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Validation of a radioimmunoassay-based fecal corticosteroid assay for Richardson's ground squirrels *Urocitellus richardsonii* and behavioural correlates of stress

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Abstract We validated a radioimmunoassay-based method quantifying fecal glucocorticoid metabolites (FGMs) from captive male and female Richardson's ground squirrels *Urocitellus richardsonii*. Blood samples were drawn to explore the correlation between plasma cortisol and FGM concentrations. We also injected groups of squirrels with normal saline (CTL; control), adrenocorticotropic hormone (ACTH; stimulating adrenal activity), or dexamethasone (DEX; suppressing adrenal activity). Potential correlations between stress and behaviour were explored through quantification of fecal pellet production and the intervention necessary to elicit defecation, as well as the behaviour of subjects in the context of handling. Changes in plasma cortisol concentration between capture (baseline), and following handling (stress-induced) were also quantified for free-living squirrels. While glucocorticoid concentrations recovered from feces during our captive-animal study were not well correlated with plasma cortisol concentrations, and uncorrelated with defecation or behaviour, FGM concentrations did reflect the activation of the hypothalamic-pituitary-adrenal (HPA) axis. FGM concentrations increased significantly during initial captivity, but declined to baseline level as individuals acclimated to the novel environment. Injection of subjects with ACTH increased FGMs above baseline, confirming activation of the HPA axis. Plasma cortisol concentrations increased significantly with induced stress, indicating that capture and handling activated the glucocorticoid stress response even among previously handled, free-living subjects. Our findings validate a non-invasive tool that will afford new insight into the physiological processes underlying social, reproductive and antipredator behaviour of Richardson's ground squirrels [*Current Zoology* 60 (5): 591–601, 2014].

Keywords Stress, Corticosteroids, Fecal glucocorticoid metabolites, Radioimmunoassay, Richardson's ground squirrels

Social interactions within group-living species buffer individuals from change in the surrounding environment (Hare and Murie, 2007). Such interactions, however, along with challenges imposed by changing and unpredictable biotic and abiotic factors, impose stress, ultimately affecting animal growth, development, reproductive performance and behaviour (Boonstra et al., 2007; Creel et al., 2013; Wingfield 2013). While both behavioural and physiological mechanisms collectively referred to as "stress responses" promote stability in the face of change (allostasis: *sensu* Sterling and Eyer, 1998), repeated adaptation to adverse physical environments and psychosocial challenges imposes costs ("allostatic load" *sensu* McEwen, 1998; Wingfield, 2005), which, if severe enough, can deregulate physiological processes, promoting disease and increasing the individual's risk of mortality (McEwen and Wingfield, 2003; but see Boonstra, 2013).

At the physiological level, vertebrate stress responses involve the activation of the hypothalamic-pituitary-adrenal (HPA) axis and the production of both corticosteroids and catecholamines (Tsigos and Chrousos, 2002). During an acute stress response, glucocorticoids suppress less essential physiological processes such as gastrointestinal, reproductive and immune systems and enhance those that promote survival such as blood glucose availability (Rivier and Rivest, 1991; Wingfield, 2005). The physiological response typically coincides with enhanced vigilance, preparing the individual for possible danger (Monclús et al., 2005). While transient stress-induced increases in glucocorticoid levels impart benefits following acute exposure to a stressful event, they come at the cost of energy storage and thus may prove deleterious during periods of physical develop-

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ment or preparation for hibernation or migration, particularly if exposure to a stressor is chronic in nature (Mateo, 2007).

Measurement of fecal glucocorticoid metabolites (FGMs) as a non-invasive method to assess the physiological consequences of environmental or life history stressors is becoming increasingly prevalent in the literature (Bosson et al., 2009; Dantzer et al., 2012, 2013; Sheriff et al., 2010, 2012; Strauss et al., 2007). Where possible, it is essential to validate the techniques used as this will substantially increase the strength of inference that can be drawn from the data produced (Millspaugh and Washburn, 2004; Palme, 2005; Touma and Palme, 2005). Here we sought to validate our methods for extraction and measurement of FGMs using Richardson's ground squirrel *Urocitellus richardsonii*. We quantified FGM levels in feces (Harper and Austad, 2000; Wasser et al., 2000; Mateo and Cavigelli, 2005; Blumstein et al., 2006; Boonstra et al., 2007) relative to baseline levels in plasma samples. We examined the daily appearance of FGMs in individuals following administration of adrenocorticotropic hormone (ACTH; stimulating cortisol release from the adrenal glands), dexamethasone (DEX; inhibiting cortisol release from the adrenal glands through increased negative feedback on higher neural centres) or normal saline (CTL; sham-manipulated controls). To explore any relationship between the stress axis and aggression, as reported previously for rats (Kruk et al., 2004), marmosets (Smith and French, 1997) and timber wolves (McLeod et al., 1996), we quantified behaviour in the context of handling individuals in the three treatment groups. In that stress is commonly associated with defecation (Hare, 1998; Miyata et al., 1998), we also quantified fecal pellet production along with the extent of manipulation necessary to induce defecation by each subject in obtaining fecal samples. Finally we contrasted baseline versus stress-induced levels of plasma glucocorticoids in an independent group of free-living squirrels to determine whether our results for captive individuals accurately reflected those obtained from free-living Richardson's ground squirrels.

