Non-invasive assessment of adrenocortical endocrine function in African wild dogs (*Lycaon pictus*) by measurement of glucocorticoid metabolites in faeces

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Abstract

Non-invasive techniques for monitoring stress in wild animals have been studied extensively over the past two decades. The aim of this study was to use an adrenocorticotrophic hormone challenge to validate the measurement of glucocorticoid metabolite concentrations in faeces with an ELISA assay as a means of monitoring adrenocortical activity in African wild dogs (*Lycaon pictus*). Faecal samples were collected from four study animals for ten days prior to, and seven days after, an injection with 50 IU ACTH (Synacthen®, Novartis RSA (Pty) Ltd, Kempton Park South-Africa). Samples were analyzed for glucocorticoid metabolite concentrations using a Cortisol-3-CMO enzyme immunoassay and a 3α,11-Oxo-etiocholanolone enzyme immunoassay. The Cortisol EIA showed an increase in glucocorticoid metabolites of 263%-325% post ACTH administration, whereas a 3α,11-Oxo-CM EIA showed an increase of only 125%-139%. These results suggest that the Cortisol EIA is more sensitive and suitable for monitoring adrenocortical activity in African wild dogs than the 3α,11-Oxo-CM EIA. However since not all animals showed a response using the Cortisol EIA, further research is necessary to fully validate this technique.

Introduction

The African wild dog (*Lycaon pictus*) is a medium sized canid from the order carnivores. They live in small packs of 2-27 animals and range over very large areas. The species is found only in Africa and has been classified, on the ICN red list, as endangered since 1990 (Mcnutt et al. 2008). Once there were approximately 500 000 African wild dogs, now the estimated number of free-ranging animals is between 3000 and 5500. The largest populations of wild dogs are found in southern Africa and the southern part of East Africa, whereas they used to be distributed throughout the continent. There are also over 500 African wild dogs in captivity in zoos and private collections, particularly in South Africa. The major threats for the species are conflict with human activities, reduced prey availability and infectious diseases, such as rabies and canine distemper (McNutt et al. 2008, Van de Bildt et al, 2002). To ensure the long-term survival of the species, breeding projects have been established. Breeding in African wild dogs is a once-a-year event and gestation lasts 70 days. Average litter size is 10-11 pups, but litters can be as big as 21 pups. On the other hand, pup mortality can be high (The IUCN/SSC canid specialist group, 1997). Captive-bred animals have been successfully introduced into the wild (McNutt et al. 2008). For breeding projects to be a success, it's important that the animals are in good health and have high reproductive rates, both of which can be negatively influenced by chronic stress (Keay et al. 2006).

If an animal is confronted with a stressor, it undergoes a stress response, one aspect of which involves activation of the hypothalamic-pituitary-adrenal (HPA) axis. This results in the release of stress hormones from the adrenal glands, primarily adrenaline and glucocorticoid hormones. Prolonged elevation of glucocorticoids due to chronic stress results in suppression of the immune system and thus poor health, rendering the animal more susceptible to infections and diseases. Cortisol also suppresses gonadal hormones, thereby predisposing to a low reproductive rate (Möstl et al. 2002b, Touma et al. 2005). A reliable technique for determining the amount of stress an animal experiences can be used to help identify the underlying causes of stress, such as aspects of the environment, disease or injury. Once the stress has been quantified, adjustments or treatments can be tested in an attempt to reduce the degree of stress. A common method for determining stress is to measure the concentration of glucocorticoids in blood (De Villiers et al. 1995, De Villiers et al. 1997). A disadvantage of blood sampling is that handling and restraining the animal, and introducing the needle to recover a blood sample, can itself cause a stress reaction and therefore enhance the concentration of cortisol, which can alter the results (Möstl et al. 2002b, Touma et al. 2005, Romero 2002).

An alternative method is to measure the concentration of glucocorticoid metabolites in excreted products, such as urine or faeces. Circulating glucocorticoids are metabolized by the liver and excreted as conjugates, partly via the urine and partly via the bile. The bile enters the gut, where part of the glucocorticoid metabolites will be reabsorbed into the bloodstream via the enterohepatic cycle. The rest will be further metabolized by intestinal bacteria and excreted via the faeces (fig. 1).

