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Contrasting stress responses of two co-occurring chipmunk species (*Tamias alpinus* and *T. speciosus*)

Talisin T. Hammond [a,b,*], Rupert Palme [c], Eileen A. Lacey [a,b]

[a] Department of Integrative Biology, 1001 Valley Life Sciences Building, University of California Berkeley, Berkeley, CA 94720-3160, USA
[b] Museum of Vertebrate Zoology, 3101 Valley Life Sciences Building, University of California Berkeley, Berkeley, CA 94720-3160, USA
[c] Institute of Medical Biochemistry, Department of Biomedical Sciences, University of Veterinary Medicine, Veterinärplatz 1, 1210 Vienna, Austria

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ABSTRACT

Glucocorticoid (GC) hormones are important mediators of responses to environmental conditions. Accordingly, differences in GC physiology may contribute to interspecific variation in response to anthropogenically-induced patterns of climate change. To begin exploring this possibility, we validated the use of fecal cortisol/corticosterone metabolites (FCM) to measure baseline glucocorticoid levels in two species of co-occurring chipmunks that have exhibited markedly different patterns of response to environmental change. In Yosemite National Park, the alpine chipmunk (*Tamias alpinus*) has undergone a significant upward contraction of its elevational range over the past century; in contrast, the lodgepole chipmunk (*Tamias speciosus*) has experienced no significant change in elevational distribution over this period. To determine if GC levels in these species vary in response to external stimuli and to assess whether these responses differ between species, we compared FCM levels for the same individuals (1) at the time of capture in the field, (2) after a short period of captivity, and (3) after adrenocorticotropic hormone (ACTH), (4) handling, and (5) trapping challenges conducted while these animals were held in captivity. Our analyses indicate that *T. alpinus* was more responsive to several of these changes in external conditions. Although both species displayed a significant FCM response to ACTH challenge, only *T. alpinus* showed a significant response to our handling challenge and to captive housing conditions. These findings underscore the importance of species-specific validation studies and support the potential for studies of GC physiology to generate insights into interspecific differences in response to environmental change.

1. Introduction

Biotic responses to environmental challenges can vary markedly, even among closely related, ecologically similar species. The reasons for such variation are poorly understood, but physiological processes seem likely to play an integral role in generating such differences (Bernardo et al., 2007; Tingley et al., 2009; Tomanek, 2012). Among the physiological systems that are expected to influence responses to environmental conditions are glucocorticoids (GCs), a group of metabolic hormones that help to maintain allostasis by mediating multiple systemic responses to abiotic (e.g., temperature, aridity) as well as biotic (e.g., social environment, predation) challenges (Bauer et al., 2013; Boonstra, 2004; Creel et al., 2013; Dantzer et al., 2013; Wingfield, 2013b; Woodruff et al., 2013). Due to their responsiveness to external conditions and their multiple, regulatory effects on an individual's biology, GCs have the potential to provide valuable information regarding the extent to which free-living animals are challenged by their environments (Bonier et al., 2009; Breuner et al., 2008; Romero, 2004; Wingfield et al., 1998).

While acute differences in GC levels provide information regarding responses to unexpected, short-term changes in environmental conditions, differences in baseline levels of GCs are more appropriate for assessing the impacts of enduring, long-term environmental challenges (Bonier et al., 2009; Busch and Hayward, 2009). Because fecal samples can be collected non-invasively and because they provide an integrated measure of hormone levels occurring over many hours, they are ideal for studies of baseline GC levels (Sheriff et al., 2011; Harper and Austad, 2000; Palme, 2012; Möstl and Palme, 2002; Touma and Palme, 2005). Prior to

* Corresponding author at: Museum of Vertebrate Zoology, 3101 Valley Life Sciences Building, University of California Berkeley, Berkeley, CA 94720-3160, USA.
E-mail address: thammond@berkeley.edu (T.T. Hammond).

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using fecal samples to measure GC response in a given species, however, a validation study should be completed to confirm that these samples reliably capture information regarding biologically significant changes in GC levels (Touma and Palme, 2005). Most studies employ a pharmacological challenge, in which animals are injected with adrenocorticotropic hormone (ACTH) and fecal samples are then collected at regular intervals to determine if an increase in fecal cortisol/corticosterone metabolites (FCM) occurs. Biological validation experiments can also be conducted by challenging animals with one or more relevant external stimuli (e.g., capture and handling, Bosson et al., 2012, 2013; Touma and Palme, 2005). Because stress physiology, including GC metabolism and excretion, can vary markedly among species (Boonstra and McColl, 2000; Clarke et al., 1988; Faure et al., 2003; Frisch and Anderson, 2005; Gomes et al., 2012; Juliana et al., 2014; Palme, 2005), taxon-specific validation studies are critical to measuring and interpreting data regarding FCM (Touma and Palme, 2005).

Chipmunks (genus *Tamias*) from the Sierra Nevada mountains of western North America provide an important system for exploring interactions between GC physiology and responses to environmental change. Two of these species – the alpine chipmunk (*Tamias alpinus*, *TA*) and the lodgepole chipmunk (*Tamias speciosus*, *TS*) – have been the subject of extensive study regarding interspecific differences in response to environmental conditions (Bi et al., 2012, 2013; Moritz et al., 2008; Rubidge et al., 2011, 2012, 2014). *TA* is a small (~30–50 g), high-elevation specialist that is found primarily above tree line in rocky habitats (Clawson et al., 1994). In contrast, *TS* is a larger (50–80 g), more generalist species that occurs mainly below tree line in a variety of habitats (Best et al., 1994). Although these species co-occur at the upper end of TS's elevational distribution and the lower end of TA's elevational range, they are characterized by strikingly different responses to climate change in this region over the past century (Moritz et al., 2008). Specifically, although *TA* has experienced an upward range contraction of over 600 m, *TS* has undergone no apparent change in elevational distribution (Moritz et al., 2008). While *TA* has experienced a concordant decrease in overall genetic diversity but increase in among-population genetic differentiation, genetic structure in *TS* has remained stable (Rubidge et al., 2012; Bi et al., 2013). Collectively, these data suggest that *TA* and *TS* differ in their responses to environmental change.

