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Fecal glucocorticoid metabolites in wild yellow-bellied marmots: Experimental validation, individual differences and ecological correlates

Jennifer E. Smith [a,b,c,*], Raquel Monclús [a,c], Danielle Wantuck [d], Gregory L. Florant [d], Daniel T. Blumstein [a,c]

[a] Department of Ecology & Evolutionary Biology, University of California, 621 Charles E. Young Drive South, Los Angeles, CA 90095-1606, USA
[b] The UCLA Institute for Society and Genetics, Box 957221, 1323 Rolfe Hall, Los Angeles, CA 90095-7221, USA
[c] The Rocky Mountain Biological Laboratory, Box 516, Crested Butte, CO 81224, USA
[d] Department of Biology, Colorado State University, Fort Collins, CO 80523, USA

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ABSTRACT

Natural selection is expected to shape phenotypic traits that permit organisms to respond appropriately to the environments in which they live. One important mechanism by which animals cope with changes in their environment is through physiological responses to stressors mediated by glucocorticoid hormones. Here we perform biological and physiological validations of a minimally-invasive technique for assessing fecal corticosterone metabolites (FCMs) in captive and wild groups of yellow-bellied marmots (*Marmota flaviventris*). Then we draw from ten years of data on these obligate hibernators at the Rocky Mountain Biological Laboratory in Colorado, USA to assess the extent to which seasonal and daily changes explain naturalistic variation in baseline levels of FCMs. Interestingly, beyond important population-level variation with respect to year, season, time of day, sex, age and reproductive state, we found repeatable inter-individual differences in FCMs, suggesting this hormonal trait might be a meaningful target of selection. FCM levels were 68% lower in captive than wild marmots, suggesting that the natural environment in which these animals occur is generally more challenging or less predictable than life in captivity. Most live-trapping events failed to represent stressors for wild marmots such that repeated measurements of traits were possible with minimal "stress" to subjects. We also document the natural ranges of annual and seasonal variation necessary for understanding the extent to which anthropogenic assaults represent stressors for wild mammals. Taken together, this study provides a foundation for understanding the evolution of hormonal traits and has important welfare and conservation implications for field biologists.

1. Introduction

Discovering the factors responsible for maintaining phenotypic diversity remains one of the most fundamental, yet unresolved, puzzles in evolutionary biology [33]. Whereas natural selection is expected to shape phenotypic traits that permit organisms to respond appropriately to the environments in which they live, conspecifics of same age and sex class often vary systematically from each other in their phenotypic responses to the same conditions [55]. Yet, until recently, these inter-individual differences were largely viewed as mere variation around a mean rather than of biological importance [81].

One important mechanism by which individual animals cope with changes in their environment is through physiological responses to stressors [42,82]. Identifying the occurrence of inter-individual differences in physiological responses to stressors therefore offers exciting opportunities for endocrinologists to explain naturally-occurring phenotypic variation in light of developing theoretical and conceptual frameworks [62,80]. Perception of a stressful situation activates the sympathetico-adrenomedullary system and the hypothalamic pituitary adrenocortical (HPA) axis [47,77]. Activation of the HPA axis stimulates the release of steroid hormones known as glucocorticoids (GCs, e.g., corticosterone, cortisol). GCs in turn activate the mobilization of energy necessary for the "fight or flight" response [24,60,77]. Chronic stressors, defined here as long-term, continuous stressors, are the result of repeated exposure to acute stressors; an acute stressor in contrast represents a relatively brief exposure to a single stressor [43]. Whereas GCs often promote adaptive responses to acute stressors, chronic stressors such as those attributed to anthropogenic disturbance, biological invasions, and climate change, however, can negatively affect populations of free-living vertebrates [21,32,59]. For example, persistent stressors can negatively impact behavior, reproduction, immune function, and growth through prolonged activation of the HPA axis ([17,54,56,66]). Thus, understanding such effects and establishing baseline measures of GCs for wild animals also

* Corresponding author. Address: Biology Department, Mills College, Oakland, CA 94613, USA. Fax: +1 510 430 3304.
E-mail address: jesmith@mills.edu (J.E. Smith).

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has potential implications for conservation biology. Moreover, because housing conditions may represent chronic stressors, understanding the effects of captivity on stress physiology is also an important animal welfare issue [7,46,70] and necessary for the meaningful integration of results from captive and field studies [22].

Biologists have come to recognize non-invasive measurements of GCs as valuable indicators of stressors encountered in laboratory, domestic, zoo and wild animals [44,63,71]. Fecal glucocorticoid metabolites are particularly useful measures of GCs because feces are easy to obtain through minimally-invasive and non-invasive sampling in animal populations [64]. This is important because handling necessary for collection of plasma can confound estimates of baseline GC measures [74]. For example, just three minutes of restraint can sufficiently elevate levels of plasma corticosterone from baseline in rodents [38]. In contrast, fecal metabolites of GCs offer excellent indicators of stressors experienced from several hours to several days because they represent an accumulated unbound fraction of GCs [53,74].

Yellow-bellied marmots (*Marmota flaviventris*), large (2–5 kg) ground-dwelling rodents of the squirrel family (Sciuridae), offer interesting opportunities for understanding naturally-occurring variation in GCs. This species is an obligate hibernator and subject to major seasonal shifts in food availability and weather conditions [6,31,73]. These animals are also exquisitely sensitive to stressors (e.g., predation risk); they modify their foraging behavior and reproductive investment in response to predator pressure [11,45]. Whereas most studies focus on the influence of social stressors on GCs (e.g., [19,23]), neither group size nor social network traits explain GCs in this species [45,79]. Moreover, although individual marmots exhibit consistent behavioral phenotypes (e.g., aggressive, social, exploratory dimensions), social factors are insufficient to explain inter-individual differences in GCs [2,69]. Yellow-bellied marmots are therefore particularly well-suited for investigations of the non-social correlates of GCs in wild mammals. A major goal of this current study is to understand the extent to which environmental variation and inter-individual differences explain GCs in these animals.