1 Methods and Materials

1.1 Validation: Captive animals

Ethics approval was granted by the Fort Garry Campus Protocol Management and Review Committee under Protocol F08-012/1. We live-trapped adult and yearling Richardson's ground squirrels (n = 24) at Winnipeg Manitoba's Assiniboine Park Zoo using Tomahawk live-traps (Tomahawk Live Trap Co., Tomahawk, WI, USA) baited with peanut butter on 8 July 2009, 26 days after the last juvenile had been weaned and emerged from its natal burrow that year. Our sample included 12 non-breeding males and 12 non-breeding females. This allowed for quantification of potential sex differences in stress responses (Touma et al., 2003), however, we were unable to accurately determine the age of individuals or if they had sired or given birth in that year's breeding season. Captured squirrels were ear-tagged through the right pinna for permanent identification with numbered metal ear tags (Monel #1, National Band and Tag Co., Newport KY, USA), weighed with a Pesola™ spring balance to the nearest 5 g, and transferred immediately to an indoor housing facility at the zoo. Squirrels were housed individually in polycarbonate "shoe-box" cages (38 cm × 33 cm × 18 cm) with pine shavings and shredded paper towels for bedding 23 h prior to any sampling, thereby attenuating any stress response induced by capture itself (Romero et al., 2008). In the holding cages, subjects were exposed to a natural photoperiod via translucent skylights in the holding room, and maintained at temperatures between 15.5 and 19.5°C over the course of the 19-day study (8–27 July). Individuals received a daily food ration of 25 g of mixed greens (leaf lettuce, raddichio, arugula) and 3 baby-cut carrots, and had *ad libitum* access to rodent blocks and water while in captivity. Squirrels were re-weighed on both the 6th and 14th day of captivity to verify their welfare, and ultimately test whether body mass differed among the treatment groups examined in the present study.

Following the 23 h acclimation period (on 9 July and daily each morning from 10–12 July thereafter), squirrels were removed from their cages in a gloved hand (as they are in the context of handling in the field), and placed in a clean shoe-box cage, where they were left for up to 1 h to produce fresh feces. From the 5th day of captivity (13 July on) rather than leaving squirrels in isolation, they were stimulated to defecate each morning through a series of escalating interventions which we recorded on an ordinal scale ranging from 1 through 11, including: 1) spontaneous defecation upon removal from the cage or initial placement in a clean shoe-box cage, 2) removal of the cage lid and approach with the gloved hand, 3) grasping of the squirrel with the gloved hand and lifting it from the cage floor, 4) rubbing the squirrel's dorsal pelage from the base of the head to the rump 3 times with the gloved hand while it remained in the cage, 5) grasping the squirrel with the gloved hand 3 times as the squirrel scrambled and thereby avoided the

grip. Where those interventions failed to result in defecation, squirrels were isolated in a clean cage for 5 min (intervention ordinal 6) followed by repetition of escalating interventions 2–5 described above (intervention ordinals 7–10), and finally, where necessary, 11) repeated rocking of the cage through 90° from horizontal for up to 1 min until feces were produced. After the first day of captivity, fecal samples were taken each morning between 0900 and 1030 hrs CDT to control for potential circadian variation in HPA activity (Coe and Levine, 1995; Raminelli et al., 2001, Reeder and Kramer, 2005).

In removing individuals from their cages from the second day of captivity (10 July on), we scored the maximum behavioural response of each subject on an ordinal scale ranging from 0 through 6 reflecting no response, through aversion and ultimately aggression, including: 0) no overt behavioural response to handling, 1) locomotory avoidance of handling, 2) alarm calling, 3) self-burying in wood shavings, 4) roll on back with forelimbs extended toward handler, 5) emission of a vocal squeal as approached, or, 6) biting the handler's glove as approached.

On the 10th day of captivity (18 July) we collected 0.3–0.4 ml of blood between 1000–1200 hrs CDT from the lateral saphenous vein of each subject using a sterile 1 ml syringe and 23 gauge needle, from which we could obtain plasma corticosteroid levels, and collected feces produced in the context of blood sampling, without recording intervention necessary to stimulate defecation or behaviour in the context of handling. From the point of removal from the cage to completion, blood sampling required between 4 to 15 minutes (8.4 ± 0.8 min; mean ± *SE*). From day 11 through day 14 (19–22 July), we continued our daily morning sampling regimen, collecting feces using the escalating interventions along with behavioural responses to handling.

On day 15 (23 July), individuals were assigned randomly to one of three treatment groups by drawing cage-card numbers with the proviso that each group included four males and four females that received subcutaneous administration of either: a) 200 µg/kg ACTH (n = 8), b) 2 mg/kg DEX (n = 8), or, c) 0.1 ml/kg 0.9 % saline CTL (n = 8). Both ACTH and DEX were administered in equivalent volumes of saline as the control group. The concentrations used in this study were based on a previous validation study for Belding's ground squirrels *Urocitellus beldingi* (Mateo and Cavigelli, 2005) that have comparable body mass to Richardson's ground squirrels. While a dose response to stimulation (ACTH) or competitive inhibition (DEX) of the endogenous production of glucocorticoids would have been ideal, these were not possible in the present study owing to the lack of experimental animals and housing limitations. Feces produced in the context of injection were collected. Fecal samples and behavioural scores were then collected each morning as above for the following 4 days, after which, squirrels were released at their site of capture (27 July). A summary timeline of experimental manipulation is included in Fig. 1.

