



Physiological and behavioral responses to anthropogenic stressors in a human-tolerant mammal

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As humans continue to alter natural habitats, many wild animals are facing novel suites of environmental stimuli. These changes, including increased human–wildlife interactions, may exert sublethal impacts on wildlife such as alterations in stress physiology and behavior. California ground squirrels (*Otospermophilus beecheyi*) occur in human-modified as well as more pristine environments, where they face a variety of anthropogenic and naturally occurring threats. This makes this species a valuable model for examining the effects of diverse challenges on the physiology and behavior of free-living mammals. To explore potential sublethal effects of habitat modification on *O. beecheyi*, we compared body masses, behaviors, and fecal glucocorticoid metabolite (FGM) levels for free-living squirrels in human-disturbed versus undisturbed habitats. Prior to these analyses, we validated the use of FGMs in this species by exposing captive *O. beecheyi* to pharmacological and handling challenges; both challenges produced significant increases in FGMs in the study animals. While FGM responses were repeatable within captive individuals, responses by free-living animals were more variable, perhaps reflecting a greater range of life-history traits and environmental conditions within natural populations of squirrels. Animals from our human-disturbed study site had significantly higher FGMs, significantly lower body masses, and were significantly less behaviorally reactive to humans than those from our more pristine study site. Thus, despite frequent exposure of California ground squirrels to human impacts, anthropogenic stressors appear to influence stress physiology and other phenotypic traits in this species. These findings suggest that even human-tolerant mammalian species may experience important sublethal consequences due to human modifications of natural habitats.

Key words: adrenocorticotrophic hormone challenge, anthropogenic change, habitat alteration, noninvasive sampling, personality, Sciuridae, stress hormones

Changes in environmental conditions may disrupt physiological homeostasis in free-living animals, leading to alterations in the production of glucocorticoid (GC) hormones (Wikelski and Cooke 2006; Busch and Hayward 2009; Dantzer et al. 2014). GCs are steroid hormones that mediate metabolic responses to biotic and abiotic challenges (Romero 2002; Bauer et al. 2013; Creel et al. 2013; Wingfield 2013; Hau et al. 2016; Hammond et al. 2018). Novel challenges (i.e., those with which animals have no evolutionary experience), including challenges associated with numerous anthropogenic activities (Frid and Dill 2002), may result in long-term alterations of GC physiology such as chronic increases in baseline GC levels (Rehner et al. 2014; Santos et al. 2018; McLennan et al. 2019) and reduced

GC responses to acute stressors (Romero and Wikelski 2002; Kleist et al. 2018). Chronic elevations in baseline GC levels are typically expected to have negative fitness consequences, although these relationships are context-dependent and differ across species (Romero and Wikelski 2001; McEwen and Wingfield 2003; Cyr and Romero 2007; Eggert et al. 2010; Jaatinen et al. 2013; Love et al. 2013; Dantzer et al. 2014). More subtle sublethal effects may manifest as changes in behavior or body condition (e.g., Creel et al. 2009; Brock et al. 2013). These changes may negatively influence demographic and community-level processes (Schmitz et al. 1997; Creel et al. 2005; Preisser et al. 2005; Sih et al. 2011), indicating that

even nonlethal effects may have significant implications for a species' biology.

The effects of anthropogenic activities on GC physiology in animals living at the human–wildlife interface have received considerable attention (Creel et al. 2002; Kersey and Dehnhard 2014; Tennessen et al. 2014; Kleist et al. 2018). Much of the existing literature focuses on species that are of particular concern due to conservation issues associated with recent exposure to anthropogenic change (e.g., Jaimez et al. 2012; Nelson et al. 2015; Carlitz et al. 2016; Hunt et al. 2019). This emphasis is understandable, but valuable insights can also be obtained from species that have persisted in close proximity to humans for numerous generations with no conspicuous negative consequences. For example, a recent study of wood frogs (*Rana sylvatica*—Tennessen et al. 2018) revealed that animals in human-disturbed areas are less GC-responsive to auditory stimuli than animals from disturbance-free habitats. Similarly, studies of European blackbirds (*Turdus merula*) indicate that baseline GCs are lower in disturbed relative to undisturbed environments (Partecke et al. 2006), leading to speculation that reduced GC response may be a common feature of species that have experienced long-term, nonlethal contact with human-modified environments. Conversely, however, fecal GC levels in many vertebrate species are elevated in response to anthropogenic disturbances (Dantzer et al. 2014). Thus, determining how GC responses vary in relation to the degree of exposure to anthropogenic activity is critical to assessing the potential for wildlife to adapt to environmental change.

Because multiple factors interact to shape GC responses (Sapolsky et al. 2000; Dickens and Romero 2013; Ensminger et al. 2014; Blumstein et al. 2016), studies that incorporate intrinsic as well as extrinsic variables are required to characterize fully the effects of different degrees of human activity on wildlife. Here, we examine responses of the California ground squirrel (*Otospermophilus beecheyi*), an ecologically generalist rodent that occurs in grassland habitats from the northern Baja Peninsula to southern Washington (Grinnell and Dixon 1918; Hall 1981). Members of this species respond behaviorally to changes in environmental conditions, including modifying foraging group size, responses to predator cues, and use of specific microhabitat features (Leger et al. 1983; Ayon et al. 2017; Ortiz et al. 2019) in relation to perceived risks. This behavioral flexibility, coupled with an ability to exploit modified agricultural landscapes (Gashwiler 1970), may underlie the success of California ground squirrels in habitats characterized by a range of degrees of anthropogenic activity (reviewed by Smith et al. 2016).

To assess potential sublethal effects of human activity on California ground squirrels, we compared patterns of GC physiology among individuals living in a moderately impacted versus a more pristine habitat. Specifically, we sought to evaluate the hypothesis that these squirrels are more physiologically stressed in areas with greater human activity. We predicted that when corrected for differences in age and sex, measures of baseline GC levels would be greater for squirrels residing in the more human-disturbed habitat (Dantzer et al. 2014). We

also predicted that baseline GCs would vary across the active season (Atkinson et al. 2006; Nunes et al. 2006), with GC levels increasing as the season progressed due to increases in vigilance behavior (Ortiz et al. 2019) and decreases in food resource abundance (Fitch 1948). To test these predictions, we conducted analytical, pharmacological, and biological validation studies to confirm that measures of glucocorticoid metabolites obtained from noninvasively collected fecal samples (Touma and Palme 2005) provide reliable measures of baseline GC levels in California ground squirrels. We then used analyses of fecal GC metabolite (FGM) levels to examine how individual life-history traits (reproductive status, sex, mass) and environmental conditions (seasonal variation, human disturbance) interact to affect the GC physiology, body masses, and behavior of the study species. Our analyses generate insights into the potential nonlethal effects of anthropogenic activity on a species that is abundant in human-modified habitats.