Here our overarching goals are to: (1) understand the extent to which seasonal and daily changes explain naturalistic variation in baseline levels of fecal GC metabolites and (2) investigate the potential for inter-individual differences in the response to stressors beyond those predicted by age, sex and reproductive state in wild yellow-bellied marmots. We capitalize on data from a long-term study at the Rocky Mountain Biological Laboratory (RMBL) in Colorado, USA for which repeated measures of GCs from individually-marked marmots of known ages, sexes, and reproductive states are available [5]. Given that marmots are obligate hibernators with limited access to food and cover and compete for mating opportunities early in the season, we expected a decrease in GC levels across the active season as marmots approached hibernation. We also predicted GCs to vary within days as these diurnal animals shift their daily patterns of activity [1]. We predicted that males should have higher GC values than females because male mammals tend to excrete more GCs through feces than do females [79]. We also expected GCs to vary with the reproductive state of adult females [76]. Finally, if this species possesses consistent inter-individual responses to stressors, then we expected to detect repeatable values of GCs for the same individuals across years after accounting for annual variation in GCs.

Because the proportion and structure of metabolized GCs differ among species, testing these predictions first requires a species-specific analytical, biological, and physiological validation of an immunoassay in yellow-bellied marmots [53,74]. We previously documented good parallelism, sensitivity and recovery of GCs from feces (specifically, fecal corticosterone metabolites (FCMs) in this species [14]) as well as low intra- and inter-assay variation of a double-antibody ^{125}I radioimmunoassay (RIA) kit (MP Biomedicals, Costa Mesa, CA). Here our goal is, for the first time, to perform biological and physiological validations necessary to extend our previous analytical validations of this assay. If this assay is valid, then we expected to detect a surge in FCM levels in response to handling and pharmaceutical manipulation after a species-specific time delay.

2. Methods

2.1. Husbandry of captive marmots for laboratory validation experiments

We used six captive, non-breeding adult marmots ($n_{male} = 1$, $n_{females} = 5$) for the validation experiments. All marmots were born in captivity and housed individually at Colorado State University. Food and water were provided *ad libitum*. Prior to the start of our current experiment, we placed animals in a cold room (-5 ± 2 °C) and complete darkness to simulate hibernation conditions from October to March [71]. Annually, from April to October, subjects were kept under natural photoperiod at room temperature (15–20 °C) to simulate the conditions of their active season in the wild. In 2011, we conducted the experiments reported here from 23 April to 1 May, a period after which marmots "emerged" from hibernation. All of the subjects used in these experiments were regularly digesting food and fully acclimated to a photoperiod resembling the active season of marmots in the wild [52]. Housing conditions and protocols complied with the policies of Colorado State University Institutional Animal Care and Use Committee (IACUC #11–2475A, approved 22 February 2011).

2.2. Adrenocortical activity in captive marmots

To assess adrenocortical activity in captive marmots, we compared baseline FCM levels to those measured after behavioral and physiological challenges. We collected feces every four hours (0800, 1200, 1600, and 2000 h) during times of day when marmots are typically active [1]. Following a 3-day habituation period, during which feces were cleared from the enclosures but not collected for analysis, we started our experiment. Whereas some experiments on wild-caught animals rely upon habituation periods of up to 4 days (e.g., [65]), 3 days was a habituation period of sufficient length given that all of our subjects were born in captivity and therefore familiar with their housing conditions.

After this 3-day habituation period was complete, we collected feces at each of these four sampling times for a total of eight days, starting on day 1 of the 8-day experiment (Fig. 1). On day 2, we conducted a "handling stress" experiment (Fig. 1A). Handling on day 2 started at 0800 h and lasted for five minutes. Each marmot was weighed, sexed, checked for reproductive status, and placed in a cloth bag to mimic handling procedures used in the live-capture of wild marmots. On day 7 (Fig. 1B), we performed an "adrenocorticotropic hormone (ACTH) challenge" test at 0800 h [74,77]. ACTH is a peptide hormone that is synthesized and released from the pituitary gland, and triggers the production of a pulse of elevated serum GCs. The same person administered an intramuscular injection of a high mass-specific dose (4.0 IU/kg, [16]) of synthetic ACTH (Synacthen® Depot, [29]) to each of the six subjects. Because marmots have slow-gut passage times [36], we expected to measure a peak in FCM roughly 6–24 h following the challenge test, the typical lag time between secretion of hormones into blood and fecal excretion for rodents ([8,18,26,34,65,67,75,76,83]). To be conservative, we collected feces every 4 h after the ACTH challenge period for a total of 36 h.

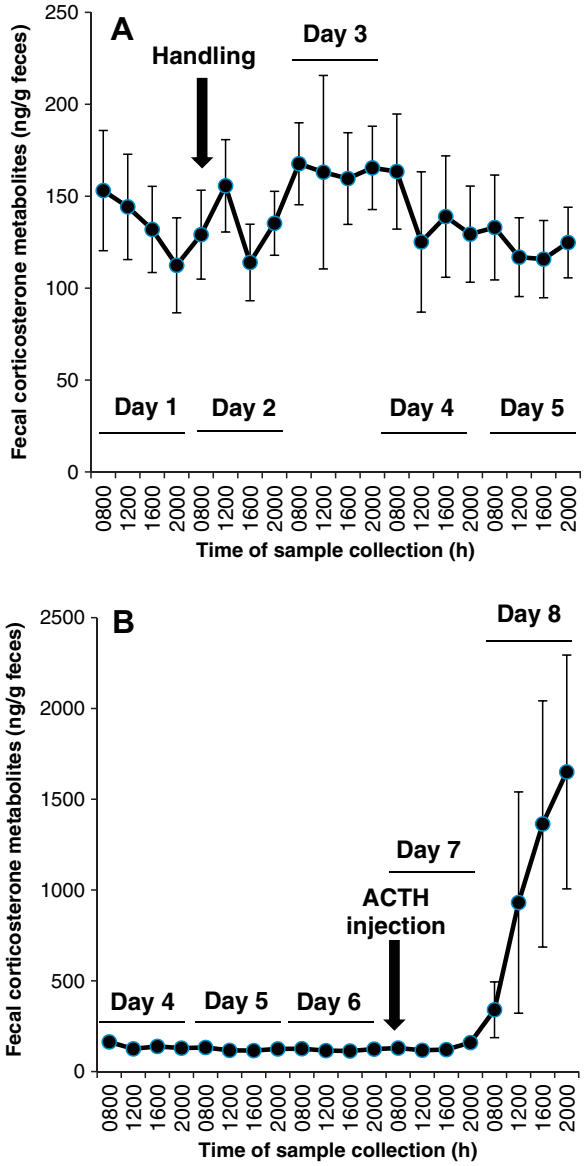


Fig. 1. Concentrations of fecal corticosterone metabolites (Mean ± S.E.) from captive yellow-bellied marmots ($n = 6$) before and after exposure to (A) biological and (B) physiological challenges. Response to treatments were measured on days 3 and 8, respectively. Arrows indicate (A) handling (0800 h on day 2) and (B) ACTH (0800 h on day 7) treatments. Baseline measurements included samples collected on day 1 (prior to handling) and days 4, 5, and 6 (prior to the ACTH challenge).