As part of efforts to understand why patterns of elevational range change differ between *TA* and *TS*, we are exploring the role of GC physiology in mediating responses of free-living mammals to environmental conditions. The primary goals of this study are to validate the use of FCM as a measure of baseline GC response in *TA* and *TS* and to complete a preliminary assessment of interspecific differences in the stress physiology of these species. Using data from captive *TA* and *TS*, we report the results of an ACTH challenge experiment and describe circadian patterns of FCM. In addition, we characterize FCM responses of each species to both handling and trapping stressors. To relate these data to information from natural populations of chipmunks, we then compare baseline FCM levels for captive and free-living members of our study species. In addition to providing the first characterization of FCM in these chipmunk species, our data provide a critical foundation for exploring the role of GC physiology in mediating differences amongst mammalian responses to environmental change.

2. Materials and methods

2.1. Study animals and sites

Lodgepole chipmunks (*T. speciosus, TS*) and alpine chipmunks (*T. alpinus*, *TA*) were live-trapped in the vicinity of Saddlebag Lake (37.966251, −119.265185; WGS 84), Inyo National Forest, Mono Co., CA during August and September of 2012 and 2013. By these dates, the annual breeding season was over, young-of-the-year were weaned, and no females that we trapped were lactating. All animals were captured using Sherman traps baited with peanut butter and oats. Traps were opened at dawn, checked approximately every 4–6 h, and closed at dusk. Each captured animal was weighed, its sex was determined, and standard external measurements (e.g., body length, tail length) were taken. In addition, each animal was marked for permanent identification by placing a numbered metal ear tag (1005–1, National Band and Tag, Newport, KY, USA) in each ear pinna. Adults were conservatively defined as individuals with developed testes or conspicuous (post-lactation) nipples, while juveniles were defined as animals with no testicular development or visible nipples; only adult animals were included in this study.

A subset of the animals captured (N = 15–16 per species) was transported to the Sierra Nevada Aquatic Research Laboratory (SNARL) located near Mammoth Lakes, Mono Co., CA. At SNARL, members of both study species were housed in a vivarium maintained at 10–20 °C (ambient outside temperature for the area). Lighting in the room (14L:10D) imitated natural lighting at the latitude of Saddlebag Lake during the study period. Animals were housed in commercially purchased cages (35.6 × 27.9 × 38.1 cm; Prevue Hendryx) containing screen floors and removable drop pans that allowed for non-disruptive collection of fecal samples. Each cage was provided with a plastic nest box, cotton bedding material, and a water bottle. Animals were fed daily at 0630 with *ad libitum* quantities of commercial rodent chow, sunflower seeds, peanut butter, oats, apples, and other fruits.

Due to spatial constraints, no more than 18 animals could be housed in this facility at the same time. As a result, all experiments were conducted twice; the first replicate was completed during August 2012 and the second during September 2012 (except for the trapping challenge, which took place in August and September of 2013). Replicates were balanced with regard to the number of individuals of each species and sex included in each round of data collection. Husbandry procedures and experimental protocols (see below) were the same for both replicates. All animal procedures were approved by the University of California, Berkeley and University of California, Santa Barbara Animal Care and Use Committees and followed the guidelines of the American Society of Mammalogists for the use of wild mammals in research (Sikes and Gannon, 2011).

2.2. Sample collection and storage

In the field, fecal samples were collected from traps as soon as captured chipmunks were removed. Pellets from the same trap were placed in a cryogenic tube and then deposited in a −40 °C freezer at SNARL later the same day, within a 4–12 h period. All fecal pellets and other materials (e.g., left over bait) were removed from traps before they were reset.

In captivity, fecal samples were collected by removing the pan beneath each cage and manually transferring pellets to cryogenic tubes. Cage pans were lined with absorbent sheets to prevent extensive urine contamination and no urine-contaminated samples were collected. Tubes were then immediately frozen at −40 °C. Prior to initiation of challenge tests (see Section 2.4, below) feces were collected daily from captive individuals at 4 h intervals from approximately 0600–2200 to habituate the animals to the sample collection process. Cage pans were cleaned completely each time that samples were collected to ensure that fecal pellets were produced within a known, 4-h sampling period; pans were cleaned in a different area of the animal facility to reduce cleaning-induced stress to the study subjects. All experimental groups

had their cages cleaned on the same schedule and in the same manner and thus any impact of this procedure should have been the same for all study animals.

Because freezing field-collected samples in liquid nitrogen is a logistically challenging procedure in backcountry field settings where no freezer is available, we used the captive study population to contrast the efficacy of storing pellets in ethanol at ambient temperature versus freezing samples immediately after collection. For a subset of samples collected from the captive study animals (*TA*: 5F, 3M; *TS*: 4F, 3M), we compared FCM data from frozen versus ethanol-stored fecal pellets collected from the same individuals. Upon collection, each pellet provided was manually split in half to ensure that the two samples were comparable, in case of heterogeneous metabolite distribution across pellets within a sample. One half of each fecal pellet was frozen immediately at −40 °C while the other half was placed in 100% ethanol and stored at ambient temperature for 1 month before freezing at −40 °C. At the end of data collection at SNARL, all samples were transported on dry ice to the to the UC Berkeley campus, where they were stored at −80 °C until analysis.

2.3. Circadian patterns of FCM excretion

In rodents, GC production typically varies throughout each 24-h period, with plasma GC levels increasing shortly before an animal becomes active and decreasing as the animal approaches the resting phase of its circadian pattern of activity (Dickmeis, 2009; Lepschy et al. 2010). To determine whether our study species exhibit circadian fluctuations in FCM excretion, we collected fecal samples from the captive study animals (*TA*: 8F, 7M; *TS*: 8F, 8M) at approximately 4-h intervals for 28 h. Pellets were collected at least 5 days after capture and 5 days before any experimental manipulation of GC physiology, thereby allowing us to use these samples both to characterize circadian patterns of FCM excretion and to provide a within-subjects comparison for our ACTH challenge experiment (see Section 2.4, below). All samples were frozen at −40 °C immediately after collection and maintained at that temperature until transport to the Berkeley campus (see above, Section 2.2).