MATERIALS AND METHODS

Capture and marking of free-living ground squirrels.—Field studies were conducted in Briones Regional Park in Contra Costa County, California (37.9377014 N, 122.1388542 W). The habitat consisted primarily of open oak savannah interspersed with more heavily vegetated riparian corridors. Since May 2013, populations of individually marked California ground squirrels have been maintained at two distinct locations within the park (Ortiz et al. 2019). These locations are separated by 120–600 m, with their geographic centers separated by 375 m; animals residing at different sites have not been seen interacting with each other (J. E. Smith, pers. obs.), suggesting that these sites are spatially distinct. Although we detected similar numbers of natural predators at these locations (J. E. Smith, pers. obs.), the two sites differ with regard to human activity; while one site (~9,596 m²) is subject to moderate disturbance due to foot traffic, off-leash dogs, and mowing (at start of summer), the second site (~9,540 m²) is dominated by tall grasses and is subject to seasonal grazing by cattle but is otherwise largely free of human disturbance (Ortiz et al. 2019). More specifically, hourly rates of incursion by humans and dogs at the disturbed site were significantly greater than those at the pristine site ($P < 0.05$); this pattern was consistent across years (for details, see [Supplementary Data SD1](#)). Given their proximity to each other, the two sites are otherwise similar with regard to environmental conditions.

Members of the study populations were captured using Tomahawk traps (Tomahawk Live Trap Co., Hazelhurst, Wisconsin) baited with sunflower seeds and peanut butter. At each study site, trapping was conducted every 2 weeks from late May to late July. Traps were opened during the daytime; each trap was shaded with cardboard and was checked at intervals of ≤ 30 min. Immediately upon detecting a captured animal, we recorded whether it was attempting to escape from the trap versus sitting quietly as well as whether it called as we approached the trap; differences in these behavioral responses are thought to reflect the extent to which individuals perceive capture as stressful (Blumstein et al. 2005). Once the behavior

of the animal had been noted, we collected fecal pellets from within and under the trap, avoiding any pellets that had been contaminated with urine. Pellets from the same individual were placed in a Ziploc freezer bag stored on ice in a cooler for 1–6 h until they could be transferred to a -80°C freezer on the Mills College campus.

Upon first capture, each animal was marked with a uniquely numbered metal ear tag (National Band and Tag Co., Newport, Kentucky) and a Passive Integrated Transponder (PIT) tag (Biomark, Inc., Boise, Idaho; for details, see [Smith et al. 2018](#)). Each individual was weighed and its sex and reproductive condition were determined based on visual inspection of the external genitalia. Body mass was used to identify animals as juveniles (weaned young of the year) or adults (≥ 1 year old—[Hanson and Coss 1997](#)). All field methods were approved by the Mills College Animal Care and Use Committee and were consistent with the guidelines of the American Society of Mammalogists for the use of wild mammals in research ([Sikes et al. 2016](#)).

Captive housing of ground squirrels.—To examine responses to different stressors under controlled conditions, we used adult squirrels (six males, five females) trapped at our human-impacted study site to assess FGM responses under captive housing conditions. No individuals were reproductive at the time of capture. One individual was trapped in April 2014, with the rest trapped in February 2015. Preliminary analyses that included year failed to detect significant interannual differences in FGM levels; thus, data from all animals housed in captivity were pooled for subsequent analyses of FGM responses to challenges.

Captured individuals were transported to the campus of the University of California, Berkeley, where they were maintained at $20\text{--}22^{\circ}\text{C}$ under a 10L:14D light cycle that replicated ambient lighting conditions at the same time of year. Squirrels were housed individually in metal cages ($41 \times 36 \times 57$ cm) with metal grid floors; a removable drop pan lined with absorbent sheets was located beneath the floor of each cage to facilitate fecal pellet collection. Cardboard sheets were clipped to the sides of each cage to prevent visual contact among the animals. Each squirrel was given a PVC tube containing cotton for nesting. Animals were provided ad libitum with water and a variety of food items, including rodent chow, sunflower seeds, and apples.

In captivity, fecal pellets were removed from drop pans and new absorbent sheets were installed every 4 h from 0800 to 2000 h, which corresponds to the active portion of the 24-h cycle for this diurnal species ([Owings et al. 1977](#)). This procedure served to habituate subjects to the sample collection process and to provide samples for quantifying circadian patterns of FGM production. The habituation period lasted for an average of 10 ± 0.4 days (range: 7–11 days, $n = 11$ squirrels). This period is comparable to those in other studies that have used wild sciurids housed in captivity to validate FGM levels ([Sheriff et al. 2012](#); [Hammond et al. 2015](#)). To determine if captive housing served as a stressor for our study subjects, baseline FGM levels for fecal pellets collected at the time of capture in the field were compared to those collected from

the same individuals after habituation but prior to exposure to any experimental stressors. Individuals were housed in captivity for no more than 4 weeks, after which they were released at the location at which they had been captured. All procedures involving captive squirrels were approved by the UC Berkeley Animal Care and Use Committee and adhered to the guidelines of the American Society of Mammalogists ([Sikes et al. 2016](#)).

Stress challenges in captive squirrels.—To confirm that GC responses could be detected in FGMs, two experimental challenges were conducted using captive squirrels. Because validation studies of closely related sciurids have revealed pronounced individual variation in response to experimental GC challenges ([Mateo and Cavigelli 2005](#); [Smith et al. 2012](#)), a within-subjects design was used. After the habituation period ended (day 0), each captive animal was subject to the following challenges:

- 1) Handling challenge: Beginning at 0800 h on day 2, each individual was coaxed from its home cage into a cloth handling bag. The animals were then weighed, sex was determined, and their reproductive status was assessed using the same procedures applied to free-living squirrels, after which each individual was returned to its cage. Mean \pm SE handling time was 25 ± 0.6 min per individual. This period mimics the handling time following initial capture of a free-living squirrel, during which body measurements are taken, ectoparasites (fleas, ticks) and tissue (DNA) samples are collected, animals are given ear and PIT tags, and the fur is painted with a unique dye mark.
- 2) Pharmacological challenge: Beginning at 0800 h on day 6, each individual was removed from its cage, weighed, and then injected intramuscularly in one hind leg with a mass-specific dose (12 IU/kg or 0.12 mg/kg—[Boonstra and McColl 2000](#)) of synthetic adrenocorticotrophic hormone (ACTH; Cortrosyn: Amphastar Pharmaceuticals Inc., Rancho Cucamonga, California) dissolved in 0.9% saline. This dosage and synthetic form of ACTH have been used successfully in previous ACTH challenge studies of rodents (e.g., [Woodruff et al. 2010](#); [Hammond et al. 2015](#)).

