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Validation of a fecal glucocorticoid assay for Steller sea lions (*Eumetopias jubatus*)

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Abstract

The Steller sea lion (*Eumetopias jubatus*) is listed as endangered in parts of its range and is suspected of suffering from ecological stressors that may be reflected by fecal glucocorticoid hormones. We validated a fecal glucocorticoid assay for this species with an adrenocorticotropic hormone (ACTH) challenge. Feces were collected from captive Steller sea lions (two males and two females) for 2 days before injection with ACTH, and for 4 or more days postinjection. Feces were freeze-dried, extracted with a methanol vortex method, and assayed for glucocorticoids. The assay demonstrated good parallelism and accuracy. All animals showed the expected peak of fecal glucocorticoid excretion after ACTH injection. However, the two males had higher baselines, higher peaks, and more delayed peaks than the females. Peak glucocorticoid excretion occurred at 5 and 28 h postinjection for the two females, and at 71 and 98 h for the two males. Correction for recoveries by the addition of tritiated hormones produced ACTH profiles that were virtually identical in pattern to uncorrected data, but with higher within-sample coefficients of variation. Based on these results, we conclude that this fecal glucocorticoid assay accurately reflects endogenous adrenal activity of Steller sea lions, and that recovery corrections are not necessary for this species when using the methanol vortex extraction method. More research is needed to address possible sex differences and other possible influences on fecal glucocorticoid concentrations.

Keywords: Steller sea lion; Pinnipeds; Glucocorticoids; Noninvasive techniques; Stress; Recovery corrections; ACTH challenge

1. Introduction

Fecal hormone analysis has recently become a common method for noninvasive studies of the physiology of free-ranging animals [1–9]. Feces of most vertebrate species contain metabolized forms of all the major steroid hormones (progestins, estrogens, androgens, glucocorticoids, and mineralocorticoids), which are secreted into the gut via bile. These fecal metabolites can be measured with slight modifications of standard extraction and hormone assay techniques. However, it is essential to validate fecal assay techniques before they enter wide use for a new species.

Fecal glucocorticoid analysis may be a particularly useful means of assessing the physiological status of pinnipeds such as the Steller sea lion (*Eumetopias jubatus*). The western population of the Steller sea lion, breeding in the Gulf of Alaska and Aleutian Islands, has declined since the mid-1970s and was declared endangered in 1997 [10–12]. Intensive research during the 1990s identified a number of ecological stressors that may limit population recovery, particularly a change in the quantity or quality of available food [10,13–15]. There is considerable speculation about what portion of the Steller sea lion population is at greatest risk as well as how such risk might vary with time of year. However, research into the population decline has been hampered by lack of cost-effective techniques to assess the physiological condition of free-living pinnipeds. Fecal hormone analyses offer a potential solution, since scat samples can be easily collected from terrestrial sites where sea lions rest (haul-outs and rookeries), and fecal glucocorticoids are known to reflect a variety of ecological stressors in terrestrial species [1,3–5,16–19]. When coupled with proper study designs, assessment of fecal glucocorticoid levels may also illuminate which ecological stressors have the greatest impact on free-ranging Steller sea lions. Thus fecal glucocor-

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ticoid analysis can potentially reduce costs and eliminate the need to capture pinnipeds for health assessment.

Fecal assays are best validated using fecal samples whose hormone content can be predicted a priori. For glucocorticoids, this is often accomplished with an adrenocorticotropic hormone (ACTH) challenge, i.e., injecting animals with ACTH, a pituitary hormone. In vertebrates, ACTH injection causes an immediate rise in secretion of endogenous glucocorticoids (cortisol or corticosterone) into blood, returning to baseline within a few hours. The same pattern also occurs in feces, although the onset of the peak in fecal metabolites is delayed by the species-specific excretion time. The fecal assay should detect a delayed increase in immunoreactive metabolites in feces following the ACTH injection [8,20–23]. If this peak is detected, the fecal assay may confidently be said to measure endogenous adrenocortical activity.