Unlike blood samples, faecal samples are easy to collect without any contact with the animal; so results are free of any effect of human contact. Faecal hormone analysis is very useful for long-term monitoring, due to the fact that the concentration of glucocorticoid metabolites in faecal samples represents the production and excretion of cortisol over a longer period of time, because of the pooling effect in the gut. This means that, with a few samples a day, it is possible to generate a reasonable overview of the concentrations of glucocorticoids in general (i.e. chronic changes), although subtle changes are likely to be missed (Möstl et al. 2002b, Touma et al. 2005).

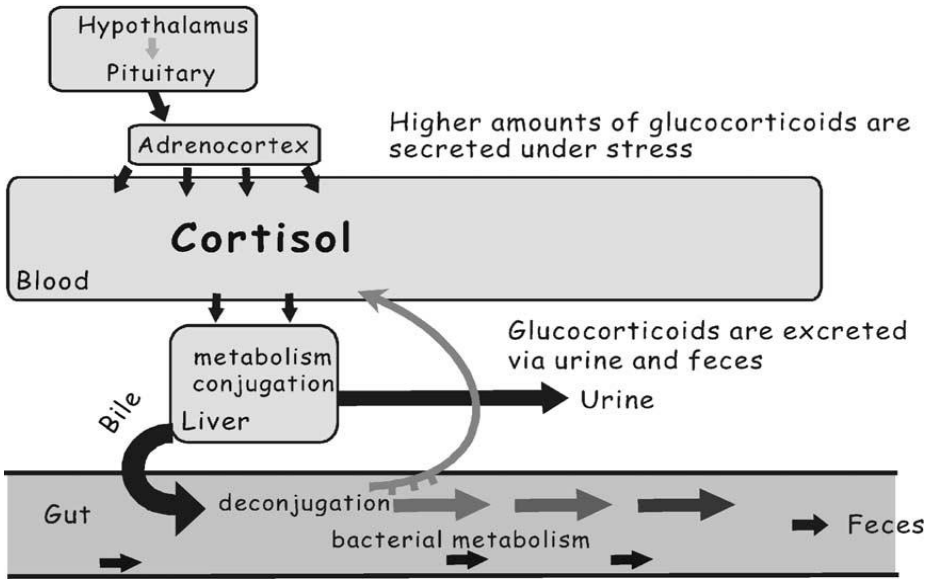

Fig 1: Scheme of the secretion, metabolism and excretion of glucocorticoids. (Möstl, Palme et al. 2002)

During the last 20 years faecal glucocorticoid metabolite analysis has been used for research in various wild and domestic animal species, including cattle, sheep, cheetahs and hyenas (Touma et al. 2005, Palme et al. 2005, Keay et al. 2006, Hodges et al. 2010).
Since there can be differences between species, and between sexes within a given species, with regard to the nature, patterns and levels of hormones excreted, it is important to evaluate which of the existing test systems can be used to reliably monitor changes in adrenocortical activity in the species of interest (Hodges et al. 2010).

There are generally two ways of assessing the reliability of a test-system: a physiological and a biological approach (Touma et al. 2005). Physiological validation means that changes in circulating glucocorticoid levels are pharmacologically induced, and then followed by evaluating faecal glucocorticoid metabolite concentrations prior to and after the test injection. The most common technique for physiological validation of glucocorticoid monitoring is the ACTH Challenge test (Monfort et al. 1998, Wasser et al. 2000). During a biological validation experiment, changes in glucocorticoid metabolite levels are monitored before and after a potentially stressful event such as transportation or electro-ejaculation, rather than ACTH challenge (Morato et al. 2004, Möstl et al. 2002a, Wells et al. 2004).

The ACTH Challenge test has been described for African wild dogs by Monfort et al. (1998). By injecting the animals with 400 IU of a long-acting ACTH preparation they showed that the glucocorticoid response could be monitored in faecal samples using a corticosterone radioimmunoassay (ICN Biomedicals, Inc., Costa Mesa, California 92626, USA). This means that this particular assay can be used to monitor changes in adrenocortical activity in African wild dogs, although the exact glucocorticoid metabolite measured in this study was not reported. In 2009, an ACTH challenge was performed on three female dogs as part of a research project by Bart Vlamings, a student from Utrecht University, the Netherlands. Each dog was injected with 25 IU ACTH (Synacthen from Novartis). Analysis of the faecal samples was performed using a double antibody cortisol-3-CMO enzyme immunoassay. This assay showed an elevation in glucocorticoid metabolites of 190-268%, which is not very prominent. Since these results were inconclusive, and only female animals were tested, a follow-up study was designed.

