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Fecal glucocorticoid metabolite analysis as an indicator of stress during translocation and acclimation in an endangered large mammal, the Grevy's zebra

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Abstract

Fecal glucocorticoid metabolite (FGM) analysis provides a non-invasive method for studying the physiological response of wildlife to a variety of stressors and is a ground-breaking monitoring technique in wildlife management and conservation. The conservation benefits of successful wildlife translocation restocking efforts are significant but understandably stressful for the animals being captured, removed from familiar habitat, held in captivity in many cases and subsequently released into an unfamiliar environment. It is imperative that we identify non-invasive methods for evaluating stress in translocated animals, especially in endangered species. Twenty Grevy's zebra *Equus grevyi* were translocated to Meru National Park as part of a Kenya Wildlife Service re-population initiative. FGMs were monitored from the time of capture, during captivity and post-release as an indicator of the stress of translocation and acclimation to the new environment. FGMs from representative non-translocated zebra were used as a further control. When held in pens at Meru Park 3–4 and 5–6 weeks after capture, the zebra had higher FGMs (25.1 ± 1.2 and 23.4 ± 1.3 ng g^{-1}) than either at the time of capture (14.6 ± 2.1 ng g^{-1}) or non-translocated controls (16.2 ± 1.2 ng g^{-1}). This suggests that the stress of captivity elevated FGMs. FGM concentrations returned to pre-capture concentrations *c.* 11–18 weeks after the zebra were released into Meru Park. The return of FGM concentrations to baseline suggests successful acclimation to the new environment. This study supports the use of FGM analysis as an assessment technique in wildlife management projects involving the movement of endangered large mammals with application for monitoring stress in a wide array of conservation projects involving translocation, reintroduction and rehabilitation.

Introduction

Several different approaches to assessing health have been employed in wildlife field studies. These have included measurements of body condition, immune status and responsiveness, physiological and metabolic variables, fitness and behavior and stress. Stress in mammals is a complex and multistage syndrome that is orchestrated by the sympathetic nervous system and glucocorticoids, a class of steroid hormones (Sapolsky, 2001). The activation of the sympathetic nervous system and the release of glucocorticoids comprise the stress response. Although short-term stress responses are thought to help an animal cope with adverse environmental conditions, long-term activation of the stress response can decrease health (Sapolsky, 1998). A common practical technique for assessing the stress response in animals is to monitor adrenocortical activity by measuring glucocorticoids and their metabolites in blood, saliva and body excretions. The measurement of glucocorticoid metabolites excreted in the feces allows for collection and analysis without handling the animals. This is especially important in large mammals and endangered species where repeated capture and handling is not possible. Another advantage of fecal analysis is that it minimizes interference with natural behaviors. Animals can be observed through binoculars from a distance, defecation locations noted and samples collected after they have moved away. Fecal glucocorticoid metabolite (FGM) analysis is an increasingly promising tool for use in wildlife field studies and conservation (e.g. Wasser *et al.*, 1997, 2000; Goymann *et al.*, 1999; Millspaugh *et al.*, 2001; Creel *et al.*, 2002; Dehnhard *et al.*, 2003; Mashburn & Atkinson, 2004). Although there are many advantages, there are also several confounding factors that need to be considered and minimized when applying

this technique in wildlife field studies and in investigating conservation issues (Millspaugh & Washburn, 2004; Palme, 2005; Touma & Palme, 2005; Keay *et al.*, 2006).

The focus of many wildlife conservation projects in recent years involves the translocation of free-living wildlife from areas of relative abundance to suitable locations and habitat as part of restocking efforts (e.g. Fischer & Lindenmayer, 2000). Capture, translocation and release into an unfamiliar environment are very stressful events. Studies in cattle and other domestic animals have documented physiological changes associated with stress during transport, including elevations in FGMs (e.g. Palme *et al.*, 2000; Morrow *et al.*, 2002). Increases in FGMs have also been observed in non-domestic species associated with the stress of capture/restraint and translocation (Goymann *et al.*, 1999; Terio, Citino & Brown, 1999; Dehnhard *et al.*, 2001; Turner, Tolson & Hamad, 2002). Monitoring the stress response in wildlife during translocation and acclimation to their new environment is critical for assessing the success of wildlife translocation projects. Animals that are chronically stressed are more susceptible to disease and unlikely to effectively reproduce (e.g. Sapolsky, 1998, 2001). Successful adaptation requires not only that the translocated individuals survive and thrive but that they go on to breed and raise offspring, ensuring species persistence.

