Determining adrenocortical activity as a measure of stress in African buffalo (*Syncerus caffer*) based on faecal analysis

A. Ganswindt[1,2*], A.S.W. Tordiffe[3], E. Stam[1], M.J. Howitt[3] & F. Jori[1,4]

[1]*Mammal Research Institute, Department of Zoology and Entomology, University of Pretoria, Pretoria, 0002 South Africa*

[2]*Department of Production Animal Studies, Faculty of Veterinary Science, University of Pretoria, Onderstepoort, 0110 South Africa*

[3]*National Zoological Gardens of South Africa, Pretoria, 0001 South Africa*

[4]*CIRAD, UPR AGIRs, Department ES, Montpellier 34398, France*

Received 9 June 2012. Accepted 22 August 2012

Little is known about the levels of stress experienced by African buffalos affected by injury, disease, or socio-ecological and anthropogenic factors. To be able to start filling this gap, we examined the suitability of two 11-oxoaetiocholanolone enzyme-immunoassays (EIAs) detecting 11,17 dioxoandrostanes (11,17-DOA) as well as faecal glucocorticoid metabolites (FGMs) with a 5β-3α-ol-11-one structure (3α,11oxo-CM), respectively, for monitoring stress-related physiological responses in African buffalo. An adrenocorticotrophic hormone (ACTH) challenge in one male and one female housed at Mokopane Biodiversity Conservation Centre, South Africa, showed a threefold increase in circulating cortisol levels in a sample taken 40 min post-injection. Corresponding 11,17-DOA levels increased tenfold (female) and 15-fold (male) above baseline, and 3α,11oxo-CM concentrations increased ninefold (female) and 12-fold (male) above pre-injection levels, indicating that both EIAs are suitable for measuring FGMs in African buffalo.In addition, 11,17-DOA levels monitored during the adaptation process of individual housing revealed an up to 14-fold elevation in FGMs. Storage of faeces at ambient temperature for up to 16 h post-defecation resulted in an significant increase in 11,17-DOA levels 2 h after defecation. Finally, higher individual baseline 11,17-DOA concentrations were found in samples defecated overnight, indicating a possible diurnal effect in excretion of FGMs in African buffalo.

Key words: faecal glucocorticoid metabolites, ACTH challenge test, animal separation, hormone degradation rate, circadian variation

INTRODUCTION

The African buffalo (*Syncerus caffer*) is a large, gregarious bovine generally common in most sub-Saharan ecosystems, and one of the most successful grazers in Africa (Estes 1999).

Susceptible to many diseases shared with cattle, it is often regarded as a constraint to the expansion of livestock activities (Bengis 2005), particularly due to their role as the main reservoirs of foot and mouth disease virus (FMDV) in southern Africa (Jori *et al.* 2009). It has been hypothesized that adult buffaloes escaping from their natural protected environment and exposed to stress (by being in a foreign and often hostile environment) could potentially be the source of outbreaks in cattle (Vosloo *et al.* 2002). In order to verify this hypothesis, a means to compare stress levels and virus excretion in adult infected buffalo is warranted. Although, this species has been intensively studied with regards to its distribution, feeding habits, and infectious diseases (Mlozsewsky 1983; Prins 1996) and two studies have touched on the effects of season and capture on adrenal activity (Brown *et al.* 1991; Morton *et al.* 1995), very little is known regarding the levels of stress experienced by African buffalos affected by injury, disease, or other socio-ecological and anthropogenic factors.

Stress can be defined as the generic term for any stimulus that threatens or appears to threaten the homeostasis of an individual (Selye 1936; Wielebnowski 2003). A stress response in turn is a series of adaptive mechanisms that are aimed at protecting an individual and restoring homeostasis (Sapolsky 2002; Bomholt *et al.* 2004). From a physiological perspective, an essential component of the response to stress is the activation of the hypothalamic–pituitary–adrenocortical axis and

*Author for correspondence. E-mail: aganswindt@zoology.up.ac.za

the symphatho-adrenomedullary system, which results amongst others in an increase in glucocorticoid and catecholamine secretion (Palme *et al.* 2005). Catecholamines are usually secreted within fractions of a second as part of the acute stress response (Palme *et al*. 2005), and although glucocorticoid responses can also be adaptive in the short term, prolonged periods of elevated glucocorticoid levels due to chronic stress can have an array of disruptive effects, including reproductive suppression, muscle atrophy, and immune suppression (Möstl & Palme 2002; Sapolsky 2002). Monitoring glucocorticoid levels can therefore be a valuable tool to provide information on the level of stress experienced by animals, which can be of central importance for related research questions as well as wildlife management.