Only freshly-produced feces, uncontaminated with urine or other foreign materials were collected from clean substrates using disposable wooden sticks throughout our study. Upon collection, feces were placed in plastic scintillation vials labeled with the date of collection and donor identity, and within 1.5 h, were transferred to a freezer at -20°C (Terio et al., 2002). Samples were only thawed when they were being immediately prepared for extraction and analysis (Khan et al. 2002). Blood samples were centrifuged for 3 min at 10,000 g to separate plasma from red cells and the plasma was then flash frozen in liquid nitrogen, and stored in a freezer at -80°C prior to extraction and analysis.

1.2 Extraction and RIA measurement of glucocorticoids from feces and plasma

Extraction and measurement of FGMs followed previously published protocols with slight modification (Mateo and Cavigelli, 2005). Briefly, samples were dried overnight at 60°C then approximately 0.2 g of dried and ground fecal matter was added to 1.5 ml 95 % ethanol. Samples were then mixed vigorously before centrifugation at 4°C for 10 min at 13, 000 g. Supernatant, the volume of which varied and was included in the final calculations, was removed and 100 µl was then transferred to an assay tube and ethanol was evaporated using a sample concentrator (Savant, Thermo-Fisher). The resulting pellet was re-suspended in RIA buffer (0.1 M phosphate buffer, 0.9% NaCl (w/v) and 0.5% bovine serum albumin (w/v)) immediately prior to measurement in a RIA.

Cortisol was extracted from plasma samples by solvent extraction as follows. 10 µl of plasma was added to 1 ml of ice-cold 95% ethanol. The sample was briefly vortexed and then centrifuged at 13,000 g for 5 minutes at 4°C. The supernatant was stored in a fresh tube and the pellet was re-suspended in 500 µl of 95% ethanol, briefly vortexed, and centrifuged as above. The two supernatants were combined and dried down in a sample concentrator and the resulting pellet was re-suspended in RIA buffer (as above) just prior to measurement.

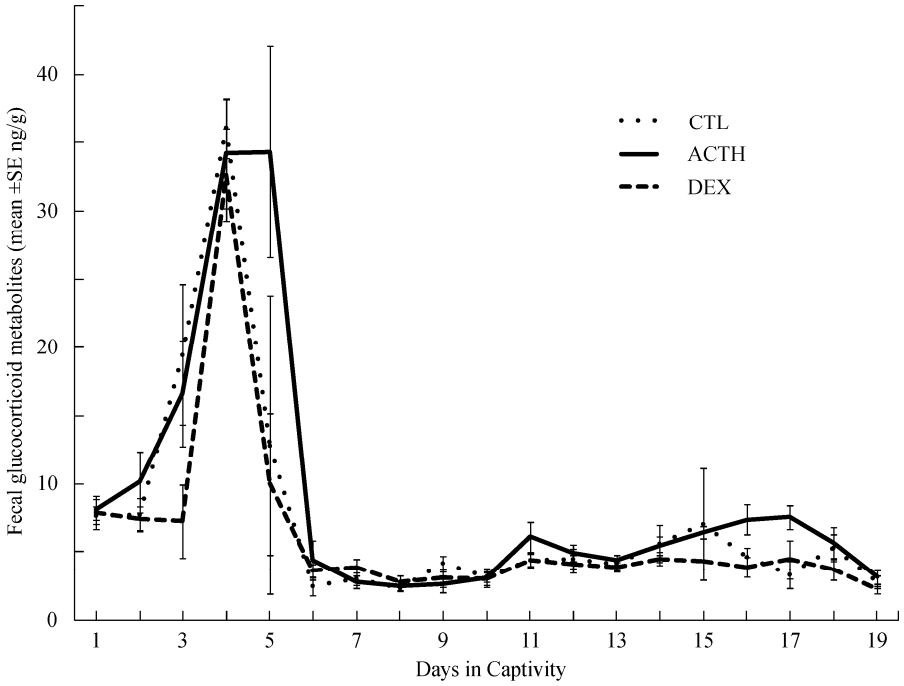

Fig. 1 Mean ± *SE* fecal glucocorticoid metabolite concentrations obtained from daily fecal sample collection over the 19-days squirrels were held in captivity, with manipulation times marked for blood sampling and injection of either saline (CTL), adrenocorticotropic hormone (ACTH), or dexamethasone (DEX)