Thirty years ago Meru National Park, Kenya, was considered one of the finest national parks in East Africa, filled with an abundance of diverse wildlife. Over the next two decades civil disruption, financial insecurity, inadequate protection, loss of habitat, poaching and land-use conflicts destroyed the park's wildlife diversity. One species that declined to non-viable numbers in the park was the Grevy's zebra *Equus grevyi*, a globally endangered species. In February 2002, Kenya Wildlife Service initiated plans to repopulate the park with Grevy's zebra as part of the Meru Park Restoration Project. The first phase of this plan was a pilot project to assess methods for translocation and evaluate success post-release.

As part of this pilot project, our objective was to develop and validate a radioimmunoassay (RIA) method for monitoring cortisol metabolites in the feces of free-living Grevy's zebra, although enzyme immunoassays have also been used effectively (Young *et al.*, 2004). Furthermore, we investigated FGM concentrations as an indicator of the stress of translocation and success of acclimation.

Materials and methods

Study site, translocation and study animals

In 2002, 20 Grevy's zebra were translocated from a privately owned park, Lewa Downs, located to the north of Mount Kenya, to Meru National Park located about 348 km northeast of Nairobi, 35 km east of Maua town in the northeastern lowlands below the Nyambeni hills. Before this translocation project there were believed to be only two Grevy's zebra in Meru Park, an adult female and her young male offspring. The translocation project followed the International Union for Conservation of Nature and Natural Resources Guidelines for Re-Introductions. Fifteen adult females, two territorial males and three bachelor males were selected and darted for capture during a 1-week period in February. Before release on 1 April they were held in pens for a total of 5 weeks, first at Lewa before transport and then at Meru. During the post-release period, one of the territorial males disappeared despite the presence of a radiocollar and the other territorial male was killed by lions. The body of one female was found following predation. One bachelor male and three females were not locatable. Through the end of the study period, 6 months following release, the 13 other individuals were consistently found in two groups, one composed of eight females with one of the young males and the other of three females with the other young male.

Sample collection

We identified individual zebra based on their unique, rear-end, stripe patterns (Ginsberg, 1988), opportunistically observed defecation and collected fecal samples. Samples were collected only when we were certain of the identity of the zebra and location on the ground. Figure 1 shows the sampling groups and time periods. When possible, at the time of darting and capture, fecal samples were collected after observation of defecation or by manual removal from

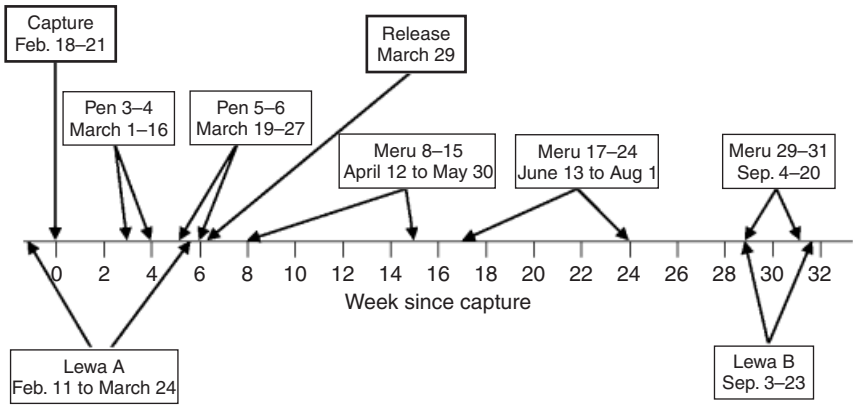

Figure 1 Timeline indicating sampling dates and groups. Each box represents a sampling period. The time of darting and capture was considered week 0. Capture, samples collected at the time of darting, reflecting pre-capture conditions. Lewa A and B, samples collected from random individuals at Lewa during two representative time periods to serve as controls. Pens 3–4 and 5–6, two sampling periods while in the pens. Meru 8–15, 17–24 and 29–31, three sampling periods post-release at Meru Park.