Nowadays, the assessment of adrenocortical function using faeces as hormone matrix is a widely accepted approach for monitoring responses to stressors, because faeces can be collected very easily, animals are usually not disturbed during sample collection, and sampling is feedback-free due to the absence of capture and handling (Touma & Palme 2005; Ganswindt *et al.* 2012). Therefore, even individual long-term monitoring *via* repeated sampling is possible without affecting the animal's endocrine status (Touma & Palme 2005). In addition, circulating hormone levels accumulate in faeces over a certain period of time, therefore faecal hormone values are less affected by episodic fluctuations or the pulsatility of hormone secretion (Creel *et al.* 1996; Touma & Palme 2005; Schwarzenberger 2007). However, respective assays for non-invasive hormone measurements need to be carefully validated in terms of applicability for the species-specific hormone matrix of interest to ensure a reliable quantification of respective glucocorticoid metabolites (Touma & Palme 2005; Hodges *et al.* 2010).

The enzyme-immunoassays (EIAs) used in this study have been shown to be potentially useful for monitoring adrenocortical function in other mammals (Möstl *et al*. 2002; Ganswindt *et al*. 2003; Heistermann *et al*. 2006; Kleinsasser *et al.* 2010; Hulsman *et al*. 2011), including cattle (*Bos primigenius*) (e.g. Palme *et al.* 1999). Although faecal glucocorticoid metabolite (FGM) levels have been successfully determined as an index of adrenal function in closely related species like cattle or bison (*Bison bison*) (Mooring *et al.* 2006), to our knowledge, a non-invasive approach has not yet been used to monitor adrenocortical activity as a measure of stress in African buffalo.

Therefore the overall aim of this study was to examine the suitability of two different group-specific 11-oxoaetiocholanolone EIAs for monitoring adrenocortical function in African buffalo based on FGM analysis. More specifically, this study aimed at: a) determining stress-related physiological responses in African buffalo faeces by performing an adrenocorticotropic hormone stimulation test (ACTH challenge test), b) comparing FGM levels of samples collected during the adaptation process of individual separation as a form of biological validation, and c) investigating the potential influence of time of collection (day *vs* night) on FGM output in African buffalo.

MATERIALS & METHODS

Study animals

The study was conducted on one male (13 months of age, ~180 kg) and one female (5 years of age, ~300 kg) African Buffalo at the Mokopane Biodiversity Conservation Centre, South Africa. The animals were fed a combination of *Eragrostis* hay, lucerne and antelope pellets daily and water was available *ad libitum*. The study was performed with approval of the Ethics and Scientific Committee of the National Zoological Gardens of South Africa, Pretoria (Reference # P10/33).

Experimental design

After separation from their herd (n = 14 animals), the two study individuals were housed singly for 10 days, while still allowing for visual and olfactory contact with former herd mates. All voided faecal samples were collected from the individually housed animals (n = 122 total from male; n = 101 total from female). Samples voided during the day (07:00 to 17:00) were collected within 1 h of defecation. Enclosures could not be accessed at night; therefore, all samples voided overnight (17:00–07:00) were collected first thing the next morning. To illustrate a more detailed temporal resolution (Fig. 1), overnight samples relatively warm to the touch (n = 4 total), indicating they were freshly voided, were classed as defecated early morning (07:00), whereas cold overnight samples were assigned to midnight. To avoid cross-contamination with urine or contamination with other faecal samples in the area, approximately 50 g of homogenized faecal material was taken from the centre of a dropping. On the 5th