Feces collected on days 1, 4, 5 and 6 served as the "baseline period" from which FCMs were compared to those collected 24 h after the "handing" and "ACTH" challenges. Feces collected on day 3 reflect the "handling" and those collected on day 8 reflect the "ACTH challenge," administered on days 2 and 7, respectively (Fig. 1). All feces were immediately stored at −80 °C until extraction. Samples were shipped on dry ice to the Blumstein Lab at the University of California Los Angeles (UCLA) for hormone extraction. Samples from the captive group were assayed with those feces collected from the wild population in 2011.

2.3. Field site and wild population of marmots

From 2002 to 2011, we studied wild marmots around the Rocky Mountain Biological Laboratory (RMBL), located in the East River Valley, in Gunnison County, Colorado, USA [5]. Marmots have been studied at this location for 50 years. We used spotting scopes to intensively observe marmots primarily at ten main colonies. We did this during the times of peak activity (0700 to 1000 h; 1600 to 1900 h) on most days from mid-April to mid-September. We used observational data to determine dates of emergence from hibernation for yearlings (1 year old) and adults (⩾2 years old) as well as the date when the pups emerged from their natal burrows.

All of the animals were live-trapped in Tomahawk traps set at burrow entrances bi-weekly during the active season (May to August). Traps were checked within 2 h of setting them. Immediately upon our arrival at each trap, we recorded the behavioral state of each captured marmot based on whether an individual marmot struggled in the trap, alarm-called and/or bit at the trap. Upon capture, we transferred each marmot into a canvas handling bag to weigh and sex it. We also recorded each subject's reproductive status (females: pregnant, lactating, non-reproductive; males: scrotal or abdominal), and if necessary, remarked it. All marmots were marked with ear tags and Nyanzol cattle dye at first capture (for details, see [1]). We collected feces in plastic bags and immediately placed these on wet ice after our arrival at each trap (⩽2 h after defecation). Samples were frozen at −20 °C within 2 h of collection. Each August, samples were shipped on dry ice to UCLA for extraction.

We determined whether adults successfully reproduced each year based on DNA parentage assignment of the newly emerged pups [49]. Adults were categorized as non-breeding if they failed to wean a litter within a given year. In yellow-bellied marmots, pregnancy lasts 30 days and offspring nurse for 25 days [4]. For reproductive females, we estimated the dates of conception based on the weaning dates of pups. Reproductive states were confirmed based on reproductive condition checked and assigned during trapping.

Field procedures were approved under a UCLA research protocol ARC 2001-191-01 (approved on 13 May 2002 and renewed annually) as well as under permits issued by the Colorado Division of Wildlife.

2.4. Fecal extraction and radioimmunoassay (RIA)

We extracted steroid hormones from the feces of captive and wild marmots. After homogenization of the fecal samples, we suspended 0.2 g of each sample in 90% aqueous ethanol (Omnisolv, EMD Chemicals Inc., NJ). Samples were boiled for 20 min at 80 °C and centrifuged for 20 min at 2500 rev/min at room temperature. We decanted the supernatant into 16 × 100 mm tubes. To ensure the total extraction of FCMs, we performed this procedure twice. Supernatants were combined and dried in a vacuum centrifuge (Savant, Holbrook, NY; Labconco Corporation, Kansas City, Missouri) for 17 h. The purified samples were reconstituted in 1 ml of Absolute ethanol (Fisher Scientific, NJ) and stored at −80 °C. We used either 12.5 or 25 ml of purified sample, making appropriate adjustments to our dilutions per the instructions of the RIA Kit (MP Biomedicals, Costa Mesa, CA). The primary antibody of this assay is raised against corticosterone and has a cross-reactivity of 100% with this steroid, but a cross-reactivity of less than 1% with other steroids (e.g., desoxycorticosterone, testosterone, cortisol, progesterone, estradiol). Because feces contain FCMs rather than native corticosterone [25,78], we hypothesized that if this assay is valid for yellow-bellied marmots that it would detect meaningful variation in FCMs. Several of the samples from the ACTH challenge had particularly high values of FCM levels and were therefore diluted and rerun to ensure that these measures fell within our standard curve. The inter-assay coefficient of variation (CV) was 8.3% and the intra-assay variation was 1.4%.

2.5. Statistical analyses

We used linear mixed models (LMM) to examine the effects of our two treatments (handling and ACTH challenge tests) on fecal production and FCM levels in captive marmots and to explain natural variation in our wild marmots. In all of our models, we included marmot "identity" as a random effect to account for repeated measures on the same individuals and used likelihood ratio tests to determine if random effects improved the fit of each model. We calculated the "repeatability" of individual and year effects as the percentage of residual variance in the LMM attributed to each of these random effects [40].

Based on data from RMBL, we assessed the extent to which life history stage, sex, and environmental factors explained variation in FCM levels in wild marmots. We included the random effect of year to examine among-year variation in FCM levels of wild marmots. Fixed effects included day of the year, hour of trapping, age class (yearling or adult), and sex (male or female). In addition, for adults, we asked whether annual reproductive success (weaned or failed to wean a litter) and female reproductive state (pregnant or lactating; days post-conception) explained significant variation in FCMs. We tested for two-way interactions when doing so increased the fit of our model (smallest AIC). Values for excluded terms are based on adding each term to our final models.