The aim of this study was, therefore, to use an adrenocorticotrophic hormone challenge to help identify an enzyme immunoassay, based on faecal glucocorticoid meansurements, suitable for monitoring adrenocortical activity in African wild dogs (*Lycaon pictus*) .

Materials and methods

Study site
This research project took place at the Ann van Dyk (a.k.a. De Wildt) cheetah centre in the northern part of South Africa. This centre houses approximately 60 African wild dogs, 60 cheetahs and various other animal species, including vultures and brown hyenas. The general feeding regime for the African wild dogs is a piece of horsemeat twice a week and a mixture of Eukanuba with horse mince on the other days. The field work of this project took place from May 24 2010 until June 16 2010. During this period, the weather was mostly dry, with an average temperature of 20 °C during the day and 5 °C during the night.

Study animals
For this project, four African wild dogs were used, two males and two females. They were all healthy animals between 4 to 8 years of age. For the duration of the project they remained in their own camps, in pairs of one male and one female. Available corner camps were used to keep the animals in sight for observation (fig. 2). They were fed the Eukanuba mix every day at around 7.00 h, supplemented with rice for the males and peas for the females, to be able to distinguish the faeces. Another purpose of the rice and pea additions was to determine the gastrointestinal transit time (GIT time) for each animal.

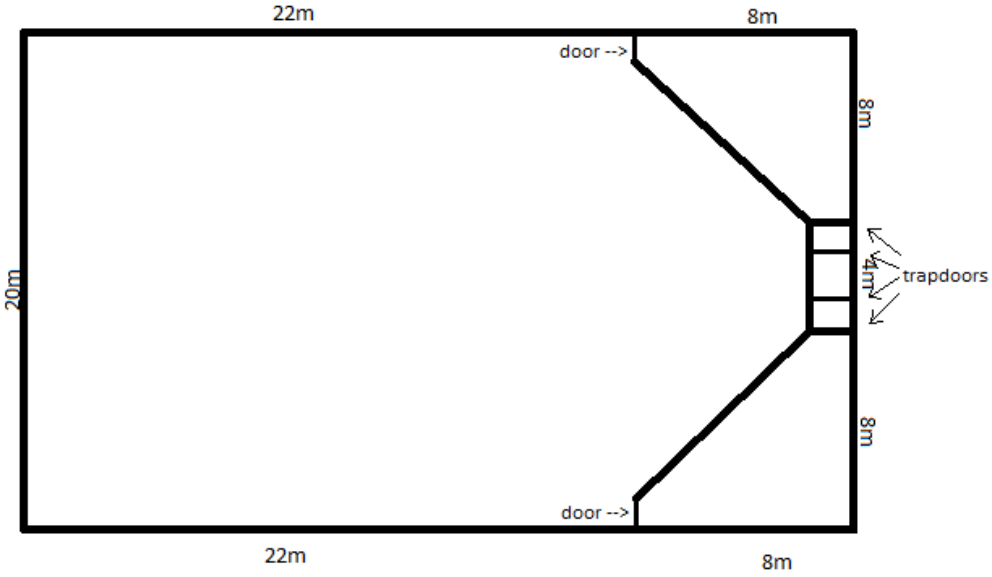

Fig 2. The camp where two African wild dogs were housed, one male and one female. Normally all the doors would be open, the dogs could go everywhere. For the duration of the project, the animals were kept in the corners with the doors closed and the trapdoors open.

Sampling procedure
The first 7 days were used to let the dogs get used to their new situation and to determine their defecation pattern. During the sampling period, the animals were observed every day from 06.45 to 17.00, and faeces was collected as soon as possible after defecation. For logistical reasons, the enclosures couldn't be entered after 17.00. Therefore the faeces produced overnight was collected at 06.45 the next morning. Because it was possible that the glucocorticoid metabolites were unevenly distributed throughout the faecal mass, the faeces were homogenized before taking a sample (Millspaugh et al. 2003). All the samples were frozen within 15 minutes after collection and stored at -20°C until analysis. This storage method ensures that the hormone concentrations in the samples will not change due to bacterial activity (Kahn et al, 2002).

