Non-invasive hormone monitoring in brown hyaenas *(Hyaena brunnea)*.

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1. Summary

In my 4 months research project I tried to non-invasively monitor the reproduction of brown hyaenas.
For carnivores it is known that most steroid metabolites are excreted into the feces. And it is also known that excreted fecal steroid metabolites accurately reflect hormonal patterns in blood with a time delay related to the time it takes for hormones to turn over into metabolites in the excreta. Advantages of collecting feces instead of blood is that you don't have to capture the animal and the excretory patterns of metabolites in the feces represents a pool of metabolites rather than reflecting the hour to hour fluctuating dynamism quantified in blood [Creel et al. 1996].

To collect brown hyaena feces, I started visiting 191 previously identified latrine sites alongside the roads in Lapalala Wilderness, South Africa. Brown hyenas are known to make use of these latrine sites for defecation and other forms of scent marking. I visited the sites at least once a week for 2 months. Since I also put up cameras at the latrine sites, I could identify the animal visiting the latrines and I could also say something about visitation rates.
After 2 months I found out that the defecation rates at the latrines I was visiting were too low to be able to monitor the reproduction of brown hyaenas. The last few weeks of my field study I tried to find a method to increase the defecation rates at the latrines. Therefore I put out blood at latrine sites and I replaced scats from one latrine to the other (as far apart as possible). I did not find an elevation in defecation rates. Since I did not put up cameras at these latrine sites, I cannot say if they visited the latrines more often, like for pasting.

After my field work in Lapalala Wilderness I went to the Lion Park in Johannesburg were they have two captive brown hyaenas. Since the animals were living (separately) in captivity it was easy to collect daily faecal samples. In the lion park I tried to validate the non-invasive monitoring of glucocorticoid levels trough faecal samples. This validation is necessary before one can use this technique in the field. Besides the validation, I also looked at the degradation rate of hormone metabolites in brown hyaena faeces and at GIT transit times.

2. Patterns of latrine use by brown hyaenas (*Hyaena brunnea*)

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The brown hyaenas current IUCN status is "Near Threatened", yet there is very limited knowledge about their biology. Collecting biological data on brown hyaenas is essential for generating appropriate conservation strategies for this species. This study was conducted from January 2009 until May 2009 in Lapalala Wilderness, South Africa. The aim of this study was to monitor patterns of latrine use by brown hyaenas. Both defecation and visitation rates, using camera traps, were monitored. Defecation rates at latrines were generally low. Visitation rates of latrines were higher than defecation rates at latrines, what could mean that brown brown hyaenas are pasting at the latrine sites more often than that they are defecating at the latrines. 39% of diel patterns of latrine use could be explained by diel patterns of activity. This could mean that they visit the latrines continuously throughout the period they are active, which suggests that the latrines are scattered throughout the brown hyaena territory. It could also mean that only during the two peaks in activity, the activity is determined by visitation of the latrines.

Keywords: Hyaena brunnea, latrine sites, visitation rates, defecation rates, activity patterns

1. Introduction

The use of latrines is common in many carnivores like golden jackals (*Canis aureus)*, striped hyaenas *(Hyaena hyaena)* [Macdonald, 1980], badgers *(Meles meles)* [Roper et al., 1993], spotted hyaenas *(Crocuta crocuta)* [Gorman et al., 1984], and aardwolves *(Proteles cristatus)* [Nel et al., 2002]. Latrines are used for scent marking.
How often carnivores visit latrines differs between species. Otters *(Lutra Canadensis)* use some latrines more frequently than others. Trusso et al.[1998] suggested that this is strongly influenced by forest-cover type, similar to the differentiation of latrine from non-latrine sites. In European badgers Roper et al. [1986,1993] found that the number of faeces per latrine was related to its proximity to territorial boundaries but also to the function of the latrine. In Namibia, Skinner et al. [1981] found that the number of faecal stools per latrine is 5 (n=10) for brown hyaenas *(Hyaena brunnea)* and 12.4 (n=5) for spotted hyaenas.
Brown hyaenas are distributed throughout the south west arid zone of Africa [Smithers 1983]. Unlike other social carnivores, brown hyaenas live in permanent clans, but adults are seldom together [Owens et al. 1996]. Since the current IUCN status of the brown hyaena is "Near Threatened" and there is very limited knowledge about their biology, collecting biological data on brown hyaenas is essential for generating appropriate conservation strategies for this species.
The aim of this study was to monitor patterns of brown hyaena latrine use, and relate latrine use to temporal patterns of brown hyaena activity in the Lapalala Wilderness, Limpopo province, South Africa.

Brown hyenas use latrine sites for scent marking. In the southern Kalahari, brown hyaenas pasted (anal gland scent marking) on average 2.6 times per km. Both chemical and behavioural evidence for brown hyaenas producing pastes with individual smell was found. Mills et al. concluded that pasting served to inform other group members of movements as well as to inform outsiders that a territory was occupied [Mills et al. 1980].

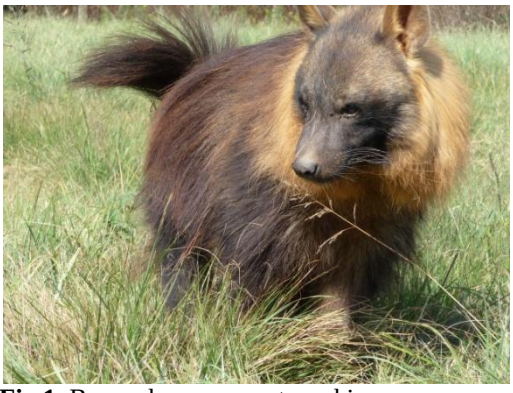

Fig 1. Brown hyaena scent marking.