2.4. ACTH challenge

To validate our EIA procedure for quantifying GC levels and to confirm that elevated GC levels are captured by analyses of fecal samples, we performed an ACTH challenge experiment. At approximately 14:00 on day 10 after capture, individuals of both species (*TA*: 4F, 4M; *TS*: 4F, 4M) were injected intramuscularly with 12 IU/kg body mass of a synthetic form of ACTH (Cortrosyn: Amphastar Pharmaceuticals Inc., Rancho Cucamonga, CA) that had been reconstituted in 0.9% saline solution. While there is no single recommended dosage for all vertebrate species, similar dosages have been used in other ACTH challenge tests for small mammals (Touma et al., 2004; Woodruff et al., 2010). Total volume injected was approximately 0.06 mL for *TS* and 0.038 mL for *TA*.

In other rodent species, response times for ACTH challenge tests range from 5 to >24 h (Mateo and Cavigelli, 2005; Smith et al., 2012; Touma et al., 2004; Woodruff et al., 2010). Accordingly, we collected fecal samples from our study subjects every four hours for the first 36 h after injection, with additional collections at 48, 60, and 72 h post-injection. Samples were stored as described above (Section 2.3).

2.5. Handling challenge

To determine if the results of our ACTH challenge tests were influenced by changes in baseline FCM levels resulting from handling of captive animals, we conducted a parallel handling challenge study. Concordant with each replicate of the ACTH challenge study, a separate subset of individuals of each species (*TS:* 4F, 4M; *TA:* 3F, 4M) was subjected to a handling stressor, in which each animal was removed from its cage and injected with 0.9% saline solution; injection volumes and handling protocols were the same as those for ACTH-injected animals. Fecal samples collected for the study of circadian patterns of FCM excretion (see Section 2.3, above), provided a within subject, no-treatment comparison for both the ACTH and handling treatments. Assignment of individuals to ACTH or saline injection treatments was randomized and the order in which animals were handled and injected was rotated among treatments, sexes, and species.

2.6. Trapping challenge

To determine whether field-trapping protocols alter baseline FCM levels in our study species, captive individuals were subjected to a trapping stressor. During August and September 2013, *TA* (N = 2F, 5M) and *TS* (N = 4F, 3M) were live-trapped and transported to the SNARL vivarium; all capture, transport, and housing procedures were the same as those used in 2012. After the animals had been held in captivity for at least 5 days, fecal samples were collected every 4 h for a total of 3 days to provide a within-subjects control set of samples. On the fourth day, the animals were placed inside Sherman traps for 4 h, which represents the typical time that individuals are held in traps in the field. The animals were then returned to their home cages and fecal samples collected every 4 h for the following 3 days.

2.7. Comparisons of baseline FCM from free-living and captive animals

Because fecal samples were collected from the same individuals at first capture and again while they were housed at SNARL, we were able to use a within-subjects design to compare FCM levels for animals living under natural versus artificial conditions. Field measures of FCM levels were based on fecal samples collected from traps (see Section 2.2, above). Traps were checked approximately every 4–6 h; cages in the lab were checked every 4 h. Thus, samples from captive and free-living animals provided information regarding baseline FCM levels over comparable time periods. Because field samples were collected at a variety of times of day, we compared the field FCM level for each individual with the temporally most similar sample (i.e. collected at the same time of day) obtained from that individual in captivity during the circadian (pre-challenge) portion of the study (see Section 2.3, above).

2.8. FCM extraction and assay

To quantify FCM levels, we first extracted GC metabolites from fecal samples following the protocol of Palme et al. (2013). In brief, samples were dried at 90 °C for 4 h, after which they were crushed using a mortar and pestle. For samples that had been stored in ethanol, the ethanol was allowed to evaporate before samples were oven-dried. Only fecal samples with dry weights $\geqslant$0.02 g were extracted, with actual sample weights ranging from 0.02 to 0.07 g due to natural variability in fecal sample size. For extractions, an aliquot of 80% methanol (1 mL per 0.05 g feces) was added to each sample, the sample was shaken using a multi-vortex for twenty minutes and then centrifuged for twenty minutes. The resulting supernatant was dried in a vacuum centrifuge and shipped to Vienna, Austria, where extracts were reconstituted in 80% methanol, diluted in assay buffer and assayed using a 5α-pregnane-3β,11β,21-triol-20-one enzyme immunoassay first described for use with laboratory mice (Touma et al., 2003). This EIA measures GC metabolites with a 5α-3β,11β-diol structure. The same

EIA has proven suitable for measuring FCM in other sciurid species (Bosson et al., 2009, 2013; Dantzer et al., 2010, 2013; Montiglio et al., 2012); because our assays produced meaningful measures of FCM, no other antibodies were tested. Intra- and inter-assay coefficients of variation were 9.1% and 14.0%, respectively. We tested for parallelism in our assays by comparing binding curves of serially diluted (4 times 1:2.5 each) samples from both species and both sexes ($N = 4$ samples total) with the binding curve of serially diluted standards.

2.9. Statistical analyses

All statistical analyses were conducted in the program R 2.15.3 (R Development Core Team, 2008). Data were not transformed prior to analysis. For two-sample analyses, the data were tested for normality (Shapiro–Wilk test) and homoscedasticity (F-test); if these assumptions were not met, non-parametric statistical analyses were used. Welch's two sample t-tests and Wilcoxon rank sum tests were used to examine the effects of replicate and sex on FCM levels in each species. An ANCOVA was used to test for parallelism between the slope of the standard curve and the binding curve for samples from each species and sex.

