Validation of faecal glucocorticoid analysis in cheetahs (*Acinonyx jubatus*)

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Abstract

Measurement of glucocorticoid metabolites in faeces is increasingly used as a non-invasive method for monitoring stress in wild animals. The aim of this study was to validate a non-invasive technique for monitoring adrenocortical activity in cheetahs (*Acinonyx jubatus*) by measuring glucocorticoid metabolites in the faeces before and after an adrenocorticotrophic hormone (ACTH) challenge. All faeces were collected from five cheetahs (two female and three male) ten days before and seven days after intramuscular injection of ACTH (50 IU). Samples were analyzed for glucocorticoid metabolite concentrations using an enzyme immunoassay (EIA). Faecal glucocortcoid metabolites increased 234 –715% above baseline within 20 hours of ACTH administration in all five cheetahs. When left at ambient temperature, faecal glucocorticoid metabolites concentrations declined over time since defaecation. Therefore it is advisable to freeze samples immediately after defaecation for reliable monitoring of faecal glucocorticoid metabolite concentrations. Our results show that non-invasive monitoring of faecal glucocorticoid metabolites can be a valid and useful tool for assessing adrenal activity in cheetahs.

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Introduction

Worldwide, cheetah (*Acinonyx jubatus*) populations are declining, with a total number of 100,000 free-ranging cheetahs counted at the end of the 19th century to approximately 10,000 counted today. Cheetahs are included on the International Union for Conservation of Nature (IUCN) list of vulnerable species, as well as on the US Endangered Species Act: threatened species – Appendix I of CITES (Convention on International Trade in Endangered Species). Cheetahs have become all but extinct in the wild in Asia, with less than 100 cheetahs surviving in Iran. Cheetahs can, however, still be found in a broad section of Africa, with the largest populations in Southern Africa (Namibia, Botswana) and Western Africa (Kenya, Tanzania). Nevertheless, cheetah populations are considered viable in less than half the countries where they still exist. Populations continue to decline from loss of habitat and prey species, conflict with humans and loss of genetic variation. Cheetahs have a gestation of approximately 95 days, and average litter size is 4-5 cubs. However, because wild populations are not faring well, captive breeding programs are considered essential for the conservation of the species. To date, however, the captive population has not been self-sustaining because of poor reproductive success and a high prevalence of unusual diseases. Chronic stress has been suspected to be an important contributing factor to both of these problems, which makes the development of a reliable glucocorticoid concentration monitoring technique in cheetahs an important project. [Marker 1998; Terio et al. 1999; Wells et al. 2004]

Stress is an imbalance in homeostasis caused by environmental stimuli (stressors). During a period of acute stress, especially catecholamines (e.g. epinephrine, norepinephrine, and dopamine) allow energy mobilization and may change an animal’s behaviour, better preparing it for a fight or flight response. Prolonged elevation of glucocorticoid (e.g. cortisol, or corticosterone) concentrations due to chronic stress however, can have several negative effects, such as suppression of the immune system and decreased reproductive success. [Möstl & Palme 2002; Touma et al. 2005]

When a vertebrate is confronted with a stressor the hypothalamic-pituitary-adrenal (HPA) axis is activated. Corticotrophin-releasing hormone (CRH) from the hypothalamus enhances the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary gland. ACTH in turn stimulates the synthesis and secretion of catecholamines as well as glucocorticoids from the adrenal cortices. In this regard, the predominant glucocorticoid produced depends primarily on the species in question, but the most prominent endogenous glucocorticoid in most large animal species is cortisol. [Möstl & Palme 2002; Touma et al. 2005]

The activity of the adrenal glands is one indication of a physiological stress response. A common method of determining the activity of the adrenal glands is to measure the concentration of cortisol in the blood. However, a blood sample represents the concentration of hormones at a given point in time. This has the advantage that small transient changes can be detected over a short period of time. However, because cortisol has a pulsatile secretion pattern and a clear diurnal rhythm in many species, a lot of blood samples would need to be recovered to produce a reliable representation of chronic changes in hormone levels. Moreover, collecting blood is an invasive approach which can itself induce a stress response (elevated glucocorticoid concentrations) due to handling and restraining procedures. [Millspaugh et al. 2004; Möstl & Palme 2002; Touma et al. 2005]