the rectum. These samples represent FGM levels before capture to serve as baseline. We collected samples while the zebra were held in pens at Meru Park before release. Opportunistic post-release fecal samples were collected following observation of defecation over a 6-month period when zebra were located and tracked. Although not all animals were represented in each post-release time period due to the opportunistic nature of sample collection, there was no systematic bias as to who we were able to collect from. Inspection of the data did not indicate any influential data points. In addition, as a control, representative samples were collected at Lewa from random individuals for comparison with the samples from the translocated animals at Meru Park. Samples were collected from territorial males, bachelor males and non-lactating females during two time periods, February–March, to ensure that baseline FGM concentrations from the translocated animals were representative of the Lewa population and to control for the stress of darting and capture, and in September to control for seasonal changes in FGMs in the translocated animals. Steroids are not evenly distributed in the feces of a number of different species (Brown *et al.*, 1994; Wasser *et al.*, 1996; Millspaugh & Washburn, 2003). To control for unequal distribution of hormones in feces, multiple small samples were collected from different portions of each defecation pile and combined. An alternative technique would have been to sample from a pre-homogenized defecation pile, but both techniques appear to be equally valid (Palme, 2005) Samples were kept in coolers on ice until stored in freezers the same day.

Sample processing, extraction and assay

To meet United States Department of Agriculture safety regulations, we heated samples for 15 min at above 60 °C before refreezing and importation to the United States. Although the impact of this particular heating process on fecal steroid metabolites has not been determined, all the field samples were subjected to this treatment so there should be no systematic bias. We processed all samples in the laboratory at Tufts University where they were lyophilized, mixed and sifted, then extracted following the method described in Wasser *et al.* (1994) with minor modifications: 0.2 g dried feces were boiled in 5 mL 100% ethanol for 20 min, centrifuged at 500 ***g*** for 20 min and the supernatant recovered. An additional 5 mL 100% ethanol was added to the fecal pellet followed by 1 min of vortexing and centrifugation. The combined supernatants were dried, re-dissolved in 1 mL methanol and diluted (1:8) in phosphate-buffered saline. The diluted extracts were assayed for FGMs using the MP Biomedicals (formerly ICN) ^{125}I corticosterone kit (Costa Mesa, CA, USA).

Validation

Although we recognize that there are many recommended tests to validate fecal glucocorticoid assays (e.g. Millspaugh & Washburn, 2004; Palme, 2005; Touma & Palme, 2005; Keay *et al.*, 2006), there are limitations on what tests can be performed on endangered species [as supported by Palme (2005) and Touma & Palme (2005)]. We performed as many of the recommended tests as were reasonable.

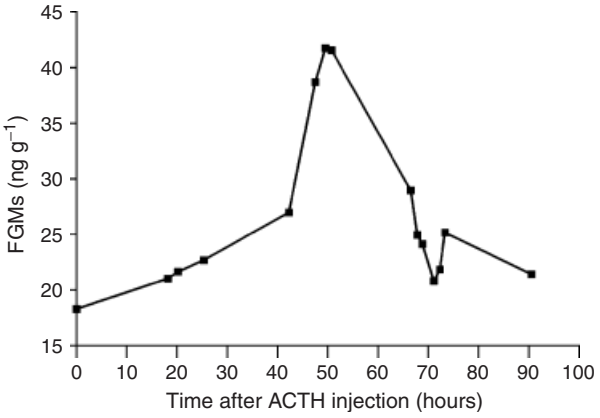

Figure 2 ACTH challenge on a captive, adult, female, Grevy's zebra *Equus grevyi*. One milligram ACTH was administered in a slow-release (ACTHAR) gel at time 0. Data points represent baseline and subsequent fecal sample collections. FGM levels were averaged when multiple defecations were on the pen floor at a collection time. ACTH, adrenocorticotropin; FGM, fecal glucocorticoid metabolite.