We performed ACTH challenges on two male and two female captive Steller sea lions to validate a fecal corticosterone assay for this species. To our knowledge, this is the first assessment of pre- and post-ACTH fecal glucocorticoid levels in a marine mammal. (One previous study did assay fecal glucocorticoid samples from the harbor seal, *Phoca vitulina richardii*, but feces were only collected pre-ACTH in this largely plasma-based study [24]). We also tested whether it is useful to correct for hormone extraction efficiency in fecal samples from Steller sea lions, given that fecal hormone metabolites may behave differently during extraction than the parent hormone typically used to monitor extraction efficiency.

2. Methods

2.1. Animals and housing

We studied two male and two female Steller sea lions housed at the Vancouver Aquarium Marine Science Centre (Vancouver, BC, Canada). All animals were hand-raised as pups and were habituated to humans. At the time of our study, Male 1 (M97KO) and Females 1 (F97HA) and 2 (F97SI) were subadults (3.5 years old) nearing reproductive age. Male 2 (M93TA) was 7.5 years old and was considered reproductively mature. All sea lions were fed herring with occasional pollock and squid, and a daily vitamin supplement. During the 6-day experiment, animals were housed in fenced, unroofed, individual dry runs with metal floor grates through which droppings fell for collection. Runs were monitored with surveillance video cameras, which recorded the exact excretion time and location of each fecal sample. As part of routine animal training and habituation, all sea lions at this facility typically spent some time each day in the dry runs beginning as pups and continuing throughout adulthood. They also experienced daily exposure to all other experimental equipment. Before our experiment, the sea lions were moved between dry runs and pools approximately two to three times per day, and time spent in the dry runs varied from a few minutes to several hours. The sea lions were motivated using positive reinforcement training techniques. All procedures were in accordance with all applicable local, provincial, and national animal welfare regulations.

2.2. ACTH injection and sample collection

ACTH (Vétoquinol; Lavaltrie, Quebec, Canada) was injected at a dose of 2 IU/kg, based on dosages used in other large mammals [21]. Injections occurred on 15 Sept 2000 (Female 1; body mass=117.3 kg), 18 Oct 2000 (Female 2; 132.9 kg), 1 Nov 2000 (Male 1; 199.8 kg), and 17 Nov 2000 (Male 2; 304.8 kg). Care was taken to minimize animal stress before and after injection, to reduce any endogenous changes in glucocorticoid secretions surrounding the time of injection. The sea lions entered the dry runs voluntarily, as they did during routine movements on other days. With the exception of the injection itself, all procedures used on the day of the ACTH injection were identical to routine procedures on all other days. It is possible that the sea lions may have experienced stress at the moment of injection, but if so, this should simply amplify the expected glucocorticoid peak. Note that each animal served as its own control in our study design of comparing glucocorticoid concentrations pre- vs. post-ACTH challenge.

Both females were healthy, active and ate normally during the study. However, Male 1 began refusing food 1 day before the ACTH injection and did not eat normally until 7 days after the injection. Consequently, he produced relatively few fecal samples (nine fecal samples before ACTH injection, and three after). His dramatically reduced defecation rate likely affected his fecal glucocorticoid concentrations. Male 2 had a mild ear infection on the day of the ACTH injection, and received a single dose of the antibiotic Baytril on that day, and two doses daily for the next week. The Baytril may have affected his gut flora. He did not eat normally on the day following ACTH injection, but was active and ate well on other days. It is common for healthy male Steller sea lions to fast one or more days between feeding trips; thus, occasional episodes of reduced food intake are not considered a health issue for captive male Steller sea lions (A. Trites, personal observation). Male 2's minor ear infection was a chronic condition that appeared to have little impact on his overall health. He was energetic, active, and fit, and was considered by Aquarium staff to be in good condition.

We collected all fecal samples from every sea lion beginning 2 days before injection (to establish baseline pre-ACTH levels) and continuing for a minimum of 4 days following injection. Sample collection was opportunistically continued beyond 4 days for the two males. Fecal samples were stored at −20 °C until processing. All samples were freeze-dried before hormone extraction to eliminate varia-

tion in water content and reduce hormone variation due to dietary fiber content [25].