An alternative technique for monitoring changes in glucocorticoid output is to measure corticoid metabolites in excreta. Circulating glucocorticoids are metabolized in the liver and excreted as

conjugates, partly via the kidneys (in the urine) and partly via the bile into the gut, where they are partially reabsorbed into the bloodstream via the entero-hepatic circulation. Any remaining corticoid metabolites in the faeces will be further converted by bacterial enzymes and excreted via the faeces. [Möstl & Palme 2002; Touma et al. 2005]

Faeces can be very easily collected without disturbing the animal. Samples can also be collected at regular intervals over time, which makes it interesting for long-term studies. Moreover, the concentration of glucocorticoid metabolites in the faeces reflects the total amount of production and excretion of cortisol over a period of time, rather than at a single point in time. [Millspaugh et al. 2004; Möstl & Palme 2002; Touma et al. 2005] This makes it a more suitable for producing a reliable representation of chronic changes in glucocorticoid secretion.

Since there are differences between species and gender, with regard to the metabolism and excretion of glucocorticoid metabolites [Millspaugh et al. 2004; Möstl & Palme 2002; Touma et al. 2005], measurement of glucocorticoid metabolites in faecal samples must be carefully validated in each species of interest and in both sexes before it can be concluded that a given technique can be used as a reliable tool for monitoring adrenal activity in that species.

There are basically two ways to assess if a respective test-system detects a reliably biological signal: a physiological and a biological validation. Physiological validation involves pharmacological induction of a change in the concentration of glucocorticoids in the blood, followed by evaluation of faecal glucocorticoid metabolites to see if the change is reflected in the faecal samples. The most common way to stimulate adrenocortical activity is to conduct an ACTH challenge test. During this test an animal will be injected with ACTH, which is expected to result in a significant increase in plasma glucocorticoid concentrations followed by a return to baseline within several hours. Samples (preferably faeces) are collected before and after the injection and subsequently analysed for glucocorticoid metabolite levels. Ideally, this procedure should result in elevated concentration of faecal glucocorticoid metabolites, with the onset of the peak delayed by the species-specific lag time (which is affected by gut passage time). [Monfort et al. 1998; Terio et al. 1999; Touma et al. 2005; Wasser et al. 2000] Biological validation is similar but involves collecting faecal samples before and after a known stressful event, like capture or transportation. [Möstl & Palme 2002; Terio et al. 1999; Touma et al. 2005; Wells et al. 2004].

Terio et al. (1999) previously validated a radioimmunoassay for quantifying faecal glucocorticoid metabolites in cheetah. ACTH was administered to two males and two females, while seven females were subjected to a variety of stressful events. Each resulted in increased faecal corticoid metabolites approximately 24-72 hours later. Wells et al. (2004) measured the stress response of cheetahs to movement between facilities. For most animals corticoid concentrations increased.

After defaecation, glucocorticoid metabolites can be further converted by bacterial enzymes. [Touma et al. 2005] This means that the faecal samples always have to be collected at the same time post-defecation, ideally as fresh as possible. Once collected, the samples must be frozen immediately to stop the microbiological activity and stored at -20˚C until analysis.[Millspaugh et al. 2004; Möstl & Palme 2002]Due to the fact that during most studies, focal animals can’t be observed 24 hours a day, faeces are sometimes not fresh when collected. To see what happens to the concentration of

glucocorticoid metabolites when faeces isn't frozen immediately, a degradation experiment can be performed.

The aim of this study was to validate a method for assessing adrenocortical activity in captive cheetahs (*Acinonyx jubatus*) by measuring glucocorticoid metabolites in the faeces using an ACTH challenge. We also measured the degradation rate of glucocorticoid metabolites in faecal samples to further evaluate if faecal glucocorticoid metabolite measurement would be a useful tool for monitoring stress in the cheetah.

Materials and methods

Study site and animals

The study took place at 'the Ann van Dyk Cheetah Centre' (also known as 'de Wildt'), which is situated near the Magaliesberg mountain range in the North West Province of South Africa.