2. Methods

Study area

The Lapalala Wilderness is a privately owned, 35000 ha game reserve in the core of the Waterberg region of the Limpopo province, South Africa. It constitutes approximately 35% of the core area of the UNESCO Waterberg Biosphere Reserve . The area consists of low but rugged mountains intersected by valleys containing mainly Waterberg mountain bushveld vegetation [Mucina et al. 2006]. The reserve currently hosts healthy populations of a range of large herbivores, including blue wildebeest *(Connochaetes taurinus)*, kudu *(Tragelaphus strepsiceros)*, zebra *(Equus burchelli antiquorum)*, giraffe *(Giraffa camelopardalis tippelskirchi)*, impala *(Aepyceros melampus)* and white *(Ceratotherium simum simum)* and black rhinoceros *(Diceros bicornis)*. It also contains three species of medium sized to large carnivores; black-backed jackal (*Canis mesomelas)*, brown hyaena and leopard (*Pantera pardus)*.

Estimation of brown hyaena defecation rates at latrines

We visited 191 previously identified latrine sites alongside the roads in Lapalala Wilderness. These sites were identified and cleaned out in September 2008. At the first visit of the latrine sites of this research in January 2009, we cleaned out the latrine sites from scats. After the first visit we selected 88 latrine sites to be revisited based on the number of scats deposited since September 2008. We revisited these sites at a minimum of once every week for 2 months to monitor defecation rates.

Estimation of brown hyaena visitation rates at latrines

In addition to monitoring defecation rates, we placed cameras at latrine sites that we had chosen to revisit to monitor visitation rates of the latrine sites. At these sites we cut the grass and the low tree/bush branches. We used Moultrie I40 digital infrared camera traps which are passive motion and heat activated and they were set 24 hr with a 1 minute delay (Moultrie feeders, Alabaster, AL, USA).

Fig 2. Putting up camera traps at latrine sites.

The cameras were left in one place for a minimum of 10 days. A total number of 22 latrine sites were

monitored. The total number of monitoring nights was 257. We checked the cameras every day. To identify the brown hyenas we made use of the characteristic stripe patterns on their legs.

Fig 3. Camera picture from which stripe pattern on leg could be used for identification.

Not all brown hyaenas could be identified by making use of these stripe patterns. Reasons for this were poor picture quality, not seeing the legs on the picture or pictures from different sides of the animals. If they could not be identified by making use of the stripe patterns, a 10 minute interval between two hyaena pictures at the same site was regarded as an independent visit.

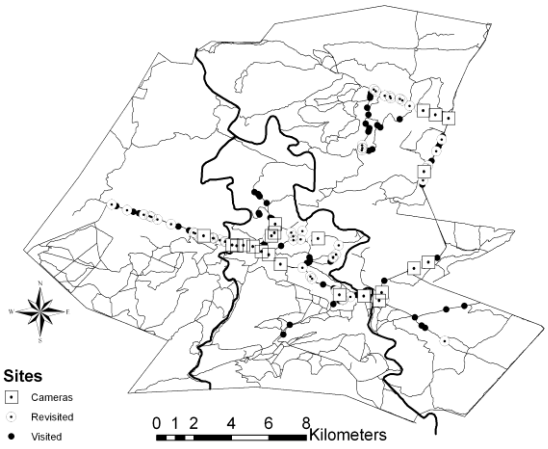

Figure 4. Map of Lapalala Wilderness including brown hyaena latrine sites monitored during the study.

Diel patterns of activity and latrine use and the correlation between these two

We obtained diel patterns (24 hour period) of activity from a camera survey in November and December 2008 in Lapalala Wilderness. In this survey, 39 camera sites were monitored for 7 days. The same cameras were used as for the monitoring of latrine sites. We regarded brown hyaena pictures that were taken at the same site with more than a 10 minute interval as independent events. We obtained diel patterns of visitation from the camera trapping at latrine sites from January-March as described previously.

Analysis

For the analysis of the visitation and defecation data we used mean +- standard deviation and median. Since the data did not show a normal distribution, the median gives a better representation of the results.

3. Results

Defecation rates at latrines

Defecation rates were generally low. However, there was substantial variation between sites, and although the median defecation rates were close to zero (both for the period September – January and January – March), they varied from zero to 2.46 and 1.67 scats per site per 30 days for the two periods, respectively (Fig 2).

From the 191 sites visited in January, we found old scats at 85 sites. Median defecation rates (scats per site per 30 days) for these sites for the period September-January was 0.65, with a maximum of 2.46 and a minimum of 0.19. At 12 from the 88 revisited sites, new scats were found. Median defecation rates for these sites for the period January-March was 1.11, with a max of 1.67 and a min of 0.55.

Visitation rates at latrines

Visitation rates at latrines were substantially higher than defecation rates. Median visitation rate (visits per site per 30 days) for the period January-March was 2.62, with a max of 30 and a min of 0 (Fig 2). For the camera sites, median defecation rate was 0, with a max of 1.11 and a min of 0. The camera monitoring from January-March resulted in 33 independent brown hyaena pictures from which 4 brown hyenas could be identified. One animal was identified at two different sites and at one latrine site more than one animal was identified. 9.7% of the nights recorded a brown hyaena visit.

