Patterns of faecal glucocorticoid metabolite levels in captive roan antelope (*Hippotragus equinus*) in relation to reproductive status and season

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Abstract

Populations of roan antelope (*Hippotragus equinus*) in southern Africa have experienced a drastic decline over the past few decades and this situation has led to the development of intensive breeding programmes to support conservation efforts. However, little is known about related welfare aspects, including stress-related physiological biomarkers. The present study set out to establish a non-invasive method to monitor faecal glucocorticoid metabolite (fGCM) concentrations as a measure of stress and determine fGCM concentrations in relation to male reproductive activity and female reproductive status in the roan antelope. An adrenocorticotrophic hormone challenge was performed using two adult roan antelope (one male and one female) at Lapalala Wilderness Nature Reserve, South Africa, to determine the suitability of five enzyme immunoassays (EIA) for monitoring adrenocortical function in roan antelope. An 11-oxoaetiocholanolone I EIA detecting 11,17 dioxoandrostanes

performed best showing 17-20 folds increases in fGCM concentrations after 12h-17h post-injection. The identified EIA was then used to monitor fGCM concentrations during active and non-active reproductive periods in males (n=3), and during periods of cyclicity, gestation, and postpartum in females (n=10). Males showed an overall 80% increase in fGCM concentrations when reproductively active and females showed a progressively significant increase in fGCM levels throughout pregnancy, with overall fGCM concentrations being 1.5 to 2.6-fold higher than the respective fGCM concentrations during periods of postpartum and cyclicity, respectively. Furthermore, fGCM concentrations remained above baseline for up to 21 days post-partum. A correlation between ecological parameters (rainfall and temperature) and fGCM concentrations revealed elevated fGCM concentrations during the dry season for males, but not females. The non-invasive method validated in this study provides a valuable tool to quantify stress-related biomarkers in roan antelope, and findings can be used to support management decisions in conservation breeding facilities.

Keywords*: ACTH challenge, conservation breeding; roan antelope; faecal glucocorticoid metabolite monitoring; reproductive status; season*

1. Introduction

Captive breeding programmes have proven to be useful for the conservation efforts of many endangered wildlife populations [16, 35]. The main objective of these programmes is to create sustainable populations, with the ultimate goal of reintroducing individuals back into their natural habitat [52]. Hence, the success of captive breeding programmes relies heavily on available resources and adequate management practices to improve the breeding outcome, as well as to ensure the animal's well-being. In captive breeding programmes, the avoidance of long-term/chronic stress is therefore an essential component to ensure animal welfare [36]. A stressor can be defined as any stimulus that threatens or appears to threaten the homeostasis of an animal [70] and while some species breed more successfully under

controlled management environments, captivity can be a distinct source of stress for others [12] [52]. Captivity requirements often vary distinctively within and between species [12]. For some species, specific conditions can be perceived as stressful, particularly for newly captured individuals due to factors such as forced proximity to humans, new or unnatural social group structures, restricted movement, as well as handling (e.g. blood collection) [40]. In those cases, quantifying stress-related physiological biomarkers can be a useful tool to evaluate both the health and welfare of captive individuals [45, 58].

In mammals, the perception of a stressor leads to a multitude of different responses of behavioural, physiological, and neuroendocrine nature, helping the individual to cope with the situation (Palme, 2019). Amongst the physiological responses is the activation of the hypothalamic-pituitary-adrenal axis leading among others to increased secretion of glucocorticoids (GCs), which are considered to be frontline stress mediators [18, 65]. Traditionally, GCs are quantified utilizing blood samples. However, non-invasive approaches using other matrices, such as urine, faeces or keratinized tissue are well-established alternative techniques for quantifying GCs or their metabolites. This is especially helpful in wildlife species as it avoids additional stress due to capture and restraint required in the case of blood collection [20, 46, 51]. Quantifying faecal glucocorticoid metabolites (fGCM) has become particularly popular when monitoring free-roaming animals because apart from the avoidance of handling stress, it is comparatively inexpensive and sample collection is relatively easy to apply [27]. The approach also enables frequent sampling from the same individual for longitudinal studies, and faecal hormone values are less affected by episodic fluctuations of GCs production since they represent cumulative signals of circulating hormones levels over several hours [65].