All data were ln-transformed to meet the assumptions of normality and homogeneity of variances prior to each statistical analysis. We used Statistica v.6.1 (StatSoft, Inc., Tulsa, OK, U.S.A.) to analyze data failing to meet assumptions of normality and/or homoscedasticity of variances despites these transformations. We compared means for two independent groups using Mann–Whitney *U* tests and compared means from dependent groups using Wilcoxon signed-ranks tests. All other statistical analyses were conducted using R (R 2.12.2, R Development Core Team, 2011). Where appropriate, we corrected for multiple testing and report corrected *P*-values [68]. We set our alpha to 0.05. For visual representation, we present data as untransformed grand mean ± standard error (S.E.) such that each individual is only represented within a single category once within a graph.

3. Results from captive marmots

3.1. Handling stressor on FCMs levels in captive marmots

Overall, the baseline measurements of FCM levels were, on average, 132 ± 20 ng/g feces for the non-reproductive marmots in the captive group ($n_{Male} = 1$, $n_{Females} = 5$). The baseline measurement for the male (Mean: 143 ng/g feces, Range: 114 to 180 ng/g) fell within the range of values for the five female subjects (Mean ± S.E. of 129 ± 23 ng/g feces, Range: 52 to 242 ng/g feces). As predicted, handling triggered a significant rise in FCM levels (Fig. 1A). Specifically, marmots excreted feces with consistently higher levels of FCMs than did those same marmots at baseline (Model estimate for effect on FCMs ± S.E.; Handling: 0.202 ± 0.042, $t = 4.809$, $P < 0.001$; Intercept: 4.902 ± 0.178, $t = 27.478$, $P < 0.0001$). FCM levels declined significantly within days as the hours progressed (Hour: −0.008 ± 0.003, $t = -2.239$, $P = 0.027$). The effects of hour of the day on FCM levels was consistent within the handling and baseline periods (Handling *Hour: −0.002 ± 0.010, $t = -0.169$, $P = 0.866$).

Marmot identity significantly improved the overall fit of our statistical model (Likelihood ratio test: $\chi^2 = 190.5$, $df = 1$, $P < 0.001$), explaining 42% of the variation in FCM levels. Thus, consistent within-individual responses to stressors across the experiment permitted us to detect a significant effect of handling in these repeated measures after accounting for these consistent inter-individual differences (Fig. 1A). After correcting the multiple testing, post hoc tests revealed a significant rise in FCM levels 24 h after handling (0800 h on day 4; Wilcoxon Sign Ranks Test: $n = 6$, $t = 1$, $P = 0.046$). At subsequent times points, measures returned to baseline levels of FCMs (e.g., 28, 32 or 36 h after handling relative to baseline measures at 1600, 2000, and 2400 h; $n = 5,4$, $t \geqslant 2$, $P \geqslant 0.173$ and 3, respectively). Samples sizes of fewer than six marmots occurred for time points at which marmots failed to defecate.

3.2. ACTH activation on FCM levels in captive marmots

As in the behavioral challenge, we detected a significant rise in FCM levels in response to the ACTH injection after a time delay (Fig. 1B). The effects of the ACTH challenge (ACTH: −0.231 ± 0.288, $t = -0.802$, $P = 0.424$) and hour of day (Hour: −0.008 ± 0.008, $t = -1.042$, $P = 0.299$) on the rise in FCM levels were non-additive (ACTH *Hour: 0.123 ± 0.020, $t = 6.143$, $P < 0.001$, Intercept: 4.904 ± 0.217, $t = 22.579$, $P < 0.001$). Whereas FCM levels significantly declined across hours of the day during the baseline period (Hour: −0.007 ± 0.004, $t = -2.013$, $P = 0.046$), FCM levels rose significantly throughout day 8 in response to the pharmaceutical manipulation (0.118 ± 0.021, $t = 5.725$, $P < 0.001$, Fig. 1B).

To identify the precise time at which there was a significant treatment effect on the rise in FCM levels, we compared baseline values to matched times of day after injection. After correcting for multiple comparisons, values at 0, 4, 8 and 12 h after injection were not statistically different from baseline measures at matched time points ($n = 5$ or 6 marmots for each matched tests, $t \geqslant 5$, $P \geqslant 0.138$ for all tests). However, we detected the first significant rise in FCM levels 24 h post-injection (compared to baseline at 0800 h; Wilcoxon Sign-Ranks Test: $n = 6$, $t = 0$, $P = 0.028$) and this treatment continued to promote a statistically significant rise at 28, 32, and 36 h after injection (compared to baseline at matched time points; $n = 5$ or 6 marmots, $t = 0$, $P \leqslant 0.043$ for all tests).

As in the handling experiment, we found consistent individual differences in response to the ACTH challenge. Marmot identity explained 28.1% of the variation in FCM levels and the inclusion of marmot identity significantly improved the fit of this model (Likelihood ratio test: $\chi^2 = 67.4$, $df = 1$, $P < 0.001$; Fig. 1B). For example, only one of the five marmots showed a peak response followed by the expected decrease in FCM levels by the end of the experiment on day 8 (Fig. 2). For this adult female marmot, the

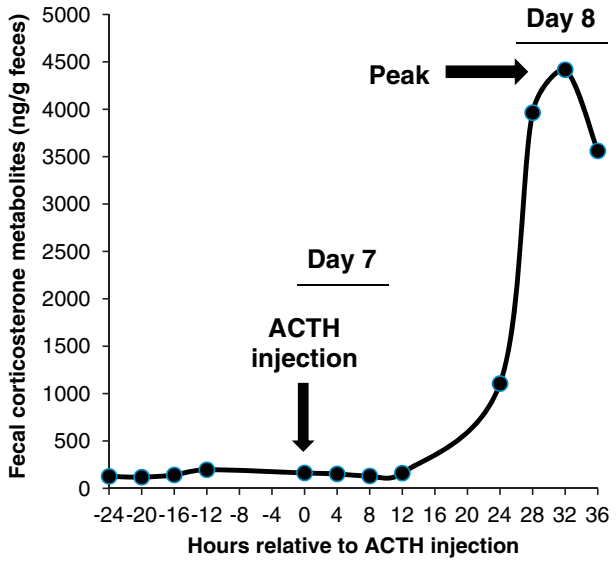


Fig. 2. Example of a peak and subsequent decline in the concentration of fecal corticosterone metabolite levels in response to the ACTH challenge. This peak response was detected 32 h post-injection at 4417 ng/g feces for one captive, non-reproductive adult female marmot.

peak extraction value of 4417 ng/g was detected at 32 h post-injection at 1600 h on day 8 (Fig. 2). This one female showed a subsequent decrease (3560 ng/g feces) in the final hour of the experiment at 2000 h (Fig. 2). Because the highest FCM levels were measured at 36 h for all but one of the six marmots, post-injection lag time to peak response was, on average, at least 35.3 ± 0.7 h. The effects of the synthetic ACTH (Synacthen® Depot) were particularly long-lasting and this peak response for five out of the six marmots occurred after 36 h.