The ACTH challenge was conducted on 5 cheetahs, 3 male and 2 female (Table 1). Animals ranged from 10 to 12 years of age at the time of the study. They were housed individually in outdoor enclosures of the size of approximately 360 m^2 (Figure 1). Two sibling males (M379 and M380) were housed together. During the day the animals were kept in the corner camp, which could be closed off from the main camp, for easier observation and sample collection. During the night (17:00 – 7:30 hours) the animals had access to their entire enclosure. Animals were fed once a day at 7:30 hours a mixture of Iams® and horse- and chicken-mince. For the two animals housed together, faeces were differentiated by mixing rice (M380) and split peas (M379) in their food. Water was available ad lib.

During the experiment there was no rainfall, temperatures were approximately 20°C during the day and approximately 5°C at night.

Cheetah	Sex	Age	Housing
F327	Female	12	Individual
F343	Female	12	Individual
M353	Male	11	Individual
M379	Male	10	Pair (with M380)
M380	Male	10	Pair (with M379)

Table 1: Study animals

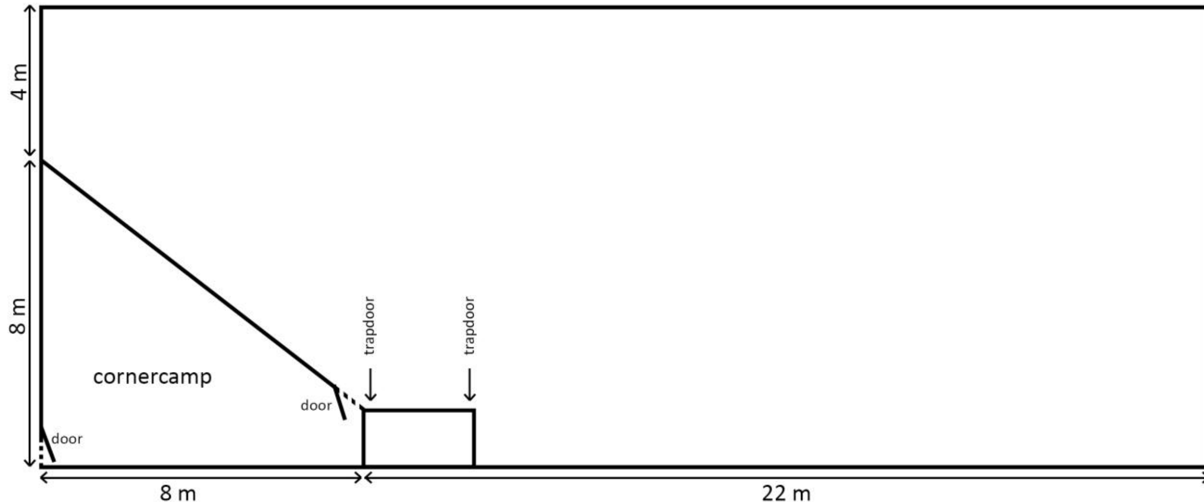

Figure 1: Ground plan of the enclosure where the cheetahs were housed. During the day the cheetahs were kept in the corner camp, at night the cheetahs had access to their entire enclosure.

Sample collection

The five cheetahs were observed during the day from 7:30 until 17:00 hours for 21 days from June 17 until July 7 2010. Before faecal sampling started, the cheetahs were getting used to the smaller corner camp for four days. Homogenized faeces were collected using rubber gloves, frozen within 10 minutes after defecation, and stored at -20°C until processing and analysis. Due to logistically restrictions, the enclosures could not be entered after 17:00 hours until 7:30 the next morning. Therefore every morning between 7:30 and 8:00 hours the faeces from the previous night were collected. Faecal samples were collected ten days pre ACTH administration to be able to establish individual baseline faecal glucocorticoid metabolite concentrations, and seven days post injection.

ACTH challenge

To stimulate adrenal activity, a single dose of 50 IU ACTH (Synacthen®, Novartis RSA (Pty) Ltd, Kempton Park South-Africa) was administered per animal into the thigh muscle between 11:00 and 11:20 hours on the 10th day of the study. Animals were physically restrained to facilitate the administration of ACTH. The cheetahs were observed that day until 18:00 hours and the next morning from 5:00 hours.

Degradation experiment

To determine the change in glucocorticoid concentration in samples post-defecation, two complete faecal samples (one 19 hours after ACTH injection and one the day before injection) were separately collected in bags and homogenized. After the zero sample was frozen immediately at -20°C, six subsamples were created from each sample and stored at ambient temperature overnight. Subsequently one subsample from each set was frozen 1, 2, 5, 7½, 10 and 12½ hours after the zero sample faeces was stored at -20°C.