Both parallelism and accuracy were demonstrated for Grevy's zebra feces using the MP Biomedicals assay kit. The curve for serial dilutions of Grevy's zebra fecal extract pool plotted against per cent bound was parallel to the RIA standard curve (data not shown). We spiked equal aliquots of Grevy's zebra fecal extract pool with the assay standards containing increasing corticosterone concentrations. A linear regression of expected versus observed indicated that the assay was accurately measuring the corticosterone added to the fecal extract ($R^2 = 0.998$; data not shown). All samples were assayed in duplicate and concentrations are reported as ng g^{-1} of dry fecal matter. Samples were run in three separate assays. The intra-assay coefficient of variation for randomly selected samples was 1.3% ($n = 10$). The inter-assay coefficient of variation was 11.1 and 11.8% for two separate fecal-extract pools run with each assay ($n = 3$).

An adrenocorticotropin (ACTH) challenge was performed on a captive female Grevy's zebra at White Oak Conservation Center in Yulee, FL. One milligram ACTH was administered in a slow-release (ACTHAR) gel to ensure sustained adrenal cortisol secretion. A baseline fecal sample was collected at the time of injection. Subsequent samples were collected for 90-h post-injection by collecting all defecation piles on the pen floor at several collection times (indicated in Fig. 2). Multiple, representative, small samples were collected from different portions of each defecation pile and combined. Samples were stored frozen and assayed with the field samples. The FGM concentration for each collection time was graphed to evaluate whether the fecal assay detected the increase in adrenal cortisol secretion (Fig. 2, see 'Results'). For those collection times with more than one

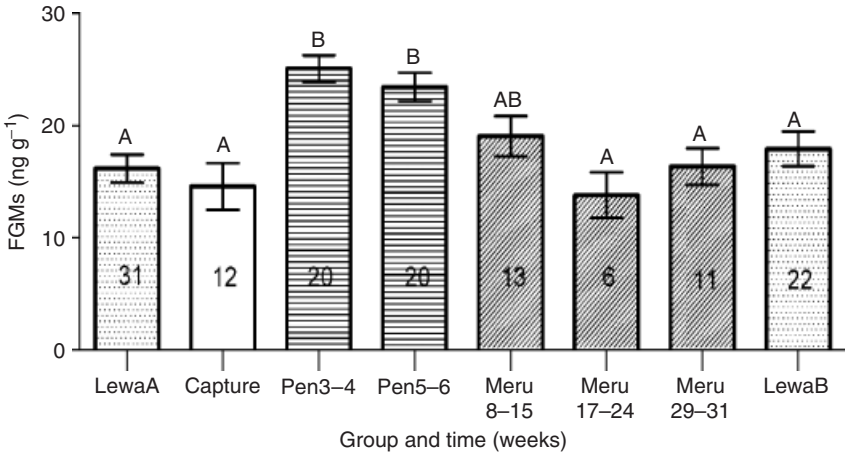

Figure 3 Comparison of FGMs between groups: before translocation, during captivity, post-release and Lewa controls within each time period. See Fig. 1 for timeline and group descriptions. Bars represent means ± SE for the sample sizes indicated. Sample sizes represent the number of individuals sampled in each time period. Bars with different letters are significantly different at the $P<0.05$ level using Tukey's *post hoc* tests. FGM, fecal glucocorticoid metabolite.

defecation pile, FGM values were averaged because the chronological order of defecations was unknown.

Baseline samples from the time of darting and the first sample collected from the same individuals while in captivity in the pens were compared by a paired *t*-test (see 'Results'). This comparison was made to determine whether the assay was sensitive enough to detect elevations from the stress of being held in captivity.