Glucocorticoids play a role in glucose metabolism, the immune response, and in reproductive processes [33, 64]. In female reproductive endocrinology, GCs are implicated in

the onset of ovulation (cyclicity, parturition (calving) and lactation (post-partum); whereas in males, GCs modulate the synthesis of testosterone by inhibiting testosterone biosynthesis enzymes, and testicular LH receptor numbers [2, 69]. Previous studies monitoring fGCM concentrations in wildlife species have provided valuable information on reproductive-related alterations in respective hormone metabolite concentrations. However, the patterns of GC concentrations during ovulation and pregnancy stages vary between species [31, 43, 57, 60]. Various studies have demonstrated differences in GC concentrations during gestation for a number of mammal species. In sheep (*Ovis aeris*) and cattle, GC concentrations do not change throughout pregnancy and only rise shortly prior to parturition, whereas in the African elephant (*Loxodonta africana*) and the pygmy rabbit (*Brachylagus idahoensis*) GC concentrations increase continuously during pregnancy [17, 57]. In males, GC levels can vary with reproductive activity; e.g. GC concentrations increase during the mating period in bison (*Bison bison*) and goral (*Naemorhedus griseus*) or decrease during periods of higher reproductive activity (musth) in the African elephant [14, 28, 39]. Generally, reproduction can be very challenging for male and female mammals in terms of energy demands [55] and therefore may stimulate an increase in GC output as a result of energy mobilisation. Likewise, elevated GC levels can also affect reproductive processes by alterating the secretion of gonadotrophins, luteinizing hormone, or follicle-stimulating hormone and impeding the normal development of pregnancy [1, 53, 63].

The roan antelope (*Hippotragus equinus*) (É. Geoffroy Saint-Hilaire, 1803) is a popular game ranch herbivore in southern Africa. Populations of roan antelope have dramatically declined in southern Africa over the last few decades due to poaching, hunting, diseases, competition with other grazers, and land transformation [19, 32, 37]. As a consequence, this antelope has been classified as “vulnerable” in this region of Africa by the IUCN, resulting in an increased focus on intensive breeding programmes [23, 32, 38]. Even

though solutions such as disease control, management of water points and habitat restoration have been applied to resolve overall population decline [19, 50], captive breeding appears to be a potential solution for the conservation of this species; especially when considering the increasing population of captive roan antelopes recorded in southern Africa [62]. In addition to captive breeding programmes, reintroduction efforts have been initiated for the roan antelope into areas where they used to occur naturally [32]. Although presumably beneficial, no method has been established for measuring stress-related biomarkers non-invasively for this species so far and thus respective data on adrenocortical activity related to reproductive management is not available for either the male or female roan antelope.

Thus, there is a need to examine the patterns of stress-related biomarkers such as GCs or their metabolites in roan antelope to investigate the influence of reproductive events on its secretion. The objectives of the present study were a) to validate a non-invasive method to reliably monitor fGCM concentrations in the roan antelope by conducting an ACTH challenge; b) to investigate the relationship between fGCM concentrations and reproductive activity as well as ecological seasons in male and female captive roan antelope by i) determining fGCM concentrations during reproductive active and non-active periods in captive male roan antelope; ii) determining fGCM concentrations in captive female roan antelope during reproductive periods cyclicity, pregnancy, and postpartum and iii) determining potential seasonal variation in fGCM concentrations in both sexes.

2. Material and Methods

2.1. Study site and animals

This study was conducted at the Lapalala Wilderness Nature Reserve (230 44'- 230 57' South; 280 09'- 280 25' East), Limpopo, South Africa, between August 2017 and July 2018. The reserve is a conservation area located within the UNESCO Waterberg biosphere;

with diverse habitat and vegetation, namely open shrubland grassland, and open to closed woodland [44]. Roan antelope were introduced into camps within the reserve in 2010 for a long-term captive breeding programme aiming to successfully reintroduce herds onto the reserve. For this study, a total of 14 roan antelope were monitored: three breeding males (7, 8.5 and 10 years; ~ 400 - 450 kg) and 11 adult females (2-6 years; ~150- 250 kg). The animals were chosen from three breeding herds (herd A, B and C), comprising of 17-18 individuals, including a breeding male, adult females, juveniles, and calves of both sexes. From herd A, the male (male 1) and one primiparous female were monitored. From herd B, the male (male 2) and five multiparous females were monitored. From herd C, the male (male 3) and six multiparous females were monitored. The herds were housed in non-adjacent 16-ha camps; with animals grazing on natural vegetation and supplemented daily with A-grade Lucerne and protein feed (160 g/kg: 0.9 kg per animal per day). Water was available *ad libitum*. The age of the study animals was determined either from the recorded date of birth or in the case of the animals that arrived on the reserve as adults, from the estimated date of birth. The study was performed with the approval of the University of Pretoria Animal Use and Care Committee (Reference V072-17).