The glucocorticoid assay was identical for both plasma and fecal material and followed a similar protocol as previously described (Ryan et al., 2012). Briefly, 100 µl of re-suspended sample was combined with 100 µl of cortisol-specific antibody (1:16,000 dilution; Fitzgerald Industries, NY, USA catalogue number 20-CR50) and 100 µl (5,000 dpm) of tritiated antigen (GE Healthcare, NJ, USA). Assay tubes were allowed to incubate at room temperature for 1 h, and then overnight at 4°C. Addition of 100 µl of dextran-coated charcoal to all assay tubes (0.5 % w/v dextran and 5 % w/v charcoal) followed by incubation at 4°C for 15 min terminated the assay. Tubes were then centrifuged at 4°C for 30 min at 2,500 g to separate bound from unbound ligand. The resulting supernatant was decanted into 7 ml scintillation vials and 4 ml of liquid scintillation cocktail (Ultima Gold AB, Perkin Elmer, MA USA) was added to each tube. Radioactivity was measured using a liquid scintillation counter (LS6500, Beckman Coulter) and glucocorticoid concentration for each sample was interpolated from standard curves using known concentrations of cold cortisol (Steraloids, Newport RI, USA). All samples were processed in duplicate and standards were processed in triplicate. According to the manufacturer cross-reactivity of the antibody used was; 5.7% for 11-deoxycortisol, 3.3% for corticosterone, 36% for prednisolone and < 0.7% for cortisone. Cross reactivity of the antibody used in this study with conjugated forms of cortisol were not available, nor were they determined.

Inter-assay and intra-assay variations for FGMs were 10.9 ± 6% and 15.3% respectively, with minimum detectable levels of 0.051 ng.ml^{-1}. Samples demonstrated good parallelism, and extraction efficiency was 90%, as determined via the addition of known amounts of tritiated cortisol (5,000 dpm) to fresh fecal material. Plasma samples for cortisol were run in a single assay, with intra-assay coefficients of 4.1%. All chemicals were purchased from Sigma-Aldrich Canada (Oakville ON, Canada) unless stated otherwise.

1.3 Statistical analyses

All statistical analyses were performed using Statistica™ 10 (Statsoft Inc., Tulsa OK, USA), with differences considered statistically significant where $P < 0.05$. Data uniformly met the parametric assumptions of normality (assessed via Kolmogorov-Smirnov tests), and homogeneity of variance (assessed via Bartlett's tests), and thus parametric tests were employed throughout, except in examining overall relationships between FGMs and defecation or behaviour where each subject squirrel contributed multiple observations to those data sets, and Spearman's rank-order correlation tests were employed.

1.4 Mass differences

We performed a repeated-measures ANOVA to test

for any sex difference, treatment group difference, or interaction between those factors on mass documented at capture, after 6 days of captivity, or after 14 days in captivity, treating body mass of individuals on those 3 days as a repeated measure. Where a significant difference was detected, we used Fisher's LSD tests to elucidate the exact nature of the difference.

1.5 Fecal glucocorticoid metabolites

Because subjects did not necessarily defecate on any given day of sampling, we could not perform repeated-measures analysis within subjects across each of the 19 days of captivity. Thus, we present a summary figure showing mean ± SE fecal cortisol concentration for each of the three treatment groups (ACTH, CTL, DEX) on each day, despite the fact that prior to injection on Day 15, members of those groups were not subject to differential treatment. We then used the emergent phases in the pre-injection cortisol concentration data (initial, early and mid-captivity; see Results) to pool daily values for subjects into means for those periods, and test for sex differences in the time course of any stress response to captivity prior to injection using repeated-measures ANOVA. For that analysis, data for one male ultimately assigned to the DEX group, and one female ultimately assigned to the CTL group were omitted, since those individuals did not produce feces during the initial two days of captivity, thus reducing our sample to 11 males and 11 females.

To test for an effect of the injection treatment and any sex difference in stress response to those, we used a repeated-measures ANOVA with injection treatment and sex as the main factors, contrasting the mean pre-injection FGM concentration in the 10 days up to and including the day of injection (mid-captivity) with the mean FGM concentration over the 3 days after injection (post-injection) as a repeated measure. All 24 subjects contributed data to this analysis, with 4 males and 4 females in each of the ACTH, DEX and CTL treatment groups.

1.6 Fecal versus plasma corticosteroids

To ascertain how FGM concentrations reflected circulating levels of corticosteroids in plasma, and the lag time for FGM to appear in feces, we contrasted the concentration of cortisol obtained from plasma on day 10, with FGMs on days 10 through 13 using simple linear regressions.

1.7 Fecal corticosteroids versus defecation

To determine whether the extent of intervention necessary to elicit defecation or the number of fecal pellets produced were associated with the stress experienced by subjects we examined the relationships between FGM concentration in feces recovered during each daily handling session from the 5th day of captivity through day 14 (excluding day 10 on which blood samples were drawn and day 15 when injections were administered as we took fecal samples opportunistically on those days), and both the number of fecal pellets produced and the ordinal level of intervention (ordinals 1 through 11, see above) necessary to obtain feces on that day. While all 24 individuals contributed multiple observations to these datasets, averaging either behavioural intervention necessary to obtain feces, fecal pellets produced or FGM concentration across days could obscure any meaningful correlation between those variables. Thus, to test for any relationship between FGM concentration and defecation, or between handling intervention necessary to obtain feces and FGM concentration, correlations were assessed using Spearman's rank-order correlation analysis for all subjects. Because elevated FGMs associated with escalating handling intervention are likely not detectable on the day of the handling experience itself, we also tested for any correlation between the ordinal level of handling intervention necessary to obtain scat, and FGM concentrations from subjects 24 and 48 hours subsequent to those handling episodes. Finally, we capitalized on the injection manipulation, testing whether the intervention level necessary to elicit defecation or the number of fecal pellets produced differed among the 3 treatment groups over the 3-days subsequent to injection using repeated measures ANOVAs.

