Journal of Zoology

Journal of Zoology. Print ISSN 0952-8369

ORIGINAL RESEARCH

Examining alterations in fGCM concentrations post-defaecation across three animal feeding classes (ruminants, hindgut fermenters and carnivores)

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Keywords
non-invasive hormone monitoring; stress; faecal glucocorticoid metabolites; enzyme immunoassays; post-defaecation; fGCM; wildlife monitoring; faecal sampling.

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Editor: Andrew Kitchener
Associate Editor: Débora Moreno Azócar

Received 17 June 2024; revised 28 December 2024; accepted 16 January 2025

doi:10.1111/jzo.13257

Abstract

Quantification of faecal glucocorticoid metabolites (fGCMs) is a popular non-invasive technique for monitoring wildlife's response to stressors, demanding an understanding of the stability of fGCM concentrations post-defaecation to ensure comparability of determined fGCM values across samples. To provide species-specific recommendations for the duration within which sampling can take place, we measured the rate at which the fGCM concentrations of nine different species changed throughout a 7-day period post-defaecation. In this study, we explored the temporal dynamics of fGCM concentrations in nine species across three feeding classes (ruminants, hindgut fermenters and carnivores): impala (*Aepyceros melampus*), giraffe (*Giraffa camelopardalis*), blue wildebeest (*Connochaetes taurinus*), plains zebra (*Equus quagga*), African elephant (*Loxodonta africana*), white rhino (*Ceratotherium simum*), cheetah (*Acinonyx jubatus*), spotted hyena (*Crocuta crocuta*) and leopard (*Panthera pardus*). Utilizing enzyme immunoassays already established for each of the focal species, we identified broader feeding class-specific patterns. All herbivores exhibited a significant decrease in fGCM concentrations over time, starting from 6 h (impala) to 48 h (giraffe, blue wildebeest, white rhino and African elephant) post-defaecation. For carnivores, concentrations remained fairly comparable for 12–24 h, after which fGCM concentrations either decreased (spotted hyena), increased (leopard) or remained stable (cheetah), with notable variation in triplicate concentrations (cheetah and leopard). These findings offer insights into scheduling faecal sampling for endocrine monitoring, particularly from free-roaming wildlife, to ensure comparability of determined hormone metabolite concentrations. Furthermore, the species-specific variation in fGCM concentration post-defaecation demonstrated in this study underlines the necessity to investigate every new species to ensure accurate and comparable results. Future studies ought to investigate how the mass of collected material, sex and drying methodologies affect the measurement of fGCMs post-defaecation.

Introduction

Quantifying glucocorticoids (GCs) in various matrices, especially faeces, is nowadays a common strategy for learning about potential stressors that animals may perceive (Ganswindt et al., 2012; Palme, 2019). This is because faeces can be comparatively easily collected and present a safer approach when working with free-roaming wildlife than the collection of urine, saliva or even blood for hormone analyses. Further, animals are typically not (or only marginally) disturbed during the procedure of faecal sample collection and thus, sampling can be considered to be feedback-free (Touma & Palme, 2005). However, faecal sampling for quantifying GC metabolites presents a number of challenges, such as a possible delay in time between defaecation and collection, and the variability of that time between sampling events (Crossey et al., 2018; Majelantle et al., 2020; Webster et al., 2018). Apart from challenges related to the sampling procedure, variations in sample preparation, including the possible challenge of irregular distribution of hormone metabolites throughout a faecal sample (Millspaugh & Washburn, 2004), and the requirement for specialized and expensive laboratory equipment to prepare samples for analyses (Postiglione et al., 2022) have to be considered. With regard to steroid quantification, species- and sex-specific

differences in steroid metabolism are also factors that need to be carefully considered (Touma et al., 2003). A study assessing fGCM concentrations in a particular species for the first time must, therefore, not only ensure to utilize an analytical, physiological and/or biologically validated assay (Touma & Palme, 2005) but also establish a reliable sampling protocol to ensure comparability of determined fGCM values.

