A technique for non-invasively detecting stress response in cougars

Frances Bonier, Howard Quigley; and Steven N. Austad

Abstract The ability to non-invasively monitor stress hormone levels in free-ranging animals could significantly aid in conservation and management efforts. Our objective in this study was to demonstrate the effectiveness of assay of fecal corticoid metabolites in detecting a stress response in cougars (Puma concolor). Fecal samples were collected from 9 captive cougars before and after an artificial stressor. Steroid hormones were extracted from the samples. Adrenal corticoid metabolite concentrations of the resulting extracts were quantified using cortisol and corticosterone assays. Results indicated that fecal glucocorticoid metabolite levels increased significantly 24 hours after the stressor in 6 of the 9 individuals. Behavior correlated with the hormonal response; all cougars that displayed a flight response to the stressor also had elevated stress hormone measures. Elevated levels of stress hormones were not observed in individuals that did not attempt to flee. We have demonstrated with this study that measurement of fecal hormone metabolites is sufficiently sensitive to detect an adrenal response to stress in cougars and could be applied in the field to monitor stress levels in free-ranging populations.

Key words cougar, glucocorticoids, non-invasive measures, Puma concolor, stress response

The link between chronic or repeated activation of the stress response and detrimental effects on multiple aspects of an animal’s health has long been established (Selye 1946). Chronic stress can have serious negative impacts on reproductive, digestive, and immune systems (Sapolsky et al. 2000). Chronic stress has been cited as the cause of low reproductive rates in captivity (e.g., in captive Felis species, Mellen 1991) as well as in the field (e.g., in snowshoe hares [Lepus americanus] Boonstra et al. 1998). It also has been linked to compromised immune function in domestic livestock (e.g., in cattle [Bos taurus] Blecha 2000), in captive animals (e.g., in the common lizard [Lacerta vivipara] Oppliger et al. 1998), and in wild populations (e.g., in bighorn sheep [Ovis canadensis] Spraker et al. 1984). The adrenal response is so sensitive that even the anticipation of a stressor can result in elevation of circulating stress hormone levels (Moberg 2000). These facts should make the stressors that affect a population a serious concern for those interested in conservation and management of wild populations, or maintenance of captive animals.

Glucocorticoids, such as cortisol and corticosterone, are released in response to internal and external challenges to homeostasis (Moberg 2000). Circulating hormone levels can fluctuate rapidly and also exhibit a circadian rhythm (Atkinson and Waddell 1997). Plasma hormone measures quantify the circulating level of the hormone at the instant blood was sampled; therefore, to examine an animal’s hormonal profile over time, several samples must be taken. Most importantly, because of the sensitivity and speed of the adrenal response, the stress of restraint and handling associated with obtaining a blood sample can result in an elevated glucocorticoid level (Sabatino et al. 1991, Le Maho et al. 1992). Non-invasive measures are increasingly being employed to monitor levels of reproductive and

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stress hormones in captive (Brown et al. 1994, Harper and Austad 2000) and free-ranging animals (Creel et al. 1997a, b; Foley et al. 2001; Millsbaugh et al. 2001; Creel et al. 2002). Fecal sampling methods have many advantages over more invasive techniques, particularly in the study of stress hormones. Non-invasive measurements of steroid hormones in feces overcome the drawbacks of plasma hormone measures. Fecal measurements are not associated with iatrogenic stress, as the animal does not need to be handled to obtain a sample. Also, because hormones are excreted at a rate slower than that at which they are secreted into circulation (Vylitová et al. 1998), fecal hormone measures integrate variation in circulating hormone levels over a longer period of time. Development of non-invasive methods for use in monitoring the stress response in large carnivores could be an invaluable tool for conservation biologists and managers who in the past have relied on sparse and unreliable data when studying rare, elusive, and intractable species. Before these methods can be applied to free-ranging animals, however, their sensitivity and accuracy in measuring the stress response should be validated.

The cougar is the widest-ranging terrestrial mammal in North and South America, occupying a wide variety of landscape types (Culver et al. 2000). The cougar has recently drawn public attention due to reported increase in conflicts with humans and their pets and livestock (Beier 1991, Danz 1999). However, much less study has been devoted to cougars as compared to other large North American carnivores, so relatively little is known about them (Kellert et al. 1996, but also see Logan et al. 2001). Non-invasive hormone measures can be used to monitor reproductive activity (Brown et al. 1994) and stress levels, increasing our understanding of this species.