1.8 Fecal glucocorticoid metabolites versus behaviour

We explored whether behaviour manifested in the context of handling was associated with subject stress by examining the correlations between FGM concentration and behaviour (scored ordinally from 0–6 as outlined above) from the 2nd day of captivity through day 14 (excluding day 10 on which blood samples were drawn and day 15 when injections were administered since behavioural observations were not recorded on those days). While all 24 individuals contributed multiple observations to these datasets, averaging corticosteroid concentrations or behavioural indices across days could obscure any meaningful correlation between those variables. Thus, correlations between FGM concentrations and the extent of aggression manifested in the context of handling were assessed using Spearman's rank-order correlation analysis for all subjects. To test whether behaviour manifested in the context of han-

dling differed among the 3 injection-treatment groups over the 3-days subsequent to injection, we used repeated-measures ANOVA.

1.9 Baseline versus stress-induced plasma glucocorticoid concentration: Free-living animals

The field-based component of our study was conducted under Fort Garry Campus Protocol Management and Review Committee Ethics Protocol F12-014, and involved 19 free-living female squirrels that had weaned a litter in 2013 from the same Assiniboine Park Zoo population as our captive study. These squirrels were live-trapped and ear-tagged as described above, and were marked earlier in the season on their dorsal pelage with human hair dye (Clairol Hydrience #52; Clairol Inc., Stamford CT, USA) for individual identification. Between 18 and 28 June 2013 they were live-trapped and as soon as possible after capture, a 0.25 ml blood sample was drawn from the medial saphenous vein(s) of each subject to obtain baseline plasma cortisol concentration. Subjects were then transported in their pillowcase-covered live trap to the on-site veterinary hospital to obtain an additional 0.25 ml blood sample allowing estimation of stress-induced plasma cortisol concentration. All samples were handled, stored, and processed to measure plasma cortisol concentration as described for captive animals above.

Baseline sampling commenced for the vast majority of subjects (14 of 19) within 3 min of capture, and was completed for 15 of 19 subjects within 5 min, though overall, baseline samples were obtained from 2.1–8.2 min post-capture. For 6 of 19 subjects, an insufficient initial blood draw necessitated a second draw from either the same or the contralateral medial saphenous vein. Thus, for the purposes of analysis, baseline samples were further divided into those obtained from the initial draw (Baseline 1: end time 2.1–7 min post-capture) versus those obtained from a second draw taken in the field within 1.3 min of initial sampling (Baseline 2: end time 3.7–8.2 min post-capture).

Drawing of stress-induced samples commenced at least 6.75 min after baseline sampling was completed, and thus ranged at the end of sampling from 9.2–19.5 min post-capture. One subject, however, escaped after her baseline sample was drawn, and had to be re-trapped for peak sampling, which was completed 51.9 min after her initial capture.

We tested for a relationship between plasma cortisol concentration and the latency from subjects being captured in live-traps to the commencement, and to the completion of baseline sampling, using simple linear regressions. For the six subjects that required a second baseline sample, we tested for a significant difference between the concentration of cortisol in the second versus first baseline sample using a paired-sample *t*-test, and whether the latency between capture and completing the draw of that second sample was related to plasma cortisol concentration with simple linear regression. We also tested for a significant relationship between the first and second baseline samples among those 6 individuals using simple linear regression.

We contrasted stress-induced versus initial baseline sample plasma cortisol concentrations using paired-sample *t*-tests either including all 19 subjects, or excluding the one subject that escaped and had to be re-trapped between baseline and stress-induced sampling. We also tested for any relationship between stress-induced cortisol concentration and the latency between initial capture and obtaining that sample using simple linear regressions including all subjects, or with all subjects except the individual that escaped between baseline and stress-induced sampling. With data limited only to the 18 individuals that were retained continuously between baseline and stress-induced blood sampling, we also tested for any relationship between the time elapsed between obtaining those samples, and the change in plasma cortisol concentration between the repeated samples using simple linear regression. The latter test was used to further explore how handling time might have affected changes in plasma cortisol.

2 Results

2.1 Validation: Captive animals

2.1.1 *Mass differences*

Repeated-measures ANOVA revealed no significant mass difference between the sexes ($F_{1,18}$ = 0.08, P = 0.78), between squirrels assigned to the ACTH, DEX or CTL treatments ($F_{2,18}$ = 3.10, P = 0.07), no significant interaction between sex and treatment ($F_{2,18}$ = 0.38, P = 0.69), no interaction between treatment and the pattern of mass change over the repeated weight measurements ($F_{4,36}$ = 1.73, P = 0.16), and no 3-way interaction between the sex of individuals, treatment and the repeated weight measures ($F_{4,36}$ = 1.27, P = 0.30). There was, however, a statistically significant difference in mass over the 3 weighing days ($F_{2,36}$ = 64.02, P < 0.01), with no significant change in mass (Fisher's PLSD, P = 0.59) from initial capture (281.9 ± 5.5 g) to day 6 (278.8 ± 6.3 g), and then a significant increase in mass (Fisher's PLSD, P< 0.001) from day 6 to day 14 (336.9 ± 9.4 g). We also detected a significant interaction between the

sex of individuals and mass change over the 3 weighing dates ($F_{2,36}$ = 5.15, P = 0.01), with males declining modestly in mass from initial capture (291.3 ± 7.8 g) to day 6 (283.3 ± 8.9 g) yet females holding steady (272.5 ± 7.8 *vs*. 274.2 ± 8.9 g), and then a less pronounced increase in mass from day 6 to day 14 for males (283.3 ± 8.9 *vs*. 328.3 ± 13.2 g) relative to females (274.2 ± 8.9 *vs*. 345.4 ± 13.2 g).