Analyses

There were no differences in FGM levels between representative territorial males, non-lactating females and bachelor males, as also shown in other studies (Lane, 2006), so these categories were pooled together for analysis of variance (ANOVA). FGM concentrations for the following groups were compared by ANOVA and Tukey's *post hoc* tests for pair-wise comparisons (see Fig. 1): before translocation (time of capture), two sampling periods while in captivity, three sampling periods post-release in Meru Park and two Lewa control sampling periods (time of capture and 6 months later during the last post-release collection period) (Fig. 3). If more than one fecal sample was collected from an individual animal within a time division, the mean for that individual was used in the analysis to avoid unequal representation of individuals.

Timelines were graphed for the five individuals (four females and one bachelor male) where sample data were available from the day of capture through the entire post-release study period (Fig. 4). This allows for visual assessment of FGM changes throughout the study period for individuals, the variability of isolated FGM levels and whether they are representative of treatment groups.

Results

FGMs gradually increased after ACTH administration, with a small elevation seen at the first collection period 18 h after injection. Peak levels were reached at about 50 h before gradually returning to baseline (Fig. 2).

Of the 12 individuals where a sample was collected at the time of capture, 11 showed a significant increase during captivity in the Meru Park pens compared with baseline (paired *t*, $t=-4.526$, d.f. = 11, $P<0.001$).

The grouped comparisons illustrate significant changes in FGM concentrations throughout the 31 weeks from capture to the end of post-release monitoring (ANOVA, $F_{7,131}=6.45$, $P<0.0005$; Fig. 3). *Post hoc* analysis showed that baseline levels for the translocated individuals are representative of the Lewa population and FGM concentrations from animals in the pre-release pens were elevated compared with either the pre-capture concentrations or the concentrations in non-translocated Lewa controls. After release, FGM concentrations decreased until they reached pre-capture concentrations, which were similar to Lewa controls for the same time of the year.

Figure 4 shows timelines for the five individuals where sample data were available from the day of capture through the entire post-release study period. There is considerable variability between single samples for each individual.

Discussion

In summary, FGM concentrations were elevated while Grevy's zebra were held in captivity, but after release FGMs returned to pre-capture levels over time.

The ACTH challenge demonstrated that an experimentally increased adrenal cortisol secretion can be detected in the feces of Grevy's zebra. In addition, FGM levels were detectably elevated by captivity following translocation, indicating the usefulness of the method for picking up stressful environmental changes (e.g. Palme, 2005; Touma & Palme, 2005). These findings are similar to those seen in white rhinoceros *Ceratotherium simum* and black rhinoceros *Diceros bicornis* where FGMs are elevated following restraint and translocation to a limited free-roaming (wildlife preserve) captive environment (Turner *et al.*, 2002). FGM concentrations in the translocated rhinoceros then decreased over the subsequent 4–6 weeks, suggesting gradual acclimation. Similarly, it is likely that FGM levels would have decreased in captivity over time in Grevy's zebra,

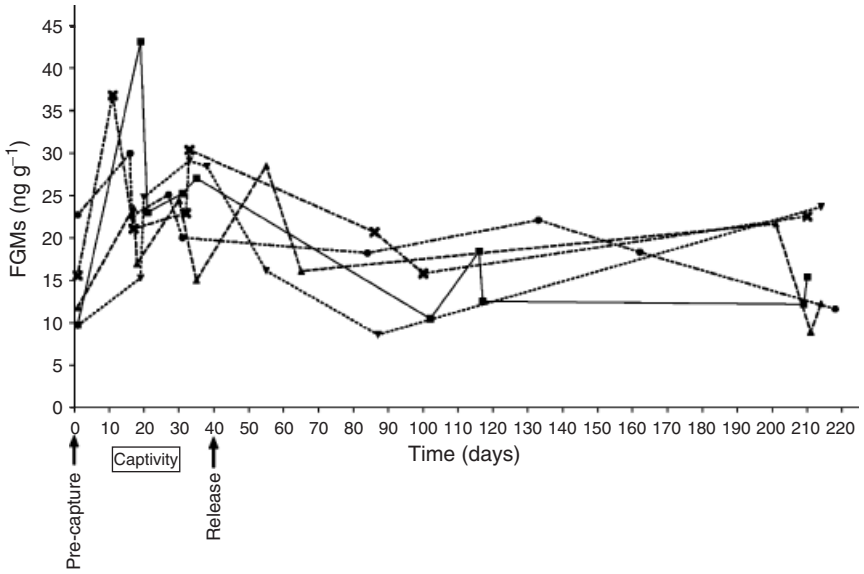

Figure 4 Timeline for the five individuals where data were available from the day of capture through the entire post-release study period. Day 0 is the day of darting and capture for that individual. The zebra were held in pens until release into Meru Park on day 40. There is considerable variability between single samples for each individual.