After each challenge, fecal samples were collected following the timeline outlined above. At each collection time point, urine-free fecal pellets from each individual were placed in a plastic bag and immediately frozen at -20°C . At the end of all captive studies, frozen samples were transported on ice from the Berkeley campus to Mills College and then stored at -80°C until FGM extraction.

Stress challenges in free-living squirrels.—As an additional biological validation, we assessed the effects of capture and handling on FGM levels in free-living squirrels. Individuals (different animals than those brought into captivity) were trapped twice with a mean \pm SE of 48 ± 0.4 h (range: 46–52 h) between successive captures. This inter-capture interval was selected based on the results of our captive studies (see below),

which suggested a rise in FGM levels ~48 h after injection with synthetic ACTH. Captures of the same animal were time-matched to control for potential circadian variation in FGMs.

Effects of habitat disturbance on behavior, mass, and GC physiology.—To examine the effects of human alteration of habitats on the behavior (response to trapping), body mass, and GC physiology of free-living California ground squirrels, we compared these traits in animals captured at the two study sites during the summers of 2013, 2014, and 2015. These traits may be affected by multiple factors and thus data regarding the sex, age, time of day, date, and year at which each capture occurred were included in analyses of each of these parameters. Because individuals vary in their tendency to be captured and because our analyses of FGM levels revealed evidence of trap-induced stress (see “Results”), analyses were limited to data collected when an individual was first captured during each biweekly sampling period.

Extraction and quantification of fecal GC metabolites.—A modified version of the protocol in [Sheriff et al. \(2011, 2012\)](#) was used to extract GC metabolites from fecal pellets. All extractions and subsequent assays were conducted at Mills College. Frozen fecal pellets were dried for 17–20 h at 0.04 mBar of ambient pressure using a lyophilizer (LabConco, Kansas City, Missouri). Freeze-dried samples were homogenized using a mortar and pestle or electric grinder. Duplicate 0.050 g (\pm 0.001 g) aliquots of each dried sample were suspended in 1 ml of 90% (v/v) aqueous methanol and placed on a multi-vortexer orbital shaker (IKA VXR basic Vibrax; IKA Works Inc., Wilmington, North Carolina) at 1,450 rpm for 30 min. Samples were then spun in a centrifuge at 1,500 rpm for 30 min, after which their supernatants were collected and frozen at -80°C until they could be assayed.

Corticosterone and cortisol enzyme immunoassay (EIA) kits from Cayman Chemical (Ann Arbor, Michigan) were used to quantify FGM levels in extracted samples. After diluting extracts with buffer following manufacturer instructions, 36 randomly selected samples representing individuals of all age and sex categories were assayed using both EIA kits. All samples analyzed had been collected from free-living animals at the time of first capture. Resulting data suggested that fecal hormone metabolites that were cross-reactive with the corticosterone antibody were significantly more prevalent than those that were cross-reactive with the cortisol antibody (see “Results”). Thus, all subsequent samples were assayed using the corticosterone EIA. The assay had a sensitivity of 30 pg/ml, with a range of 8.2–5,000 pg/ml (equivalent to 0.164–100 ng/g feces in our assay). Fecal extracts were thus typically diluted to 10% (100 μl sample + 900 μl buffer).

Analytical validation.—Parallelism in corticosterone EIAs was assessed by comparing binding curves for a serially diluted sample from our study animals to the slope of the curve for standards provided in the assay kit. Parallelism of these slopes suggests that corticosterone metabolites in feces are measured by the assay in the same dose-dependent manner that the corticosterone standard curve samples are measured. A single test sample was produced by mixing equal portions of extracts from

six different squirrels ($n = 1$ juvenile male, 1 juvenile female, 2 adult males, 2 adult females). This pooled extract and 13 serial dilutions (1:1.5) of this solution were assayed for comparison with kit standards.

Statistical analyses.—Fecal glucocorticoid metabolite values were natural log-transformed prior to all analyses so that the data met the assumptions of normality and homogeneity of variances required for parametric analyses. All statistical analyses were performed in R ([R Development Core Team 2018](#)). An analysis of covariance (ANCOVA; R function *aov*) was used to test for parallelism between the slopes of the binding curves for the assay kit standard and the serially diluted sample from our study animals. Paired *t*-tests were used to compare cortisol versus corticosterone metabolites in a subset of the fecal samples analyzed. Paired *t*-tests were also used to compare FGMs for captive squirrels at the time of capture in the field versus at the end of the habituation period as well as to compare FGMs for free-living squirrels at the time of initial capture and recapture 48 h later.

To explore potential predictors of FGM levels, generalized linear mixed models (GLMMs) were constructed using the *lmer* or *glmer* functions in the *lme4* package in R ([Bates et al. 2007](#)). To allow for comparisons of effect sizes, all continuous variables were mean-rescaled prior to analysis. Animal identity was always included as a random effect to account for repeated observations of the same individuals and to test for interindividual differences in FGM levels. We also calculated the adjusted repeatability (considers all fixed effects included in our models) of individual identity for all models using the *rptR* package ([Stoffel et al. 2017](#)). Repeatability is defined as the intraclass correlation coefficient, which measures the proportion of total variance that can be explained by between- rather than within-individual differences. Year was also included as a random effect in all models for data from free-living squirrels. The *P*-values for fixed effects were calculated using the Satterthwaite approximation in the *lmerTest* package (version 3.1.0—[Kuznetsova et al. 2017](#)), with values of $P < 0.05$ considered statistically significant. Likelihood Ratio Tests were applied to assess whether each random effect significantly improved the fit of a model. Graphs (QQ plots, plots of residuals versus predicted values and versus fixed effects) and statistical tests (uniformity, outliers) implemented in the R package *DHARMA* were used to assess residuals to validate all models ([Hartig 2019](#)).