From the 22 camera sites, 8 sites were visited. Median visitation rate for these 8 sites were 20.71, with a max of 30 and a min of 2.14.

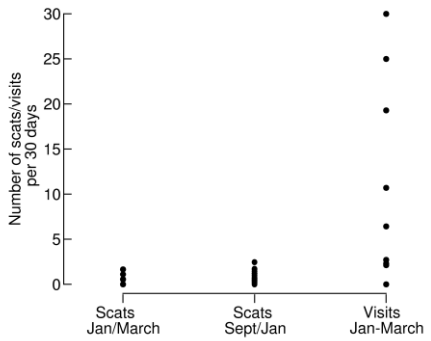

Figure 5. Brown hyaena defecation and visitation rates per latrine during September 2008 - March 2009

Diel patterns of activity and latrine use

Diel patterns of latrine use showed two distinct peaks in time at 9:00 PM and 01:00 AM (Fig 3A). No use of latrines was observed between 05:40 AM and 05:35 PM. No activity as seen from one hour after sunrise until one hour before sunset. 51% of each 24hr period general brown hyaena activity was seen. Latrine use was linearly related to the overall activity patterns of brown hyaenas. ($y=0.6817x + 1.8726$, $R^2 = 0.39$, Fig 3B), which generally showed a very similar temporal pattern to latrine use (Fig 3B.)

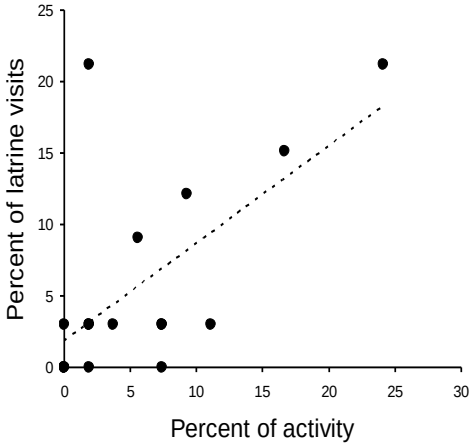

Figure 6. Diel patterns of brown hyaena activity and latrine use. The grey box represents the dark period.

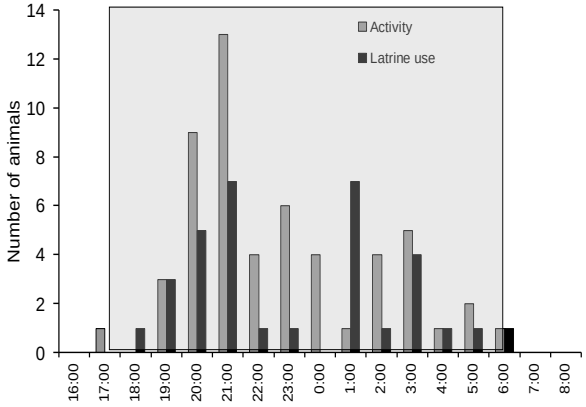

Figure 7. The relationship between activity and latrine use.

4. Discussion

The defecation and visitation rates at the latrine sites monitored in this study were generally low, but there is little information on brown hyaenas to compare with. We can think of different explanations for these low defecation and visitation rates. Firstly, it can be due to the use of many different latrine sites. Secondly, some of the supposed latrines monitored

may no longer have been in use, since some latrines are used only for a short period [Skinner et al. 2005]. A third possible explanation could be that the latrines are cleaned out by dung beetles. This is not a likely explanation if we assume that the following data of spotted hyaenas resemble those of brown hyaenas. Calcium rich spotted hyaena feces is not attractive to dung beetles [Cambefort 1984, Krell et al 2003] and can remain visible for 14 months [Bearder et al 1978]. Only 15 percent of the spotted hyaena feces are brown [Sillero-Zubiri et al 1992], and these scats are produced after a meat rich meal and are disintegrated within 3 days [Bearder et al 1978]. By visiting the latrines weekly, we missed some of these brown scats which might have resulted in a lower estimation of the defecation rate at latrines. A final explanation could be that by cutting the grass and tree/bush branches some of the conspicuous landmarks were taken out of the latrine which might have caused the latrine site being less visited since it has been shown that a lot of carnivores prefer depositing their scent marks at conspicuous landmarks and/or above ground level [Macdonald 1980; Macdonald 1985: Robinson & Delibes 1988: Kruuk 1978: Gorman et al 1984, 1989] . Mills et al. [1980] showed that brown hyaena scent marking may be carried out on grass stems, bushes or rocks. It does not explain the low defecation rates, since only the latrine sites where we put up a camera were cleaned out.

The visitation rates at the latrine sites are higher than the defecation rates at the latrine sites found in this study. An explanation for this could be that the brown hyaenas are pasting at the latrine sites more often than that they are defecating at the latrines.

We found that identification of brown hyaenas by the stripe patterns on their legs is a feasible method for the identification of brown hyaenas, but one should preferably get pictures of both sides of the animals or one should use camera footage for a better chance of getting an identification. The vegetation in between the cameras should be kept short so the legs of the brown hyaenas are visible. The camera survey from January-March showed that more than one hyaena was making use of the same latrine site and one hyaena made use of more than one latrine site which is consistent with data from aardwolves [Net et al. 2002].