3.3. Stressors reduced fecal production in captive marmots

Marmots produced an average of 691.0 ± 61.6 g of feces per day throughout the experiment, but decreased fecal production in response to stressors. Whereas fecal production did not consistently decrease across the days of the study (day: 0.467 ± 0.296, $t = 1.580$, $P = 0.116$), it was significantly lower on days in which marmots responded to experimental treatments (days 3 and 8, Fig. 1, see below) than during the baseline period (Challenge: −3.813 ± 1.752, $t = -2.176$, $P = 0.031$). Feces production also generally decreased throughout each day (Time: −1.992 ± 0.163, $t = -12.226$, $P < 0.001$; Intercept ± S.E.: 52.004 ± 3.208, $t = 16.209$, $P < 0.001$). Interestingly, we also found consistent individual differences in the amount of feces produced by captive marmots (Likelihood ratio test: $\chi^2 = 23.4$, $df = 1$, $P < 0.001$).

4. Results from wild marmots

4.1. Live-trapping promotes a rise in FCM levels, but only for "stressed" individuals

We next assessed the effects of trapping and handling wild marmots on FCM levels by comparing values for feces from marmots live-trapped at two sequential trapping events at RMBL. To control for hour of the day, trapping history, and to avoid pseudoreplication, we limited this set of analyses to the first paired trapping events that occurred 24 h apart for each focal marmot. At the population level, trapping and handling generally failed to promote a significant rise in FCM levels between the first and second trapping events in matched pairs of wild marmots (Wilcoxon Sign-Ranks Test: $z = 0.640$, $P = 0.640$, $n = 42$ marmots). However, marmots differed in their behavioral responses to this potential stressor. The majority of individuals (79% of our sample) failed to display one or more of behavioral indicators that trapping represented a stressor. For these "calm" individuals, we found no detectable rise in FCMs between the first trapping event and the second sequential trapping event ($z = 0.652$, $P = 0.644$, $n = 33$, Fig. 3). In contrast, however, when we limited our analysis to only those "stressed" individuals (21% of our sample) that struggled, alarm-called, and/or bit the trap at the first trapping event, we detected a significant rise (131% higher on the second capture) in FCM levels 24 h after trapping and handling, even after correcting for multiple comparisons ($z = 2.547$, $P = 0.033$, $n = 9$, Fig. 3). These findings could not be explained by a difference in baseline FCM levels between "calm" and "stressed" individuals at first capture (Mann–Whitney U-test: $U = 111$, $P = 0.250$, $n_c = 33$, $n_s = 9$ marmots, respectively). We therefore limited all subsequent analyses to feces collected at the first time an individual was captured (within each bi-weekly sampling period) to avoid the possible confounding effects of repeated trapping on FCM levels.

4.2. Consistent inter-individual differences in FCMs across years for wild marmots

Samples collected at the first trappings during our bi-weekly sampling from ten years of data on wild marmots at RMBL ranged from 22.8 ng/g to 940.0 ng/g with a mean value of 191.8 ± 3.5 ng/g ($n = 1335$ samples from 352 different marmots). Fecal samples included in this reduced data set were therefore collected at first capture events an average of 3.7 ± 0.2 times from each marmot across the ten year study. Females ($n = 182$) and males ($n = 170$), respectively, were sampled an average of 5.2 ± 0.3 and 2.3 ± 0.1 times. Our data set included samples from up to 8 years of repeated measures on the same marmot. Repeated measures, on average, included data from 2 ± 1 years on each marmot. The addition of the random effects of marmot identity (Likelihood ratio test: $\chi^2 = 47.5$, $df = 1$, $P < 0.001$) and year ($\chi^2 = 955.1$, $df = 1$, $P < 0.001$) significantly improved the fit of the model. Year of the study accounted for almost half (46%) of the variation in FCM levels in free-living yellow-bellied marmots. Individual differences explained an additional 12% of the variation in FCM levels.

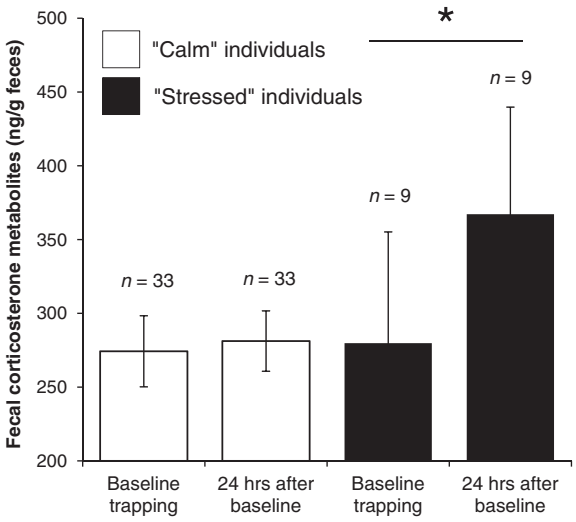


Fig. 3. Concentrations of fecal corticosterone metabolites (Mean ± S.E.) for matched pairs of feces collected from wild marmots at two sequential trapping events separated by 24 h. Baseline trapping and 24 h after trapping for which "calm" individuals ($n = 33$ marmots) failed to elicit any of signs that trapping represented a stressor and for which "stressed" individuals ($n = 9$ marmots) struggled, alarm-called, and/or bit the trap upon the first capture of the year. The asterisk above the bars represent statistically significant differences after correcting for multiple testing.

4.3. Seasonal and daily variation in FCMs for wild marmots

Sampling dates of fecal samples ranged from 30th April (day 120) to 7th September (day 250) of the year (Mean ± S.E. on June 25th (day 176 ± 1). As predicted, FCM values significantly decreased as the active season progressed (Day of year: −0.004 ± 0.001, $t = -7.456$, $P < 0.001$; Intercept: 5.472 ± 0.179, $t = 30.554$, $P < 0.001$, $n = 1335$ samples from 352 marmots). Moreover, FCM levels increased significantly throughout the day such that FCM levels were generally higher in feces collected in the evening than in the morning from wild marmots (Hour: 0.016 ± 0.003, $t = 5.420$, $P < 0.001$).