The ACTH challenge test
Baseline faecal samples were collected for ten consecutive days. On the afternoon of the tenth day, each dog was given an intramuscular injection of 50 IU ACTH (Synacthen®, Novartis RSA (Pty) Ltd, Kempton Park South-Africa) diluted with saline. The dogs were observed until 18.00 that day and from 05.00 onwards the next morning. Subsequently, all faecal deposits were collected for another seven consecutive days.

The degradation experiment
To be able to determine the change in GCM concentrations in samples not frozen immediately post-defecation, a glucocorticoid degradation experiment was performed. Faeces were collected from one dog in the morning the day after injection (peak sample) and from another dog on the last day of sampling (baseline sample). The samples were homogenized and frozen at -20°C within 15 minutes after collection. Then they were taken out of the freezer and were defrosted at 17.00. This time point was chosen to reflect the time period when faeces weren't freshly collected (during the night). From the large samples smaller samples were taken and put back into the freezer after 1, 2, 5, 7.5, 10 and 12.5 hours.

Hormone extraction and steroid assays
The faecal samples were lyophilized, pulverized and sieved through a mesh to remove peas, rice, hairs and other undigested material. Approximately 0.05 gram of faecal powder was weighed and used for extraction. This was done according to the procedure described by Ganswindt et al (2005). The faecal powder was mixed with 80% ethanol in water (3 ml) and vortexed for 15 minutes, followed by centrifugation for 10 minutes at 3,300 g. The supernatants were transferred into eppendorf cups and stored until analysis.
Faecal extracts were measured for immunoreactive glucocorticoid metabolites using a Cortisol-3-CMO EIA (first described by Palme and Möstl 1997) and a 11-Oxo-etiocholanolone EIA (first described by Möstl et al. 2002a). For the characteristics of these EIA's see table 1.

Table 1: characteristics of the EIA's used for this study.

EIA	Cortisol	11-Oxo-etiocholanolone
Antibody	Cortisol-3-CMO	5β-Androstane-3α-ol-11-one-17-CMO
Label	Cortisol-3-CMO	5β-Androstane-3α-ol-11-one-17-CMO
Standard	Cortisol	5β-Androstane-3α-ol-11,17-dione
Specificity	Cortisol (ring A reduced)	3a,11-Oxo-cortisol metabolites
Sensitivity	1.5	3
Intraassay CV	7.7 – 11.6 pg/well	6.6 – 11.0
Interassay CV	13.2 – 13.3	10.8 – 17.7

Data analysis
For each individual animal a baseline was determined by calculating the mean hormone concentration of all samples in the 36 hours prior to the ACTH administration.
A period of elevation was defined as two or more consecutive samples above this baseline, after time zero when the ACTH was administered. The peak duration reflects this period of elevation, beginning with the first sample above baseline and ending with the last sample before the concentration returned to baseline. To be able to compare the two assays, all results were converted to a percentage of baseline concentrations.

Results

On average 2.1 samples per day per animal were collected. Out of a total of 143 samples, 71.5 % were collected fresh. (See table 2).

Table2 : an overview of the samples collected.

Animals	Samples	Pre injection	Average per day pre injection	Post injection	Average per day post injection	% fresh
F364	38	22	2.20	16	2.29	73.7
F423	28	15	1.50	13	1.86	75.0
M368	42	25	2.50	17	2.43	71.4
M401	35	21	2.10	14	2.00	65.7
Total	143	83	2.08	60	2.15	71.5

An elevation of 125%-325% post ACTH administration was detected in three out of four study animals, with 263%-325% within 0 to 38 hours when the Cortisol assay was used, compared to 125%-139% within 0-38 hours when the 3α,11-Oxo-CM assay was used (See figures 3 and 4 and tables 3 and 4).
M401 showed unexpected results, with no elevation post-ACTH injection shown by the Cortisol assay, whereas the 3α,11-Oxo-CM assay showed an elevation of 223%, a significantly higher response than detected using this assay in any other animal.