2.1.2 *Fecal glucocorticoid metabolites*

FGM levels varied considerably over the 19-day period of captivity, with concentration in the first two days of captivity (initial captivity) distinct from those on days 3–5 inclusive (early captivity), and from the relatively low concentrations of FGMs from days 6–15 (mid-captivity) leading up to the injection with ACTH, DEX or CTL on day 15 (Fig. 1). Repeated-measures ANOVA treating mean FGM concentrations quantified during initial, early, and mid-captivity as a within-subjects repeated measure revealed no significant sex difference ($F_{1,20}$ = 1.72, P = 0.20) between males and females throughout the overall pre-injection period (10.52 ± 1.04 *vs*. 12.45 ± 1.04 ng·g^{-1} respectively), and no significant interaction between sex and the repeated measure ($F_{2,40}$ = 2.13, P = 0.13), confirming that males and females did not differ in the time course of their stress response to captivity and daily handling. There was, however, a statistically significant repeated measures difference ($F_{2,40}$ = 70.56, P = 0.00), reflecting the precipitous increase and then decline in FGMs during early and mid-captivity respectively (Fig. 1), though post-hoc contrasts with Fisher's LSD revealed significant pairwise differences only for early captivity relative to both initial and mid-captivity, and not between initial and mid-captivity for either males or females.

Repeated-measures ANOVA contrasting FGMs in mid-captivity (days 6–15) to those obtained in the ACTH, DEX and CTL groups during the post-injection period (days 16–18) for male versus female subjects revealed no significant difference in FGM levels between males and females ($F_{1,18}$ = 0.81, P = 0.38). There was, however, an overall significant difference in FGM concentration among injection treatments ($F_{2,18}$ = 5.35, P = 0.02), with FGM levels of the ACTH group significantly higher than both the CTL (P = 0.02) and DEX (P = 0.01) groups, though surprisingly the CTL and DEX groups did not differ significantly (P = 0.57), and no significant interaction was detected between injection treatment and sex ($F_{2,18}$ = 0.62, P = 0.55). FGM levels also differed significantly between the mid-captivity and post-injection periods ($F_{1,18}$ = 8.08, P = 0.01), with higher levels overall in the post-injection period. A significant injection treatment x repeated measure interaction ($F_{2,18}$ = 5.46, P = 0.01), however, revealed that only the ACTH-treated group showed a significant increase in FGM concentration from the pre-injection to post-injection period, with Fisher's LSD tests confirming that only post-injection FGM levels in the ACTH-treated group were significantly higher (all P < 0.0004) than those FGM concentrations for all other treatment groups in both the pre- and post-injection periods (Fig. 2). A significant interaction was also detected between sex and time period ($F_{1,18}$ = 5.89, P = 0.03), with females showing a more pronounced rise in FGM concentration from the pre-injection to post-injection period than males (Fig. 2), though no 3-way interaction between sex, injection treatment type, and time period was evident ($F_{2,18}$ = 1.08, P = 0.36), so this difference does not confound the assessment of the injection treatment effect.

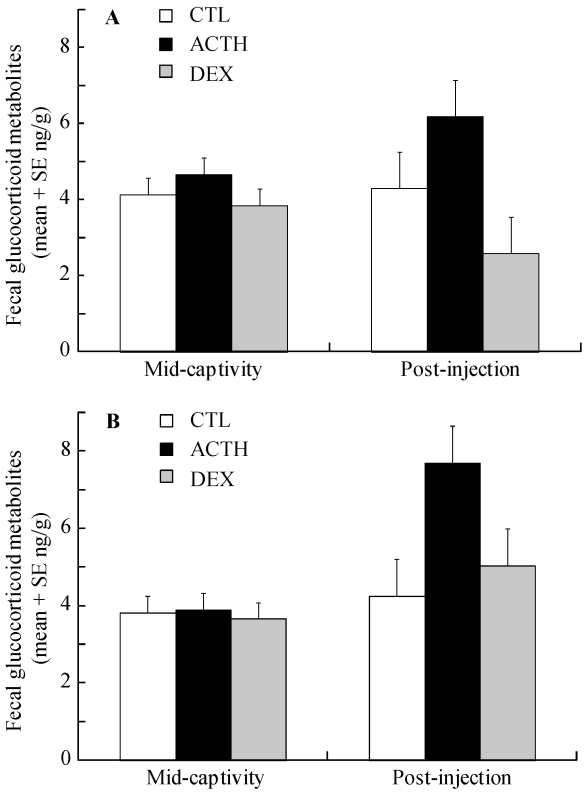

Fig. 2 Mean ± *SE* fecal glucocorticoid metabolite concentrations obtained from A) male, and, B) female Richardson's ground squirrels in the 3 treatment groups for the 10 days prior to injection (mid-captivity) and for the three days subsequent to injection with saline (CTL), adrenocorticotropic hormone (ACTH), or dexamethasone (DEX) in the captive validation study

2.1.3 *Fecal versus plasma corticosteroids*

Plasma cortisol concentrations from the blood samples drawn on day 10 were not correlated with FGMs recovered on that same day, or for up to 3 days after blood sampling (days 11–13; Table 1).