day of the study, an adrenocorticotropic hormone (ACTH) challenge test was successively performed on the two individually housed animals (male at 09.00, female at 10.00). Each study animal was anesthetized using a combination of etorphine hydrochloride (M99, Novartis South Africa (Pty) Ltd) and azaparone (Stresnil, Janssen Pharmaceutica) and subsequently a total of 150 IU of synthetic ACTH (Synacthen Depot©, Hoffman La Roche AG) was injected intramuscularly. To provide a control of the actual effects of the challenge on circulating glucocorticoid levels, an initial blood sample was taken from each animal just before ACTH administration and a second blood sample 40 min later (n = 2 samples/individual). The effects of the immobilization drugs were then reversed within approximately five minutes *via* the intravenous administration of a combination of diprenorphine (M5050, Novartis South Africa (Pty) Ltd) and naltrexone hydrochloride (Naltrexone, Kyron Laboratories (Pty) Ltd). Subsequently, an aliquot of each voided faecal sample from the two animals was taken over the next five days for FGM analysis. On day 10 of the study, the two animals were released and joined their former herd.

In a separate study to determine the stability of FGM post-defecation, a fresh faecal sample from one adult herd member was additionally collected after the experiment. The collected material was homogenized and divided into 18 equal subsamples, which were stored at room temperature. Subsequently, three subsamples were frozen at –20°C after 0, 1, 2, 4, 8, and 16 h post-defecation, respectively.

Sample processing and extraction

Immediately after collection,faecal samples were placed on ice and within one hour stored at –20°C until further processing. The frozen samples were lyophilized, and the resulting dried faecal matter pulverized and sieved through a mesh to remove fibrous material (Fieß *et al*. 1999). Approximately 0.1 h of the faecal powder was then extracted by vortexing for 15 min with 3 ml of 80% ethanol. Following centrifugation for 10 min at 1500 *g*, supernatants were transferred into microcentrifuge tubes and stored at –20°C until analysis. Blood samples were stored on ice for approximately 60 min until clotted, and then centrifuged at 2350 *g* for 5 min. After transferring the blood serum into glass tubes, the serum was stored at –20°C until analysis.

Hormone analysis

Serum cortisol levels were determined by using a Coat-A-Count© Cortisol RIA (Siemens Medical Solutions Diagnostics). In brief, 25 μl of standards, controls, and samples followed by the addition of 1 ml of ^{125}I cortisol solution was added to precoated antibody tubes, vortexted for approximately 30 s, incubated at 37°C for 45 min, decanted, and counted for one minute in a gamma counter (2470 Wizard2; Perkin Elmer). Sensitivity of the assay was 5.5 nmol/l, its intra-assay coefficient of variation (CV) was 3.0–6.4%, and major cross-reactivities,as given in the company's pamphlet, were cortisol, 100%; prednisone, 76%; 11-deoxycortisol, 11.4%; and corticosterone and cortisone, <1%.

Faecal extracts were measured for immunoreactive FGM concentrations using two different 11-oxoaetiocholanolone enzyme-immunoassays (EIA) detecting 11,17 dioxoandrostanes (11,17-DOA) and FGMs with a 5β-3α-ol-11-one structure (3α,11oxo-CM) according to the procedure described by Ganswindt *et al*. (2002). Serial dilutions of faecal extracts gave displacement curves that were parallel to the respective standard curve in both assays (relative variation (%) of the slope of respective trendlines <5%). Sensitivity of the assay was 3.0 pg/well for both EIAs. Intra-assay CV was 5.2–7.6% for the 11,17-DOA and 4.2–9.1% for the 3α,11oxo-CM EIA, and the inter-assay CV, for the 11,17-DOA EIA was 8.5–10.0% (no inter-assay CV could be determined for the 3α, 11oxo-CMEIA as samples were not analysed across several plates). Details of the two EIAs, including cross-reactivities are described by Palme & Möstl (1997) for 11,17 DOA and by Möstl *et al*. (2002) for 3α,11oxo-CM.