Analyses of circadian patterns of FCM concentrations as well as temporal comparisons of FCM levels for ACTH, handling, and trapping challenge experiments were completed using general linear mixed-effect models (GLMM), implemented in the R package 'lme4' (version 1.1–6). For these analyses, FCM level was used as the response variable, with collection time and treatment (circadian pattern, ACTH, handling, trapping) as fixed effects and individual as a random effect. Significance tests were performed by comparing these models to null models that included only time and the random effect (circadian study: random effect only) using a log-likelihood ratio test (R function 'anova') as described by Rimbach et al. (2013). To corroborate the GLMM results and to examine treatment-specific FCM effects in greater detail, post hoc significance tests were used to compare specific time points that appeared to differ between treatment groups. Specifically, Welch's two-sample t-test was used if data met the associated assumptions of homoscedasticity and normality; Wilcoxon rank sum tests were used whenever these assumptions were not met. For analyses involving multiple comparisons, False Discovery Rate (FDR) adjustments (Benjamini and Hochberg, 1995) to p-values were made separately for each species. For comparisons of baseline (circadian) data versus data from ACTH and handling challenges, t-tests were used to compare FCM levels from samples collected at the same time of day.

Within-individual comparisons of mean FCM levels for field- versus vivarium-collected samples were completed using paired t-tests. Between-species comparisons of these data were completed using two-sample (unpaired) t-tests. Comparisons of frozen versus ethanol stored samples were conducted using one-sample t-tests in which the difference in FCM levels between the two portions of each sample were compared to an expected value of 0 (no difference between storage methods).

3. Results

In both study species, FCM binding curves for males and females were parallel to the standard curve for the assay (all $F < 0.51$, all $P > 0.49$, Fig. 1). Based upon comparisons of mean circadian (pre-challenge) hormone levels, we found no significant inter-sexual differences in FCM levels in either species (Welch two sample t-tests, *TA*: $t = 0.62$, df = 12.95, $P = 0.55$; *TS*: $t = -1.91$, df = 12.79, $P = 0.078$). In *TA*, males averaged 171 ng FCM/0.05 g fecal powder ($N = 7$, S.D. = 66) and females averaged 193 ng FCM/0.05 g fecal powder ($N = 8$, S.D. = 71). Male *TS* averaged 136 ng FCM/0.05 g fecal powder ($N = 8$, S.D. = 60) and females averaged 84 ng FCM/0.05 g fecal powder ($N = 7$, S.D. = 45). While there does appear to be some effect of sex on baseline captive FCM levels in captive *TS*, the difference between the sexes was not significant and we do not have enough data here to make any strong conclusions. Because the distribution of males and females was balanced across experimental replicates and because all experimental manipulations employed a within-subjects design, we pooled data from males and females of each species for subsequent analyses. When data from both sexes were combined, we found no significant differences in FCM levels between the first and second replicates of our study for any of the variables examined (circadian, handling, ACTH, and trapping challenges; two-sample t-tests, all $P > 0.08$), and thus data were from both replicates were pooled for subsequent analyses.

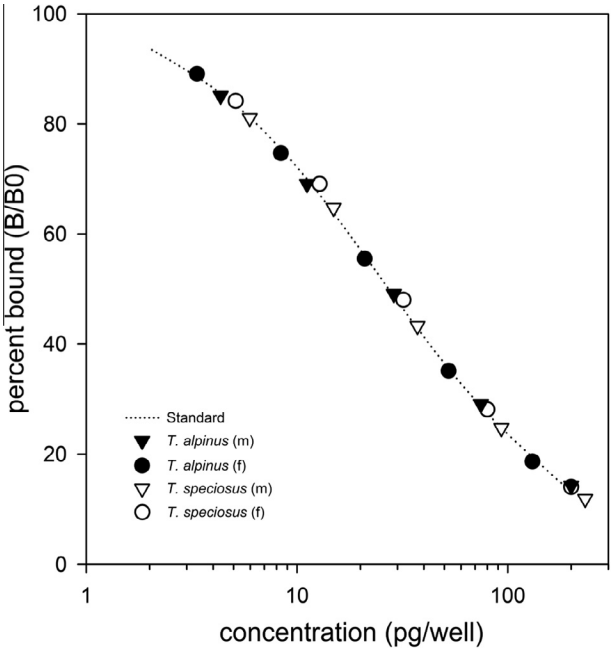

Fig. 1. Parallelism between FCM levels obtained from fecal extracts versus a serially diluted standard. Fecal extracts were assayed for one male (triangles) and one female (circles) each for both *T. alpinus* (filled symbols) and *T. speciosus* (open symbols). Slopes for FCM binding curves were not significantly different from the standard curve ($P > 0.49$).

3.1. Comparison of storage methods

The effects of storage method on baseline FCM levels differed between the study species. In *TA*, ethanol storage produced significantly higher GC metabolite levels than immediate freezing of samples (one sample t-test: $T = 3.24$, $N = 5$F, 3M, $\mu = 0$, $P = 0.01$; Fig. 2). In *TS*, however, no significant difference was found between ethanol preserved and frozen samples (one sample t-test: $T = -0.80$, $\mu = 0$, $N = 4$F, 3M, $P = 0.46$; Fig. 2). Because all samples used in the remaining analyses reported here were frozen after collection, this apparent interspecific difference in response to fecal pellet preservation in ethanol did not impact the analyses of FCM levels reported below.