Faecal extraction and hormone analysis

The faecal samples were lyophilized, pulverized and sieved. Between 50 -60 mg of faecal powder was extracted with 3 ml of 80% ethanol by vortexing for 15 minutes and subsequent centrifugation for 10 minutes at 3,300 g. The supernatant was then recovered and stored at -20°C until hormone analysis.

The glucocorticoid metabolite concentrations were quantified using an enzyme immunoassay (EIA), as first described in Palme & Möstl (1997), using a polyclonal antibody against 4-pregnene-11ß,21-diol-3,20-dione -3CMO:BSA and cortisol-3-CMO-DADOO-biotin as label. The sensitivity of the assay at 90% binding was 4 pg per well. Inter- and intra-assay coefficients of variation ranged between 4.7% and 17.8%.

Results

In total 167 faecal samples were collected, 101 baseline samples and 66 post ACTH injection samples. Table 2 gives an overview of all the samples. Of the faecal samples 33,5% were collected fresh and frozen immediately. All peak samples were freshly collected.

Cheetahs	Total samples	pre injection	mean per day pre injection	post injection	mean per day post injection	% fresh
F327	27	18	1,8	9	1,3	7,4
F343	33	21	2,1	12	1,7	21,2
M353	31	19	1,9	12	1,7	35,5
M379	35	19	1,9	16	2,3	34,3
M380	41	24	2,4	17	2,4	34,1
Total	**167**	**101**	**10,1**	**66**	**9,4**	**33,5**

Table 2: overview of the samples collected

The ACTH injection resulted in a 234 to 715% increase in glucocorticoid metabolites concentration in all study animals 17 to 20 hours post injection compared to pre-injection values (Figure 2 and 3). The glucocorticoid metabolite level in the faeces increased 497-715% above baseline after ACTH injection in females and 234-403% above baseline in males (Table 3). The peak samples in all cheetahs were the first faecal sample after ACTH injection. The peak samples in all five cheetahs had similar delays: 17 to 20 hours after ACTH injection and returned to their baseline in the second faecal sample after injection (between 20 and 44 hours post ACTH injection).

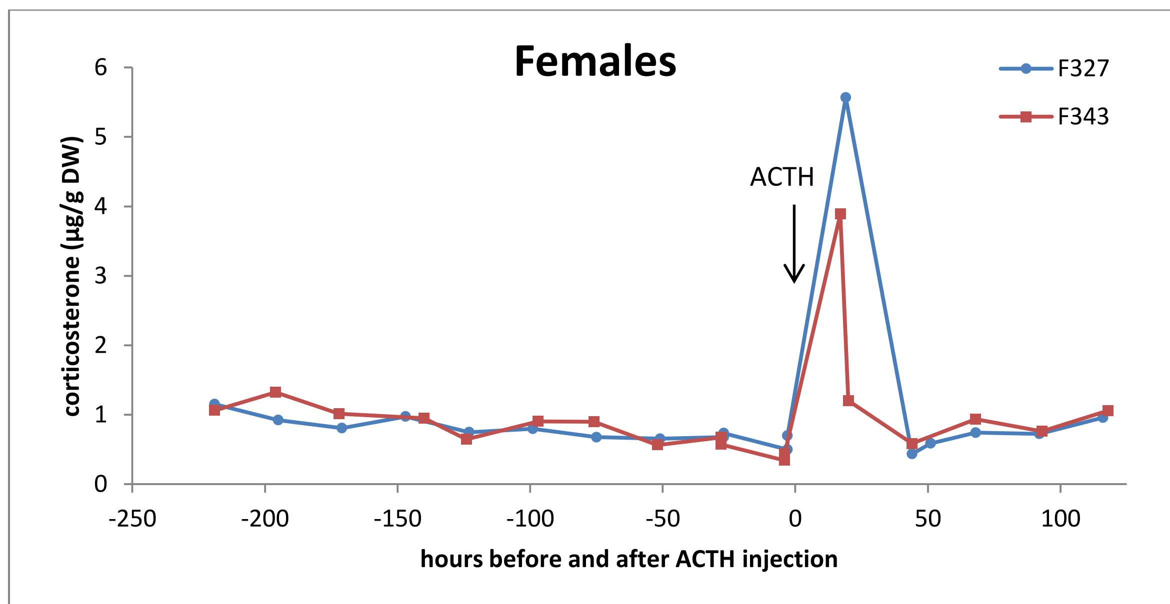

Figure 2: Glucocorticoid metabolite levels before and after ACTH injection for the two female cheetahs.