As part of the reserve's management regime, the focal males were removed from their respective herds and housed individually in adjacent camps (10 to 20-ha) for a period of four to five months once all the females of their respective herds were presumed to be pregnant, i.e. when no more mating behaviour was observed. They were later returned to the breeding camps after all the females of their respective herds had given birth.

2.2. Classification of male and female reproductive activity

For the males, two reproductive periods were categorized, namely the active and the non-active reproductive period. Based on behavioural observations and recordings [25, 26] the active reproductive period represents the time during which 70% of copulatory events

occurred during the study; whereas the non-active period corresponded to the time during which only 30% of copulatory events occurred.

For females, three reproductive periods were categorised: cyclicity, pregnancy and postpartum. Among the females, the only primiparous female was part of the ACTH challenge, whereas the other females were multiparous (n=10). At the beginning of this study, all the adult females were lactating. The cyclicity period was defined as the period following birthing when the reproductive males were placed into breeding enclosures until the occurrence of the last acts of mating. For each female, the gestation period was defined as the period from four weeks following the last mating act (when males showed little interest in her) until birthing, whereas the postpartum period covered the three weeks following birthing when the females were housed without males and lactating exclusively.

2.3. Monitoring adrenocortical function during male and female reproductive activity

All females were separated from the males during the postpartum (lactation) period (only olfactory and visual contact was possible). The males were later introduced to the herds from September 2017 until 4 months prior to calving when they were moved back to their individual camps. Faecal samples for fGCM quantification were collected twice per week from the three breeding males between August 2017 and July 2018 (male 1 and 3) and between August and December 2017 (male 2). Respective samples were collected from females, during the periods of cyclicity (n=5 females), pregnancy (n=10 females) and post-partum (n=5 females). In females, samples were collected three times per week during the cyclicity period, twice per week during the pregnancy period, and daily during the post-partum period. All the females were selected from two of the three breeding herds and were monitored from August 2017 to early October 2017 (except for one multiparous female that was only monitored until late November 2017). For postpartum, only five of the ten focal

females that exhibited reduced horn threats (considered here as aggressive behaviour) after birthing were monitored.

2.4. Environmental data

Average monthly ambient temperature (°C) and cumulative rainfall (mm) at the study site were obtained from the South African weather services (Mokopane station; https://www.weathersa.co.za) from August 2017 to July 2018. Additional photoperiod data were obtained from the weather service ClimaTemps (https://www.ClimaTemps.com) with minutes of daylight per day recorded for the area of Limpopo South Africa). Seasons were defined as the 'wet season', which occurs during the southern hemisphere spring and summer (from September to February) and the 'dry season', which occurs in autumn and winter (March to August) [41].

2.5. ACTH challenge

An adrenocorticotropic hormone (ACTH) challenge was conducted on two adult roan antelope, a breeding male (7 years of age; ~350 kg) of herd C and a non-gravid primiparous female (2 years of age; ~200 kg) from herd A. At the time of the experiment, the male was individually housed. The female remained within her respective herd during the experiment to retain the hierarchy within the herd. Each of the two animals received an intramuscular injection (1 IU/kg) with Synacthen depot (Novartis, South Africa). Drugs were delivered in 3-ml single-use darts (Pneu-dart Inc) with a 3/4" gel-collared needles. Darts were fired from a cartridge-fired projector (Pneu-dart Inc.). To ensure continuous observation and frequent timely sample collection, the animals were injected approximately 70 days apart. Based on previous reports on other ungulates species indicating peak values post-ACTH injection after 10 - 12 hours [47], the Synacthen was administered in the afternoon, between 16:30 and 17:30 to ensure collection of samples with assumed peak fGCM concentrations during daylight. Four days prior to the ACTH injection, samples were collected once daily to

determine individual fGCM baseline levels. On the day of the ACTH administration and for three consecutive days post-injection, all voided faeces were collected between 6:00 and 18:00. Since the male was housed alone, samples voided throughout the night (between 18:00 and 6:00) were collected the next morning, with samples warm to the touch classified as early morning samples. For the female, the day following ACTH injection, the first faeces voided after waking up early in the morning were collected. Daytime samples were collected within 5 - 45 min post-defecation, once the animal had moved away from the area of faecal deposition. All collected samples were immediately placed on ice, and stored at -20 °C within 4 hours after collection and kept frozen until analysis.