To analyze data from the handling and ACTH challenges conducted in captivity, a single GLMM was constructed that contained the fixed effects of treatment (handling versus ACTH), sex, hour of day, hours post-stressor, and the square of hours post-stressor. Including the time squared term allowed us to account for the expected inverse-U-shaped, nonzero-centered relationship between hours post-stressor and FGM levels ([Uhart et al. 2006](#); [Hammond et al. 2018](#)) and to test the prediction that following exposure to a stressor, an individual's FGM levels would increase but then decrease back toward baseline over the course of the sampling period.

To examine relationships between human disturbance and the phenotypic and environmental parameters recorded for free-living ground squirrels, three separate GLMMs were constructed using the following response variables: 1) FGM levels; 2) body mass; and 3) behavior. FGM levels and body mass were modeled with a Gaussian distribution. Behavioral reactivity was modeled as a binary response (yes = attempted to escape the trap or alarm called or no = neither behavior elicited by researcher's approach) with a binomial distribution. Each model included capture site (pristine versus human-disturbed), age category (juvenile or adult), sex (male or female), mass (only included in models of FGMs and behavior), date (day of the year), and hour of the day as fixed effects. An interaction term between day and age was also included to account for potential changes in juvenile phenotypes over the course of each field season. The model with FGM levels as the response variable allowed us to test for relationships between phenotypic (sex, age) and external (season, hour) factors and FGMs in free-living squirrels. For this model, we also included an interaction term between sex and capture site, based on previous work suggesting that sensitivity to human disturbance may vary by sex (Dantzer et al. 2014); this term did not explain significant variation in models of mass or behavior, and thus was not included in those models.

RESULTS

Analytical validation of corticosterone EIA.—Paired measurements from the same fecal samples revealed that concentrations of fecal hormone metabolites that cross-reacted with the corticosterone antibody were significantly higher than concentrations of fecal hormone metabolites that cross-reacted with the cortisol antibody (paired *t*-test, $t_{35} = 7.763$, $P < 0.0001$; [Supplementary Data SD2](#)). We found no significant difference between the slope of the corticosterone binding curve for the assay kit standard and the slope of our serially diluted samples, indicating parallelism between the standard curve and our samples (ANCOVA, $F_{1,18} = 0.280$, $P = 0.603$; [Supplementary Data SD3](#)). Mean \pm SE intra- and inter-assay coefficients of variation were $7.0 \pm 0.4\%$ and $24.0 \pm 5.3\%$, respectively ($n = 17$ plates).

Biological and pharmacological validation of EIA.—Fecal pellets were rarely ($< 20\%$ of collection attempts) available at

the 2000 h collection time point and thus assays were limited to samples collected at 0800, 1200, and 1600 h. Handling of captive animals and injection with synthetic ACTH produced statistically significant increases in FGM levels ([Table 1](#); [Fig. 1](#)). FGM concentrations were positively associated with time since exposure to these stressors, with FGM response to ACTH being significantly greater than the response to handling ([Table 1](#); [Fig. 1](#)). FGM levels peaked approximately 51.6 ± 9.7 SE h after injection with ACTH. Inclusion of animal identity significantly improved the overall fit of the model ([Table 1](#)) and FGM levels were significantly repeatable within individuals (repeatability: 0.69 ± 0.12 SE), suggesting interindividual differences but intraindividual consistency in FGM responses to the challenges employed ($n = 24.25 \pm 2.83$ fecal samples per individual, $\bar{X} \pm SE$). In contrast, neither sex nor hour of day explained variation in FGM responses to handling or pharmacological challenges ([Table 1](#)).

Effects of captivity and trapping.—For nine of the 11 ground squirrels housed in captivity, fecal samples were obtained at both the time of capture in the field and at the end of the captive habituation period (9–11 days after capture). FGM levels at the end of the habituation period were significantly higher than those at the time of capture (paired *t*-test: $t_8 = -3.42$, $P = 0.009$, $n = 4$ males and 5 females; [Fig. 2A](#)), suggesting that captive housing was a stressor for the study animals. Free-living squirrels that were captured twice (~ 48 h between trapping events) had significantly higher FGM levels upon second capture (paired *t*-test: $t_{28} = -3.44$, $P = 0.002$, $n = 16$ males and 13 females; [Fig. 2B](#)), suggesting that capture was a stressor for these animals.

Effects of phenotypic traits and temporal rhythms on FGM levels.—Among free-living squirrels ($n = 136$ males and 145 females), we found no significant effects of sex or body mass on FGM levels ([Table 2](#)). In contrast, there was a significant effect of age, with adults exhibiting significantly higher FGM levels than juveniles ([Table 2](#); [Fig. 3](#)). FGM levels increased significantly across the course of the day and across the course of the field season ([Table 2](#)), indicating that production of FGMs in this species shows circadian and seasonal rhythmicity; the effect of sampling date was more pronounced among adults than juveniles ([Table 2](#); [Fig. 3](#)). Adding year as a random effect significantly improved the fit of the model ($n = 160, 120, \text{ and } 326$

Table 1.—Effects of adrenocorticotropic hormone (ACTH) and handling challenges on fecal glucocorticoid metabolite (FGM) levels in California ground squirrels (*Otospermophilus beecheyi*). Results are from a generalized linear mixed model (GLMM); significant terms are shown in bold. “Hour” indicates the time of collection, while the “Time” terms indicate the number of hours after exposure to a stressor. For categorical fixed effects, the level of the variable being compared is shown in parenthesis (e.g., for “Challenge,” handling is being compared to ACTH, and a negative estimate indicates that handling is associated with lower FGMs than ACTH). Significant terms indicated in bold.

	Estimate	SE	df	t-value	P-value
Intercept	0.17	0.39	9.52	0.44	0.67
Challenge (handling)	-0.38	0.08	198.83	-4.66	<0.00001
Time	0.15	0.07	198.02	2.22	0.03
Square of time ²	-0.14	0.10	198.16	-1.42	0.16
Sex (males)	0.36	0.52	9.00	0.69	0.51
Hour	0.03	0.04	198.25	0.77	0.44
Random effect	Variance	SD		X ²	P-value
Identity	0.73	0.85		175.04	<0.00001