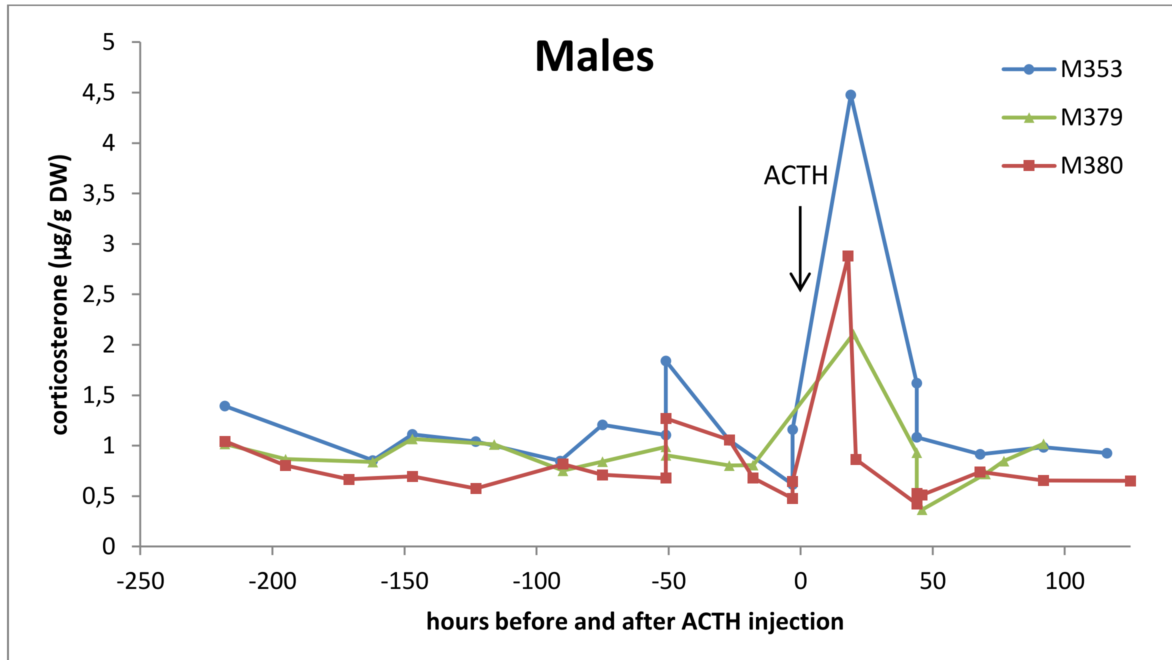

Figure 3: Glucocorticoid metabolite levels before and after ACTH injection for the three male cheetahs.

Cheetah	Mean baseline ± SEM (ng/g)	Peak concentration (ng/g)	Elevation (%)	Time of peak (h)	Decline to baseline after injection (h)
F327	779 ± 49,5	5567	715	19	44
F343	783 ± 83,3	3888	497	17	20
M353	1112 ± 96,0	4478	403	19	44
M379	900 ± 31,7	2107	234	20	44
M380	779 ± 61,2	2883	370	18	21

Table 3: results of the enzyme immunoassay

For the degradation experiment two fresh samples were used: one baseline sample from the male cheetah M380 and one peak sample from the female cheetah F327. Faecal glucocorticoid levels degraded during the night (Figure 4 and Table 4). The sample of the female cheetah declined faster (more than 20% decline at 5 hours post defaecation) than the sample of the male cheetah (more than 20% decline at 12,5 hours post defaecation). The degradation rate for the female cheetah is 2.9% decline of faecal glucocorticoids per hour and for the male cheetah 1.9% decline per hour.