We observed no activity from after sunrise until 1 hour before sunset, this is consistent with Mills et al. [1978] that stated that brown hyaenas are almost exclusively nocturnal. We observed activity from 17h30 until 06h00 in the summer period from November-December compared to brown hyaenas in the south-western Kalahari who are active from 18h00 until 08h30 during summer and from about 16h30 until 06h00 during winter [Mills et al. 1977]. Along the Namib coast they are active from sunset to sunrise [Skinner et al. 1995]. Mills et al. also states that brown hyenas are active 42.6% of the 24hr period in the southern Kalahari [Mills et al. 1978]. In this study we did not follow an individual hyaena, so we cannot say what percentage of time a brown hyaena is active. What we can say is that 51% of each 24hr period general brown hyaena activity was seen. 39% of diel patterns of latrine use in this study could be explained by diel patterns of activity. First of all, this could mean that they visit latrines continuously throughout the period they are active. This suggests that the latrines are scattered throughout the brown hyaena territory like stated by

Milles et al. 1980. It could also mean that only during the two peaks in activity, the activity is determined by visitation of the latrines.

5. Conclusion

Defecation rates at brown hyaena latrine sites were generally low. The visitation rates at brown hyaena latrines were substantially higher than defecation rates. An explanation for this could be that the brown hyaenas are pasting at the latrine sites more often than that they are defecating at the latrines.
39% of diel patterns of latrine use could be explained by diel patterns of activity, this could mean that they visit the latrines continuously throughout the period they are active. Which suggests that the latrines are scattered throughout the brown hyaena territory. It could also mean that only during the two peaks in activity, the activity is determined by visitation of the latrines.

6. Acknowledgments

I wish to thank the Institute for Breeding Rare and Endangered African Mammals (IBREAM) for giving me the opportunity to perform this brown hyaena study. I thank my supervisors Fredrik Dalerum and Monique Paris for their support and advice during this project. I also wish to thank the management of Lapalala Wilderness: Duncan Parker, Mike Gregor, Tom Hugo, Anton Walker, Roger Collinson, and Anthony and Erin Roberts for allowing me to perform my research in Lapalala Wilderness and for all their help and support. And thanks to John Hanks (IBREAM) who visited us in Lapalala and really encouraged and motivated us with his enthusiasm and optimism!

7. References

BEARDER S.K. & RANDALL R.M. 1978. The use of fecal marking sites by spotted hyenas and civets. *Carnivore* 1: 32-48.

CAMBEFORT Y. 1984. Étude écologique des Coléoptères Scarabaeidae de Côte d'Ivoire. *Travaux des Chercheurs de la Station de Lamto* 3: VIII + 294 + 13 pp.

GORMAN M.L., MILLS M.G.L. 1984 Scent marking strategies in hyenas (Mammalia). *Journal of Zoology* 202: 535–547

GORMAN M.L., TROWBRIDGE B.J. 1989 The role of odor in the social lives of carnivores. In *Carnivore behavior, ecology and evolution. (eds. Gittleman J.L.),* pp: 57–139: Cornell University Press, Ithaca.

GORMAN M. L. 1990. Scent-marking strategies in mammals. *Revue Suisse de Zoologie* 97:3-29.

KRELL F.T., KORB, J., WALTER P., 2003 The beetle fauna of hyaena latrines: coprocenoses consisting of necrophagous beetles (Coleoptera Trogidae Scarabaeidae) *Tropical Zoology* 16:145-152

KRUUK H. 1978 Spatial organisation and territorial behaviour of the European badger, *Meles meles*. *Journal of Zoology* 184:1–19

MACDONALD D.W. 1980 Patterns of scent marking with urine and faeces amongst carnivore communities. *Symposia of the Zoological Society of London* 45: 107–139

MACDONALD D.W. 1985 The carnivore: Order Carnivora. In *Social*

odours in Mammals (eds Brown R.E., Macdonald D.W.) pp 619–722 Clarendon Press, Oxford.

MILLS, M.G.L., MILLS, M.E.J. 1978 The diet of the brown hyaena *Hyaena brunnea* in the southern Kalahari. *Koedoe* 21:12-149

MILLS, M.G.L., GORMAN, M.L., MILLS, M.E.J. 1980, The scent marking behaviour of the brown hyaena *Hyaena brunnea, South African Journal of Zoology* 15:240-248

MILLS, M.G.L. 1982 The mating system of the brown hyaena, *Hyaena brunnea* in the southern Kalahari. *Behav. Ecol. Sociobiol.* 10:131-136

MUCINA, L. & RUTHERFORD, M.C. (eds.) 2006 The vegetation of South Africa, Lesotho and Swaziland. *Sterlitzia 19*. South African Biodiversity Institute, Pretoria, South Africa.

NEL, J.A.J., BOTHMA, J.DU P., 2002 Scent marking and midden use by aardwolves (*Proteles cristatus*) in the Namib Desert. *Journal of Ecology* 21.1:25-39

OWENS D., OWENS M. (1996) Social dominance and reproductive patterns in brown hyaenas, *Hyaena brunnea*, of the central Kalahari desert *Anim. Behav.* 51: 535–551

ROBINSON I.H., DELEBIS M. 1988 The distribution of faeces by the Spanish lynx (*Felis pardina*). *Journal of Zoology* 216:577–582

ROPER T.J., SHEPERDSON D.J., DAVIES J.M. 1986 Scent marking with faeces and anal secretion in the european badger (*Meles meles*): seasonal and spatial characteristics of latrine use in relation to territoriality. *Behaviour* 97: 94–117

ROPER T.J., CONRADT L, BUTLER J, CHRISTIAN S.E., OSTLER J, SCHMID T.K. 1993 Territorial marking with faeces in badgers (*Meles meles*): a comparison of boundary and hinterland latrine use. *Behaviour* 127: 289–307