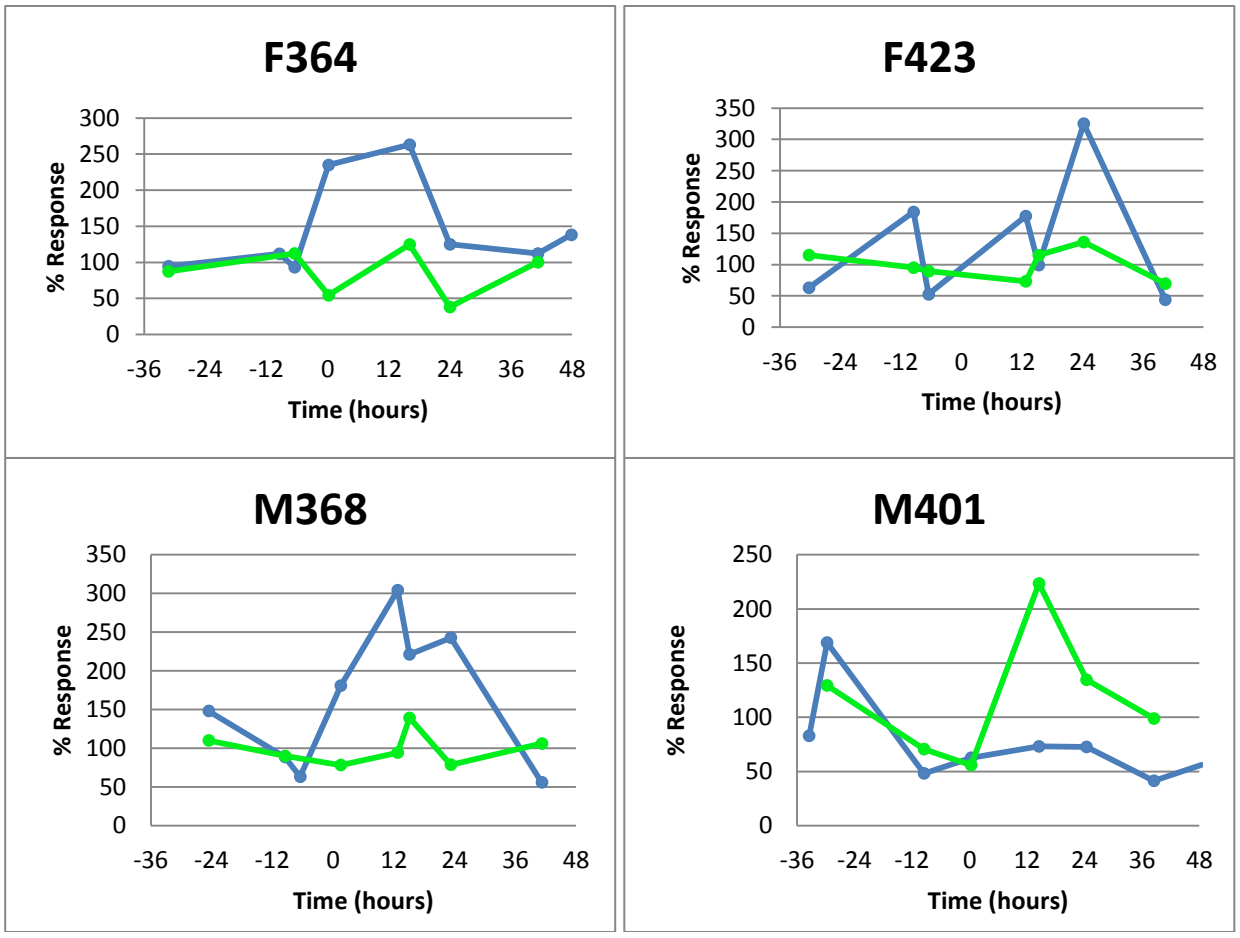

Fig.s 3 and 4. The results of the cortisol EIA (blue line) and the 3α,11-Oxo CM EIA (green line) for the African wild dogs. 0 h represents the time of the ACTH injection.

Table 3: results of the Cortisol EIA.

Cortisol					
Animal	Mean baseline (ng/g)	SEM (ng/g)	Peak concentration (ng/g)	Time of peak (h)	Elevation (%)
F364	103.03	10.67	271.15	0-24	263
F423	202.17	147.85	657.73	14-38	325
M368	162.43	70.83	493.93	0-38	304

Table 4: results of the 3a,11-Oxo-Corticoid Metabolites EIA.

