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Faecal glucocorticoid metabolite concentrations and their alteration post-defaecation in African wild dogs *Lycaon pictus* from South Africa

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Non-invasive techniques for the monitoring of animal well-being, such as faecal hormone analysis, are increasingly becoming some of the most desirable methods for addressing practical conservation questions. Despite the widespread use of faecal hormone measurements for monitoring responses to stressors and its known applicability to African wild dogs *Lycaon pictus*, the potential influence of time of collection post-defaecation on stress-steroid concentrations in faecal matter has not yet been investigated. In the present study, we determined the rate at which African wild dog faecal glucocorticoid metabolite (fGCM) concentrations change over a 16-day period post-defaecation, in order to provide recommendations for best sampling practice. No significant changes in fGCM concentrations were found for the first 48 h post-defaecation. However, an approximately 30% increase in fGCM concentrations were already noted between day 1 and day 2, followed by a significant 150% increase at 96 h post-defaecation. We therefore suggest that respective faecal material should be collected within the first 24 h post-defaecation to ensure the reliability of fGCM analysis. In addition, we collected baseline data denoting the fGCM concentrations of captive African wild dogs sampled across three South African captive sites. Determined baseline fGCM concentrations differed between African wild dogs at the sites sampled. These data could be used in future studies aimed at identifying the key stressor complexes perceived by captive African wild dogs in order to improve management strategies.

Keywords: captive housing conditions, fGCM stability post-defaecation, fGCM baseline, captive management

Hormone monitoring has become a widely-used approach to gain insights into factors that may act as animal-perceived stressors (Ganswindt et al. 2012). One of the most popular matrices for non-invasive hormone monitoring is faeces, which, in comparison to blood, saliva or urine analysis, facilitates feedback-free sampling as animals are not usually disturbed during sample collection (Ganswindt et al. 2012). In addition, the use of faecal steroid analysis allows for the assessment of a more cumulative hormone signal than other matrices (Hulsman et al. 2011). This is largely due to the fact that faecal hormone metabolite (fGCM) concentrations represent an accumulation of the fluctuating secretions and eliminations of hormones circulating in the blood (Möstl et al. 2005, Touma and Palme 2005).

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There are several challenges associated with faecal steroid analyses, including species and sex-specific differences in steroid metabolism (Touma et al. 2004) and the potential uneven distribution of hormone metabolites throughout a faecal sample (Millspaugh and Washburn 2004). The stability of faecal steroid concentrations post-defaecation also needs to be investigated to ensure the comparability of determined hormone values from differently aged sample material. Glucocorticoids are metabolized in the liver and gut prior to excretion, and bacterial enzymes present in the faeces continue this process post-defaecation (Palme 2005). Standardized collection procedures, usually involving the freezing of faecal material directly post-defaecation, have thus been developed to ensure the comparability of determined faecal steroid concentrations (Hulsman et al. 2011). This is achieved through the removal of moisture from the faeces (for example by freezing), thereby halting the action of bacterial enzymes in the faeces which alter assay-sensitive steroid metabolite concentrations post-defaecation(Washburn and Millspaugh 2002). Under field conditions however, where

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the collection of samples directly after defaecation is either not possible or the freezing of collected samples is delayed, this approach becomes more challenging.

The African wild dog *Lycaon pictus* is a medium-sized social carnivore which was once widely distributed over most of sub-Saharan Africa (Skinner and Chimimba 2005). However, African wild dogs are now considered the second most endangered carnivore on the African continent and the most endangered carnivore in South Africa (IUCN 2016). This has necessitated the artificial management of the South African *L. pictus* population, which has been mediated through the use of a metapopulation dynamic structure to avoid the deleterious factors directly related with a genetic bottleneck (Mills et al. 1998). Ultimately, such management relies on the use of 'source and sink' populations where the captive breeding of African wild dogs could play an important role in replenishing free-ranging packs (Mills et al. 1998).Thus, a greater understanding of the factors which contribute to increased stress in African wild dogs is extremely important, particularly in ensuring the success of captive breeding programs, which need to minimize the negative reproductive and immuno-suppressive effects associated with prolonged glucocorticoid production.