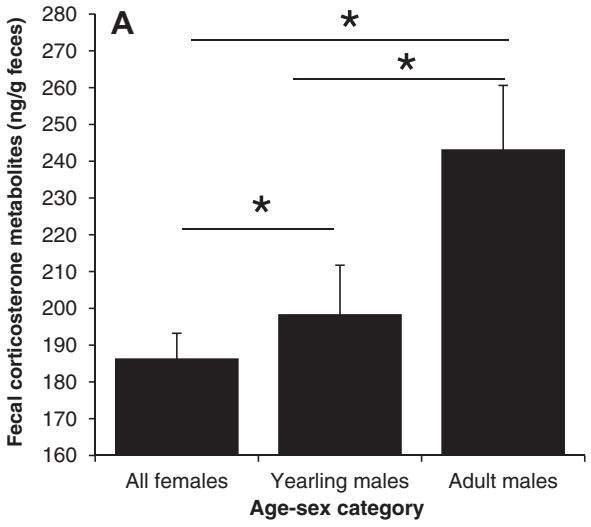
4.4. Sex and age effects on FCMs in wild marmots

After accounting for these seasonal and daily effects in our model, the effects of age (Yearling: −0.064 ± 0.038, $t = -1.692$, $P = 0.091$) and sex (Male: 0.172 ± 0.043, $t = 4.020$, $P < 0.001$) on FCM levels were non-additive (Age class * Sex: −0.168 ± 0.060, $t = -2.800$, $P = 0.005$, Fig. 4A). Whereas yearling males had significantly lower FCM levels than did adult males (Yearling: −0.267 ± 0.053, $t = -5.067$, $P < 0.001$, $n = 392$ samples on 170 males), we detected

no consistent differences for females in FCM levels between yearlings and adults (Yearling: −0.036 ± 0.035, $t = -1.052$, $P = 0.293$, $n = 943$ samples on 182 females Fig. 4A). On average, FCM levels were 131% and 123% higher in adult males than in females (yearlings and adults) and yearling males, respectively.

4.5. Effects of reproduction on FCM levels

We next built a new statistical model for which we focused only on the FCMs of those adults of the population capable of reproduction (⩾2 years old). We first asked whether adults that weaned offspring differed in their FCM levels from those adults that failed to do so ($n = 990$ samples for 197 adults). After accounting for day of the year (Day: −0.004 ± 0.001, $t = -6.344$, $P < 0.001$), sampling hour (Hour: 0.016 ± 0.003, $t = 4.728$, $P < 0.001$), and sex (Male: 0.177 ± 0.044, $t = 4.054$, $P < 0.001$; Intercept: 5.486 ± 0.184, $t = 29.655$, $P < 0.001$), we found no consistent difference in FCM levels between samples from adults that weaned or failed to wean offspring in a given year (Reproduced: −0.029 ± 0.032, $t = -0.898$, $P = 0.369$). Moreover, we detected no interaction between the effects of sex and this measures of annual reproductive success (Sex * Reproduced: 0.016 ± 0.079, $t = 0.200$, $P = 0.842$).



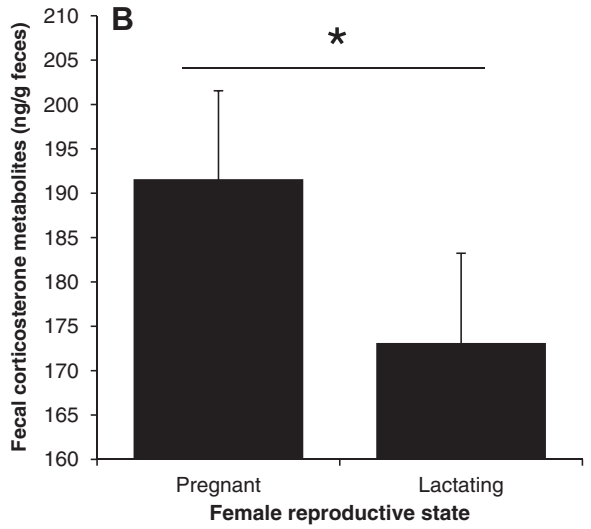


Fig. 4. Concentrations of fecal corticosterone metabolites (Mean ± S.E.) of wild marmots of different (A) age-sex categories ($n = 377$ marmots) and (B) reproductive state for pregnant ($n = 69$) or lactating ($n = 78$) females. Asterisks above the bars represent statistically significant differences after correcting for multiple testing.

Among females that successfully weaned offspring, pregnant females had significantly higher FCM levels (111% higher, on average) than did lactating females (Pregnancy: 0.191 ± 0.043, $t = 4.480$, $P < 0.001$, Fig. 4B, $n = 315$ samples on 92 females) after accounting for effects of hour of sampling (Hour: 0.026 ± 0.005, $t = 4.885$, $P < 0.001$) and day of the year (Day: −0.005 ± 0.002, $t = -2.403$, $P = 0.017$; Intercept: 4.669 ± 0.171, $t = 27.262$, $P < 0.001$) as well as the random effects of year and marmot identity. Because females are seasonal breeders and because marmots belonging to all age-sex categories showed a general decrease in FCMs throughout the active season, we next ruled out the possibility that the effect of pregnancy was simply driven by sampling date. That is, in a second model, we found that the days since conception (−0.007 ± 0.001, $t = -4.995$, $P = 0.001$), but not day of the year (−0.188 ± 0.6362, $t = -0.295$, $P = 0.768$), predicted FCM levels in reproductive females; this model also controlled for hour of sampling (0.025 ± 0.006, $t = 4.424$, $P = 0.001$; Intercept: 4.983 ± 0.169, $t = 29.376$, $P < 0.001$). As before, year effects were particularly strong predictors of FCMs in reproductive females, accounting for roughly half (47%) of the variation. Nevertheless, we again found consistent inter-individual variation in these females, accounting for an additional 16% of the variation. Thus, whereas FCMs generally decline as the active season progresses, the effects of female reproductive status trump those of day of the year *per se*. Above and beyond seasonal effects, pregnancy itself promotes an initial rise in FCM levels followed by a decrease as the date of pup emergence and weaning approaches.