2.6. Faecal sample collection and storage

Apart from the collection regime for the ACTH challenge, faecal samples were collected from identified females three times per week for a period up until four weeks post-mating and then twice per week until parturition. After parturition, faecal sample collection was carried out daily until day 21 post-partum. Sample collection took place between August 2017 and July 2018. For the males, faecal samples were collected twice per week throughout the study period. Faecal samples (15-20 g) were collected within one hour of defecation using gloves and subsequently placed in a 30 ml plastic container. Each container was stored in a cooler box immediately and within one hour in a freezer (-20° C) until analysis.

2.7. Steroid extraction and analysis

All frozen samples collected during the ACTH challenge (male: n=46, female: n= 40) and the long-term monitoring (males: n=274, females: n=529) were lyophilised, pulverised and sieved through a mesh to remove fibrous material [11]. Subsequently, 0.10 to 0.11 g of the faecal powder was extracted by adding 3 ml of 80% ethanol. The suspension was vortexed for 15 min and subsequently centrifuged for 10 min at 1500 x g. The resulting supernatant was decanted into 1.5 ml microcentrifuge tubes and stored at -20 °C until analyses.

Faecal extracts resulting from the ACTH challenge were measured for fGCM concentrations using five different enzyme immunoassays (EIAs) namely a) 11-oxoaetiocholanolone I (detecting 11, 17 dioxoandrostanes), b) 11-oxoaetiocholanolone II (detecting fGCMs with a 5α-pregnane-3α-ol-11one structure), c) 5α-pregnane-3β-11β,21-triol-20-one (measuring 3β,11β-diol CM), d) cortisol and e) corticosterone. Detailed EIA characteristics including full descriptions of the assays components and cross-reactivities are described for the 11-oxoaetiocholanolone I, cortisol and corticosterone EIA by Palme and Möstl (1997), for the 11-oxoaetiocholanolone II EIA by Möstl et al. (2002) and the 5α-pregnane-3β-11β,21-triol-20-one EIA by Touma et al. (2003). The sensitivities of the EIAs were 0.06 ng/g dry weight (DW) for the cortisol, 11-oxoaetiocholanolone I, and the 11-oxoaetiocholanolone II, EIA, 1.8 ng/g DW for the corticosterone EIA, and 2.4 ng/g DW for the 5α-pregnane-3β-11β, 21-triol-20-one EIA. The Intra-assay coefficient of variance (CV) of high and low-concentration quality controls were: 2.15% and 2.21% for the 11-oxoaetiocholanolone I EIA, 5.65 % and 6.11% for the 11-oxoaetiocholanolone II EIA, 6.62% and 6.70% for the 5α-pregnane-3β-11β,21-triol-20-one EIA, 5.67% and 6.90% or the Cortisol EIA, and 4.15% and 5.41% for the corticosterone EIA respectively. The Inter-assay CV of high- and low-concentration quality controls were 8.99 % and 14.33 % for the oxoaetiocholanolone I EIA, 7.37% and 11.88% for the 11-oxoaetiocholanolone II EIA, 4.16% and 6.30% for the 5α-pregnane-3β-11β,21-triol-20-one EIA, 6.59% and 8.59% for the Cortisol EIA, and 7.06% and 10.93 % for the corticosterone EIA, respectively. As the 11-oxoaetiocholanolone I EIA appeared to be the most suitable, the remaining faecal extracts of the longitudinal sample set were analysed using only the 11-oxoaetiocholanolone I EIA. Serial dilutions of faecal extracts gave displacement curves that were parallel to the respective standard curve (relative variation (%) of the slope of respective trend lines < 5%). All analyses were conducted at the Endocrine

Research Laboratory, University of Pretoria, South Africa following already established protocols (Ganswindt et al., 2012).

2.8. Data analysis

Individual baseline fGCM concentrations were determined using an iterative process following Brown et al. (2001). For that, all fGCM concentrations of an individual data set exceeding the mean plus 2 standard deviations (SD) were discarded and the mean and SD recalculated until no values exceeded mean plus 2 SD. The remaining mean value yielded the respective individual baseline fGCM concentration.