Although hormone monitoring is widely used to examine responses to stressors in captive and free-ranging African wild dogs (Creel 2005, Vlamings 2011, van der Weyde et al. 2016), potential differences in faecal glucocorticoid metabolite (fGCM) concentrations post-defaecation have not been investigated. As a result, previous studies that aimed to identify perceived stressor complexes in African wild dogs have, by necessity, relied on intense observation of individuals for the collection of faecal samples as soon as possible post-defaecation. Considering the preferred habitat for free-ranging African wild dogs (Skinner and Chimimba 2005), maintaining visual contact can become increasingly difficult and labour intensive. It is therefore not surprising that many stress-related physiology studies on African wild dogs utilize captive settings (Creel et al. 1997, Monfort et al. 1998, Santymire and Armstrong 2010).

With this in mind, we aimed to determine to what extent the collection of African wild dog faecal samples directly post-defaecation should be considered a necessity by examining changes in fGCM concentrations over time after defaecation. We also collected baseline data to gain insights into how fGCM concentrations may vary among captive African wild dogs housed in different South African captive facilities.

Material and methods

This research project commenced with the approval of the University of Pretoria Animal Ethics Committee (AEC) (Ethics clearance number: EC023-17), as well as the National Zoological Gardens (NZG) (Pretoria) Research Ethics and Science Committee (RESC) (Ethics clearance number: P17/08).

Study sites and sampling

For the alteration of steroid concentration post-defaecation experiment, we collected fresh faecal material (from 12–14 June 2017) at the Johannesburg Zoo (Jhb Zoo), South Africa, as well as (from 12–14 July 2017) from the Hoedspruit Endangered Species Centre (HESC), South Africa. After observing defaecation events, we collected seven faecal samples immediately post-defaecation; four from males housed at the HESC, as well as two from males and one from a female housed at the Jhb Zoo, which we froze on site at –20°C. In the laboratory, we defrosted the samples at 4°C and subsequently thoroughly mixed them over a cool surface by hand. This mixed faecal material was then divided into 33 equal sub-samples and stored, in containers exposed to open air, at room temperature (12–20°C). Subsequently, three sub-samples (triplicates) were frozen at –20°C at 0, 0.5, 1, 2, 6, 12, 24, 48, 96, 192 and 384 h post-homogenization until hormone analysis. The use of this drying regime allowed a prolonged drying of the faecal material as to what would be expected under much warmer conditions in the field (throughout the course of our study, field temperatures ranged between 11 and 32°C). This consequently allowed for an analysis which was able to encapsulate a longer timeframe over which bacterial enzymes could theoretically remain active and continue to metabolize fGCMs within the faeces (Galama et al. 2004). Thus mimicking the 'worst case scenario' in terms of possible field sampling conditions.

In addition, we collected 35 faecal samples from three different sites across South Africa: The Jhb Zoo and the National Zoological Gardens in Pretoria (NZG Pta) in Gauteng Province, and the HESC in the Limpopo Province (Fig. 1). At the HESC facility, sampling occurred in two separate enclosures (one about an area of 7700 m^2 and the other of about 4000 m^2). Adequate fresh water and shelter where always provided and each enclosure was separated by other fenced areas that housed other predators such as lion *Panthera leo* and cheetah *Acinonyx jubatus*. Thick vegetation and other infrastructure also provided visual barriers between enclosures. The African wild dog enclosure at Jhb Zoo was located on the periphery and neighboured an enclosure housing a single cheetah on one side and a busy street on the other. The enclosure at the NZG Pta was located closest to an Arabian oryx *Oryx leucoryx* enclosure and was more centrally located than the enclosure at the Jhb Zoo. Gender ratios, social composition and group size of African wild dogs varied per site sampled (Table 1). All samples were collected and frozen at –20°C within 24 h post-defaecation until hormone analysis. We determined all of the samples collected to be less than 24 h old by clearing out all faecal material present in each enclosure and subsequently collected only the faecal material found the following day. As a result of this sampling approach, we were not able to identify individual African wild dog samples. However, based on the fact that African wild dogs defaecate, on average, once a day (Creel et al. 1997), we are confident that the number of samples collected from each enclosure over three consecutive days are sufficient to encapsulate at least one sample per individual present.

