a bacterial, ectoparasite or viral infection. It is also possible that other measures of immunity would have reacted differently to chronically raised GCs. Stress affects each branch of the immune system differently and our results may not be translatable to the adaptive branch of the immune system (Adamo, 2004; Matson et al., 2006a; Palacios et al., 2012; Versteegh et al., 2012). In future studies, measuring bacteria killing ability alongside a similarly integrative and functional assessment of adaptive immune function would help establish a complete picture of how chronic stress regulates immune function in Belding's ground squirrels.

5. Conclusions

These results highlight the variability of the immune system's response to chronic stress. We found that chronically high GCs suppress innate immune function after an immune challenge. Immune function, was not suppressed, however, in the absence of a challenge. It is possible, then, that chronic stress is not detrimental to Belding's ground squirrels, or other taxa, until an infection infiltrates the body. If this is the case, we may be measuring an adaptive response to chronic stress in *U. beldingi*. Past research in our lab suggests that the *U. beldingi* HPA axis responds to differences in local predation risk by adjusting baseline GC levels (Mateo, 2007). Because the immune system is so closely linked to the stress response, it may also adapt to local stressors and be able to maintain normal functioning in the face of chronic stressors. Our results add to the growing body of work suggesting that immune system functioning and reactions to stress depends on individual condition, infection status, reproductive state and time of year (French et al., 2007; Owen and Moore, 2008; Prendergast et al., 2008; Zysling et al., 2009). In future studies on stress and immune function, researchers should consider infection status of their study animals, especially when conducting field studies. It would be good practice to measure ectoparasite loads and note any wounds, both of which can stimulate the immune system and moderate immune responses to stress (Boughton et al., 2011; French et al., 2009). Accounting for these variables in our studies will help us to better understand the complex relationship between the stress response and the immune system.

Acknowledgments

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