Linear mixed-effects models (lme4 package in RStudio (version 3.6.1, R core team 2013) were used to investigate the effect of ecological season and reproductive periods on faecal glucocorticoid metabolite concentrations. Males and females were analysed separately, whereby the fixed effects in the male model were season (wet vs dry), and reproductive activity (active vs non-active), while in the female model they were season (wet vs dry), and reproductive status (cycling vs first-trimester of pregnancy vs second-trimester of pregnancy vs third-trimester of pregnancy vs post-partum). Both models included time of collection (week) and individual (id) as a random effects. The faecal GCM concentrations data set was log-transformed and normality was tested visually using quantile comparison plots and Levene's test on model residuals.

Male: log(fGCM) ~ season + reproductive event + (1 | week) + (1|id).

Female: log(fGCM) ~ season + reproductive state + (1|week) + (1|id).

A paired-sample t-tests with a Bonferroni correction were performed post-hoc to determine which of the female reproductive periods were significantly different from each other. All statistical analyses were carried out using RStudio (version 3.6.1, R core team 2013). The

results are presented as mean ± standard error (SE) and differences between data sets were found to be significant at $p < 0.05$.

3. Results

3.1. ACTH challenge

For the female, four of the five tested EIAs detected increase in fGCM concentrations >100% post-ACTH administration; with only the corticosterone EIA revealing a comparatively moderate increase of about 59%. For the male, three of the five tested EIAs detected increases in fGCM concentrations > 100% post-ACTH administration, with only the Cortisol and corticosterone EIA showing moderate increases of about 43% and 98%, respectively (Table 1). Overall, the 11-oxoaetiocholanolone I EIA performed best, with a 20-fold increase in fGCM concentrations post-ACTH injection in the male (baseline: 0.38 ± 0.08 µg/g DW; peak: 8.59 µg/g DW) and a 17-fold increase in the female (baseline: 0.32 ± 0.06 µg/g DW, peak: 7.28 µg/g DW), measured 17- and 12-hours post-administration, respectively (Figure 1). The time for fGCM concentrations to return to baseline levels post-injection varied between assays and the individuals (Table 1). For the female, it took between 37 to 48 h (42.63 ± 5.63 h) for the fGCM concentrations to return to baseline, whereas for the male, baseline fGCM concentrations were reached again after 37 to 61 h post-injection (49.75 ± 16.97 h).

Table 1: Faecal glucocorticoid metabolite (fGCM) concentrations (µg/g, DW) in a male and a female roan antelope during an ACTH challenge test using 5 different enzyme immune assays. Baseline concentrations before injection, maximum rise after injection, percentage increase compared to fGCM baseline levels, time to peak concentrations, and time to return to fGCM baseline levels are presented in the table.

Enzyme immunoassays	**11-oxoaetio-cholanolone I**	**11-oxoaetio-cholanolone II**	**5α-pregnane 3β-11β,21-triol-20-one**	**cortisol**	**corticosterone**
Male					
Baseline concentration (µg/g DW)	0.38	0.60	0.70	0.06	0.66
Max concentration post-injection (µg/g DW)	8.58	6.32	4.13	0.13	0.95
% increase	2131	949	486	98.00	43.14
Time of peak after injection (h)	17.00	15.50	15.50	16.50	18.00
Time to return to baseline after injection (h)	61.75	37.75	64.50	24.75	38.25
Female					
Baseline concentration (µg/g DW)	0.32	0.64	0.43	0.03	0.39
Max concentration post-injection (µg/g DW)	7.28	6.18	2.16	0.09	0.62
% increase	2146	872	395	166	59
Time of peak after injection (h)	12.75	13.25	12.75	13.75	12.75
Time to return to baseline after injection (h)	48.25	48.00	37.75	37.75	37.00