2.3. Hormone extraction and assay

Following freeze-drying, all samples were sifted through a stainless steel mesh, mixed and subsampled twice to assess intrasample variation in hormone content (except for a single sample from Male 1 that was too small to subsample). The mesh did not remove any components from the feces because the fecal samples had a very fine-grained consistency, pulverizing readily and passing entirely through the mesh. Steroids were extracted by adding 2.0 ml of 90% methanol (10% water) to ~0.2 g of dried feces (weighed to the nearest 0.0001 g) and vortexing for 30 min, followed by centrifugation [21]. The supernatants (containing hormones) were stored at −20 °C until assay.

Extraction efficiency was 67.9±0.9% for corticosterone and 64.4±1.1% for cortisol (means±S.E.M.), based on the recovery of tritiated hormone from three samples taken from each of the four sea lions for both hormones. These extraction efficiencies were lower than those seen in other species with this extraction method [26]. However, variation in extraction efficiency was very low (within the range of normal intra-assay variation), enabling relative comparisons to be made across samples.

We assayed fecal glucocorticoid concentrations with a double-antibody ^{125}I radioimmunoassay kit (MP Biomedicals, formerly ICN Diagnostics, Costa Mesa, CA; catalog #07-120103). The primary antibody in this kit was raised against corticosterone, and also binds well to a variety of fecal metabolites of both corticosterone and cortisol in many mammal and bird species (see Ref. [21] for a detailed discussion of this antibody). The manufacturer's reported cross-reactivities for common plasma steroids are all below 1%. Specific cross-reactivities are: desoxycorticosterone 0.34%, testosterone 0.25%, cortisol 0.05%, aldosterone 0.03%, progesterone 0.02%, androstenedione 0.01%, 5α-dihydrotestosterone 0.01%, and <0.01% for all other tested steroids. We used the manufacturer's protocol except with half-volumes throughout. Nonspecific binding tubes and maximum-bound tubes were assayed in quadruplicate, and standards, samples, and controls were assayed in duplicate. Any samples that fell outside 15–85% on the standard curve or that had >7%CV between duplicate assay tubes were reassayed. Combined intra- and interassay variation for this assay was 3.6% for the manufacturer's low control and 9.2% for the high control. Serial dilutions of a pooled Steller sea lion fecal extract showed good parallelism; that is, the slope of the line for log [relative dose] against percent bound was not significantly different from the slope of the standard curve; t_8=1.11, P=.30 [27]. The assay also had good accuracy for Steller sea lion extracts diluted 32-fold. The slope of the true standard dose vs. the apparent dose of standards spiked with diluted fecal extract was not significantly different from 1.0; t_4=1.79, P=.15.

2.4. Recovery corrections

To assess whether correcting for extraction efficiencies improved fecal glucocorticoid profiles, we measured percentage recovery of tritiated hormone during extraction of each sample. We added 500 μl of tritiated corticosterone to the weighed fecal samples before extraction. Samples were left at room temperature for 1 h to allow the tritiated hormone to soak into the fecal powder, and then stored for 48 h at −20 °C before extraction. After extraction, we dried 500 μl of the methanol extract, reconstituted it in 500 μl distilled water, added 3.5 ml Ultima Gold scintillation cocktail (PerkinElmer, Boston, MA), and counted radioactivity for 10 min/vial using a Beckman LS6500 liquid scintillation counter with quench curve correction. Radioactivity in fecal extracts was converted to percent of the average total radioactivity added, and assay results were corrected accordingly. Finally, we compared corrected vs. uncorrected hormone profiles for the full set of ACTH challenge fecal samples from all four sea lions. Percentage hormone recovery was measured for each sample separately rather than using an average recovery value because of the need to assess inter- and intrasample variation as part of this test (see also below).

To further investigate the effect of recovery corrections on variation in fecal hormone data, we extracted and assayed five separate subsamples from each of 16 fecal samples (four from each animal's ACTH challenge, spanning a wide range of fecal glucocorticoid concentrations), measuring percentage recovery of tritiated corticosterone for each subsample as described above. We calculated two coefficients of variation (CVs) for each set of five subsamples—one for hormone data calculated with recovery corrections and one without. The resulting CVs for all 16 samples (with vs. without recovery corrections) were compared with paired t tests.