SILLERO-ZUBIRI C., GOTTELLI D 1992 Feeding ecology of spotted hyaena (Mammalia: *Crocuta crocuta*) in a mountain forest habitat. *Journal of African Zoology* 106: 169-176

SILLERO-ZUBIRI C., Macdonald D. W. 1998. Scent-marking and territorial behaviour of Ethiopian wolves Canis simensis. *Journal of Zoology* 245:351-361

SKINNER J.D. VAN AARDE R.J. 1981 The distribution and ecology of the brown hyaena, *hyaena brunnea*, and spotted hyaena, *crocuta crocuta*, in the central Namib desert. *Madoqua* 12:231-239

SKINNER J.D., VAN AARDE R.J., GROSS R.A. 1995 Space and resource use by brown hyaenas, *Hyaena brunnea*, in the Namib desert. *J. Zoology London* 237:123-131

SKINNER J.D., CHIMIMBAC.T. 2005 Hyaena brunnea. In *Mammals of the Southern African Subregion* 365-370 Cambridge University Press, Cambridge

TRUSSO, SAMARA 1998 Lake shore latrine site selection by river otters (Lutra canadensis) *Masters Abstracts International* 1998, Vol. 36 Issue 02, p446-446

3. Non-invasive glucocorticoid measurement in brown hyaenas *(Hyaena brunnea)*

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Non-invasive hormone measurement techniques have been widely developed the last two decades. These techniques are especially interesting to use in wildlife. Before one can use such a technique, it has to be biologically validated. The aim of this study was to validate an assay for non-invasive monitoring of glucocorticoid levels in brown hyenas through faecal samples. This study was conducted in March 2009, on two brown hyaenas in the Lion Park in Johannesburg. An AcTH challenge test was performed and hormone metabolites were measured with the 11-Oxo-etiocholanolone II enzyme immuno assay. This study provides biological validation for measuring fecal glucocorticoid metabolites in brown hyaenas.
GIT transit times and fecal glucocorticoid metabolite degradation rate were also studied. GIT transit times (15 hours and 23-24 hours) in this study do not give a good indication of the delay in fecal glucocorticoid excretion in brown hyaenas. Glucocorticoid metabolite levels decline significantly over time. This highlights the need to collect fresh samples and freeze these immediately for accurate detection of GCM levels.
Keywords: Hyaena brunnea, fecal glucocorticoid metabolites, AcTH callenge test, GIT transit times, degradation rate

1. Introduction

Stress can be defined as the physiological response elicited when an individual perceives a threat to its homeostasis. One of the responses to physiological or behavioural stressors is the secretion of glucocorticoids (GC's) by the hypothalamic-pituitary-adrenal axis. Although GC responses are adaptive in the short term, chronically elevated GC levels can produce an array of pathologies, including reproductive suppression, ulcers, muscle wasting and immune suppression [Sapolsky 1992, Munck 1984].
Methods for non-invasive measurement of GC levels in urine or faeces have been widely developed the last two decades [Wasser et al. 1988, 1997; Creel et al. 1991, 1996; Monfort et al. 1997; Palme et al. 1998]. Excreted urinary or faecal hormone metabolites accurately reflect hormonal patterns in blood with a time delay related to the time it takes for hormones to turn over into metabolites in the excreta. Whether the hormone is primarily passed into urine or faeces is species dependent. In spotted hyaenas for instance, glucocorticoids can be monitored trough fecal sampling [Goymann et al 1999]. The advantage of collecting urine or feces instead of blood is that there is no need to capture the animal and thereby disturbing the animal subject. In addition, another advantage is that the faecal glucocorticoid metabolites represent a pool of metabolites rather than reflecting the hour to hour fluctuating dynamism quantified in

blood [Creel et al 1996]. However, each non-invasive hormone monitoring technique must be biologically validated. The aim of this study was to validate an assay for non-invasive monitoring of glucocorticoid levels in brown hyenas through faecal samples. This was done by AcTH injection into 2 captive study animals and faecal glucocorticoid levels pre- and post injection were measured. When extraneous ACTH is administered to a mammal it acts upon the adrenal pathways to cause release of glucocorticoids, which cause a rise in plasma levels of these steroids followed by a return to normal baseline levels. This rise and fall is reflected in the feces after the glucocorticoids are metabolized and excreted [Keay et al 2006]. Such a validation experiment was already performed by Goymann et al. on spotted hyenas [Goymann et al. 1999]. Naturally occurring bacteria and their enzymes degrade steroid metabolites in feces [Keay et al 2006]. In the field, reluctance to disturb study animals, behavioural sampling protocols, the presence of other large mammals such as lions, and the distance back to basecamp can make it difficult to quickly collect, process, and freeze samples [Dloniak et al 2004]. Therefore, in this study we also analyzed the degradation rate of hormone metabolites in faeces whilst kept outside where temperatures varied between 15 and 25 °C, without rainfall. This information will yield valuable information from which we can define guidelines on the needed freshness of field samples that are still suitable for analysis.
Finally, gastrointestinal transit times were determined by making use of party glitters as a digestive marker.

2. Methods

Field study site, subjects
Data were collected in the Lion Park in Johannesburg, South Africa in March 2009. Subjects were 2, individually kept, adult brown hyenas of unknown gender and age, referred to as animal 1 and 2.

Fig 1. Brown hyaena (animal 1) in the Lion Park in Johannesburg.