3a,11-Oxo-CM					
Animal	Mean baseline (µg/g)	SEM (µg/g)	Peak concentration (µg/g)	Time of peak (h)	Elevation (%)
F364	3.62	0,63	4.52	0-24	125
F423	4.55	0.61	6.19	14-38	136
M368	2.24	0.32	3.11	12-24	139
M401	2.10	0.87	4.68	3-24	223

The samples in which the highest concentrations were measured were freshly collected for each animal except M368, who defecated during the night between 2 and 13 hours after the ACTH administration. One thing that stands out is that for M368 the timing and duration of the peak was different for the two assays (0-38 h for the Cortisol assay versus 12-24 h for the 3α,11-Oxo-CM assay).
The GIT-time for each dog was determined by feeding rice or peas to the animals and registering when this was first evident in the faeces (see table 5). Both males (M368 and M401) defecated during the night, which is why for these animals the GIT-time is only an estimate.

Table 5: Gastrointestinal transit time.

Animal	GIT-time (h)	Time of Cortisol peak (h)	Time of 3α,11-Oxo CM peak
F364	**26**	**0-24**	**0-24**
F423	**33.5**	**14-38**	**14-38**
M368	**10-24**	**0-38**	**12-24**
M401	**34-48**	**/**	**3-24**

Discussion

In the previous study with African wild dogs described in the introduction, injecting 25 IU ACTH induced a faecal glucocorticoid elevation of 190%-268% when using the Cortisol EIA. With the same assay in this study, an elevation of 263%-325% at 0- 38 hours post ACTH injection was detected using 50 IU ACTH. This suggests that the Cortisol EIA is suitable for measuring changes in faecal glucocorticoid metabolite levels in the African wild dog and might be used for monitoring adrenocortical activity in future studies. The 3α,11-Oxo-CM EIA seems less suitable for this purpose, since it showed an elevation of only 125%-139% 0- 38 hours post ACTH injection. Schatz and Palme (2001) similarly concluded that a Cortisol EIA was the most suitable for faecal glucocorticoid analysis in the domestic dog, since it detected the highest amounts of immunoreactive metabolites when compared with other EIA's.

For M401, one of the male animals, the results were contrary. With the Cortisol EIA, no elevation in glucocorticoid metabolites was found post ACTH injection, whereas with the 3α,11-Oxo-CM EIA an elevation of 261% was found 14.5 hours post-ACTH injection, which was a significantly higher elevation then when this assay was used for the other animals.
Because of the differing results for this one male animal, there are only two females and one male left to use for comparison between sexes and, partly as a result, there were no clear differences seen between the two sexes. However, one would need many more animals to be able to draw a firm conclusion.

Both assays detected an elevation in glucocorticoid metabolite concentrations after ACTH injection, but this was not very prominent in all animals. An explanation for the inter-animal variation could be that the dose administered ACTH was not high enough to cause an optimal reaction from the adrenal glands in all animals. In this respect, Monfort et al (1998) used 400 IU ACTH in wild dogs, compared to only 50 IU ACTH administered in the current study. Terio et al (1999) also used 400 IU ACTH in a study with cheetahs, while Goymann et al (1999) were successful in validating a system for monitoring glucocorticoid levels in spotted hyenas using only 50 IU ACTH.
Since this study was not more conclusive than the previous pilot study, even though the dose of ACTH was doubled, it seems that the ACTH dose is not the only determinant of the level of the elevation in faecal glucocorticoid metabolite concentrations. This theory is supported by De Villiers et al (1997), who found no significant difference in maximum cortisol concentrations in blood samples within 2 hours post ACTH injection, using 8 versus 16 IU ACTH (Acthar Jel, Fisons Pharmaceuticals (Pty) Ltd., Chloorkop, South Africa).

Another aspect which could influence the results is the overall stress level of the animals during the sampling period. Even though the animals were exposed to their new situation and human interaction from seven days prior to the sampling period, to allow accommodation, they still showed excitement. After defecation, the animals had to be chased into one corner, to make it safe to collect the faeces. This resulted in considerable vocalisation and activity and it is possible that it became stressful for the animals to defecate, as a result of conditioning. That is, it is possible that the animals experienced chronic stress during this project and it is conceivable that because the HPA axis was already stimulated extensively, that stimulation with exogenous ACTH didn't give the prominent response expected from the adrenal glands.