Data analysis

Descriptive statistics were used to determine the initial rise, peak and fall in FGM following separation and the ACTH challenge. To achieve this, an iterative process was used to determine individual baseline FGM values as described by Brown *et al*. (2001). For that, all FGM concentrations of an individual data set exceeding the mean plus two standard deviations (S.D.) were excluded, the average then recalculated, and the elimination process repeated until no values exceeded the mean plus 2 S.D. To determine the influence of FGM degradation the relative degradation rate (%) of FGMs post-defecation were calculated for each subsample separately, using the mean

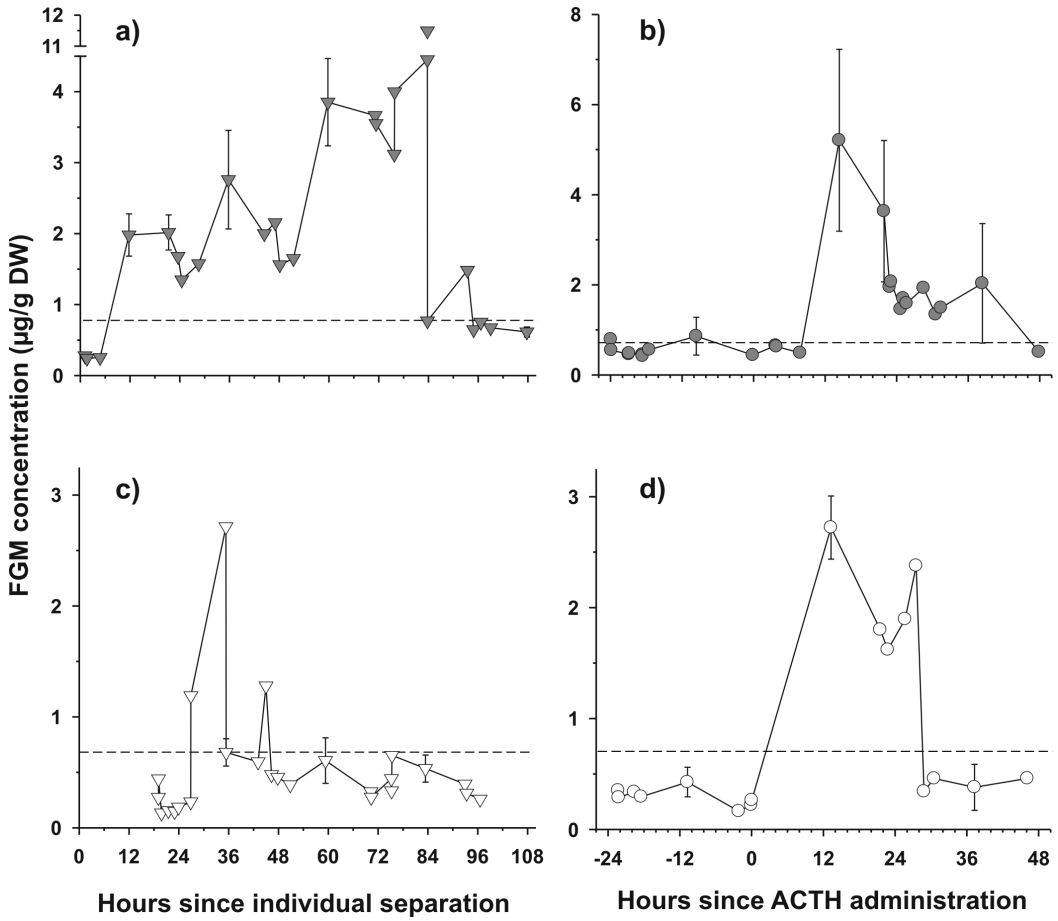

Fig. 1. Immunoreactive faecal glucocorticoid metabolite (FGM) levels using the 11,17-DOA EIA, following separation (**a**, **c**) and ACTH administration (**b**, **d**) in one male (solid symbols) and one female (open symbols) captive African buffalo. Dashed lines indicate the individual baseline FGM levels.

hormone value determined at $t = 0$ as 100%. Differences in relative degradation rate between samples stored at $t = 0$ h and 1–16 h post-defecation were examined by one-way repeated measures ANOVA, followed by *post hoc* analysis using Bonferroni t-test, with application of Bonferroni correction. To determine the influence of time of sample collection, a subset of baseline samples (collected >55 and >127 h post-separation and >30 and >48 h post-ACTH administration for the female and male, respectively) were classed as day or night and differences in FGM concentrations were examined by two tailed Wilcoxon signed rank test. Data were statistically analysed using Jandel Sigma Stat, version 2.0 and KyPlot, version 2.0 beta 13, respectively, and significance was considered achieved when $P < 0.05$.