Sample collection and storage
We collected faeces from the study animals during a 5 day pre-injection period to provide a measure of baseline glucocorticoid levels in the animals. We darted the subjects with 50 IU of AcTH (*Synacthen*, Novartis, Australia) on the fifth day of faecal sample collection. The time of darting was 07:50 and16:40 respectively. After AcTH injection, we collected faeces for another 6 days. We checked the enclosures between 8 and 9 AM and if possible again between 4 and 5 PM.
For the purpose of defining the rate of hormone metabolite degradation, we observed the animals from early morning until defecation took place. We than removed the scat from the hyaena enclosure and placed it outside the enclosure on the grass. Since it was summer, temperatures varied from 25°C during the day to 15°C during the night. There was no rainfall. After about 0, 0.5, 1, 2, 4, 8, 16 and 32 hours we took a part

of the scat and put it in the freezer at -20 degrees until analysis took place.
We fed a table spoon of plastic red party glitter (Pick and Pay, South Africa) as a digestive marker to determine GIT transit times just after the animal was darted with the AcTH. It was mixed with the normal diet of these specific hyenas. This normal diet consist of horse and cow meat/bones. We watched the animals until they started eating, to be sure of the exact time of eating. Because we could only enter the enclosure twice a day, we can not tell the exact GIT transit time.
We used plastic cloves to pick up the scats and we than put it in glass sample cups. The glass sample cubs were stored frozen at -20°C until transported to the Veterinary Faculty of Onderstepoort, University Pretoria. They were transported for one hour in an isolating box without ice. At Onderstepoort, the samples were put in a freezer at -20°C until assayed.

Extraction
Faecal samples were extracted and analysed at the Veterinary Faculty Onderstepoort, University Pretoria, South Africa.
We freeze dried the faecal samples in a vacuum oven (Instruvac Freeze-drier from Air & Vacuum Technologies, model: VFDT 02.50, manufactured and purchased in South-Africa) at -48°C for 48 hours to reduce water content variability between samples. After the samples were freeze dried, the samples were pulverized using tweezers and a sieve. The tweezers, sieve and working surface were cleaned with 80% EtOH (prepared from Ethanol Absolute 99%, Merck, Saarchem, diluted with distilled water). Hand gloves were changed between samples.
Between 330 and 340 mg freeze dried faeces was put in a sample tube (Kimble Borosilicate Glass, Disposable Culture Tubes, 12x75mm) and used for the extraction. The precise sample weight was recorded for each sample. For the extraction we added 3 ml 90% methanol to the sample tubes. The tubes were than vortexed for 15 minutes, whereafter centrifuge for 10 minutes at 3300g. The supernatant was transferred to a fresh tube and stored at -20 °C until analysis.

Enzyme immunoassay
The enzyme immuno assay (EIA) used in this study is the 11-Oxo-etiocholanolone II as described by Ganswindt et al. (2003) who validated the assay for usage in the African elephant *(Loxodonta Africana).*
Antibodies were raised against 5b-androstane-3a -ol-11-one-17-CMO. 5b-androstane-3a -ol-11-one-17-CMO was used as label and 5b-androstane-3a -ol-11,17-dione was used as standard. The sensitivity of the assay at 90% binding was 3 pg per well, and the inter- and intra-assay coefficients of variation ranged between 4.21% and 17.27%. The assay was performed on microtiter plates with a double antibody technique according to the methods described by Palme and Möstl (1997) and Ganswindt (2002). Sample concentrations are expressed as µg/g DW.

Data analysis
We used a mixed linear model to test the effect of time on measured GCM levels. In the model, we used time since defecation as a fixed continuous predictor and added each scat event nested within individual as a random factor to control for interdependence both within samples and within individuals.

3. Results

Validation of non-invasive monitoring of glucocorticoid levels in brown hyenas (Hyaena brunnea) through faecal samples

Pre-injection glucocorticoid levels for animal 1 varied between 2.98-9.61 µg/g DW (mean 5.44 +- 2.88) and for animal 2 between 0.05-6.78 µg/g DW (mean 2.35 +- 2.60). After AcTH injection on day 4, glucocorticoid levels raised up to 21.13 µg/g DW (388 % compared to baseline) for animal 1 and up to 63.15 µg/g DW (2687% compared to baseline) for animal 2. Peak glucocorticoid level of animal 2 was much higher than of animal 1. The peak glucocorticoid level for animal 1 was measured in a fresh scat (defecation was seen), 24 hours and 46 minutes after AcTH injection. Peak glucocorticoid level for animal 2 was measured more than 24 hours and less than 39 hours and 32 minutes after AcTH injection at which time the sample was collected, but defecation took place during the night and was therefore not timed. Before peak glucocorticoid level for animal 2 was measured, a clear increase in glucocorticoid level up to 12.30 µg/g DW could be measured in a sample that was collected 24 hours and 8 minutes after AcTH injection. Glucocorticoid levels returned to 7.54 µg/g DW for animal 1 and 8.82 µg/g DW for animal 2 both one day after glucocorticoid peaking. For animal 1 this showed that the glucocorticoid level returned to pre-injection level range as expected. The glucocorticoid level of animal 2 did not completely return to pre-injection level range as yet, but a drastic decline could be measured (fig 1). Both animals showed a second peak, lower than the one after AcTH injection (animal 1 on day 10, animal 2 on day 11).