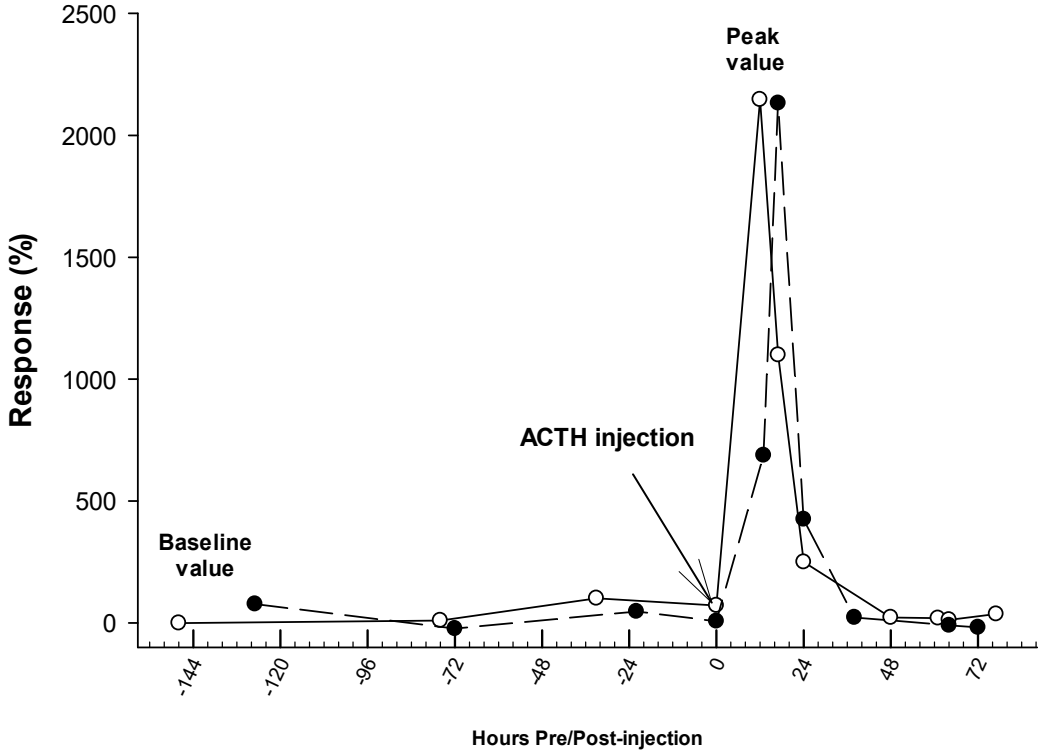

Figure 1: Changes in immunoreactive faecal glucocorticoid metabolite (fGCM) concentrations (%) in a female (—○—) and male (---●---) roan antelope pre-and post-ACTH administration measured by an 11-oxoaetiocholanone EIA (detecting 11,17 dioxoandrostanes).

3.2. fGCM concentrations during reproductive events in male roan antelope

The linear mixed-effects model explained 44% (conditional R^2) of the variation in fGCM concentrations, season and reproductive period explained 30% (marginal R^2) of the variation in the fGCM concentrations. During periods of reproductive activity, individual fGCM concentrations (0.749 ± 0.075 µg/g DW) showed a significant increase (X^2=37.81; P<0.001) between 59% and 143% compared to respective periods of reproductive inactivity (0.405 ± 0.020 µg/g DW). When mating activity was observed (in male 1 and male 2) during the reproductively active periods, fGCM concentrations were 7.60 % higher compared to the

time when such behaviour was not observed (0.64 ± 0.02 µg/g DW vs 0.56 ± 0.14 µg/g DW). During the periods of reproductive inactivity, overall individual fGCM concentrations were 49% higher during the calving period compared to respective hormone metabolite levels during the lactation/gestation period (0.57 ± 0.02 µg/g DW vs 0.38 ± 0.05 µg/g DW).

3.3. fGCM concentrations during reproductive events in female roan antelope

The linear mixed-effects model explained a large proportion of the variation (conditional R^2 = 78%), season and reproductive period explained 72% (marginal R^2) of the variation in fGCM concentrations. There was a significant difference (X^2= 741.06; P< 0.001) between female reproductive statuses (cycling vs first trimester of pregnancy vs second trimester of pregnancy vs third trimester of pregnancy vs post-partum) (Table 2). For the 10 pregnancies monitored, nine females showed a progressive increase in fGCM concentrations from the first trimester, through to the second and third trimester, with peak values occurring close to parturition. Individual fGCM concentrations during the third trimester (1.36 ± 0.03 µg/g DW) were significantly higher compared to fGCM concentrations of all other reproductive periods (P < 0.001). Faecal GCM concentrations were significantly higher during post-partum compared to the cycling period (p = 0.001).

Table 2: Model estimates from the linear mixed-effects model of the effect of season and reproductive activity/status on log (faecal glucocorticoid metabolite concentrations). Bold indicates significant p-value < 0.05.