RESULTS

Separation

Using the 11,17-DOA EIA, FGM levels increased up to 14-fold (male; Fig. 1a) and fourfold (female; Fig. 1c) above baseline after separation, with highest levels noted 77–91 h post-separation for the male and 29–43 h for the female (overnight samples in both cases). Concentrations returned to baseline after 95 h in case of the male (Fig. 1a), and after 35 h in case of the female (Fig. 1c). Samples were not analysed with the 3α,11oxo-CM EIA, as the assay showed a slightly less pronounced increase in FGM levels after ACTH treatment compared to the 11,17-DOA EIA (see below).

ACTH challenge

The 11,17-DOA EIA detected a 15-fold (male;

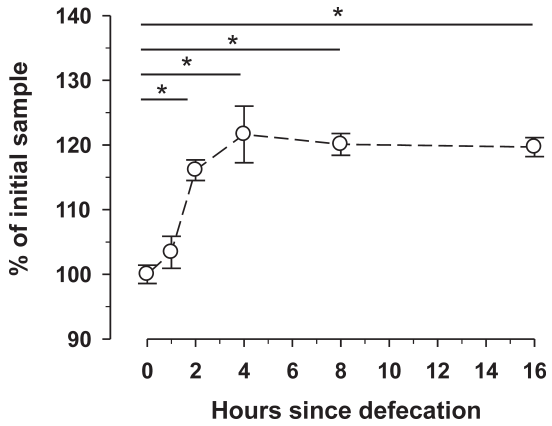

Fig. 2. Relative change (%) of FGMs (mean ± S.E.M.) in African buffalo faeces over time (0, 1, 2, 4, 8, and 16 h since defecation).

Fig. 1b) and tenfold (female; Fig. 1d) elevation in FGM levels above baseline, with peak levels noted in the interval 8–22 h (male) and 7–21 h (female) after ACTH treatment. FGM levels returned to baseline within 48 h (male) and 29 h (female), respectively. The 3α,11oxo-CM EIA revealed a 12-fold (male) and ninefold (female) increase in FGM concentrations above pre-injection levels, with highest FGM concentrations found 22 h post-ACTH administration for the male and 7–21 h for the female (data not shown). Administration of synthetic ACTH resulted in a threefold increase in circulating cortisol levels 40 minutes after injection in both animals (cortisol concentration pre-/post-administration; female: 56/232 nmol/l, male: 34/146 nmol/l).

Storage experiment

Mean FGM levels increased between 16.1 and 21.6% in samples frozen 2–16 h post-defecation (Fig. 2), with an overall maximum increase of 28.8% (comparing the lowest 0 h and highest 4 h value). Variation in FGM concentration between subsamples ranged from 4.6 to 13.9% for respective measuring points in time. A statistically significant difference was found between 0 h and 2–16 h post-defecation samples ($F = 18.27$, $P = 9.65\text{E-}05$, *post hoc* analysis: $P = 2.03\text{E-}03$, 1.90E-04, 3.52E-04, 4.20E-04, for 2, 4, 8, and 16 h, respectively).

Influence of collection times

Baseline FGM levels of overnight samples were significantly higher than samples collected during the day ($T = 378$ and 465; $P = 5.69\text{E-}06$ and 1.77E-06, for the male and female, respectively). Mean baseline FGM concentrations increased by 62% (0.50 *vs* 0.81 μg/g dry weight) and 33% (0.36 *vs* 0.48 μg/g dry weight) for the male and female, respectively (Fig. 3).