3. Results

3.1. Response to ACTH injection

All four animals exhibited pronounced increases in fecal glucocorticoid excretion following ACTH injection (Fig. 1). However, the timing of peak excretion varied. The peak for Female 1 occurred just 5 h after injection, and at 28 h for Female 2. The peak of Female 2 was also less pronounced than that of Female 1. Both males showed a long delay between ACTH injection and the peak of immunoreactive fecal hormones (71 h for Male 1, 98 h for Male 2). All animals showed additional peaks in fecal glucocorticoid excretion before injection. This was especially prominent in Male 1 at 48 h before injection. Male 2 also had a prominent secondary peak ~190 h after injection.

Differences were noted between sexes and individuals in the absolute values of fecal glucocorticoids. Both females

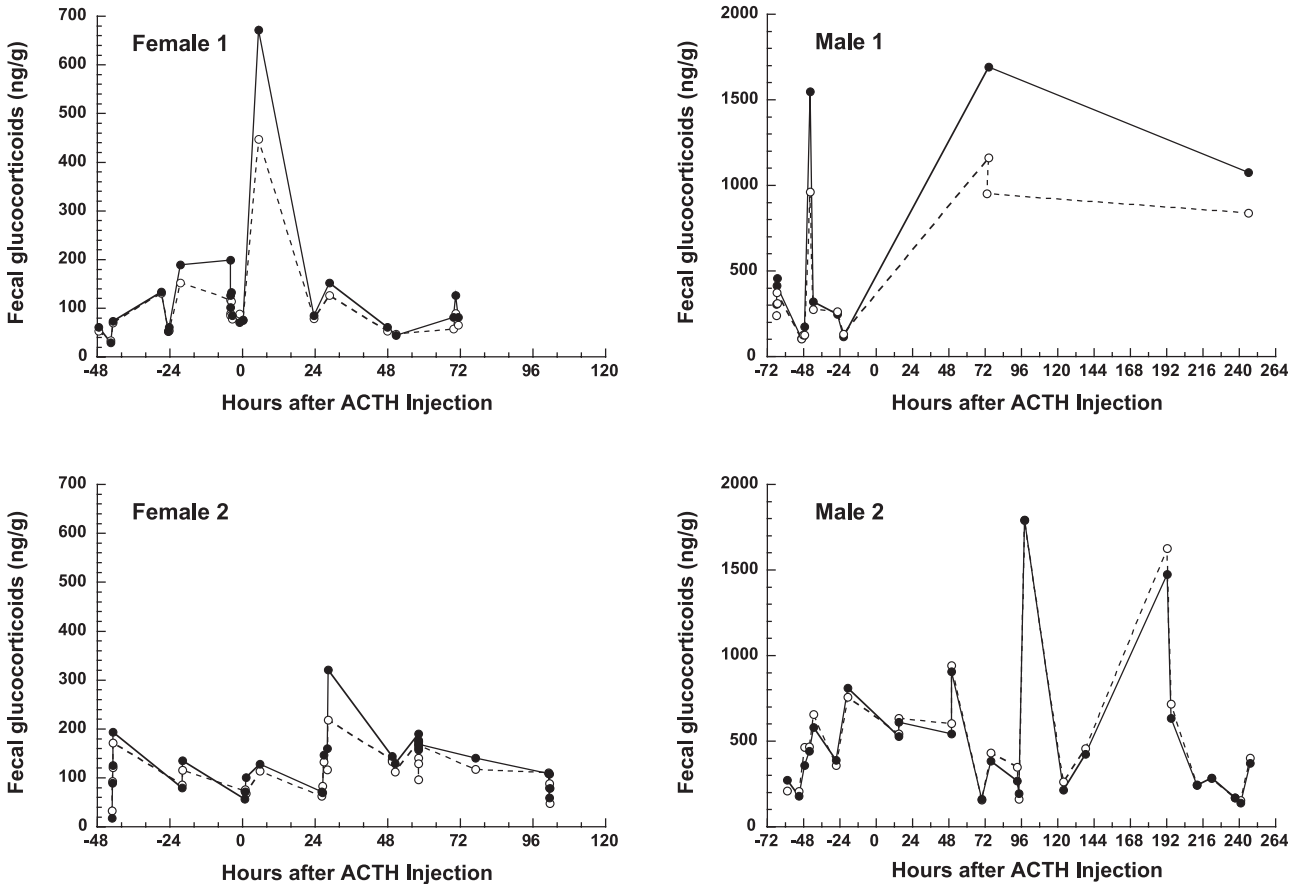

Fig. 1. Immunoreactive fecal glucocorticoid profiles before and after ACTH injection in four Steller sea lions. For each animal, results are graphed from two independent subsamples from every sifted, freeze-dried fecal sample. Note different scales of *x*- and *y*-axes for different animals. Values shown here are not corrected for extraction recoveries.