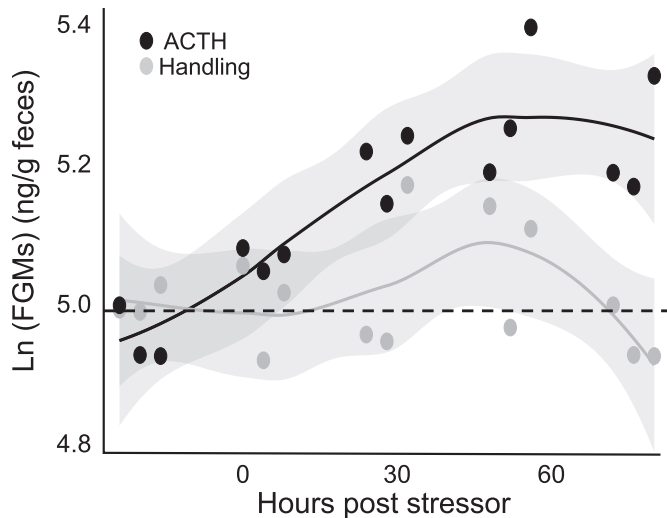


Fig. 1.—Ln-transformed concentrations of fecal glucocorticoid metabolites (FGMs) from captive California ground squirrels (*Otospermophilus beecheyi*) before and after handling (gray) and injection with synthetic adrenocorticotrophic hormone (ACTH; black). Both challenges took place immediately after the sample collected at time 0 (different 0 time points for each challenge). The dotted line indicates approximate average baseline FGM concentrations (calculated based on fecal samples collected in captivity after the habituation period but before any experimental manipulations). Points represent mean values for each treatment at each time, and gray shading indicates the 95% confidence interval. Both time since challenge and challenge type had significant effects on FGM levels (Table 1).

for 2013, 2014, and 2015, respectively). Adding animal identity did not affect model fit.

Effects of habitat disturbance.—Individuals from our human-disturbed ($n = 170$) and pristine ($n = 123$) sites displayed significant differences in FGM levels, body masses, and behavioral responses to capture. Specifically, animals from the more human-disturbed site exhibited significantly higher FGM levels (Table 2; Fig. 4A) and lower body masses (Table 3; Fig. 4B), even after controlling for age and sex. There was also a significant interaction between sex and study site, such that while both sexes had higher FGM levels in disturbed habitats, this pattern was stronger in females than in males (Table 2; Fig. 4A). Ground squirrels at the more human-disturbed site were also less behaviorally reactive to trapping: the proportion of times that each individual animal called and/or attempted to escape a trap was significantly higher at the pristine site (mean number of behavioral measures per individual \pm SE: 2.53 ± 0.10 ; Table 4; Fig. 5). Including animal identity significantly improved the fit of models for both behavior and body mass (mean number of measures of mass and behavior per individual \pm SE: 2.53 ± 0.10), and both behavior and body mass were repeatable within individuals (repeatability: 0.56 ± 0.04 SE for body mass, 0.19 ± 0.01 SE for behavior; Tables 3 and 4). Individual identity did not significantly improve model fit for FGM levels (mean number of FGM measures per individual \pm SE: 2.16 ± 0.09) and FGM levels were not repeatable within free-living individuals (repeatability: 0.02 ± 0.03 SE; Table 2).

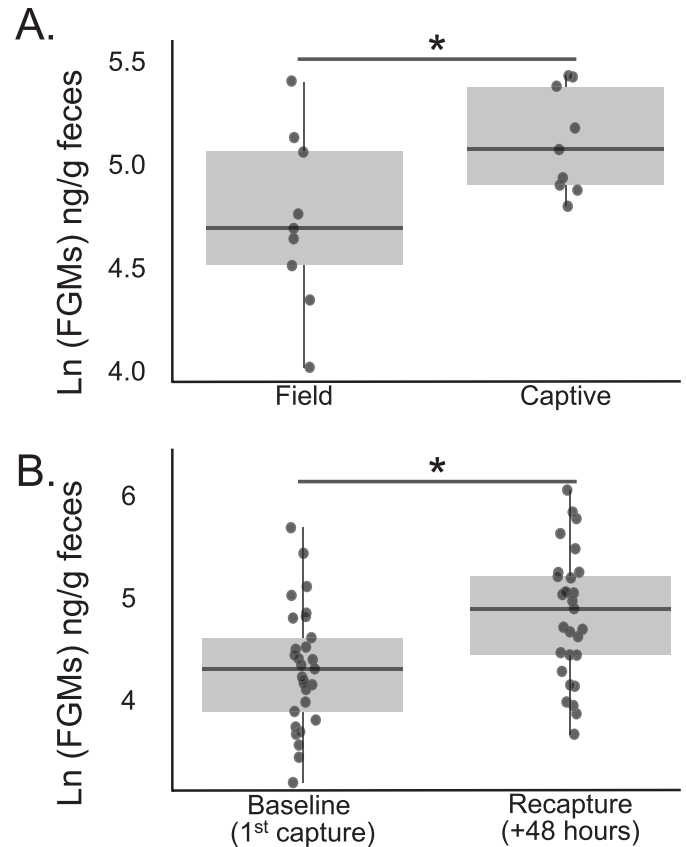


Fig. 2.—Fecal glucocorticoid metabolite (FGM) levels of California ground squirrels (*Otospermophilus beecheyi*) before and after (A) captivity and (B) trapping. (A) For captive animals, samples collected upon first capture of an individual in the field are compared to samples from the same animals at the end of the captive habituation period \sim 9–11 days later. (B) For free-living animals, samples collected upon the first capture of an individual in the field are compared to samples from the same animals upon recapture in the field \sim 48 h later. For the box and whisker plots, the central line shows the median value while the bottom and top edges of the box show the 25th and 75th percentiles, respectively. Whiskers extend up to 1.5 times the interquartile range. Individual data points are overlaid. Asterisks indicate that FGMs were significantly different between groups (paired t -tests, A: $t_8 = -3.42$, $P = 0.009$, $n = 9$ paired samples; B: $t_{28} = -3.44$, $P = 0.002$, $n = 29$ paired samples).

DISCUSSION

Assay validation and characterization of FGM response.—Our analytical, pharmacological, and biological challenges confirmed that EIA analyses of corticosterone concentrations provide reliable information regarding FGM levels in California ground squirrels. Specifically, handling, injection with synthetic ACTH, and laboratory housing each produced significant increases in FGM levels in captive animals, with handling also resulting in a significant increase in FGM levels among free-living animals. The time interval between injection with synthetic ACTH and detection of peak FGM levels ($\sim 11.6 \pm 9.7$ SE h) was longer than the 4–24 h response time documented for some rodents (Touma et al. 2003; Mateo and Cavigelli 2005; Bauer et al. 2008; Bosson et al. 2009; Soto-Gamboa et al. 2009;

Table 2.—Fecal glucocorticoid metabolite (FGM) levels in free-living California ground squirrels (*Otospermophilus beecheyi*) from a human-modified versus a more pristine habitat. Results are from a generalized linear mixed model (GLMM) testing for effects of environmental and phenotypic variables on FGM levels; significant terms are indicated in bold. “Hour” indicates the time of collection.