We examined the sensitivity of non-invasive fecal hormone measures to detect an adrenal response to stress in captive cougars. The magnitude of the stress response can be considered an objective indicator of an animal’s physiological reaction to its environment.

**Methods**

Cougars (3 intact females, 6 neutered males) were housed at the Wildlife Rescue and Rehabilitation facility in Boerne, Texas. They were either rescued from the exotic pet trade or acquired from other captive facilities. They ranged in age from <1 year to approximately 10 years. The animals were group-housed in enclosures ranging in area from 0.25–1 ha.

The cougars were hand-fed equal amounts of meat twice a day at regular times. Because the animals were group-housed, we added food-dye-colored seeds to the meat to differentiate each individual’s fecal samples. We collected fecal samples during 2 1-week periods in May of 2000 and January of 2001. During the second collection period, 1 cougar that was sampled in May was re-sampled in January, increasing effective sample size to 10 (treating the stress events individually). We opportunistically collected samples during regular checks of enclosures during daylight hours. All samples were <12 hours old at the time of collection and were immediately preserved.

Following methods described in Wasser et al. (2000), we homogenized samples prior to collection. After mixing, we obtained a 25-g subsample, wrapped it in a coffee filter, and placed it in a bag containing approximately 5 g of silica. We stored samples at -20°C until extraction.

**Stressors**

We exposed each cougar to an artificial stressor on the same day we collected ≥1 pre-stress fecal sample. All pre-stress samples were collected from group- or adjacent-housed animals prior to any stressor exposure. We designed artificial stressors to elicit a hormonal response similar to that expected in response to a natural acute stressor. Duration of stressors ranged from <5 minutes to 2 days. Stimuli were varied and included noise, social, and visual stressors (Table 1). Except in the case of the social stressor, the animal was confined to a portion

![Young male cougar housed at the Wildlife Rescue and Rehabilitation Center in Boerne, Texas. Photo by F. Bonier.](image-url)
of the enclosure measuring approximately 10 m², and we varied the type of stressor presented until it elicited a behavioral response (such as attempting to flee or climb the fence, or flattening ears and growling). We presented the stressor at random intervals for approximately 15 minutes per hour over a 4-hour period. The cougars remained confined throughout the period. All animals sharing an enclosure were stressed on the same day, either simultaneously (but in separate lockouts) or sequentially. It should also be noted that “pre-stress” hormone levels cannot be equated with baseline levels for cougars in general. The stress of captivity and group housing, as well as other unmeasured stressors, prevented measurement of a true baseline hormone level.

**Sample preparation and extraction**

We transported fecal samples back to the lab overnight on ice and then lyophilized them to control for variation in water content (Wasser et al. 1994). We pulverized samples and homogenized them with a mortar and pestle and sifted them through a fine-mesh strainer to remove any nonfecal material. We then placed a 0.5-g subsample of the resulting powder in a 125-mL glass vial. To extract steroid hormones, we boiled the sample twice in 10 mL of 95% ethanol. Between boiling steps we centrifuged the sample (5 minutes at 2,500 rpm), combined the resulting supernatants from each step, and transferred them to a clean culture tube. We then dried down the extracts in a water bath at approximately 40°C under airflow for 4 hours (Wasser et al. 1994). We rinsed tubes with 5 mL of ethyl acetate:hexane (3:2) to remove any hormone remaining on the tube walls. The ethyl acetate:hexane was evaporated, and the hormone was resuspended in 2 mL of 95% ethanol.

**Radio-immunoassay**

We assayed cortisol metabolites in triplicate using both cortisol and corticosterone antibodies from commercially available radio-immunoassay (RIA) kits (ICN Diagnostics, Irvine, Calif.). Assays were counted on a Packard Cobra gamma counter (Packard Instrument Company, Meriden, Conn.), and resulting values were converted to hormone concentration using a standard curve (run with each assay) and RIAPC curve-fitting software (Rieger 1988). Means of values from triplicate assays were used for statistical analyses. Assay protocols were based on product information, except that all volumes were halved for the corticosterone assay.