Males					
Fixed Effects	Estimate	SE	t-value	df	P value
Intercept	-0.724	0.132	-5.482	2.4	0.021
Season: Wet	0.576	0.093	6.209	124.1	<0.001
Reproductive activity: Active	-0.403	0.073	-5.495	125.6	<0.001
Random Effects	Variance	SD	Levels		
ID	0.041	0.201	3		
Week	<0.0001	<0.0001	56		
Residuals	0.161	0.402			
Females					
Fixed Effects	Estimate	SE	t-value	df	P value
Intercept	-1.266	0.067	-18.865	33.8	<0.001
Season: Wet	-0.006	0.060	-0.103	415.3	0.918
Reproductive period					
First trimester	0.159	0.080	1.986	297.6	0.047
Second trimester	0.728	0.074	9.835	152.4	<0.001
Third trimester	1.517	0.062	24.629	57.5	<0.001
Post-partum	0.440	0.108	4.073	202.1	<0.001
Random Effects	Variance	SD	Levels		
ID	0.018	0.133	10		
Week	0.013	0.113	57		
Residuals	0.124	0.352			

3.4. Seasonal variation in male and female fGCM concentrations

This study covered two periods of male reproductive activity as well as two periods of male reproductive inactivity, which occurred in almost equal parts during the wet season and the dry season (Kamgang et al. 2020). In the present study, pregnancy began during the wet season and ended (late pregnancy) during the dry season. Post-partum and birthing occurred during the dry season while cyclicity occurred in the dry season.

Male fGCM concentrations were significantly influenced by season and reproductive activity (table 2), with significantly higher fGCM concentrations during the dry season (0.554 ± 0.040 µg/g DW; $X^2 = 30.19$; $P<0.001$) compared to the wet season (0.395 ± 0.027 µg/g DW).

Female fGCM concentrations differ not significantly between seasons ($X^2= 0.0105$; $P = 0.918$; Table 2), although overall fGCM concentrations in the dry season (0.970 ± 0.035 µg/g DW) were over 80% higher than respective hormone metabolite concentrations during wet season (0.536 ± 0.023 µg/g DW).

4. Discussion

ACTH challenge

In this study, three EIAs have been identified to reliably monitor fGCM concentrations in captive male and female roan antelope. The finally chosen 11-oxoaetiocholanolone assay measuring 11,17 dioxoandrostane showed variations in fGCM concentrations linked to certain reproductive periods in male and female roan antelope. Further, fGCM concentrations were significantly increased during the dry season in males but not in females.

Although three EIAs for the male and four EIAs for the female out of the five EIAs tested seemed suitable for monitoring fGCM concentrations in the roan antelope, the two group-specific 11-oxoaeticholanolone EIAs revealed the highest increases in fGCM concentrations for both sexes following an ACTH challenge, with the 11-oxoaetiocholanolone I (Palme and Möstl 1997) performing best. The two EIAs have also been reported to be suitable for the measuring of fGCM concentrations in other ungulates species such as African buffalo (*Syncerus caffer*) and rocky mountain goat (*Oreamnos americanus*) [3, 9, 15]. With the 11-oxoaetiocholanolone I being the best performing assay, it can be speculated that 11,17 dioxyoandrostanes are abundant in roan antelope faeces as demonstrated for cows (*Bos taurus*) and sheep (*Ovis areis*) [42].

Peak fGCM concentrations in the male and the female were detected at 17 and 12 hours post-injection of ACTH. These intervals are comparable to those reported for sheep [48], African buffalo (*Syncerus caffer*) [15, 61], red deer (*Cervus elaphus*) [22], and goat (*Capra aegagrus*) [30], which demonstrated peak fGCM values between 10 to 20 hours post-injection of ACTH.

fGCM concentrations during reproductive events in male

In males roan antelope, fGCM concentrations were elevated when males were reproductively active compared to when they were not. Increased fGCM concentrations during the mating season were found in other ungulate species such as the goral (*Naemorhedus griseus)* [28] and bison (*Bison bison*) [39]. These species showed an increase in GC levels just prior to, or during the mating season. In many species, the breeding period is associated with behavioural challenges in males such as those for territory or harem defence, which incur an energetic cost (Romero 2002The energy mobilization hypothesis might not apply to the males in this study, as they received supplementary feed throughout the year. However, even though challenges linked to reproduction are conspicuously reduced in a captive setting, activities such as male courtship might be reflected in an increase in adrenocortical activity, as e.g. a study in rats show that sexual arousal can elicit an increase of plasma cortisol [4]. This may also explain the slightly higher fGCM concentrations found when actual copulation was observed within the reproductively active periods. Kamgang et al (2020) demonstrated that male roan antelope show higher faecal androgen metabolite levels during their reproductively active periods. This could be indicative of a potential interactions between the hypothalamic-pituitary-adrenal and hypothalamic-pituitary-gonadal axis, as seen for example in African elephants [54] and other mammals (Viau 2002).