3.2. Circadian patterns of FCM excretion

Because not all individuals provided fecal pellets during each collection period, sample sizes varied somewhat across collection time points for the circadian study (Fig. 3). For *TA*, a full model

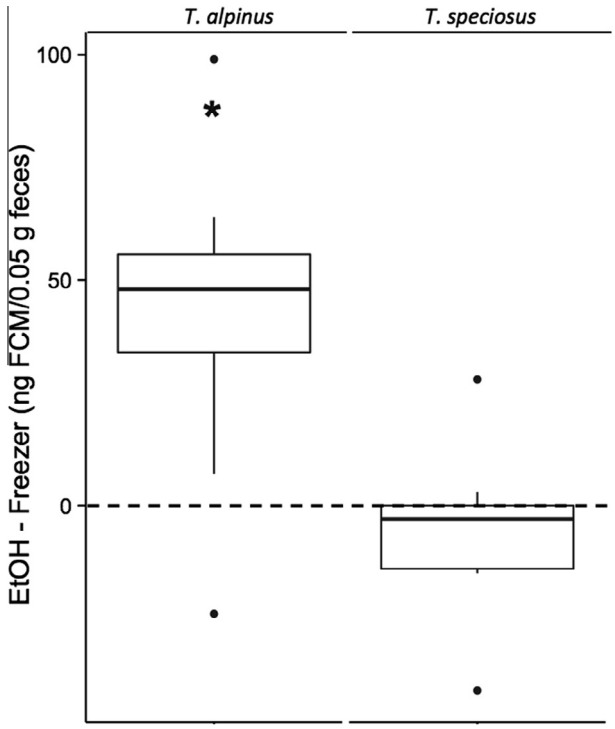

Fig. 2. Comparison of baseline FCM levels for ethanol-stored versus frozen samples. Data are from 8 *TA* and 7 *TS*. For each individual, fecal samples collected in captivity were divided in half; one half was stored in 70% ethanol and the other was frozen at −80 °C immediately after collection. The difference in baseline FCM levels (EtOH – frozen) was calculated for each animal and these values compared to an expected difference of 0 (dotted line). The difference between observed values and 0 was significant for *TA* ($P = 0.01$) but not for *TS* ($P = 0.46$); statistical details are provided in the text.

containing time as a fixed factor and individual as a random factor outperformed a null model containing the random effect alone ($\chi^2 = 8.52$, $P = 0.0035$), suggesting that time-of-day had some effect on FCM levels in this species. Visual inspection of these data (Fig. 3) indicated that FCM levels tended to be highest in the late morning (~11:00) and early afternoon (~15:00) and lower during the rest of the day. In contrast, for *TS* the full model did not significantly outperform the null model, although visual inspection of these data suggested that FCM production tended to be highest during the middle of the day, during the period when FCM production was lowest for *TA* (Fig. 3; $\chi^2 = 3.54$, $P = 0.06$). It is possible that the lack of a strong circadian pattern for either species was due to the 4-h inter-collection period, which could have masked the assay's ability to detect a more obvious rhythm. For both species, circadian samples were used as a baseline against which to compare the results of ACTH and handling challenges. While these samples do not represent a truly un-manipulated baseline because they were obtained after animals were transferred to captivity, they provide a reasonable basis for comparison with samples collected after more targeted manipulations such as our ACTH and handling challenges (see below). In all cases, comparisons were restricted to fecal pellets collected at the same time of day, thereby avoiding potential confounds resulting from any circadian variation in FCM production in either study species.

3.3. Response to ACTH and handling challenges

Injection with ACTH had a significant effect on FCM levels in both species (Fig. 3). Because not all individuals provided fecal pellets at each time point, sample sizes varied across sampling intervals. Due to limited numbers of pellets, only the following time points were assayed for each study species: −24 h (pre-injection), 0 h (injection), and 4, 16, 20, 24, 28 and 48 h post-injection. For both species, the full model (individual, time, experimental treat-

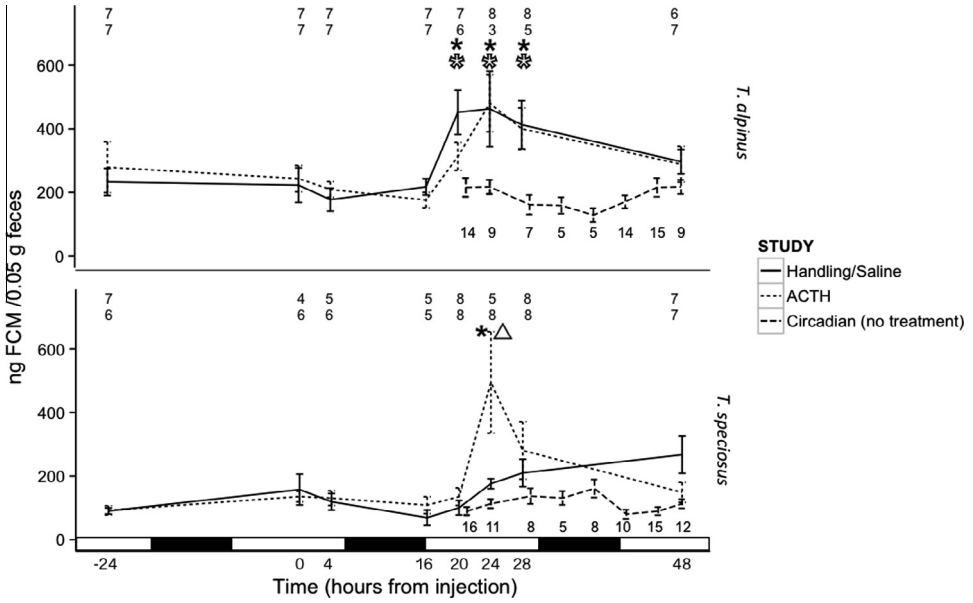

Fig. 3. Comparisons of mean (±1 SE) baseline FCM levels for circadian (no treatment, pre-stressor) samples (dashed line) and samples collected post-challenge with ACTH (dotted line) or handling (solid line). Sample sizes for each time point are indicated on the plot in the following order: ACTH, handling/saline (above curves) and circadian (below curves). Black and white bars along the bottom of the figure indicate the dark/light cycle during sample collection. Time = 0 h occurred at approximately 14:00, the time of injection with ACTH or time of handling treatment. Circadian (no-treatment) samples were collected from 15–16 animals per species beginning 5 days prior to challenge and served to establish unmanipulated circadian patterns of FCM production; ACTH and handling challenges were each conducted on a subset of 7–8 of these individuals. For purposes of visual contrast, the circadian data are presented starting at hour +20, with the time (hour) of sample collection matched to the times at which post-challenge samples were collected. Asterisks denote significant contrasts between circadian (pre-challenge) and ACTH (solid symbol) or handling (open symbol) FCM levels, or between ACTH and handling/saline (triangles).