While cortisol is the primary stress hormone released by the adrenal cortex in response to stress in felids (Graham and Brown 1996), it varies in structure from corticosterone only by the presence of one hydroxyl group. Cortisol appears to be converted into corticosterone-like metabolites prior to excretion (Sencill et al. 1991, 1992; Wasser et al. 2000). Therefore, we assayed extracts using cortisol and corticosterone antibodies to determine which had a higher affinity for the cortisol metabolites found in cougar fecal extracts.

**Method validation**

We tested our collection, preservation, extraction, and assay techniques to ensure both precision and effectiveness of the methods. To determine the effects of variation in time to collection and preservation of samples, we collected 9 fresh samples, homogenized them, and collected a subsample as described above. The remaining sample was left in an area outside of the cougar enclosures (where it would not be disturbed or contaminated), and subsamples were collected after 120 hours. We compared hormone measures of subsamples to detect degradation of hormone over time.

Only a small portion (0.5 grams in this study) of each collected scat is typically assayed. While most

<table>
<thead>
<tr>
<th>Cougar</th>
<th>Stressor</th>
<th>Response</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Noise (chainsaw) &lt;5 minutes</td>
<td>Minimal, occasionally flattened ears</td>
</tr>
<tr>
<td>2</td>
<td>Social (enclosed w/ new individual)</td>
<td>Aggression towards individual 3</td>
</tr>
<tr>
<td>3</td>
<td>Same as 2</td>
<td>Attempted to flee, avoid individual 2</td>
</tr>
<tr>
<td>4</td>
<td>Same as 1</td>
<td>Attempted to flee, climbed fence</td>
</tr>
<tr>
<td>5</td>
<td>Noise (chainsaw) and shown tranquilizer gun</td>
<td>Flattened ears, growled</td>
</tr>
<tr>
<td>6</td>
<td>Same as 5</td>
<td>Attempted to flee, paced at gate</td>
</tr>
<tr>
<td>7</td>
<td>Same as 5</td>
<td>Flattened ears, growled</td>
</tr>
<tr>
<td>8</td>
<td>Same as 5</td>
<td>Attempted to flee, climbed fence</td>
</tr>
<tr>
<td>9</td>
<td>Noise (chainsaw) and sprayed with water</td>
<td>Attempted to flee, climbed fence</td>
</tr>
<tr>
<td>10</td>
<td>Same as 9</td>
<td>Attempted to flee, climbed fence</td>
</tr>
</tbody>
</table>
researchers mix entire fecal samples prior to taking a subsample for extraction and assay (e.g., Creel et al. 1997a), several do not (e.g., see Whitten et al. 1998, in which only top portion of sample was taken and then homogenized). This relies on the assumption that the hormone is evenly distributed throughout the sample. To test this hypothesis, we took 3 random 0.5-gram subsamples from 7 whole cougar scats. Each subsample was identically extracted and assayed for cortisol. We then compared measured levels to determine whether the assumption that the hormone is evenly distributed was supported.

We homogenized all samples included in this study prior to collection. To test the effectiveness of this procedure, we mixed 19 samples, took subsamples, and then stored the remainder. We assayed both the subsample and the remainder and compared measured hormone levels.

To ensure that our preservation technique was effective, we compared preservation in silica to another common method: preservation in ethanol. We collected 9 samples as described above, but for each sample we retained 2 large subsamples: 1 we preserved in silica and the second we preserved in ethanol. We stored samples at -20°C for a period of 120 days. Subsamples were assayed for cortisol at 4 time points: 0 (initial assay), 60, 90, and 120 days. We compared resulting hormone levels to determine effectiveness of preservation.

To examine the effectiveness of the extraction method used, we subsampled 10 scats and spiked them with radiolabeled cortisol. They were allowed to incubate overnight, extracted, and then aliquots of extracts were counted on a gamma counter in triplicate. The measured counts of each spiked sample, divided by counts of straight radiolabel, are reported as the percent of hormone recovery.