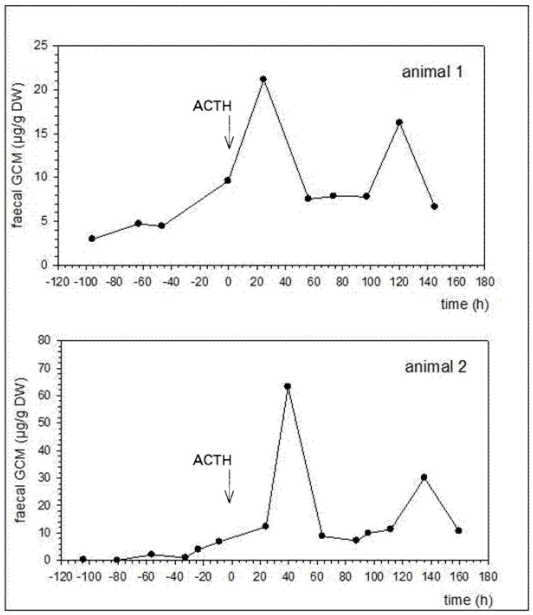

Fig 2. Fecal glucocorticoid metabolite concentrations during the AcTH challenge test. Arrows indicate time of AcTH injection.

Degradation rate of hormone metabolite concentrations in brown hyaena faeces

Of both brown hyaenas, two fresh scats were collected. Defecation time of these four scats were recorded. For these four scats, GCM declines significantly over time ($\beta = -1.3 \times 10^{-3}$, $t = 2.29$, $p = 0.03$) and the decline was more pronounced in samples with high GCM levels (Fig 2).

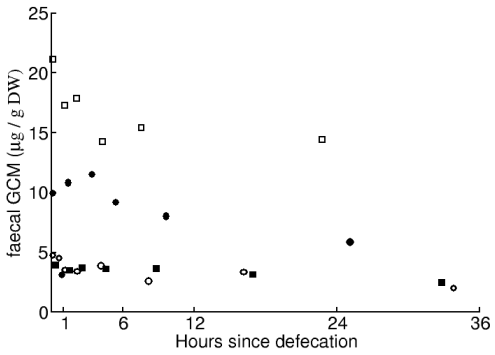

Fig 3. Degradation curve of glucocorticoid hormone metabolites in faeces

GIT transit time in brown hyaenas
Time feeding until time collecting scat with glitter for hyaena A was 15 hours (17h30 until 08h36 the next day). Time feeding until time collecting scat with glitter from hyaena B was 23-24 hours (17h00-18h00 until 16h48 the next day). GIT transit times for these two animals are therefore less than 15 hours and 23-24 hours, assuming that the GIT transit time for the glitter, which was mixed with the food, is the same as the GIT transit time of the food that was fed with the glitter. The glitter came out with a minimal change in brightness of the red colour.

4. Discussion

AcTH challenge test
Although the exact identity of the metabolites measured in this study remains unknown, the AcTH challenge test suggests that the metabolites measured were relevant indicators of adrenal activity, because administration of exogenous AcTH led to a clear increase in these fecal metabolites in both subject animals.
An explanation for the much higher increase in glucocorticoid level in animal 2 could be that the animal was stressed during the darting. First we tried to chase it out of its burrow and after that the dart would not go in at once, because of all the fur on the animal. There were no difficulties darting animal 1.
In spotted hyaenas, fecal glucocorticoid metabolites peaked 26 +- 5 hours after AcTH injection [Goymann et al. 1999]. This is similar to the results found in this study on brown hyaenas, in which fecal glucocorticoid metabolites peaked 24 hours and 46 minutes after AcTH injection for animal 1 and more than 24 hours but less than 39 hours and 32 minutes after AcTH injection for animal 2.
Both animals show a second peak in fecal glucocorticoid metabolites after AcTH injection (subject A 5 days after AcTH injection, subject B 6 days after AcTH injection). No recorded stressful event happened during that period. Although diurnal fluctuations can occur [Keay et al 2006], all samples around and at the day of the second peak of fecal glucocorticoid metabolite concentration were collected around 8.00AM, thus diurnal fluctuations do not explain the second peaks. Another explanation for, short-term, fluctuations in glucocorticoid metabolite concentrations could be diet, especially in carnivores, because the diet itself may contain glucocorticoids [Keay et al 2006]. There is no information on the glucocorticoid content in the diet of these brown hyaenas.

GIT transit time in brown hyaenas
The delay of fecal glucocorticoid excretion in sheep, ponies, and pigs roughly corresponds with the food transit time from duodenum to rectum in these animals [Palme et al. 1996]. In spotted hyenas, GIT transit time was approximately 24 hours and fecal glucocorticoid excretion increased within 24–50 h after injection of ACTH. Taking gut passage time as an approximate measure of food passage time between duodenum and rectum, the time lag of glucocorticoid excretion in feces appears to correspond with food transit time in spotted hyenas as well [Goymann et al 1999].
The GIT transit time for animal 1 in this study was less than 15 hours and 6 minutes, compared to a delay in fecal

glucocorticoid excretion of 24 hours and 46 minutes. The GIT transit time for animal 2 in this study was less than 23-24 hours, compared to a delay in fecal glucocorticoid excretion of 24 hours and 8 minutes, although glucocorticoid levels were still rising at that time. Thus the time delay of glucocorticoid excretion in feces appears to roughly correspond with food transit time in one of the two brown hyaenas in this study. From this study we therefore cannot say GIT transit time gives a good indication of the delay in fecal glucocorticoid excretion. Measuring the GIT transit time in more brown hyaenas and if possible, measuring the food passage time between duodenum and rectum instead of measuring the GIT transit time will give more conclusive results on the relationship between the delay in fecal glucocorticoid excretion and the food transit time from duodenum to rectum.