ment) outperformed the null model containing only individual and time (*TA*: $\chi^2 = 39.45$, $P = 5.6e{-}8$, *TS*: $\chi^2 = 29.70$, $P = 5.6e{-}6$), indicating that some time points differed between experimental treatments. Visual inspection of the data revealed that FCM response to ACTH appeared more quickly (at +20 versus +24 h) and lasted longer (+20 through +28 h versus +24 h only) in *TA* than in *TS*. Averaged across individuals, peak FCM level occurred at +21.5 h for *TA* and at +28.5 h for *TS*. Post-hoc comparisons of specific time points revealed that for *TA*, ACTH-injected and circadian baseline FCM values differed significantly at +20, +24, and +28 h (Fig. 3, Table 1). For *TS*, ACTH-injected and circadian baseline FCM values were significantly different only at +24 h, although this difference disappeared when the associated *p*-value was corrected for multiple comparisons (Fig. 3, Table 1).

In response to handling, FCM levels for *TA* were significantly greater than circadian (pre-challenge baseline) levels at +20, +24, and +28 h (Fig. 3, Table 1). Results of the ACTH challenge were similar, with FCM levels for ACTH-challenged animals differing significantly from circadian baseline values at +24 and +28 h (Fig. 3, Table 1). In contrast, post-handling FCM levels for *TS* were never significantly higher than circadian (pre-challenge baseline) levels; although post-handling FCM levels in this species were significantly lower than ACTH-injected levels at +24 h, this difference disappeared when the associated *p*-value was corrected for multiple comparisons (Fig. 3, Table 1). Thus, while both species responded to injection with ACTH, only *TA* displayed a significant response to handling.

3.4. Response to trapping challenge

Neither study species displayed a significant response to our trapping challenge. Based on the timeline for response to injection with ACTH, we assayed samples collected −72, −48, −44, −20, 0, +24, +28, and +52 h from the start of the trapping challenge to provide a comprehensive series of pre- and post-challenge FCM values for each individual. A model including individual, time, and treatment did not significantly outperform the null (individual and time) model for either study species (*TA*: $\chi^2 = 0.028$, $P = 0.87$, *TS*: $\chi^2 = 2.78$, $P = 0.095$), suggesting that trapping did not have a significant effect on FCMs in captive animals. Further, no significant differences were found for paired *t*-tests between pre-challenge and post-challenge FCM levels at any sample time point (all $P > 0.16$ for unadjusted values, all $P > 0.61$ for FDR adjusted values).

3.5. Comparisons of FCM in captive and free-living animals

Within-individual comparisons of time-matched samples collected in captive versus field settings (mean difference between captive and field times of day = 96 min, range = 0–300 min, paired $N = 15$ for *TA*, 15 for *TS*) revealed that FCM levels for *TA* but not for *TS* were significantly higher in captivity (paired *t*-tests, *TA*: $T = 3.28$, $N = 15$, $P = 0.0054$; *TS*: $T = 1.46$, $N = 15$, $P = 0.17$, Fig. 4). Among captive animals, mean baseline FCM levels were significantly higher in *TA* than in *TS* (Welch's two-sample *t*-test: $T = 3.08$, $N = 15$, 15, $P < 0.005$, Fig. 4). In the wild, however, there was no significant difference between mean baseline FCM levels for the study species (same individuals as those sampled in captivity; Welch's two-sample *t*-test: $T = 1.38$, $N = 15$, 15, $P = 0.18$, Fig. 4). Thus, while captivity produced a significant increase in baseline FCM levels in *TA*, the same response to captive conditions was not detected for *TS*.

4. Discussion

Our analyses indicate that the EIA procedure employed was able to detect experimentally induced changes in fecal glucocorticoid metabolites in alpine and lodgepole chipmunks. Specifically, injection with synthetic ACTH induced significant increases in FCM levels in both study species beginning around 24 h after administration of this physiological challenge. With regard to the other challenges employed, *TA* exhibited a more pronounced response than *TS* to both handling and captivity. While multiple factors may have contributed to this difference in outcomes, one general interpretation of this finding is that *TA* possesses a more responsive hypothalamic–pituitary–adrenal axis (HPA, the neuroendocrine pathway that regulates production of GC hormones). Future studies that examine the FCM responses of these species to a greater variety of challenges as well as studies that explore HPA function in these animals in greater detail will be valuable in revealing the extent to which the findings reported here reflect more generalized differences in endocrine response between *TA* and *TS*.

4.1. Interspecific comparisons of FCM responses

The two study species displayed several intriguing differences in FCM response to the challenges administered here. First, ethanol storage resulted in significantly higher FCM levels than freezer storage for *TA* but not for *TS*. While this difference did not impact

Table 1
Comparisons of post-challenge FCM levels. Data are from pharmacological and handling challenge experiments conducted on alpine and lodgepole chipmunks; data from both challenges were compared to pre-challenge (circadian) data from the same individuals. Based on visual inspection of FGM levels (Fig. 2), post hoc statistical comparisons were conducted for data collected +20, +24, and +28 h post-challenge to test for differences between the these treatments. For each comparison, the name of the statistical test employed is given, as is the test statistic and both unadjusted and adjusted (false-discovery rate) *p*-values; significant contrasts are indicated in bold.