4.6. FCM levels higher for wild than captive marmots

All of the captive marmots were non-reproductive adults ($n_{Male} = 1$, $n_{Females} = 5$). Therefore, to understand the relative effects of captivity on FCM values, we focused on the subset of fecal samples collected from non-reproductive adult wild marmots at first captures ($n_{Males} = 33$, $n_{Females} = 84$). Wild marmots had 147% higher FCM levels than did captive marmots (Environment: 0.516 ± 0.174, $t = 2.974$, $P = 0.003$, Fig. 5). This effect emerged after we controlled for the effects of sampling day (−0.005 ± 0.001, $t = -4.293$, $P < 0.001$), sex (0.166 ± 0.098, $t = 1.696$, $P = 0.091$) and hour of the day (−0.002 ± 0.005, $t = -0.496$, $P = 0.620$; Intercept: 5.355 ± 0.217,

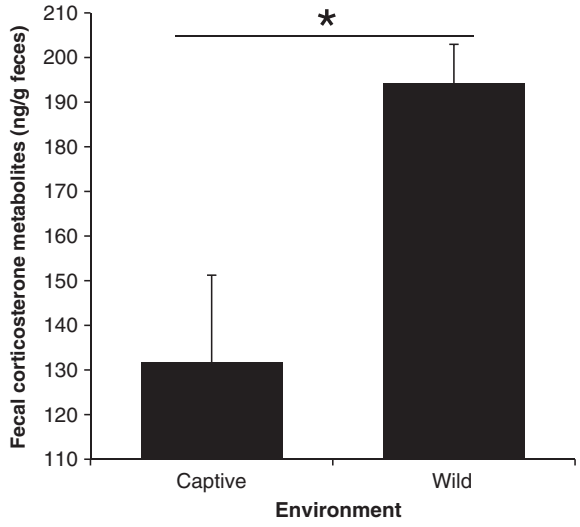


Fig. 5. Baseline concentrations of fecal corticosterone metabolites (Mean ± S.E.) of non-reproductive adults in captive ($n_{Male} = 1$, $n_{Females} = 5$) and wild ($n_{Males} = 33$, $n_{Females} = 84$) environments. The asterisk above the bars represents a statistically significant difference. Differences remained statistically significant between categories even when the analysis was limited to only adult females (see text).

$t = 24.627$, $P < 0.001$). We ruled out the possibility that this difference was driven by variation in the proportion of males in the captive and wild groups by restricting this analysis to non-reproductive adult females. As before, wild female marmots had significantly higher FCM levels than did captive female marmots (Environment: 0.493 ± 0.185, $t = 2.666$, $P = 0.007$); inclusion of marmot identity and year, respectively, significantly improved the fit of this model (Likelihood ratio test: $\chi^2 = 72.1$ and 74.0, $df = 1$ and $P < 0.001$ for both).

5. Discussion

5.1. Biological and physiological validation of the assay

The results of the experiments support the suitability of this minimally-invasive technique for assessing the physiological responses of yellow-bellied marmots to stressors. That is, the results of the handling and pharmacological experiments in the captive group as well as a trapping effect in wild marmots provide biological and physiological evidence for the validity of applying this RIA to measure stress-reactivity in this species. Interestingly, the magnitude of response to handling in captive marmots was considerably lower and more transient than that triggered by the ACTH challenge. Handling effects were presumably low because these subjects were born in captivity. Moreover, our finding here that physiological stress-responses induced by ACTH were more pronounced than those imposed by handling is likely due to the fact that pharmaceutical challenges act directly on the HPA axis without requiring the animal to perceive a stimulus in its environment as a stressor [74].

Here we found an initial response to ACTH after 24 h and first detected a peak response at 35 h after injection. Even though this post-injection lag time to the peak FCM is a low estimate (only 5 out of 6 marmots peaked by the end of our experiment), this value is still longer than that of most previous studies of most rodents. For instance, the lag time from injection to peak concentration was higher than that found in Arctic ground squirrels (*Urocitellus parryii*, 4 h [65], common degu (*Octodon degus*, 6 h: [67]), Columbian ground squirrels (*Urocitellus columbianus*, 7 h: [18]), laboratory mice (10 h: [76]), North American red squirrels (*Tamiasciurus hudsonicus*, 11 h: [26]), guinea pigs (*Cavia aperea* f. *porcellus*, 18 h: [8]), and tuco-tucos (*Ctenomys sociabilis*, 24 h: [83]). Although subjects from the captive group were foraging normally at the time of the experiment, gut passage times likely vary with diet and metabolic rate (e.g., [72,73]). Yellow-bellied marmots have particularly slow metabolisms, allowing these animals to accrue enhanced energy savings during hibernation compared to other members of the Genus Marmota [6]. Whereas numerous previous studies emphasize the need to collect feces within several hours (e.g., within 4 h of capture) to avoid the effects of trapping and handling stress, our study is novel in that it highlights the need to also account for potentially slow-acting effects of stressors on changes in fecal metabolites of GCs. We suggest that, to be conservative, baseline measures of FCMs should be limited to those collected at the first trapping event within a weekly (or bi-weekly) trapping session for animals with slow-gut passage times.

In wild marmots, only a small fraction (less than one quarter) of individuals displayed behaviors signaling that trapping represented a stressor and we only detected a significant surge in FCMs in marmots that were "stressed" in the trap. Because animals in this study population have been continuously live-trapped and released for 50 years, it is possible that trapping failed to represent a stressor because of habituation to the field methods used here. However, differences in behavioral responses to trapping were independent of trapping history of each individual marmot because data here were limited to the first pair of sequential captures of the year for "calm" and "stressed" individuals. Whereas trap-stress may induce GCs by 108% in meadow voles spending up to 16.5 h in the trap [30], subjects here typically only spent 2 h or less in the traps at RMBL prior to their release. Our multiple capture data on marmots has important welfare implications because they suggest that the majority of live-trapping events do not represent stressors for these wild mammals.