Degradation rate of hormone metabolite concentrations in brown hyaena faeces
No previous research was found on fecal glucocorticoid metabolite degradation in other hyaena species. Fecal androgen concentrations in spotted hyaenas do not seem to be affected by delays up to 48 h between depositing and freezing (there is no information on temperature during the delay between depositing and freezing) [Dloniak et al 2004]. But a research on fecal glucocorticoid metabolite degradation in baboons showed that degradation rates were low, representing no more than 9.3% over 30 days of storage at ambient temperature and when stored at −10 °C, no significant steroid changes were observed for up to 400 days. In our study, we found a significant decline in GCM levels over time. Therefore, low GCM levels are not conclusive for low circulating GCM levels. This highlights the need to collect fresh samples and freeze these immediately for accurate detection of GCM levels.

5. Conclusion

The aim of this study was to validate an assay for non-invasive monitoring of glucocorticoid levels in brown hyenas through faecal samples. Our results show that the 11-Oxo-etiocholanolone II EIA tested in this study, could detect a clear rise in glucocorticoid metabolite concentration after AcTH injection. Therefore, this study provides biological validation for measuring fecal glucocorticoid metabolites in brown hyaenas. GIT transit time of only one of the two brown hyaenas in this study roughly corresponds to the delay of fecal glucocorticoid excretion. From this study, we therefore cannot say that GIT transit time gives a good indication of the delay in fecal glucocorticoid excretion in brown hyaenas.
Our result on degradation showed that detectable GCM levels declined significantly over time. This highlights the need to collect fresh samples and freeze these immediately for accurate detection of GCM levels.

Since all these results are based on only 2 brown hyaenas, performing this study on more brown hyaenas is recommended to get a more accurate indication of GIT transit time in brown hyaenas and GCM degradation in brown hyaena feces. Putting up video cameras at the enclosures will help to get the exact time of defecation. It might also be useful to compare different EIA's in future studies, like in the study performed by Goymann et al [1999].

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7. References

CREEL S., MOONFORT S.L., WILDT D.E., WASER P.M., 1991 Spontaneous lactation is an adaptive result of pseudopregnancy. *Nature* 351:660-662

CREEL S., CREEL N.M, MONFORT S.L., 1996 Social stress and dominance. *Nature* 379:212

DLONIAK S.M., FRECNH J.A., PLACE N.J., WELDELE M.L.GLICKMAN S.E., HOLEKAMPA K.E. 2004 Non-invasive monitoring of fecal androgens in spotted hyenas (*Crocuta crocuta*) *General and Comparative Endocrinology* 135: 51–61

GANSWINDT A., HEISTERMANN M., BORRAGAN S., HODGES J.K., 2002 Assessment of testicular endocrine function in captive African elephants by measurement of urinary and fecal androgens. *Zoo Biol*. 21: 27–36.

GANSWINDT A., PALME R.,HEISTERMANN M., BORRAGAN S., HODGES J.K. 2003 Non-invasive assessment of adrenocortical function in the male African elephant (Loxodonta africana) and its relation to musth *General and Comparative Endocrinology* 134: 156–166

GOYMANN, W., MÖSTL, E., VAN T HOF T., EAST M.L., HOFER H. 1999 Noninvasive fecal monitoring of glucocorticoids in spotted hyenas, Crocuta crocuta. *Gen. Comp. Endocrinol*. 114: 340–348

KEAY J.M., SINGH J.,GAUNT M.C., KAUR T. 2006 Fecal glucocorticoids and their metabolites as indicators of stress in various mammalian species. *Journal of Zoo and Wildlife Medicine* 37(3):234-244

MONFORT S.L. WASSER S.K. MASHBURN K.L., BURKE M., BREWER B.A., CREEL S., 1997 Steroid metabolism and validation of non-invasive endocrine monitoring in the African wild dog *(Lucaon pictus). Zoo Biology* 29:176-183

MUNCK A., GUYRE P.M., HOLBROOK N.J 1984 Physiological functions of glucocorticoids in stress and their relation to pharmalogical actions. *Endocrine revieuws* 5 (1): 25-44

PALME R., MÖSTL E., 1997 Measurement of cortisol metabolites in faeces of sheep as a parameter of cortisol concentrations in blood. *Int. J. Mammal. Biol*. 62: 192–197.

PALME R.C., BOBIA C., MESSMAN S., MOSTL E., 1998 Measuring faecal

cortisol metabolites: a non-invasive tool to evaluate adrenocortical activity in mammals. *Advances in Ethology* 33:27-46

PUKAZHENTHI, B.S., WILDT, D.E. 2004 Which reproductive Technologies are most relevant to studying, managing and conserving wildlife? *Reproduction, Fertility and Development* 16: 33-46

SAPOLSKY R. 1992 Neuroendocrinology of the stress response. In *Behavioral andocrinology* (eds Becker J.B., Breedlove S.M., Crews D.), pp 287-324 MIT Press, Cambridge, Massachusetts.

WASSER S., RISLER L., STEINER R.A., 1988 Excreted steroids in primate feces over the menstrual cycle and pregnancy. *Biological reproduction* 39:862-872

WASSER S., BEVIS K., KING G., HANSON E., 1997 Noninvasive physiological measures of disturbance in the Nothern Spotted owl. *Conservation Biology* 11:1019-102