Steroid hormone extraction and analysis

All frozen faecal samples were lyophilized and then pulverized in order to separate any undigested material (Fiess et al. 1999). Faecal powder (0.050–0.055 g) was then extracted

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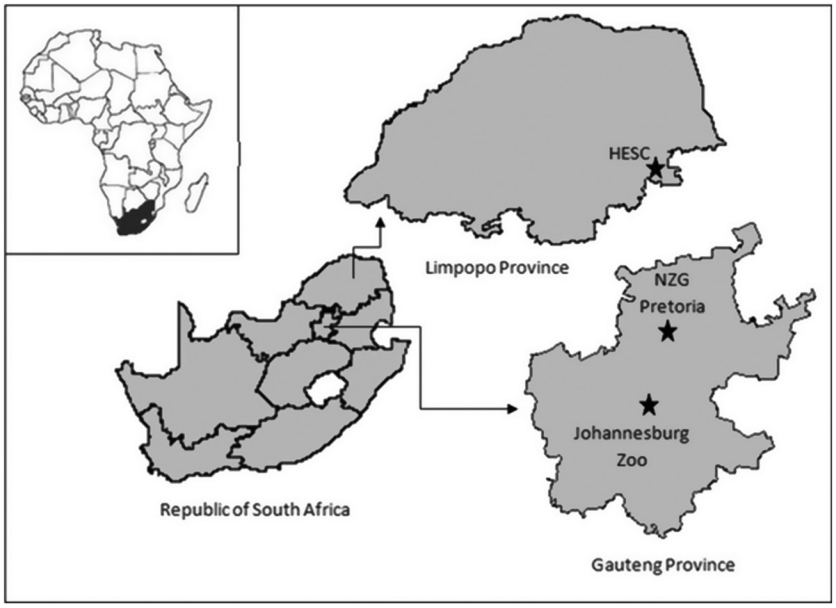


Figure 1. Sampling sites from which wild dog faecal samples were collected: Johannesburg Zoo (Jhb Zoo), National Zoological Gardens Pretoria (NZG Pta) and the Hoedspruit Endangered Species Centre (HESC) (two sites), South Africa.

with 3 ml of 80% ethanol in water. After vortexing for 15 min, the mixture was centrifuged at 1500 × g for 10 min. The supernatant was transferred into sealed micro-centrifuge tubes for storage at –20°C until hormone analysis (Ganswindt et al. 2010). A competitive enzyme immunoassay (EIA), cortisol-3-CMO:BSA antibody and cortisol-3-CMO-DADOO-biotin label, previously validated for African wild dogs (Vlamings 2011) was used to measure fGCM concentrations in the steroid extracts. Detailed assay characteristics, including a full description of the assay components and cross-reactivities are provided in Palme and Möstl (1998). Sensitivity of the assay was 1.2 ng g^{-1} dry weight (DW). Intra- and inter-assay coefficients of variation, determined by repeated measurements of high and low quality controls ranged between 4.8–5.8% and 9.1–12%, respectively. Assay procedures followed published protocols (Ganswindt et al. 2002) and were conducted in the Endocrine Research Laboratory, Univ. of Pretoria, South Africa.

Statistical analyses

A repeated measures analysis of variance (ANOVA) was used to test for differences in fGCM concentrations post-defaecation. Here, each time interval at which freezing occurred was considered a separate treatment. Pair-wise *t*-tests were conducted post hoc in order to identify between which of these treatments significant differences in fGCM concentrations could be found. Simple linear regression was utilized in order to assess differences in fGCM concentrations determined for the various sampling sites. This was followed by Tukey's post hoc tests conducted at 95% family-wise confidence levels. In cases of all pair-wise multiple comparison procedures, the α-level was adjusted by applying the procedure described by Holm (1979). Statistical significance for all tests was set at alpha (α) = 0.05 and inferred at $p < 0.05$. All statistical analyses were run using algorithms in *R* (<www.r-project.org>) with the use of the *R Studio* (<www.r-project.org>) interface.

Table 1. The number of samples collected as well as the gender composition of African wild dog packs sampled across three South African captive sites.

Site	n Males present	n Females present	n Sample collected
Jhb Zoo	3	2	8
NZG Pta	3	0	13
HESC(A)	3	0	7
HESC(B)	1	1	7
Total	10	3	35

Results

There was a statistically significant change in fGCM concentrations over the 16 day post-defaecation period ($F_{10,22} = 22.55$, $n = 33$, $p < 0.001$) (Fig. 2). While respective fGCM concentrations did not change significantly for the first 48 h (from an initial mean concentration of 57.2 ng g^{-1} DW at $t = 0$), an overall increase of approximately 30% was observed in fGCM concentrations between day 1 (mean fGCM concentration of 57.8 ng g^{-1} DW) and day 2 (mean fGCM concentration of 75.56 ng g^{-1} DW) (Fig. 2). Subsequently, fGCM concentrations increased significantly by approximately 155%, to a mean of 146.30 ng g^{-1} DW, 96 h post-defaecation ($p < 0.001$) before declining steadily until the end of the experiment (384 h post-defaecation). At 384 h post-defaecation, fGCM concentrations reached comparable levels with fGCM concentrations determined at 48 h post-defaecation (Fig. 2).