DISCUSSION

For a reliable monitoring of adrenocortical activity in mammals using FGMs for the first time, a physiological validation of the involved assay, which is usually achieved by an ACTH challenge test, is nowadays obligatory (Touma & Palme 2005; Kleinsasser *et al.* 2010). We measured a substantial increase in circulating cortisol levels 40 minutes after ACTH administration and detected the corresponding increase in FGM levels 7–22 h post-injection with both 11-oxoetiocholanolone EIAs, respectively. Although the time of peak FGM excretion could not be determined more exactly, the noted time delay between the measured elevation in blood cortisol concentrations and the related increase in FGMs is within the range reported for other ruminants (Dehnhard *et al.* 2001; Huber *et al.* 2003; Kleinsasser *et al.* 2010). As the two group-specific 11-oxoaetiocholanolone EIAs recognize different groups of metabolites, it is not surprising that their suitability varies across species as hormone metabolism is highly species specific. In European (*Lepus europaeus*) and snowshoe hares (*Lepus americanus*) for example, the 11,17-DOA EIA appear to be suitable for monitoring adrenocortical function(Teskey-Gerstl *et al.* 2000; Sheriff *et al.* 2009), whereas, for example, in African elephants (*Loxodonta africana*) and mountain hares (*Lepus timidus*) only the 3α,11oxo-CM EIA (Ganswindt *et al*. 2003; Rehnus *et al*. 2009), and in goats both EIAs (Kleinsasser *et al.* 2010) reflect adrenocortical activity adequately. Although the

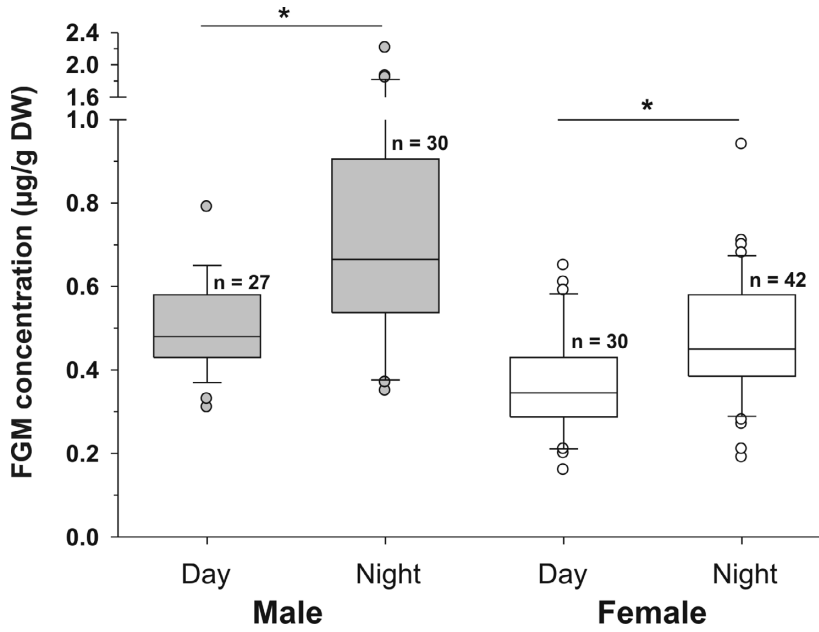

Fig. 3. Variation in baseline FGM levels in one male and one female captive African buffalo from samples voided overnight (17:00–07:00) and during the day (07:00–17:00).*$P < 0.0001$.

EIA measuring 11,17-DOA seems to perform slightly better and was therefore used for all subsequent analyses, both assays would be suited to provide information on the level of stress experienced by African buffalos. The suitability of at least the 11,17-DOA EIA gets further support by the clear alteration in FGM levels detected during the first days of separation of the two study animals. The more pronounced signal, in terms of higher magnitude and duration found in the male, could have been caused by individual variation in restoring homeostasis or additional stress experienced by the bull during the experiment. African buffalo calves are weaned around six months of age, but have been reported suckling for up to 18 months (Ryan *et al.* 2007). The 13-month-old study male might therefore have been more dependent on some of the herd members than the 5-year-old female, a circumstance which could have acted as an additional individual stressor throughout the study. This hypothesis gets additional support from behavioural recordings during the first few days of separation. Throughout the study, we recorded potentially stressful behavioural alterations (fence pacing, limited feeding and drinking) during the day, using *ad libitum* sampling (Altmann 1974). Apparently, the male frequently paced up and down along the fence, ate less than usual, and probably did not drink for the first 3.5 days post-separation, whereas the female appeared very calm, ate and drank as usual, and was lying down the same afternoon (unpublished data). The quantitative differences in FGM output between the two study animals in response to ACTH stimulation, however, is most probably a result of the different dose of synthetic ACTH administered. Both animals received 150 IU, but the study male weighed only ~180 kg while the female weighed ~300 kg. This circumstance results in a difference of 0.33 IU/kg of administered ACTH between the two study animals. Such dose-dependent differences in peak FGM excretion are in line with findings from other studies monitoring the effect of ACTH administration on adrenocortical activity (Palme *et al.* 1999; Touma *et al.* 2004).