To quantify assay precision, we used standard techniques to estimate 3 quality-control measures. First, to ensure that there was no source of interference with antibody binding in fecal extracts, pooled extract was serially diluted and assayed. Deviation from parallelism could be caused by interference of extract contents with normal antibody or hormone binding. We tested the resulting hormone levels for parallelism to the standard curve, which would indicate that extracts were binding similarly to hormone standards used to generate the curve. We also included aliquots of 3 standard hormone solutions (low, medium, and high hormone concentration) in each assay. We compared hormone measures for these between assays. Resulting coefficients of variation reflect estimated inter-assay variation. Finally, we assayed each sample in triplicate, including the hormone standards. Coefficients of variation between triplicate sample hormone measures are reported as a measure of intra-assay variation. We used these 3 tests of precision, combined with the ability to detect a stress-related change in hormone measures, to assess the 2 different hormone assays.

**Statistical analyses**

In cases where we collected >1 sample in a 24-hour period, the mean hormone measure was used for statistical analysis. Results of assay for the 2 hormone metabolites were analyzed separately for significant effects of stress at 3 time periods: pre-stress ($t=0$), 24 hours post-stress ($t=24$), and 48 hours post-stress ($t=48$). Only samples collected $\leq 48$ hours after stress were included in the analysis, given the expected excretion lag times of about 24 hours for felids (Brown et al. 1994). We conducted paired $t$-tests of all orthogonal contrasts to test for significant differences in hormone measures between specific time periods. Because the stressors elicited 2 distinct types of responses, we analyzed hormone measures for animals that displayed a notable flight response to the stressor separately from those for animals that responded aggressively. We determined absence of gender and season (May or January) effects on hormone measures using a repeated-measures analysis of variance (ANOVA). We analyzed method validation data using paired $t$-tests or repeated-measures ANOVA where appropriate. If data failed tests of normality, they were square-root transformed prior to analysis. All statistical analyses were completed using SigmaStat version 2.0 computing software (SPSS Incorporated, Chicago, Ill.).

**Results**

The range in hormone levels was 163–2,234 nanograms of cortisol per gram dry fecal material (ng/g) and 125–3,625 ng corticosterone/g (Figures 1 and 2). The ANOVA analyses revealed that there was no significant gender or seasonal effect on hormone measures.

The corticosterone assay provided a better indicator of the adrenal response to stress. In animals that attempted to flee ($n=7$), glucocorticoid
metabolites increased by 24 hours post-stress (Figure 1, \( t_7 = -3.23, P = 0.01 \)). Forty-eight-hour corticosterone assay measures were still somewhat elevated above pre-stress levels (\( t_7 = -1.76, P = 0.12 \)). When animals that did not attempt to flee (\( n = 3 \)) were included in the \( t \)-tests, no comparisons were statistically significant.

Metabolites measured by the cortisol assay also resulted in significantly different cortisol measures (\( F_{2,5} = 7.21, P = 0.03 \) for cortisol; corticosterone not measured). Subsampling of homogenized samples, on the other hand, did not result in significantly varied hormone measures (\( t_{18} = 0.12, P = 0.91 \) for cortisol; \( t_{18} = 0.18, P = 0.86 \) for corticosterone). The 2 preservation methods that we compared also did not result in varied hormone measures, even after 120 days in storage (\( F_{5,8} = 0.78, P = 0.57 \) for cortisol; corticosterone not measured). The 2 methods did not differ in preservation effectiveness. Mean recovery of radiolabeled hormone was 87%. Serial dilution of pooled extracts resulted in a curve that was parallel to the standard curve. Coefficients of inter-assay variation were 9 and 13%, and 8 and 15% for intra-assay variation, for the corticosterone and cortisol assays, respectively.

**Discussion**

We have shown that non-invasive hormone measures can be used to detect an adrenal stress response in increased in animals that attempted to flee, but not to the same extent as those measured by corticosterone assay. These differences approached significance (\( t_7 = -1.75, P = 0.12 \)), but only for the comparison of pre-stress to 24 hours post-stress levels.

In the test of hormone degradation in samples, for all 3 conditions there was no significant change in hormone levels between initial collection and 120 hours (\( t_8 = -0.08, P = 0.935 \) for cortisol; \( t_8 = 0.749, P = 0.476 \) for corticosterone). Assay of repeated samples of unhomogenized scats

![Figure 1](image1.png)

Figure 1. Mean plus 1 standard error of corticosterone assays for animals that fled (\( n = 7 \), shaded bars) and did not flee (\( n = 3 \), stippled bars) from stressor.