African wild dog fGCM concentrations ranged between: 80.41 ± 38.99 ng g^{-1} DW (mean ± SE) at Jhb Zoo and 40.52 ± 11.35 ng g^{-1} DW at NZG Pta (Fig. 3). Determined baseline fGCM concentrations differed significantly between sites ($F_{3,31} = 7.86$, $n = 35$, $p = 0.001$). Respective steroid concentrations from NZG Pta were significantly

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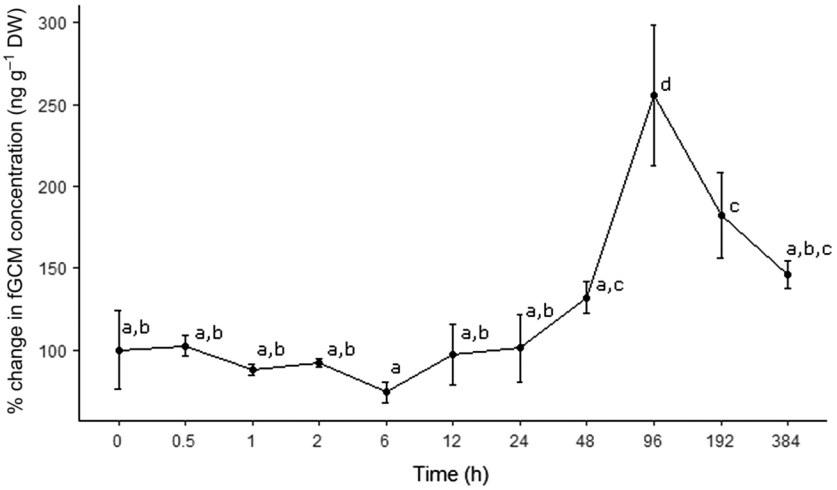


Figure 2. Changes in fGCM concentrations of African wild dogs (mean ± SD in %) post-defaecation (0–384 h). Different superscripts indicate statistically significant differences in % change of fGCM concentrations between time intervals.

lower compared to fGCM concentrations of African wild dogs from Jhb Zoo ($p = 0.001$) and HESC site 1 ($p = 0.01$) (Fig. 3).

Discussion

Our results demonstrate that African wild dog fGCM concentrations determined by our extraction and assay protocol remain fairly stable until 24 h post-defaecation. However, the generalizability of these results require further investigation by including conditions which more closely resemble those likely to be found in the field. Exposure to sunlight, high and highly variable temperatures, rainfall, and differences in humidity are likely to result in the samples being dried out at different rates, influencing the duration of bacterial enzyme activity. For example in African elephants

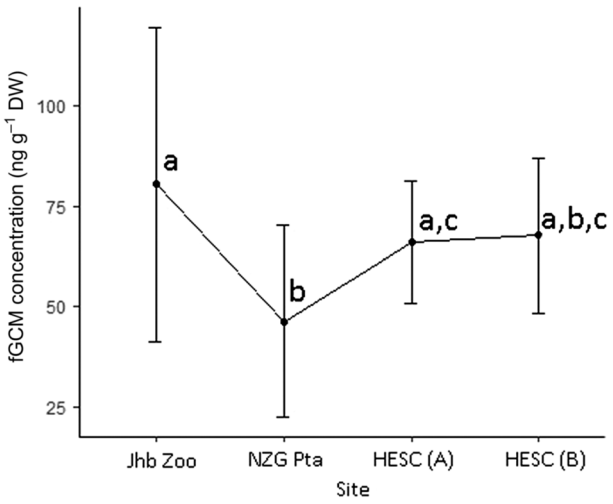


Figure 3. fGCM concentrations (ng g^{-1} DW) of African wild dogs located at Johannesburg Zoo (Jhb Zoo), National Zoological Gardens in Pretoria (NZG Pta), and the two sites at Hoedspruit Endangered Species Centre (HESC) in South Africa. Different superscripts indicate statistically significant differences of fGCM concentrations between sites.

Loxodonta africana it has been shown that fGCM concentrations are less affected in sun-dried samples compared to steroid concentrations revealed from shade-dried material (Webber et al. 2018). Similarly, a study conducted on leopards *Panthera pardus* showed that alteration in fGCM concentrations was significantly less in sun-dried faeces compared to material which were left to dry in the shade (Webster et al. 2018). The exposure of wild bear faeces to cumulative precipitation demonstrated noticeable, although not significant, effects on fGCM concentration (Stetz et al. 2013).