reflecting acclimation to the captive environment. To our knowledge, this is the only investigation where zebra FGMs were monitored following capture and placement in a captive environment. Both the ACTH challenge results and elevations in captivity help validate the ability of the assay to detect physiological and biologically important changes in adrenocortical activity and demonstrate its usefulness as an index of stress in Grevy's zebra.

Baseline FGM levels for the translocated individuals were representative of the Lewa population and were indistinguishable from Lewa controls both at capture and several weeks after release in the new habitat. Seasonal differences in glucocorticoid levels have been documented in a number of wildlife species but very little work has been done in large wild mammals (e.g. Romero, 2002). Measurement of FGMs in Lewa controls from the two different times of year was important to control for possible seasonal changes in FGMs during the study period. Results indicate no detectable seasonal changes between the two time periods (Fig. 3).

There was considerable variability between individual samples (Fig. 4). This indicates that relying on individual samples could yield false results as shown for other species (Scheiber, Kralj & Kortrschal, 2005). Single samples may not be representative of the overall condition that necessitated our grouping of multiple samples from each individual.

FGM concentrations returned to pre-capture levels by 11–18 weeks following release, suggesting that the zebra acclimated fairly rapidly to the new environment (Fig. 3). If the zebra had not acclimated and the Meru Park environment was persistently stressful, we would expect to see it reflected in their FGM concentrations. For example, higher FGMs are associated with increased snowmobile activity in elk *Cervus elaphus* and wolves *Canis lupus* and with proximity to clear-cutting logging practices in northern spotted owls *Strix occidentalis caurina* (Wasser *et al.*, 1997; Creel *et al.*, 2002). Although adrenocortical activity is a good indicator of physiological acclimation, it alone does not provide enough information about the long-term survival probability of translocated animals. However, animals with compromised physiology are unlikely to cope with environmental stressors adequately, ultimately impacting fitness and survival.

Elevated FGM concentrations in the Meru pens show that the soft phase pre-release approach to translocation does little to alleviate stress and is actually very stressful. Because FGM concentrations decreased after release, a quicker release approach may be less stressful. On the other hand, captivity before release allows health screens, time for long-acting tranquilizers to wear off, and adjustment to local diet if provided while in the pens. In any translocation operation, it is important to carry out a cost benefit analysis. FGM measurement and evaluation can help guide translocation methods by identifying the least stressful approaches.

FGMs have been measured in both captivity and the field in a variety of wildlife studies on a diversity of topics such as social interactions and status, anesthesia, capture and transport, environmental enrichment, radio-transmitters, logging and snowmobile activity (e.g. Wasser *et al.*, 1997; Whitten *et al.*, 1998; Boinski *et al.*, 1999; Goymann *et al.*, 1999; Creel *et al.*, 2002; Dehnhard *et al.*, 2003; Wells *et al.*, 2003; Sands & Creel, 2004). FGM analysis has potential as a tool for monitoring adrenocortical activity as an indicator of stress under a variety of conditions in Grevy's zebra with considerable management implications. It is a promising tool for assessing and optimizing captive environments, evaluating procedures for transport and handling and monitoring the health of wild populations. It should thus be a useful complementary technique to other physiological and behavioral measures of stress (reviewed in McLaren, Bonacic & Rowan, 2007) in behavioral and ecological studies in this species. This study will hopefully help initiate and guide protocols for monitoring stress in a wide array of wildlife conservation projects involving translocation, reintroduction and rehabilitation.

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