had lower baseline and peak values of fecal glucocorticoids compared to the males, especially Female 2. In some cases, male baselines were above female peak values (e.g., pre-ACTH levels of Male 2 compared to peak levels of Female 2). In general, Female 2 had the weakest response to the ACTH injection.

3.2. ACTH challenge results with vs. without recovery correction

ACTH challenge profiles were virtually identical when calculated with or without the correction for percentage of recovered tritiated corticosterone (data not shown), although absolute values were approximately doubled after correction for recovery. However, comparison of corrected vs. uncorrected values for 16 selected samples extracted in quintuplicate revealed that recovery corrections significantly increased intrasample variation in hormone values [paired *t* test, $t(19)=3.32$, $P<.01$]. Average % CV was $5.7\pm2.6\%$ without recovery corrections vs. $12.1\pm8.5\%$ with recovery corrections (see examples in Fig. 2). This effect was minor for half of the samples, with recovery corrections increasing variation by less than 5% or not at all (e.g., sample A from Male 2, Fig. 2). In contrast,

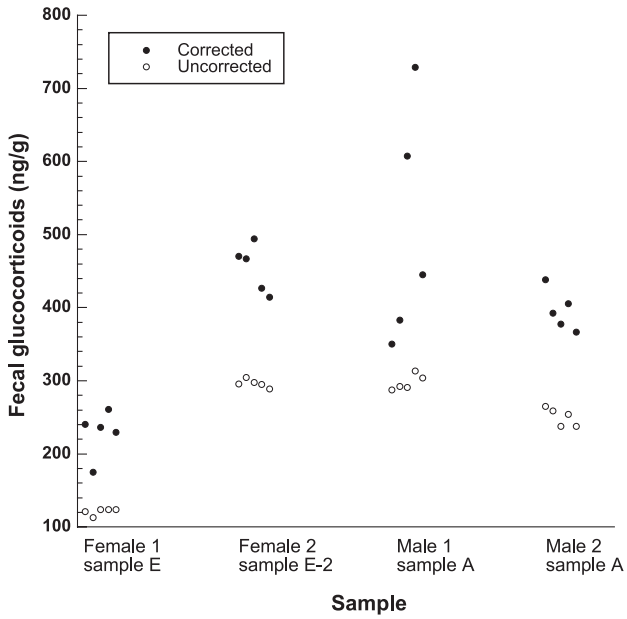

Fig. 2. Examples of intra-extract variation in fecal glucocorticoid concentrations of four fecal samples from captive Steller sea lions, with vs. without recovery corrections. Each sample was extracted in quintuplicate, with percentage recovery of tritiated corticosterone measured separately for each subsample.

recovery corrections increased CV by more than 5% in the other half of the samples, and in three samples by more than 20% (e.g., sample A from Male 1, Fig. 2). These three samples did not differ noticeably from other samples in appearance, odor, percentage recovery, darkness of extract, glucocorticoid concentration, or hours post-ACTH.

4. Discussion

Our results demonstrate that fecal glucocorticoid metabolites of Steller sea lions can be accurately measured in freeze-dried feces with a commercially available corticosterone radioimmunoassay kit. Assay results from all four sea lions showed the predicted peaks in hormone excretion after ACTH injection, indicating that the immunoreactive hormones measured by this assay reflect biologically meaningful adrenal activity.

Details of the ACTH profiles revealed interesting differences between individuals. Excretion peaks occurred earlier in the two females than in the two males (i.e., 5 h after injection for Female 1 and 28 h for Female 2, compared to 71 and 98 h, respectively, for Males 1 and 2). Both female peaks were comparable to known gut passage times of fish bones recovered from the same females' feces in a separate study [28]. Interestingly, the gut passage times of Female 1 were consistently faster than those of Female 2 in that study [28], which corresponds to our results. Finally, Male 2 also showed a secondary peak another 96 h after his first 98-h peak. A double-peak pattern has been seen in some other species after ACTH challenges (e.g., yellow baboon [21]), and may reflect enterohepatic recirculation of hormones [29].