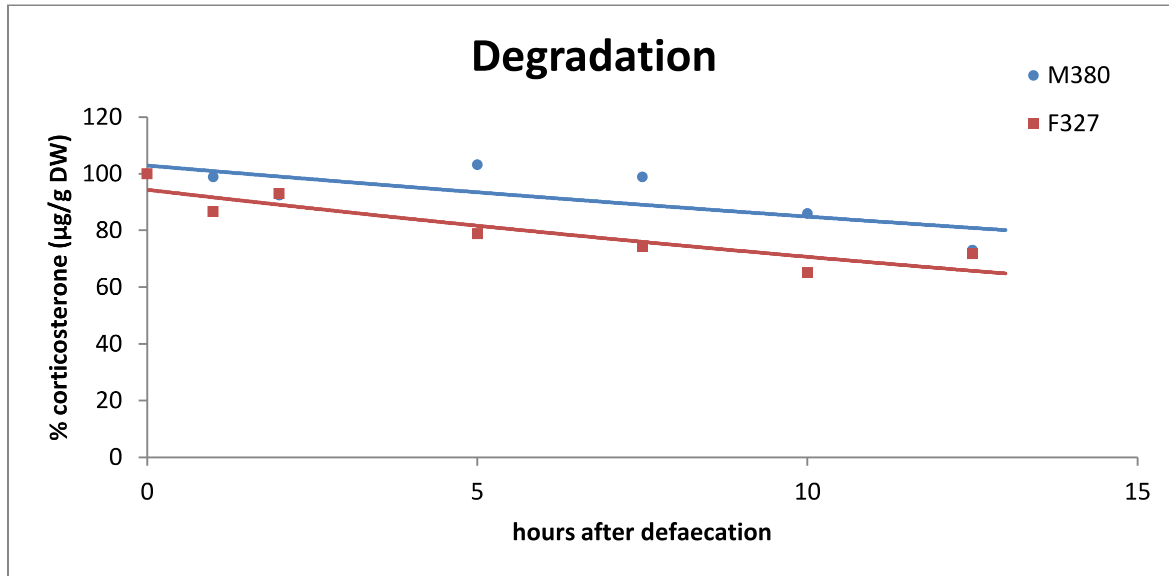

Figure 4: Degradation experiment of a fresh faecal sample of the male cheetah M380 and the peak sample of the female cheetah F327

Degradation (post defaecation)	1 h	2 h	5 h	7,5 h	10 h	12,5 h
F327	-13,29%	-6,82%	-21,18%	-25,49%	-34,83%	-28,19%
M380	-1,08%	-7,53%	+3,23%	-1,08%	-13,98%	-26,88%

Table 4: Decline in faecal glucocorticoid metabolite levels after defaecation in % compared to the fresh zero sample

Discussion

An increase in faecal glucocorticoid metabolites following the ACTH challenge was measured in all five cheetahs. In this study, ACTH induced a 234-715% rise above baseline concentrations of faecal glucocorticoid metabolites. These results are similar to other studies. Graham and Brown (1996) found a 238-826% increase over individual baseline values in domestic cats. In the study of Schatz and Palme (2001) the increase was 355% (median) above baseline in domestic cats and 702% (median) in domestic dogs. Goymann et al (1999) found a 198-3748% increase above baseline in spotted hyenas. In the study Terio et al (1999) conducted the increase was 690-4194% compared to the baseline in four cheetahs. Young et al (2004) found a 1145-1361% increase after an ACTH challenge in two cheetahs. They both used 400 IU ACTH, compared to the 50 IU used in this study, which could explain the higher increase.

In this study, the glucocorticoid peak of both females (497-715% increase) was higher than the glucocorticoid peak of the males (234-403% increase). This can be due to individual variation and/or additional stress to the injection. The cheetahs in our study were restraint in a squeeze cage in order to administer the ACTH injection. Terio et al (1999) found elevations of faecal glucocorticoid concentrations in cheetahs following restraint in a squeeze cage. The two males housed together were more used to people than the other cheetahs, which may have resulted in a lower glucocorticoid peak due to less additional stress to the injection. The difference could also be because of gender differences, since gender can have an effect on the glucocorticoid concentrations found in faeces. Differences in metabolism and route of excretion of glucocorticoids are reported in