71.5% of the samples were fresh when collected. To be able to use the samples that weren't fresh, it is important to ascertain the expected rate of glucocorticoid degradation. A lot of previous studies concerning faecal sampling don't contain suitable metabolite degradation experiments. Huber et al. (2003) found the concentration of glucocorticoid metabolites in faeces collected six hours after defecation did not differ significantly compared to freshly collected faecal samples. However, some reviews about this topic emphasize the importance of bacterial activity in faecal samples, which can elevate or decrease measured hormone concentrations (Keay et al. 2006, Touma et

al. 2005, Möstl et al. 2002b, Sheriff et al. 2011). A degradation experiment shows the changes in glucocorticoid metabolite concentrations in faeces that aren't frozen immediately. These changes are influenced by factors such as temperature and humidity and, therefore, it's important to perform the degradation experiment at the same time as the ACTH challenge. Unfortunately the samples from the degradation experiment in this study weren't analyzed, because of the inconclusive results of the two assays.

It's important to know when fluctuations in stress level are expected to be reflected in the faeces (i.e. the lag time). This information enables correlation between potentially stressful events and higher GCM levels measured in the faeces (Keay et al. 2006). In the current study, the GIT-time was determined to see if there was a correlation between this time and the time of the highest GCM levels measured after ACTH injection. Although this is difficult, because there is a wide range in peak time for both assays, it can be estimated that, with the exception of M401, the animals showed a GIT-time of roughly 10 hours longer than the time of the peak concentration. An explanation for this could be that the lag-time for the glucocorticoid levels represents the gut passage time from duodenum to rectum instead of the total food GIT-time.

The aim of this study was to identify a suitable enzyme immunoassay for monitoring adrenocortical activity in African wild dogs, using faecal samples. The results suggest that the Cortisol EIA is more suitable than the 3α,11-Oxo-CM EIA, however further research is necessary to be able to confirm this suspicion, because the results for one animal were inconclusive. Once the test is validated, it will be a very useful means for monitoring stress in animals in captivity since it is non-invasive, sample collection is easy, and it can be performed over long periods of time. Before this technique can be used on wild ranging animals, extensive knowledge must be obtained about suitable methods for identification of animals, storage of samples, hormone degredation rates and analysing samples in the field.

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Literature

De Villiers M.S, Meltzer D.G.A, van Heerden J, Mills M.G.L, Richardson P.R.K, van Jaarsveld A.S. *Handling-induced stress and mortalities in African wild dogs (Lycaon pictus).* Proc. R. Soc. Lond. B 262; 215-220 (1995)

De Villiers M.S, van Jaarsveld A.S, Meltzer D.G.A, Richardson P.R.K. *Social dynamics and the cortisol response to immobilization stress of the African wild dog, Lycaon pictus.* Hormones and Behaviour 31; 3-14 (1997)

Ganswindt A, Heistermann M, Hodges K. *Physical, physiological, and behavioral correlates of musth in captive African elephants (Loxodonta Africana).* Physiological and Biochemical Zoology 78 (4), 505-514 (2005)

Goymann W, Möstl E, Van 't Hof T, East M.L, Hofer H. *Noninvasive fecal monitoring of glucocorticoids in spotted hyenas, Crocuta crocuta.* General and Comparative Endocrinology 114, 340-348 (1999)

Hodges K, Brown J, Heistermann M. Endocrine monitoring of reproduction and stress. Wild animals in captivity: principles and techniques for zoo management, chapter 33, 447-468 (2010)

Huber S, Palme R, Zenker W, Möstl E. *Non-invasive monitoring of the adrenocortical response in red deer.* The Journal of Wildlife Management, Vol. 67, No. 2 (Apr 2003), pp. 258-266

Keay J.M, Singh J, Gaunt M.C, Kaur T. *Fecal glucocorticoids and their metabolites as indicators of stress in various mammalian species: a literature review.* Journal of Zoo and Wildlife Medicine 37 (3), 234-244 (2006)