Species	Time point	ACTH versus Circadian	Handling versus Circadian	ACTH versus Handling
Tamias alpinus	+20 h	Welch, $t = 3.2428$, df = 8.597, ***P* = 0.011, FDR *P* = 0.020**	Wilcoxon, $W = 78$, ***P* = 0.0016, FDR *P* = 0.0072**	Welch, $t = 1.6837$, df = 8.594, $P = 0.13$, FDR $P = 0.17$
	+24 h	Wilcoxon, $W = 68$, ***P* = 0.00099, FDR *P* = 0.0072**	Wilcoxon, $W = 27$, ***P* = 0.0091, FDR *P* = 0.020**	Welch, $t = -0.117$, df = 4.488, $P = 0.91$, FDR $P = 0.91$
	+28 h	Welch, $t = 3.2906$, df = 9.89, ***P* = 0.0083, FDR *P* = 0.02**	Welch, $t = 3.0684$, df = 5.343, ***P* = 0.026, FDR *P* = 0.039**	Welch, $t = 0.1273$, df = 9.259, $P = 0.90$, FDR $P = 0.91$
Tamias speciosus	+20 h	Wilcoxon, $W = 82$, $P = 0.29$, FDR $P = 0.57$	Wilcoxon, $W = 63$, $P = 0.98$, FDR $P = 0.98$	Welch, $t = -0.971$, df = 13.5, $P = 0.35$, FDR $P = 0.57$
	+24 h	Wilcoxon, $W = 48$, ***P* = 0.019**, FDR $P = 0.17$	Welch, $t = 0.566$, df = 15.473, $P = 0.58$, FDR $P = 0.65$	Wilcoxon, W = 6, ***P* = 0.045**, FDR $P = 0.20$
	+28 h	Wilcoxon, $W = 45$, $P = 0.20$, FDR $P = 0.57$	Wilcoxon, $W = 41$, $P = 0.38$, FDR $P = 0.57$	Wilcoxon, W = 25, $P = 0.51$, FDR $P = 0.65$

Welch = Welch two sample *t*-test for parametric comparisons.
Wilcoxon = Wilcoxon rank-sum test for non-parametric comparisons.

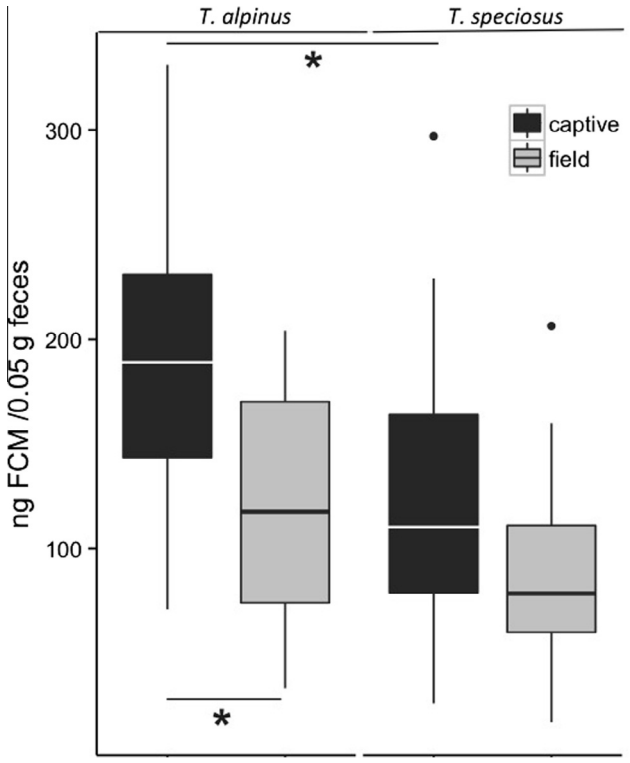

Fig. 4. Comparisons of baseline FCM levels for captive versus free-living chipmunks. Data are from 15 (8F, 7M) *TA* and 15 (7F, 8M) *TS* captured in Inyo County, CA and subsequently housed in captivity. Baseline FCM levels were measured upon capture and again after at least 5 days in captivity. Significant contrasts ($P < 0.006$) within and between species are indicated; details of the statistical analyses are provided in the text.

our subsequent analyses, this outcome should be considered as part of future field studies that require remote storage of fecal samples for endocrine assays. Second, while *TA* exhibited a significant increase in FCM levels after handling, a similar response was not detected for *TS*. It is intriguing that our handling stressor produced as marked an increase in FCM levels in *TA* as our pharmacological (ACTH) challenge; in most species, saline-injection with handling results in lower overall increases of FCMs in comparison to ACTH challenge (Chelini et al., 2010; Sheriff et al., 2009; Touma et al., 2004; Woodruff et al., 2010). Additionally, the response to ACTH challenge in *TA* was more rapid and more enduring, with this species exhibiting significantly higher ACTH-induced FCM levels earlier (at +20 versus +24 h) and for a longer period (at +20, +24, +28 versus only +24 h) than *TS*. Finally, although baseline FCM levels for free-living animals did not differ between the study species, levels for captive individuals were significantly higher for *TA*. Relatedly, only *TA* displayed a significant difference in baseline FCM levels between the field and captivity. These findings suggest that *TA* may be more responsive to some forms of seemingly diverse environmental challenges.

Several factors may have contributed to the apparent interspecific differences in FCM response reported here. For example, the sensitivity of the HPA axis may vary among species such that the same stimulus produces markedly different levels of response in different taxa. This includes potential interspecific variation in perception and evaluation of potential stressors (Malmkvist et al., 2011). At the same time, the suite of environmental conditions – the fundamental niche (Grinnell, 1914) – to which *TA* is adapted may be more limited; *TA* is thought to be more of a habitat specialist (Best et al., 1994; Clawson et al., 1994), with the result that homeostasis in this species may be more easily perturbed than in *TS*. These potential explanations are not mutually exclusive and it seems likely that the interspecific differences in FCM responses reported here reflect a combination of causal factors.