	Estimate	SE	df	t-value	P-value
Intercept	53	0.09	4.22	49.61	<0.00001
Colony (pristine)	-0.36	0.06	257.87	6.46	<0.00001
Day of year	0.33	0.03	568.93	10.99	<0.00001
Hour	0.05	0.02	592.72	2.17	0.03
Body mass	-0.06	0.04	464.02	-1.56	0.12
Sex (males)	0.07	0.07	386.46	1.02	0.31
Age (juveniles)	-0.22	0.07	487.01	-2.89	0.004
Colony * sex (males)	-0.21	0.09	333.31	-2.36	0.02
Day * age (juveniles)	-0.17	0.04	594.24	-3.72	0.0002
Random effects	Variance	SD		X ²	P-value
Identity	0.004	0.06		0.13	0.72
Year	0.02	0.12		8.23	0.004

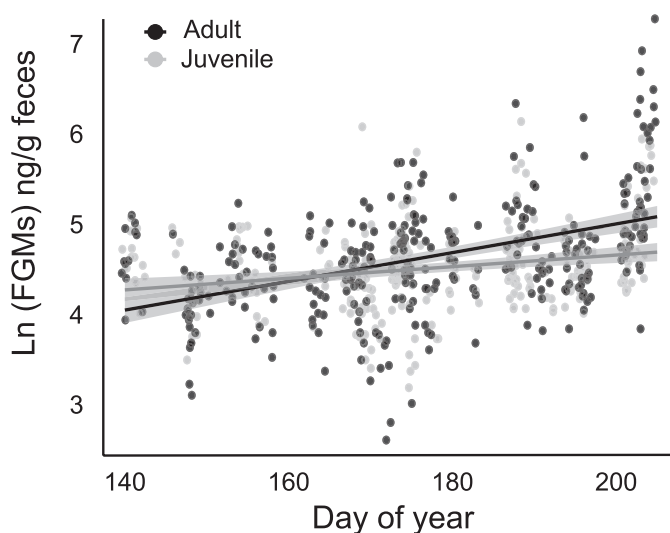


Fig. 3.—Relationships between fecal glucocorticoid metabolite (FGM) levels, age, and day of year for free-living California ground squirrels (*Otospermophilus beecheyi*). FGM levels for adult (black) and juveniles (gray) are shown as a function of day of year. Adults displayed significantly higher FGM levels than juveniles, with a significantly greater increase over the course of the field season (Table 2).

Danzter et al. 2010; Woodruff et al. 2010; Sheriff et al. 2012; Hammond et al. 2015). Our findings, however, are comparable to those for pharmacological challenges of Belding’s ground squirrels (*Urocitellus beldingi*) and yellow-bellied marmots (*Marmota flaviventris*), which resulted in peak FGM levels at 30–66 h and > 36 h after injection, respectively (Mateo and Cavigelli 2005; Smith et al. 2012). Although additional phylogenetically controlled studies are needed, it is possible that peak FGM response times vary across rodent taxa, with sciurids displaying particularly extended responses to exposure to stressors.

Inter- and intraindividual variability of FGM levels.—Our analyses of captive animals suggest that individual ground squirrels display repeatable differences in FGM levels in some contexts. For example, including individual identity significantly improved the fit of our model for FGM responses to handling and ACTH challenges. Further, among captive animals, we

found relatively high repeatability ($\sim 0.69 \pm 0.12$ SE) for individual identity, indicating pronounced differences among but considerable consistency within individuals (Taff et al. 2018). In contrast, FGM levels were not repeatable within free-living individuals. Repeatability in GC responses has been reported for other mammals (Pereira et al. 2006; Dantzer et al. 2010; Montiglio et al. 2012; Clary et al. 2014; Schoenemann and Bonier 2018) and is typically greater under more controlled, less dynamic captive settings (Smith et al. 2012; Dantzer et al. 2016; Hau et al. 2016; Baugh et al. 2017). Although the greater number of fecal samples collected for captive individuals may have contributed to the greater repeatability for the animals in our study (Wolak et al. 2012), it is also possible that this outcome reflects the more dynamic, unpredictable series of challenges associated with natural environments. Future studies that place greater emphasis on repeated sampling of free-living California ground squirrels should provide additional insights into the consistency of individual GC responses in natural settings.

Factors affecting FGM levels.—Fecal glucocorticoid metabolite responses to external challenges were influenced by multiple factors, including the context in which animals were exposed to stressors. For example, the relative effect of handling was greater for free-living than for captive individuals. This difference likely reflects the elevated baseline FGM levels detected among captive animals, which may have inhibited hypothalamic-pituitary-adrenal axis activity, thereby resulting in a reduced response to challenge (Busch et al. 2008). Although our handling and ACTH challenges resulted in significant increases in FGM levels, the magnitude of the ACTH challenge effect was relatively low in comparison to other studies (e.g., ~ 2 -fold increase in FGMs in this study versus ~ 2 - to 8-fold increases in Woodruff et al. 2010; Smith et al. 2012; and Hammond et al. 2015). This difference in relative response may reflect elevated baseline FGM levels in our study animals associated with their recent transition from the field to captivity. Differences in baseline GC levels in free-living versus captive animals have been reported for other species (Calisi and Bentley 2009; Woodruff et al. 2010; Danzter et al. 2016), underscoring the importance of the environmental context in which FGM levels are measured.