The most likely explanation for the greater excretion lag times in the males compared to the females is that excretion time was affected by reduced food intake and defecation of Male 1, and reduced gut flora due to antibiotics in Male 2 [30]. However, it is also possible that these differences in peak timing may reflect a general difference between the sexes, possibly related to the substantial sexual dimorphism that exists among Steller sea lions and its corresponding influence on their digestive physiologies [31]. We cannot rule out the existence of secondary peaks occurring in the two females after 96 h. Sampling for females ended at 96 h, close to the time when pronounced peaks occurred in the males.

There were marked sex differences in baseline and in peak fecal glucocorticoid values, with both females generally having baseline glucocorticoids below 200 ng/g and peak values in the range of 200–700 ng/g. In contrast, the males' baselines were often over 200 ng/g, with some peaks over 1000 ng/g. Comparison to fecal glucocorticoid concentrations of wild Steller sea lions in a separate study revealed that the two females in our study had fecal glucocorticoid levels typical of wild Steller sea lions, while the two males had unusually high fecal glucocorticoid levels, even before ACTH injection (471 wild fecal samples collected 1999–2000, from multiple haul-outs, all seasons, and both sexes: mean fecal glucocorticoids ±S.E.M.= 148±12 ng/g; median=91 ng/g; range 4–4733 ng/g; A Trites, K Wynne, K Hunt, and S Wasser, unpublished data). This indicates that the two males in our study were possibly already stressed at the time of ACTH injection. However, the possibility of sex differences in fecal glucocorticoid levels in this species should still be further investigated as this could affect interpretation of glucocorticoid levels of wild populations. Further research is also needed on other factors that may affect fecal glucocorticoid levels in this and other pinniped species, such as effects of diet, diurnal and seasonal rhythms, and eating frequency.

4.1. Recovery corrections

Uncorrected and corrected hormone data produced virtually identical profiles of glucocorticoid elevation in response to the ACTH injection. For Steller sea lion feces, this indicates that it is not necessary to apply recovery corrections (when using the methanol vortex extraction method), particularly if data analysis concentrates on relative patterns rather than absolute values. Intrasample CVs actually became higher when data were corrected for recovery in our study, compared to very low intrasample CVs in the original uncorrected data. We suspect that this occurred because the fecal glucocorticoid metabolites likely have differing polarities from pure corticosterone [6,32–34], and thus probably behave differently during methanol extraction. The use of tritiated hormones as proxies for measuring extraction efficiencies is a technique originally developed for plasma samples, which contain hormones chemically identical to the tritiated hormones, i.e., pure corticosterone or pure cortisol. However, fecal glucocorticoid metabolites are often heavily modified from the original parent hormones [6,32–34], and so the use of tritiated parent hormones as proxies may actually introduce inaccuracies into the data.

The methanol vortex extraction technique we used is particularly simple, involving only one solvent, no drying or resuspension steps, and only one transfer between tubes. The simplicity of this method may account for the low intrasample variation we saw in the uncorrected data. However, thorough mixing and pulverizing of samples, as well as extracting and assaying samples in duplicate, may be essential to forego the need to correct for extraction efficiencies. More complicated extraction techniques may result in a larger and potentially more variable loss of hormones that may require corrections for extraction efficiency.

4.2. Summary

Our results show that endogenous adrenal activity of Steller sea lions can be measured noninvasively from freeze-dried feces with a straightforward methanol extraction technique and a commercial corticosterone radioimmunoas-

say kit. This paves the way for using noninvasive fecal glucocorticoid assays to address the role of nutritional stress and other physiological stressors in the decline and lack of recovery of the western population of the Steller sea lion. Further research is also needed on other factors that may affect fecal glucocorticoid levels, particularly effects of sex, diet, and season. This assay may also be useful in noninvasive physiological studies of other pinnipeds as well, though we recommend that similar validations be performed for each new species wherever possible.

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