Table 1 Correlations between plasma cortisol concentrations (ng·ml^{-1}) assayed from blood drawn on day 10 and FGM concentrations recovered from feces (ng·g^{-1}) on the same day blood was sampled, and each of the 3 days thereafter (all $P > 0.14$) in the captive validation study

Sample Day	Relationship	*r*
Day 10	Positive	0.141
Day 11	Negative	0.164
Day 12	Negative	0.240
Day 13	None	0.003

2.1.4 *Fecal corticosteroids versus defecation*

There was no correlation between FGMs and the number of fecal pellets produced (Spearman's $r = -0.13$, $t_{169} = -1.69$, $P = 0.09$), nor between FGM levels and the ordinal level of intervention necessary to stimulate defecation (Spearman's $r = 0.12$, $t_{169} = 1.54$, $P = 0.12$) from day 5 through day 14 of captivity. Similarly, no significant relationship was detected between the ordinal level of handling intervention necessary to elicit defecation and FGM concentrations measured from fecal pellets produced 24 hours (Spearman's $r = 0.12$, $t_{147} = 1.41$, $P = 0.16$) or 48 hours (Spearman's $r = 0.08$, $t_{126} = 0.86$, $P = 0.39$) after handling.

The number of fecal pellets produced was unaffected by injection treatment ($F_{2,18} = 0.92$, $P = 0.42$), subject sex ($F_{1,18} = 0.32$, $P = 0.58$), or the interaction of those factors ($F_{2,18} = 0.88$, $P = 0.43$). Further, the number of fecal pellets produced did not change significantly among the 3 days after injection ($F_{2,36} = 0.58$, $P = 0.57$), and no interaction among the repeated measure and either subject sex or injection treatment was detected (all $P > 0.17$). Similarly, the intervention necessary to elicit defecation was unaffected by injection treatment ($F_{2,18} = 0.12$, $P = 0.89$), or subject sex ($F_{1,18} = 0.01$, $P = 0.93$), and no significant interactions among injection, sex and/or the repeated measure were evident (all $P > 0.16$). There was, however, a significant difference in the level of intervention necessary to elicit defecation across the 3 days post-injection ($F_{2,36} = 6.90$, $P < 0.01$), with a significantly higher level of intervention ($P < 0.01$) necessary on the third day (3.38 ± 0.37) relative to the second day (2.33 ± 0.34) following injection to obtain feces.

2.1.5 *Fecal corticosteroids versus behaviour*

FGM concentrations were not correlated with behaviour manifested in the context of handling during the pre-injection period (Spearman's $r = 0.01$, $t_{233} = -0.18$, $P = 0.86$). Further, repeated-measures ANOVA revealed no significant effect of injection treatment, subject sex, day since treatment or interactions among those variables on subject behaviour (all $P > 0.22$).

2.2 Baseline versus stress-induced plasma cortisol: Free-living animals

Neither the length of time between trapping and the beginning of baseline blood sampling ($F_{1,17} = 1.15$, $P = 0.30$), nor between trapping and the completion of baseline sampling ($F_{1,17} = 0.37$, $P = 0.55$) were associated with plasma cortisol concentration. For the 6 subjects where drawing a second baseline sample in the field proved necessary, second baseline cortisol concentrations (110.14 ± 16.68 ng.ml^{-1}) did not differ significantly from those measured in the initial baseline samples (104.99 ±10.65 ng.ml^{-1}; $t_5 = -0.85$, $P = 0.44$). Further, the time elapsed between initial capture and drawing the second blood sample was not significantly associated with plasma cortisol concentration in those second baseline samples ($F_{1,4} = 0.73$, $P = 0.44$). Indeed, plasma cortisol concentrations from the first and second baseline samples derived from those 6 individuals were significantly positively correlated ($F_{1,4} = 20.52$, $P = 0.01$).

As anticipated, plasma cortisol concentrations (161.04 ± 6.51 ng.ml^{-1}) in stressed individuals were significantly higher than initial baseline values ($t_{18} = -3.65$, $P = 0.002$). This remained the case when the one female that escaped temporarily between baseline and peak sampling (latency of 51.9 minutes from initial capture with a measured peak plasma cortisol concentration of 339.84 ng·ml^{-1}) was excluded from the analysis ($t_{17} = 3.84$, $P = 0.001$). Stress-induced and baseline plasma cortisol concentrations for free-living squirrels bracket plasma cortisol concentrations reported for subjects in our captive validation study, and those from previous studies of Richardson's ground squirrels (Delehanty and Boonstra, 2009).

While the relationship between the latency from trapping to sampling and stress-induced plasma cortisol concentration was statistically significant when data for that escapee were included in our regression analysis ($F_{1,17} = 35.30$, $P < 0.0001$), exclusion of that outlier's data revealed that the relationship was, in fact, non-significant ($F_{1,16} = 0.15$, $P = 0.70$). Individual changes between baseline and stress-induced plasma cortisol were unrelated to individual differences in time between

those two sampling points ($F_{1,16} = 0.44$, $P = 0.52$).