The revealed changes in FGM concentrations in samples stored unpreserved at ambient temperature are in line with findings from studies on e.g. domestic mammals (Möstl *et al.* 1999; Lexen *et al.* 2008). In these studies, FGM levels determined using the 11,17-DOA EIA also increased within a few hours after storage at room temperature in samples from cattle, horses, pigs, and sheep. As possible changes in measured FGM levels in unpreserved samples surely depend upon the species, they also seem to depend on the EIA used. By determining changes in FGM concentrations in

unpreserved post-defecation samples using the 3α,11oxo-CM EIA, a distinct decrease in hormone levels 4–8 hours post-defecation was observed in faeces from e.g. sheep (*Ovis aries*) and brown hyaena (*Hyaena brunnea*) (Lexen *et al*. 2008; Hulsmann *et al.* 2011). Although still not conclusively explained, bacterial enzymes present in the faeces may further metabolize FGM (Millspaugh & Washburn 2004; Möstl *et al*. 2005) and the resulting metabolites showing then more or less cross-reactivity with the antibodies used in the respective EIAs. In this respect, even different species seem to follow the same pattern, with an increase in FGM values in samples stored at room temperature when 11,17-DOA are measured, and a decrease when the 3α,11oxo-CM EIA is used. However, further research would be necessary to corroborate this hypothesis as there is always an exception to the rule (banded mongoose; Laver *et al.* 2012). If applicable for the African buffalo however, it may obscure the results of our ACTH challenge test, as peak concentrations in samples defecated overnight may show 'artificially' higher 11,17-DOA concentrations, but lower 3α,11oxo-CM levels after storage for an indefinite time outside during the night. Thus the determined increase after ACTH administration would be slightly overestimated in the former and underestimated in the latter.

The changes in FGM levels demonstrated in the storage experiment were with a maximum increase of 28.8% below the differences in FGM concentrations found between day and night samples, as individual mean baseline FGM levels increased by 62% and 33% in faeces voided overnight. Although a potential alteration in FGM composition caused by microbial activity in the unpreserved overnight samples can be assumed (see above), it might not be the only influencing factor responsible for the differences in hormone concentrations found between day and overnight samples. Like many other hormones, plasma glucocorticoids have a circadian rhythm in most vertebrate species (Möstl & Palme 2002; Touma & Palme 2005), and diurnal variations of FGMs was already demonstrated for at least some mammals (Touma & Palme 2005). Although respective data for ruminants is limited and rather conflicting (Bubenik *et al*. 1983; Ladewig & Smidt 1989), we cannot exclude the possible existence of circadian rhythm of FGM excretion in African Buffalo as an additional contributor to the differences in hormone levels found between samples voided overnight and during the day. However, future studies have to show whether diurnal changes of circulating glucocorticoid levels can be detected in African buffalo faeces.

The ability to reliably assess adrenocortical function in African buffalo now provides a solid basis to further examine endocrine responses to putative stressful circumstances in this iconic African species. Potential applications could for instance be the possibility of measuring the potential effect of stress on FMD virus excretion in faecal material of adult infected buffalo, or the ability to measure potential variability of FGM levels in natural populations of buffalo facing ecological challenges such as shortage of grazing or water sources.

ACKNOWLEDGEMENTS

We thank National Zoological Gardens of South Africa for allowing us to perform the research and Alfred Thole, Thomas Mashile, Johannes Kekana, Ephraim Tlhako, George Kekana, and Renee Brabants (from KATHO College, Belgium) for logistical help during the experiment. We also thank Lauren Snyman for her help in collecting faecal material and Stefanie Ganswindt for expert help with laboratory techniques.

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Responsible Editor: J.H. van Wyk