![Figure 2](image2.png)

Figure 2. Individual values from corticosterone assay for 9 cougars. Individuals 4 and 9 are the same animal, sampled twice. Dashed lines represent the 3 animals that did not flee from the stressor. The 3 cougars represented by open symbols (C2, 3, and 10) are female while the remaining 6 are male. In cases where more than one sample was collected within a 24-hour period, the mean of the resulting hormone levels was taken. These values are displayed plus one standard error (shaded bars).
individual cougars. Physiological and behavioral responses to stress can be extremely variable between species and individuals (Moberg 2000). This fact is evident in this study. Nevertheless, adrenal and behavioral responses to stressors were clearly linked. The perception of threat was illustrated by the animals' attempts to flee, or lack thereof, and was directly related to the release of glucocorticoids. The individuals that did not attempt to flee from the stressor had higher pre-stress hormone measures than post-stress, except in the case of the corticosterone metabolite assay for individual 5 (Figure 2). A different explanation seems plausible for the lack of hormonal and behavioral response in each of the non-fleeing individuals. Individual 5, a low-ranking male (as determined by observation of conflicts with other individuals) housed with 3 other males, might already have been experiencing chronic stress, making him physiologically unresponsive. The 2 other nonfleeing individuals appeared relatively undisturbed by the stressor as evidenced by their minimal behavioral response. This was especially true for individual 1, the highest-ranking male in the same enclosure with individual 5. Individual 7 may have been hormonally responding to a previous environmental stressor (possibly a severe rainstorm the previous day), which would explain the elevated pre-stress corticoid metabolite levels. Regardless of the individual explanations, it is evident that none of these 3 cougars experienced an elevation of stress hormone levels in response to the artificial stressor.

Our findings are encouraging for future applications of these methods in the field. Method validation provides guidelines for field collection and lab analysis. Samples up to 5 days old may be collected without concern of significant hormone degradation, though methods for determining age of samples for field collection need to be developed and validated, and the effects of varying environmental conditions on hormone degradation should be explored. The use of detection dogs for locating cougar fecal samples in remote terrain is feasible and reduces sampling bias and ineffectiveness associated with standard transect methods for visual search (F Bonier, unpublished data). The corticosterone assay of fecal corticoid metabolites appears to be a more effective tool for detecting an adrenal response to stress in this species than the cortisol assay because it revealed a response to relatively acute and minor stressors. Additionally, the corticosterone assay was more precise, with lower inter- and intra-assay variation than the cortisol assay. As employed in this study, the corticosterone assay is expected to be even more sensitive to significant and chronic stressors of the sort that would impact population health. Repeated sampling from individual cougars would be necessary to differentiate acute versus chronic hormone elevation, though this should not present a particular challenge, given the fact that cougars are territorial and tend to use latrine areas within their territories. Individual identity of cougars could be determined using knowledge of cougar home ranges or, preferably, using genetic analyses of fecal-extracted DNA that are increasingly being applied in non-invasive studies (e.g., Taberlet et al. 1997, Gerloff et al. 1999, Ernest et al. 2000). Application of these methods in the field, particularly in combination with reproductive hormone measures and genetic analyses, could be used to address a wide range of questions and could aid in assessing and focusing management and conservation efforts in populations at risk.

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Literature cited


Frances Bonier (photo) is a Ph.D. candidate in the Department of Biology at the University of Washington. She received her B.S. from Lesley College and M.S. from the University of Idaho at Moscow. Her work explores the ways animals respond to anthropogenic disturbances in both the short and long term. To address these questions, she employs a variety of approaches, including field endocrinology, evolutionary physiology, and population genetics methods. Howard Quigley is a senior ecologist with Beringia South, a nonprofit science and education group in Kelly, Wyoming. He received his B.S. from the University of California at Berkeley, M.S. from the University of Tennessee at Knoxville, and Ph.D. from the University of Idaho at Moscow. He has worked on a variety of large carnivores around the world and continues to coordinate multi-carnivore research and conservation efforts in the Greater Yellowstone Ecosystem. Steven N. Austad is a professor in the Department of Biological Sciences at the University of Idaho. He received Bachelor’s degrees from University of California, Los Angeles and California State University at Northridge and his Ph.D. from Purdue University. He was an assistant and associate professor in organismic and evolutionary biology at Harvard University prior to joining the University of Idaho. He has done field work on birds and mammals in the United States, Venezuela, Papua New Guinea, and the western Pacific Islands.

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