Khan M.Z, Altmann J, Isani S.S, Yu J. *A matter of time: evaluating the storage of fecal samples for steroid analysis.* General and Comparative Endocrinology 128, 57-64 (2002)

McNutt J.W, Mills M.G.L, McCreery K, Rasmussen G, Robbins R, Woodroffe R. *Lycaon pictus*. IUCN 2010. IUCN Red List of Threatened Species. (2008)

Millspaugh J.J, Washburn B.E. *Within-sample variation of fecal glucocorticoid measurements.* General and Comparative Endocrinology 132, 21-26 (2003)

Monfort S.L, Mashburn K.L, Brewer B.A, Creel S.R. *Evaluating adrenal activity in African wild dogs (Lycaon Pictus) by fecal corticosteroid analysis.* Journal of Zoo and Wildlife Medicine 29(2), 129-133 (1998)

Morato R.G, Bueno M.G, Malmheister P, Verreschi I.T.N, Barnabe R.C. *Changes in the fecal concentrations of cortisol and androgen metabolites in captive male jaguars (Panthera onca) in response to stress.* Brazilian Journal of Medical and Biological Research 37, 1903-1907 (2004)

Möstl E, Maggs J.L, Schrötter G, Besenfelder U, Palme R. *Measurement of cortisol metabolites in faeces of ruminants.* Veterinary Research Communications 26, 127-139 (2002a)

Möstl E, Palme R. *Hormones as indicators of stress.* Domestic Animal Endocrinology 23, 67–74 (2002b)

Palme R, Möstl E. *Measurement of cortisol metabolites in faeces of sheep as a parameter of cortisol concentration in blood.* International Journal of Mammalian Biology 62, 192–197 (1997)

Palme R, Rettenbacher S, Touma C, El-Bahr S.M, Möstl E. *Stress hormones in mammals and birds: comparative aspects regarding metabolism, excretion, and noninvasive measurement in fecal samples.* Annals of the New York Academy of Science 1040, 162-171 (2005)

Romero L.M, Romero R.C. *Corticosterone responses in wild birds: the importance of rapid initial sampling.* The Condor, 104 (1): 129-135 (2002)

Schatz S, Palme R. *Measurement of faecal cortisol metabolites in cats and dogs: a non-invasive method for evaluating adrenocortical function.* Veterinary Research Communications 25, 271-287 (2001)

Sheriff M.J, Dantzer B, Delehanty B, Palme R, Boonstra R. *Measuring stress in wildlife: techniques for quantifying glucocorticoids.* Oecologia DOI 10.1007/s00442-011-1943-y (2011)

Terio K.A, Citino S.B, Brown J.L. *Fecal cortisol metabolite analysis for noninvasive monitoring of adrenocortical function in the cheetah (Acinonyx jubatus).* Journal of Zoo and Wildlife Medicine 30 (4), 484-491 (1999)

Touma C, Palme R. *Measuring fecal glucocorticoid metabolites in mammals and birds: the importance of validation.* N.Y. Acad. Sci. 1046, 54–74 (2005).

The IUCN/SSC canid specialist group. *African wild dog status survey and action plan*. International Union for the Conservation of Nature and Natural Resources, 1997.

Van de Bildt M.W.G, Kuiken T, Visee A.M, Lema S, Fitsjohn T.R, Osterhaus A.D.M.E. *Distemper outbreak and its effect on African wild dog conservation.* Emerging infectious diseases Vol. 8, No. 2, 211-213 (2002)

Wasser S.K, Hunt K.E, Brown J.L, Cooper K, Crockett C.M, Bechert U, Millspaugh J.J, Larson S, Monfort S.L. *A generalized fecal glucocorticoid assay for use in a diverse array of nondomestic mammalian and avian species.* General and Comparative Endocrinology 120, 260–275 (2000)

Wells A, Terio K.A, Ziccardi M.H, Munson L. *The stress response to environmental change in captive cheetahs (Acinonyx Jubatus).* Journal of Zoo and Wildlife Medicine 35(1), 8-14 (2004)

Young K.M, Walker S.L, Lanthier C, Waddell W.T, Monfort S.L, Brown J.L. *Noninvasive monitoring of adrenocortical activity in carnivores by fecal glucocorticoid analyses.* General and Comparative Endocrinology 137: 148-165 (2004)