3 Discussion

Squirrels did not gain weight over their first 6 days in captivity, coincident with increasing FGM levels recovered from feces collected over the first 5 days of captivity. Diminished FGM levels from Day 6 on were accompanied by weight gain among subjects prior to injection on Day 15, so that no mass difference was evident among squirrels assigned to the three treatment groups. Therefore, variation in individual mass did not confound our assessment of other factors of interest in the present study.

Measured concentrations of FGMs recovered from feces in the captive-animal component of our study were lower than those reported for congeners in previous studies (Mateo and Cavigelli, 2005; Mateo, 2007), though plasma cortisol concentration was higher than that reported among congeners (Boonstra et al., 2001; Mateo and Cavigelli, 2005), and yet almost identical to the mean concentration reported in an earlier study of this same species (Delehanty and Boonstra, 2009). Correlations between FGMs and plasma cortisol concentrations were weak at best, even allowing up to 3 days lag time for FGMs to appear in the feces, which is peculiar given that FGMs typically track changing free cortisol levels in mammalian plasma (Sheriff et al., 2010). We did, however, experience considerable difficulty in drawing blood from our subjects in the captive study, such that it typically took in excess of 8, and sometimes up to 15 min to obtain the desired 0.3–0.4 ml of blood, well over the roughly 3 min time limit commonly cited as necessary to ensure baseline plasma levels are obtained (Kenagy and Place, 2000; Mateo and Cavigelli, 2005). Indeed, data from the field component of our study are not only in accord with the concentrations reported for our captive animals in the initial validation phase of our study, but reveal that trapping and handling activated the HPA axis, as reported previously for Richardson's ground squirrels by Delehanty and Boonstra (2009). Our data, however, reveal a significant increase in plasma cortisol levels from true baseline within roughly 9 min of initial capture and handling, indicating that plasma-based glucocorticoid concentrations reflect acute activation of the HPA axis. The volatility of plasma cortisol concentration in response to handling also reveals that handling itself constitutes an acute stressor (as demonstrated in eastern grey squirrels; Bosson et al., 2013), even for animals that have a long-term history of live-trapping and handling, as is the case for the subjects employed in the field component of our study.

The systematic rise and then fall to a steady baseline level in FGMs during early captivity suggests fecal cortisol concentrations do reflect the stress experienced by subjects, since stress levels are expected to rise when animals are placed in a novel environment and eventually decline once animals acclimate to their surroundings (Bosson et al., 2009; Vera et al., 2011). That said, it is unclear which aspect(s) of the captive experience precipitated these changes, as even dietary changes can have a pronounced influence on fecal steroid hormone metabolite concentrations (Dantzer et al., 2011). In that treatment with ACTH, however, resulted in a significant increase in FGMs, while neither CTL nor DEX-treated individuals showed any significant change in FGM concentrations over that same period, we can conclude that FGM concentrations reflect the level of activation of the HPA axis, which provides a time-integrated record of exposure to environmental stressors. The failure of DEX to promote significant reductions in FGMs likely reflects the fact that after 15 days in captivity, activation of the HPA axis was already at a minimum, and thus the dose of DEX used in the present study was not high enough to further suppress adrenal activity. It is also possible that the antibody used in the present study cross-reacted with a dexamethasone metabolite, thereby artificially raising the measured FGM levels. Cross reactivities between the antibody used and dexamethasone metabolites were not confirmed in the present study.

While we predicted that both defecation and the behaviour of subjects in the context of handling would be related to their current stress level as indicated by FGM concentrations, neither of these predictions was borne out. Clearly, the propensity of individuals to defecate along with their willingness to escalate their behaviour from escape, through alarm, to aggression is a product of a multiplicity of factors transcending recent time-integrated stress reflected in fecal glucocorticoid metabolites, as is the case for the integration of multiple stressors affecting FGMs of Arctic ground squirrels (*Urocitellus parryii*; Sheriff et al., 2012). Further, individual variability in the stress response underscores the fact that not all individuals find the same environmental challenges stressful (e.g. Love et al., 2013). The very fact that the extent of handling intervention necessary to elicit defecation had no apparent effect on subsequent FGM concentration in feces supports this assertion. It remains possible, however, that more frequent sampling could have detected handling-induced changes in FGMs,

as labeled cortisol has been shown to appear in fecal matter in less than 24 hours in a variety of mammals (Montiglio et al., 2012; Palme et al., 2005).

That fecal glucocorticoid levels follow a predictable time course as animals first experience and then ultimately acclimate to captivity, and increase significantly with stimulation of the HPA axis via ACTH injection, suggest that our methods are robust in resolving FGM concentrations reflective of underlying activation of the HPA axis associated with environmental stressors. Further, given the paucity of sex differences in the present study, it is apparent that these measurements apply meaningfully to both males and females. Taken together with our earlier finding that variation in FGM levels measured using the same methodology are correlated with adaptive variation in litter size and sex ratio (Ryan et al., 2012), our present results confirm that our methods will prove informative for future studies of stress, sociality, personality variation and reproductive performance of Richardson's ground squirrels.

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