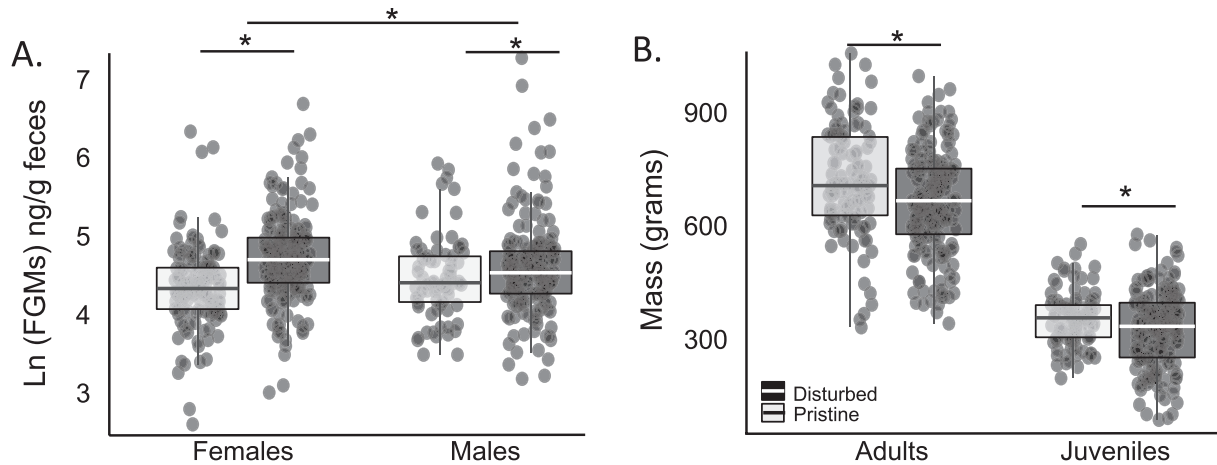


Fig. 4.—Relationships between human disturbance and (A) fecal glucocorticoid metabolite (FGM) levels and (B) body mass for free-living California ground squirrels (*Otospermophilus beecheyi*). Data are from pristine (left plot of each pair) and human-disturbed habitats (right plot of each pair). For (A) data are shown for females (left) and males (right), in (B) data are shown for adults (left) and juveniles (right). For the box and whiskers plots, central lines show median values, while the bottom and top edges of boxes show the 25th and 75th percentiles, respectively. Whiskers extend up to 1.5 times the interquartile range. Individual data points are overlaid. Asterisks indicate significant differences (Tables 2 and 3).

Table 3.—Masses of free-living California ground squirrels (*Otospermophilus beecheyi*) from a human-modified versus a more pristine habitat. Results are from a generalized linear mixed model (GLMM); significant terms are indicated in bold.

	Estimate	SE	df	t-value	P-value
Intercept	666.36	15.90	6.82	43.31	<0.00001
Colony (pristine)	37.52	11.58	404.91	-3.24	0.001
Day of year	-9.05	5.21	396.50	-1.74	0.08
Sex (males)	30.81	12.56	252.80	2.45	0.01
Age (juveniles)	-314.51	9.69	578.83	-32.45	<0.00001
Day * age (juveniles)	58.47	7.72	438.90	7.58	<0.00001
Random effects	Variance	SD		X ²	P-value
Identity	7,461.9	86.38		135.11	<0.00001
Year	350.4	18.72		6.57	0.01

Table 4.—Behavioral responses of free-living California ground squirrels (*Otospermophilus beecheyi*) from human-modified versus a more pristine habitat. Results are from a binomial generalized linear mixed model (GLMM). Significant terms are indicated in bold.

	Estimate	SE	df	P-value
Intercept	-0.14	0.39	-0.38	0.71
Colony (pristine)	1.24	0.22	5.52	<0.00001
Day of year	-0.16	0.12	-1.37	0.17
Mass	-0.17	0.18	-0.91	0.36
Sex (male)	0.11	0.21	0.51	0.61
Age (juvenile)	-0.81	0.36	-2.27	0.02
Day * age (juvenile)	0.02	0.18	0.12	0.90
Random effects	Variance	SD	X ²	P-value
Identity	0.83	0.91	17.21	<0.00004
Year	0.28	0.53	19.34	<0.00002

In this study, FGM levels in free-living animals tended to increase over the course of the day and, more notably, across the course of the summer (May–July). These findings are consistent with circadian and seasonal patterns of GC production reported for other mammals (Nunes et al. 2006; Dickmeis 2009) and likely reflect temporal variation in the physiological demands placed on members of these species (Holekamp

and Nunes 1989). FGM levels in our free-living study animals also varied with age, with adults displaying higher levels than juveniles. This outcome is consistent with those of classic laboratory analyses (Sapolsky et al. 1983) as well as studies of other free-living mammals (Creel et al. 2002; Smith et al. 2012; Rimbach et al. 2013a). It is also consistent with data suggesting that young animals are less attentive to risk than adults to predation risk, since reduced sensitivity to risk may also manifest in GC physiology (Owings and Coss 1977; Hanson and Coss 1997; Putman et al. 2015; Ortiz et al. 2019).

We found that California ground squirrels of both sexes exhibited higher FGM levels in disturbed compared to pristine habitats, with this pattern being stronger for females. This outcome is counter to the findings of Dantzer et al. (2014), who reported that it is generally males in free-living populations of mammals that show greater response to anthropogenic disturbance. Our data, however, appear to be consistent with the life history of California ground squirrels, in which females, particularly reproductive individuals, are more behaviorally responsive to risk in some contexts (Leger and Owings 1978). We did not, however, find sex-related differences in FGMs in other contexts. Although somewhat surprising, this outcome is

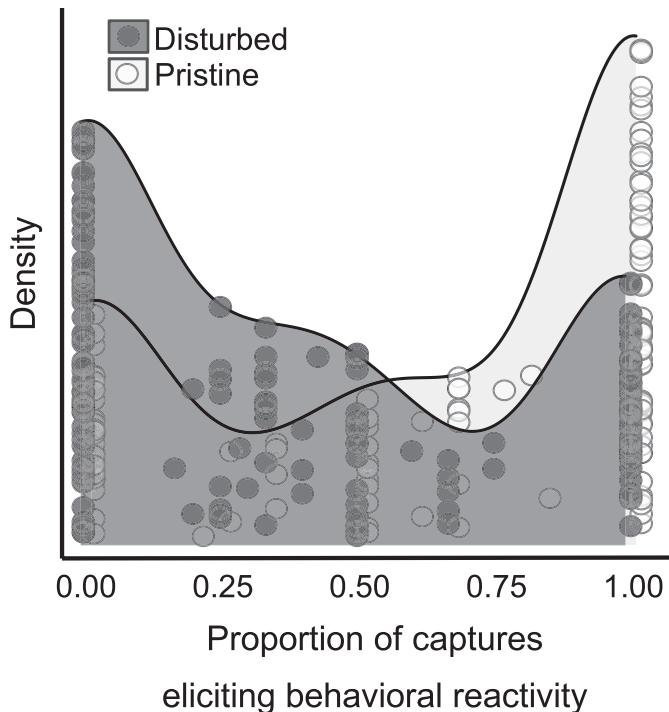


Fig. 5.—Relationships between human disturbance and behavioral responses of captured California ground squirrels (*Otospermophilus beecheyi*). Density plots depicting frequencies of the proportion of captures during which individual squirrels called or attempted to escape from traps (e.g., a value of 0.5 indicates that an individual displayed these behaviors one-half of the times that they were captured). On average, individual squirrels were captured 2.53 ± 0.10 times (mean \pm SE). Density plots are shown for a pristine (top) and a more human-disturbed (bottom) site. Frequencies of behavioral responses differed significantly between habitats (Table 4).