males and females. [Millspaugh et al. 2004; Möstl & Palme 2002; Touma et al. 2005] It has also been reported that cheetahs that were described as more 'nervous' by zookeepers showed higher baseline faecal glucocorticoid metabolites than cheetahs that were described as more 'calm'. [Jurke et al 1997; Terio et al 1999] This has also been described in clouded leopards. [Wielebnowski et al 1999] The females in our study and one male (M353) were more nervous than the other two males (M379 and M380). However, the glucocorticoid metabolite baselines of the female cheetahs (F327: 779 ± 49,5 ng/g and F343: 783 ± 83,3 ng/g) were lower compared to two of the three male cheetahs (M353: 1112 ± 96,0 ng/g and M379: 900 ± 31,7 ng/g). One male cheetah (M380) had similar baseline concentrations as the females (779 ± 61,2 ng/g). This contradicts what was found in the previous studies mentioned above. Wells et al (2004) found that cheetahs with low baseline faecal glucocorticoid metabolite concentrations had a significantly higher glucocorticoid peak after movement compared to cheetahs that had high baseline glucocorticoid levels. They suggest that this might be due to the fact that animals with high baseline levels might already be at maximal production, precluding further stimulation. We found similar results in this study, since both females had lower baseline glucocorticoid levels and higher glucocorticoid peaks compared to the other animals. Since in all studies only small numbers of animals were used, the differences between gender and temperament need further evaluation.

It is important to know when a glucocorticoid metabolites peak, as a reaction to a stressor, will appear in the faeces in order to correlate the stressful event to the faecal glucocorticoid peak. The delay between the stressor and the elevated concentration of faecal glucocorticoid metabolites (lag time) is closely related to the gut transit time in an animal. This lag time is species-specific. [Monfort et al. 1998; Terio et al. 1999; Touma et al. 2005; Wasser et al. 2000] In this study, all increases in faecal glucocortcoid metabolite concentration were observed in the first faecal sample collected after the ACTH challenge. The delay between the ACTH injection and appearance of peak concentrations was 17-20 hours. These results are in line with previous research. Terio et al (1999) also observed the glucocorticoid metabolite peaks in the first faecal sample after ACTH injection in three out of four cheetahs. Similar delay times were also found in the domestic cat (25 hours, Schatz and Palme 2001; 24-48 hours, Graham and Brown 1996), domestic dog (22 hours, Schatz and Palme 2001), African wild dog (24-30 hours, Monfort 1998) and the spotted hyena (16-50 hours, Goymann et al 1999).

In this study 33,5% of the faecal samples were fresh on the time of collection. After defaecation glucocorticoid metabolites can be further converted by bacterial enzymes, which can increase or decrease the measured faecal concentrations. [Keay et al 2006; Touma et al. 2005; Möstl and Palme 2002] This can be influenced by factors as temperature and rainfall. [Washburn and Millspaugh 2002; Palme 2005] To test the stability of faecal glucocorticoids in samples post-defaecation , a degradation experiment was performed under the same environmental conditions as during the experiment. The degradation experiment in our study shows that the glucocorticoid metabolite levels of faecal samples decline over time since defaecation, when left at night. This contradicts previous data on other animals. In a study Huber et al (2003) conducted on red deer the concentration of glucocorticoid metabolites in faeces collected within approximately six hours from defaecation did not differ significantly compared to faeces that were collected fresh. Rehnus et al. (2009) also found that neither different storage conditions (10 and 25°C) nor time intervals resulted in a significant

change in glucocorticoid concentrations in faeces of Mountain hares. Considering our results, it is advisable to freeze faecal samples immediately after defaecation to accurately determine the levels of glucocorticoid metabolites in the faeces.

Conclusion

The aim of this study was to evaluate a method for assessing adrenocortical activity in captive cheetahs (*Acinonyx jubatus*) by measuring glucocorticoid metabolites in the faeces. An increase in faecal glucocorticoid metabolites following the ACTH challenge was measured in all five cheetahs. This suggests that the EIA used in this study can reliably detect stress-related changes in glucocorticoid metabolite levels in male and female cheetahs. This can be a very useful tool for monitoring stress in animals in captivity since it is a non-invasive technique, faecal samples can be very easily collected and it is suitable for long-term evaluations of adrenal activity.

Since our results show a decline in faecal glucocorticoid metabolites when left at ambient temperature, due to bacterial degradation, it is important to freeze samples immediately after defaecation for reliable monitoring of faecal glucocorticoid metabolite levels using this EIA in cheetahs.

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