The association between behavioral indicators of "stress" and a rise in FCMs in those wild marmots that did struggle, alarm-call, or bite at the trap demonstrates the ability of this assay to detect meaningful responses to acute stressors in field conditions. Thus, the handling (Fig. 1A), pharmaceutical (Fig. 1B and 2), and trapping assays (Fig. 3) all indicated statistically significant rises in adrenocortical activity starting as early as 24 h after treatments. We also detected a decrease in fecal production, suggesting that captive marmots reduced their food intake and/or gut passage rates in response to stressors. In contrast, deer mice (*Peromyscus maniculatus*) increased their fecal production when exposed to the stressor of novel caging [35]. This contrasting result emphasizes the need for species-specific investigations of the stress-response. Moreover, our findings merit further investigation in ecological settings in which marmots face varying degrees of predation risk and weather conditions.

5.2. Consistent inter-individual differences across years in hormonal traits

Interestingly, beyond the importance of population-level variation with respect to year, season, time of day, reproductive state, and age, we found repeatable inter-individual differences in FCMs. Evidence of consistent inter-individual variation of hormonal traits in wild vertebrates is rare (but see [26,50,51]). Documenting its occurrence presents the opportunity for future investigations. For example, our data provide an important first step in suggesting that this hormonal trait might be a meaningful target of selection in natural populations [10,20,57]. Because the pleiotropic actions of hormones could potentially shape multiple traits, additional understanding of the relationships between adrenocortical function and consistent individual differences for wild animals is needed. Such data should provide novel insights and promote an integrative understanding of endocrinology, evolutionary biology, and ecology. Application of quantitative genetics [39] and reaction norm approaches [28] to long-term data on marked individuals for which lifetime reproductive success and genealogies are known should prove particularly fruitful in these efforts.

5.3. Patterns of seasonal and daily variation in FCMs for wild marmots

The results from wild marmots suggest that adrenocortical activity might play an important role in the seasonal and daily regulation of their physiological states.

FCM levels from sampling dates reported here (30th April to 7th September) capture the start of the breeding season because the average date that we first detected any of the marmots to emerge from hibernation in this population was on 22nd April [12]. Roughly 50% of all marmots in this population emerge from hibernation by 4th May and 21st May at low and high elevation sites, respectively [12]. Nonetheless, the precise factors responsible for the decline in FCMs as marmots approach hibernation remain unknown. A seasonal peak in early FCM levels might be due to intense early season breeding followed by the need to gain mass before entering hibernation at the end of the breeding season. Either seasonal variation in competition over mates or access to food/habitat might explain this decline in FCMs because yellow-bellied marmots engage in competitive searching for mates early in the active season [3],

but access to high quality food and suitable cover (protection from predators) also increases as the active season progresses [13]. In some sciurids, such as in arctic ground squirrels, male-male competition for mates early after hibernation explains the decline in GCs across the growing season [27,41,65]. In contrast, experimental evidence from other sciurids, such as red squirrels, suggests that seasonal changes in FCM levels simply reflect shifts in food availability and composition [25]. Whereas both food and habitat quality increase as the active season initially progresses for marmots, FCMs levels here continued to decreases even as habitat quality deteriorated again prior to hibernation, raising the possibility that mating competition rather than ecological factors might be the most important drivers of this seasonal decline in FCMs. Future research is needed to understand which of these two hypotheses best explains seasonal variation documented here.

Surprisingly, FCM levels increased throughout the day for wild marmots, but were highest in the morning in captivity. Different behavioral patterns of foraging in the captive and wild groups might explain this discrepancy. For example, captive marmots sometimes forage at night whereas wild marmots do not [1]. These patterns might also be attributed to differences in diet, and associated gut-passage rates, between the two groups. Because the results of the ACTH challenge here suggests a 36-h delay between the surge of GCs in plasma and that detected in feces, our finding that FCM levels increased throughout the day for wild marmots is consistent with the finding that plasma GCs are generally highest in the morning for other sciurids for which daily patterns of plasma GCs are available (e.g., [48]).

5.4. Female reproductive state explains variation in FCMs for wild marmots

Above and beyond these seasonal effects, FCM levels were higher for pregnant than for lactating females. This finding that GCs also vary with female reproductive state is consistent with data for other mammals, such as bats, squirrels, and whales [26,37,58]. Elevated GCs in reproductive females is consistent with the idea that GCs act to mobilize energy stores required for reproduction (reviewed by [42]). Importantly, the reduction in FCM levels following parturition was most apparent when we considered female reproductive condition as a quantitative, continuous trait based on days past conception. These data on marmots underscore the need to view reproduction, and its associated trade-offs, along a continuum rather than as a categorical trait [80].

5.5. Welfare and conservation implications

Our findings that FCM levels were 68% lower in captivity than in the wild suggests that the natural environment in which these animals occur is generally more challenging or less predictable than life in captivity. Similarly, baseline FCM levels, such as those of tuco-tucos [83], were lower in captive than in wild rodents [15]. Our data here may in part reflect the fact that captive marmots were born in captivity and habituated to their housing conditions. Additional studies are therefore needed to assess whether this finding is generalizable to wild-caught marmots housed in captivity. Nevertheless, our finding that wild marmots are sensitive to environmental stressors is consistent with previous research on yellow-bellied marmots [9,14,45]. Validation of this minimally-invasive technique should therefore permit for the discovery of new insights from yellow-bellied marmots into the relationships among ecological factors and behavioral endocrinology. For example, FCMs play an important, yet underappreciated role, in alarm-call production in marmots, predicting the probability that a marmot will emit a call as well as the acoustical structure of calls [9,14]. Maternal FCMs also appear central to mediating reproductive strategies in mothers faced with varying degrees of predation risk [45]. Taken together, these findings suggest that although handling of wild mammals represents an acute stressor, predictable food, water, and shelter in captivity is less stressful than conditions characterized by lack of predictability and control faced by animals in the wild.

Our findings have crucial welfare implications for field biologists because they suggest that repeated measurements of traits are possible with minimal "stress" upon wild animals. The tremendous importance of year effects found here also underscore the need for multi-year studies to assess the effects of stressors and flexible management plans in the conservation of wild animals. There is growing recognition in the emerging field of conservation physiology for the need to make links among physiological theory, changing environments, and ecological outcomes explicit [61]. Our integrative understanding of the natural ranges of annual and seasonal variation in wild mammals therefore provides important baseline estimates for understanding how anthropogenic assaults influence GCs and an important tool for elucidating the consequences of such disturbances on the persistence of wildlife populations and species.

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