not unprecedented, as the effects of sex on FGMs levels vary among mammalian species (Kudielka and Kirschbaum 2005; Goymann 2012; Dantzer et al. 2016). Notably, our studies took place outside of the breeding season. Although studies conducted during the annual breeding season are required to understand fully the extent to which sex shapes stress physiology in California ground squirrels (Kenagy and Place 2000; Boonstra et al. 2001; Edwards and Boonstra 2016), we predict that sex differences in FGMs should be more apparent in samples collected during February–April, when the animals are actively breeding and caring for young. Collectively, these findings underscore the importance of characterizing the effects of multiple parameters on the GC physiology of a given species.

Effects of anthropogenic disturbance.—Human activity may have pervasive and sometimes unpredictable effects on wildlife (Beale and Monaghan 2004; Ciuti et al. 2012; Dickens and Romero 2013), leading to variable behavioral responses as well as changes in GC physiology (Walker et al. 2006; Van Meter et al. 2009; Kleist et al. 2018). We found significant differences in FGM levels, body mass, and behavioral reactivity between ground squirrels inhabiting our more human-disturbed versus our more pristine study site. These relationships were still evident after controlling for sex and age as well as annual,

seasonal, and circadian differences among individuals. Animals at the more human-disturbed site displayed higher FGM levels and lower body masses, suggestive of potentially negative responses to human activity. Animals at the more disturbed site, however, were also less behaviorally responsive when captured, suggesting that they may be more habituated to humans. Previous studies have demonstrated that antipredator behavior in California ground squirrels differs depending on whether animals are exposed to anthropogenic noise, supporting the assertion that this species responds behaviorally to human-related activities (Rabin et al. 2006).

At present, potential causal connections between elevated GC levels and reduced behavioral reactivity in animals from more disturbed sites remain unknown. Such relationships could apply within the lifetime of an individual, but also between generations, as maternal stress can shape offspring behavioral phenotypes (e.g., Hinde et al. 2015). Multiple studies have demonstrated links between GC levels and consistently identifiable patterns of behavior (i.e., behavioral syndromes) such as the tendency for animals that are more GC-reactive to be less bold when exploring novel settings (Carere et al. 2010; Hau and Goymann 2015). In the context of anthropogenic activity, many organisms show evidence of behavioral and physiological habituation to humans (Walker et al. 2005, 2006; McCleery 2009) and individuals with different behavioral phenotypes may be nonrandomly distributed across anthropogenically modified landscapes (Martin and Réale 2008). Our finding that California ground squirrels from more disturbed habitat display reduced behavioral reactivity appears to be consistent with these analyses. The relationship of GC levels to these reduced behavioral responses, however, is less clear. While previous studies have demonstrated decreased acute stress reactivity in animals from human-disturbed habitats (e.g., Walker et al. 2005, 2006), they have also revealed a consistent elevation of baseline GC levels associated with human disturbance (Dantzer et al. 2014). Future studies of California ground squirrels will explore causal relationships among GC levels, behavioral responses, phenotypic condition, and anthropogenic disturbance in greater detail.

Distinguishing the effects of human activity from those of other environmental factors can be challenging (Dickens and Romero 2013). As with previous studies that have employed similar methodologies (e.g., Van Meter et al. 2009; Jaimez et al. 2012; Nelson et al. 2015; Carlitz et al. 2016), the scope of our inference is limited given that data were obtained from only two locations. Consequently, we cannot distinguish the effects of human activity from other differences between study sites. Future studies that encompass additional field locations as well as comparisons with other mammal species occurring at the two study sites (e.g., Rimbach et al. 2013b; Rakotoniaina et al. 2016) will help to clarify the effects of human disturbance on the biology of California ground squirrels.

Because California ground squirrels often co-occur with humans, they have been presumed to be resilient to habitat perturbations and are often deemed a pest species (Swaigood et al. 2019). Due to their ability to persist in human-modified

habitats, these rodents provide a valuable system for exploring physiological and other nonlethal responses to anthropogenic activity. Our results suggest that California ground squirrels exhibit significant differences in GC physiology, body mass, and behavior in a human-disturbed environment. Whether the changes documented in our study are adaptive, allowing animals to make the best of suboptimal habitats (Boonstra 2013) is unknown. While chronically elevated GCs stimulated by anthropogenic factors are often considered maladaptive (Cyr and Romero 2007; Tennessen et al. 2014), such changes may reflect mechanisms for coping with environmental change that serve to increase fitness under challenging conditions. To address this issue, future work should explicitly explore links among anthropogenic disturbance, GC levels, and fitness. Given their persistence in human-modified habitats, studies of California ground squirrels that encompass multiple sites representing a range of degrees of exposure should generate important insights into the behavioral and physiological consequences of anthropogenic activity on an apparently human-tolerant species.

ACKNOWLEDGMENTS

We are grateful to the East Bay Regional Park District, especially to J. Miller and D. Bell, and to the California Department of Fish and Wildlife for their cooperation. C. E. Jang, L. M. Gearhart, H.-H. Lin, S. Y. Reed, and C. L. Working were enormously helpful in performing extractions and assays in the lab. We are also grateful to numerous members of Team Squirrel for help in the field. TTH was supported by an NSF graduate research fellowship. MV and CTB were supported by Jill Barrett Biology Research and Undergraduate Research Opportunity Fellowships from Mills College. JES received funding from the Undergraduate Education Program of the W. M. Keck Foundation, Barrett Foundation, Contra Costa Fish and Wildlife Propagation Grant, Joseph and Vera Long Foundation, Faculty Development Funds, Sandra S. Greer Scholar Award and from the Provost's Office at Mills College to support this research.

SUPPLEMENTARY DATA

Supplementary data are available at *Journal of Mammalogy* online.

Supplementary Data SD1.—Variation between sites in disturbance rates by humans and dogs.

Supplementary Data SD2.—Concentrations of fecal cortisol and corticosterone metabolites.

Supplementary Data SD3.—Parallelism of fecal glucocorticoid metabolites.

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Submitted 26 May 2019. Accepted 7 August 2019.

Associate Editor was Loren Hayes.