Based on our findings, we suggest that faeces not older than a day should be used for monitoring responses to stressors in African wild dogs. This generally supports Vynne et al. (2012), who reported that faeces should be collected fresh to minimize methodological errors which can compromise the interpretation of hormone profiles. In the case of individual sample collection from free-ranging African wild dogs, the practice of following individuals by vehicle may thus still be required, however, the present study demonstrates that African wild dog faecal samples do not need to be collected immediately post-defaecation. As a result, deposition of faecal material from known individuals can be recorded using a geographic positioning system (GPS) and the collection of samples can be conducted up to 24 h later, thus allowing for a more continuous and less disruptive monitoring of African wild dogs by vehicle. In cases where faecal samples are required but the animals are resident and no direct identification is required, the observation of African wild dogs is not a necessity. In such a case, a site can be cleared of all African wild dog faecal material and then all newly deposited samples can be subsequently collected within a 24 h period whilst still being considered valid for fGCM analysis.

The temporary increase in fGCM concentrations found at 96 h (four days) post-defaecation is likely a result of on-going glucocorticoid metabolism by bacterial enzymes present in the faeces (Möstl et al. 1999, Washburn and Millspaugh 2002). Such bacterial activity which results intermittently in an increase of immunoreactive compounds as has also been demonstrated in cattle *Bos taurus*, horses

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Equus caballus, pigs *Sus scrofa domesticus* (Möstl et al. 1999) and orangutans *Pongo pygmaeus morio* (Muehlenbein et al. 2012).

It is important to acknowledge that, as demonstrated by Lexen et al. (2008), antibody specificity should not be underestimated when interpreting the results of a specific immunoassay. The use of different EIAs has yielded similarly distinct increases and decreases in the same faecal matter when detecting different fGCMs in mammalian faeces (e.g. fGCMs with a 5β-3α-ol-11-one structure versus 11,17 dioxandrostanes in sheep *Ovis aries* (Lexen et al. 2008)).To clarify the context of our results, we would like to conclude that African wild dog fGCM concentrations are subject to substantial alteration by the presumed presence of faecal bacteria enzymes when all moisture is not removed from a sample within about 24 h post-defaecation. When active in the faeces, bacteria continue to metabolize available fGCMs and thus compromises the reliability of results obtained from an EIA analysis (Washburn and Millspaugh 2002). The EIA used in the present study showed an increase in African wild dog fGCM concentrations after a 24 h period. However, this may be reflected by a similarly decreasing trend in fGCM concentrations when used in conjunction with a different assay and extraction protocol (Lexen et al. 2008).

In addition, determined baseline fGCM concentrations exhibited by African wild dogs were shown to differ across captive facilities sampled. Considering the limited sample size and the inherent effect of pseudoreplication within our study, differences in stress-related hormone levels exhibited by African wild dogs across the different sites need to be interpreted with some caution. Overall differences may be driven by factors known to be acting as stressors for other captive species, including noise disturbance (Owen et al. 2004), on-exhibit display (Terio et al. 2004), or social instability (Creel et al. 1997, van der Weyde et al. 2016); the latter of which could be a key stressor complex for African wild dogs in captivity given their reliance on complex social structures and pack dynamics in free-ranging conditions (Creel et al. 1997, Spiering et al. 2010). Considering the vast range of biological and anthropogenic factors which may act as perceived stressors of African wild dogs (van der Weyde et al. 2016), future studies building on the data presented in this study will be necessary to pin-point key stressor complexes and improve captive management strategies.

As samples were obtained from unknown individuals in our study, it was not possible to determine potential sex-specific differences in fGCM alterations in the African wild dogs monitored. The finding of any such differences would, in any case, need to be further investigated for African wild dogs as the possibly confounding effect of sex-specific variations in metabolites found within the faeces may be inherent (Touma et al. 2004). It cannot however be ruled out that within the sites exhibiting higher overall fGCM concentrations, sampling may have been skewed towards the collection of several samples from dominant individuals that have been shown to consistently exhibit higher stress levels than subordinates (Creel et al. 1997). However, the role of social instability on fGCM concentrations for African wild dogs, and in particular dominant individuals, is one that requires further attention. Particularly when considering that the possible link between dominance behaviour and elevated glucocorticoid levels has been found to be inconsistent between studies and likely depends on the social stability of the pack in question (Goymann and Wingfield 2004).

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