fGCM concentrations during reproductive events in female

In female roan antelope, fGCM concentrations were lowest during the cyclicity periods, which could be a consequence of the higher energetic demands required during gestation and lactation (postpartum), as seen in other mammals [59, 66, 68]. Furthermore, low GC levels could be favourable for oestrus display or ovulation as shown for sheep [8].

Female fGCM concentrations progressively increased throughout pregnancy, peaking during the third trimester. These findings are in contrast to studies on other artiodactyls such as the cow [49], and sheep [5], which demonstrated that plasma cortisol levels only rise close to parturition. However, a similar pattern of pregnancy-related elevations in GC concentrations as seen in this study have been found for other mammals such as the Asian elephant [24] and the red deer (*Cervus elaphus*)[67]. The underlying mechanisms of GC increases during pregnancy are still not well understood, but it is speculated that the increase of GCs during pregnancy is necessary to fulfil the maternal energetic demands required to stimulate the growth of foetal organs [13, 34]. However, in some artiodactyls, there is no increase in GCs during pregnancy despite assumed similar energetic requirements by the mother. Hence, the energetic cost of pregnancy may not be the sole explanation for the increase in GCs [10, 59].

In the current study, although fGCM concentrations dropped rapidly after parturition, they remained above baseline for three weeks post-partum, which corresponds to the lactation stage. Such a pattern has also been demonstrated for the Dorset sheep and the North American squirrel [7, 29]. It is well known that lactation is energetically demanding, therefore the delay in GCs concentrations returning to baseline levels post-partum could be seen as a period for additional mobilization of energy required by the mother to feed her calf [55].

Seasonal variation in male and female fGCM concentrations

In male roan antelope, fGCM concentrations were elevated during the dry season when there is less/no rain and cooler temperatures. These results are similar to findings reported for species such as the yak (*Poephagus grunniens L.*), the impala (*Aepyceros melampus*) (Hunninck et al., 2020) and bedouin goat (*Capra aegagrus*) [6, 56]. However, the findings in our study may not support the energy mobilization theory (Sakar et al. 2009), as the study animals were provided with supplementary feed throughout the year. The comparatively higher fGCM concentrations found during the dry season might also not be linked to male reproductive activity, as those periods occurred during the wet as well as the dry season (Kamgang et al. 2020). However, photoperiod might play a role in the synthesis of GCs, as a respective link for faecal androgen metabolite concentrations was demonstrated for the study animals (Kamgang et al. 2020), and e.g. a similar pattern of androgen and GC concentrations has been demonstrated for the pygmy goat (*Capra aegagrus hircus*) [21]. However, further studies should be carried out to confirm this hypothesis and provide basic data on cortisol metabolism and excretion for the Roan antelope, as it has been shown that the EIA used in this study can cross-react with androgen metabolites (Ganswindt et al., 2003).

In contrast to the males, female fGCM levels, although overall 80% higher during the dry season, were not significantly different between the ecological seasons. This implies that in females roan antelope, seasons may not influence glucocorticoids levels. The females monitored in the present study went from the cycling state to the pregnancy, birthing and postpartum states. Even though pregnancy mostly occurred during the wet season, birthing which is also characterised by high fGCM concentrations occurred in the dry season. Thus, females' reproductive state may have masked a potential seasonal variation in fGCM levels

5. Conclusion

The present study shows that monitoring adrenocortical function in the roan antelope can be successfully achieved using various EIAs including an 11-oxoaeticholanolone EIA measuring 11,17 dioxoandrostanes in faecal matter. This study further demonstrated higher fGCM concentrations during pregnancy, and parturition, as well as during reproductively active periods of males. The knowledge obtained is valuable for conservation breeding programmes of roan antelope, as it identifies potentially stressful circumstances which should be carefully monitored to ensure animal welfare.

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8. Data base

The data generated and subsequently analysed during this study are available on the repository of the University of Pretoria. The digital object identifier assigned to the data is https://doi.org/10.25403/UPresearchdata.14073662

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