In addition to these differences in FCM response, the study species also shared a number of important elements of GC physiology. For example, in response to ACTH challenge, both species' FCM levels peaked at approximately 24 h after injection. Across sciurids, there appears to be marked interspecific variation in the timing of this peak, with some species exhibiting significantly higher FCM only 8–12 h after injection (Bosson et al., 2009; Montiglio et al., 2012; Sheriff et al., 2012) but others requiring over 24 h to exhibit a strong response (Bosson et al., 2013; Mateo and Cavigelli, 2005; Smith et al., 2012). Such differences are generally attributed to interspecific variation in gut passage times and diet (Palme, 2005), although the specific assay methodology employed may also play a role.

Neither study species exhibited significant sex-dependent differences in FCM levels, although such differences have been reported for other chipmunk species (Montiglio et al., 2012). It is possible that our relatively small sample sizes precluded the ability to detect differences in FCM levels between males and females. Alternatively, differential effects of captivity and our experimental challenges may have served to mask typical inter-sexual differences in FCM in these animals. Sample collection during this study occurred after the end of the annual breeding season and it is possible that stronger sex differences in FCM levels would have been detected if samples had been obtained during the portion of the year when individuals are reproductively active, as has been found for other species of sciurids (Dantzer et al., 2010; Kenagy and Place, 2000).

Somewhat surprisingly, neither study species displayed a significant response to our trapping stressor. It is possible that our challenge protocol was not appropriate for eliciting a strong GC response, particularly given that in *TA*, individuals were already exhibiting increased FCM levels as a result of being housed in captivity. Trapping-induced changes in GC levels in other small mammals have typically been measured using blood plasma samples (Bosson et al., 2012; Fletcher and Boonstra, 2006), which are likely better suited to detecting acute (i.e., rapid, short-term) changes in GC levels initiated by experiences such as our trapping challenge (Sheriff et al., 2011; but see Bosson et al., 2013). In contrast, although injection with ACTH likely also represents an acute challenge, the extreme nature of this pharmacological stimulus may make it detectable in FCM despite the more extended timeline over which GC metabolites accumulate in fecal samples. Thus, additional analyses targeting acute rather than more baseline FCM responses may be required to determine whether trapping fails to elicit a GC response in our study species or the more likely possibility that this response was simply not detectable using FCM analysis in captivity.

4.2. Implications for response to environmental change

The variation in baseline FCM levels reported here has potentially important implications for exploring the impacts of environmental change. Baseline GC levels may be useful as indicators of environmental challenges for several reasons (Wingfield, 2013a). Within species, differences in baseline FCM levels may provide insights into the relative severity of the environmental challenges to which individuals or populations are exposed. For example, some species undergoing elevational range shifts have exhibited varying GC responses across their distributions, with expanding populations characterized by higher baseline and acutely stressed GCs (Addis et al., 2011; Liebl and Martin, 2012) and enhanced GC receptor frequencies (Liebl and Martin, 2013). Applying the same logic, in species undergoing range contraction it is possible that

trailing-edge populations may exhibit elevated GCs; to the best of our knowledge, this aspect of range contraction has not been studied.

More generally, assays of samples collected throughout a species' distribution may reveal populations that are subject to challenges substantive enough to elicit a physiological response (Busch et al., 2011; Sheriff et al., 2012). Across species, differences in GC sensitivity may be useful in predicting which taxa are most likely to be challenged by changes in environmental conditions; for example, if increased GC sensitivity is associated with a reduced tolerance for change, then species with more responsive GC physiologies may be particularly vulnerable to anthropogenic or other sources of environmental modification (Jessop et al., 2013; Wikelski and Cooke, 2006; Wingfield, 2013a). In sum, because GCs are integrally involved in homeo- and allostasis and in moderating trade-offs between survival and reproduction (Angelier and Wingfield, 2013; Boonstra, 2004; Busch and Hayward, 2009; Wingfield, 2013a), they should provide valuable indicators of response to environmental change.

Given the complexity of the GC response, use of this physiological system as an indicator of environmental change requires basic knowledge of a species' neuroendocrine response to challenge. Pharmacological validation studies provide critical information regarding the ability of fecal GC levels to capture information regarding response to external challenges (Touma and Palme, 2005). As demonstrated here, the addition of biologically relevant challenges (e.g., trapping, handling) as well as comparisons of captive-housed and field-caught individuals can generate further insights into relationships between environmental conditions and physiological responses. Critical next steps include experimental quantification of the relative sensitivity of the GC response in our study species as well as sampling along an elevational transect of free-living chipmunk populations to determine if baseline GCs vary systematically with environmental conditions. Coupled with environmental data from localities occupied by *TA* and *TS*, this information will allow us to explore potential biotic and abiotic correlates of intraspecific variation in baseline FCM levels. At the same time, analyses that examine patterns of glucocorticoid and mineralocorticoid receptor activity may be important in further quantifying interspecific differences in response; these receptors – both of which mediate GC responses – have been found to differ in other species experiencing range changes (Addis et al., 2011; Liebl and Martin, 2012, 2013). Because our study species are closely allied phylogenetically and geographically yet exhibit clearly divergent responses to habitat conditions over the past century (Moritz et al., 2008; Bi et al., 2013; Rubidge et al., 2012), we believe that these animals provide a particularly important system within which to explore the potential for baseline GC responses to serve as indicators of response to environmental change.

5. Conclusions

This study documents marked differences in baseline GC physiology between two species of chipmunks, thereby emphasizing the importance of species-specific validation studies. Although congeneric and partially sympatric in the central Sierra Nevada, our analyses indicate that *TA* and *TS* respond differently to several external challenges, including pharmacological administration of ACTH, captive housing conditions, and handling by humans. Specifically, *TA* appears to be more responsive to these challenges than *TS*, a finding that may have significant implications for understanding documented differences in range change in these species over the last century. We believe that this study confirms the importance of conducting species-specific validation studies of FCM and outlines the potential for using GC physiology to monitor responses to environmental change. Future analyses of these species will explore these themes in greater detail, with the intent of improving our understanding of the role of stress hormones in mediating response to evolutionary as well as anthropogenic change.

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