When sampling cannot take place directly after defaecation, as is often the case in wild or free-ranging settings, the stability of immunoreactive fGCM concentrations post-defaecation is an important factor to consider when quantifying fGCMs to ensure the comparability of respective steroid concentrations from samples collected at different times post-production. Following defaecation, the presence of still active bacterial enzymes in faeces could lead to further alteration in the composition of fGCMs, which might then be reflected in differences in the immunoreactive readout depending on the cross-reactivity of the antibody involved (Lexen et al., 2008; Palme, 2005). Therefore, to maintain consistency in steroid concentration measurements across different samples, standardized collection protocols are implemented. This typically involves gathering faecal material within a defined timeframe (Hulsman et al., 2011) and preventing ongoing bacterial enzyme activity, often by removing moisture from the sample through freeze-drying (Fraňková et al., 2012; Washburn & Millspaugh, 2002).

Previous studies have examined potential changes in immunoreactive fGCM concentrations post-defaecation for different species. For example, a trend of increasing fGCM concentrations over time was observed in cattle, horses, pigs (Möstl et al., 1999), African buffalo (*Syncerus caffer*; Ganswindt et al., 2012), African clawless otter (*Aonyx capensis*; Majelantle et al., 2020) and African wild dogs (*Lycaon pictus*; Crossey et al., 2018). The opposite trend of declining fGCM concentrations post-defaecation has been shown for brown hyenas (*Hyaena brunnea*; Hulsman et al., 2011), and banded mongoose (*Mungos mungo*; Laver et al., 2012), Depending on the assay used, an increase or decrease in fGCM concentrations has also been observed in sheep (*Ovis aries*; Lexen et al., 2008) and cattle (*Bos taurus*; Morrow et al., 2002). However, some studies demonstrated little-to-no change in fGCM concentrations over the monitored period, as has been seen in Nile crocodiles (*Crocodylus niloticus*) (Ganswindt et al., 2014), Jaguars (*Panthera onca*; Mesa-Cruz et al., 2014) and Leopards (*Panthera pardus*; Webster et al., 2018). Previous studies have examined potential changes in immunoreactive fGCM concentrations post-defaecation for different species, usually focusing at a single species level.

Therefore, this study aimed to examine alterations in fGCM concentrations post-defaecation for nine species where enzyme immunoassays for fGCM quantification have been already established. More specifically, the study describes and evaluates alterations of fGCM concentrations post-defaecation across three animal feeding classes' (ruminants, hindgut fermenters and carnivores). Lastly, a respective degradation study has already been done for four of the nine species (in four different studies) and we compared the previous with the current results.

This study offers comparable measurements across nine species using a consistent experimental design, making it more directly comparable than some other studies. It seeks to establish trends by feeding class, proposes tentative cut-offs based on fGCM concentration changes over time and considers variation between samples.

Materials and methods

Ethical considerations

This study was undertaken with the approval of the University of Pretoria Research Committee and Animal Ethics Committee (AEC) (Ethics clearance number: NAS286/2021).

Study species

Three species from each of the three feeding groups investigated were included in our study, four of which have already been investigated, namely; (1) ruminants: (a) impala (*Aepyceros melampus*), (b) giraffe (*Giraffa camelopardalis*), (c) blue wildebeest (*Connochaetes taurinus*) (Wolf et al., 2021); (2) hindgut fermenters: (d) plains zebra (*Equus quagga*), (e) African elephant (*Loxodonta Africana*) (Webber et al., 2018), (f) white rhino (*Ceratotherium simum*) (Nhleko et al., 2022); and (3) carnivores: (g) cheetah (*Acinonyx jubatus*), (h) spotted hyena (*Crocuta crocuta*) and (i) leopard (*Panthera pardus*) (Webster et al., 2018).

Sampling sites and sample collection

One fresh faecal sample was collected from 10 individuals per species during 18 March–20 April 2022 in the Limpopo Province, South Africa, from free-ranging herbivores at Voluvista Game Reserve [impala (n = 10), blue wildebeest (n = 10), giraffe (n = 10) and zebra (n = 1)] and Zinnshoek Private Game Reserve [white rhino (n = 10) and zebra (n = 9)] and individuals held semi-captive from Adventures with Elephants [elephant (n = 10)]. Fresh faecal samples from captive carnivores were collected between 30 May and 09 June 2022 at Predator World, North-West province, South Africa [spotted hyena (n = 10), cheetah (n = 10) and leopard (n = 10)].

All samples were collected within 15 min after observing defaecation while wearing single-use gloves. During sampling, faecal material was collected from the centre of a sample to avoid contamination with urine or surrounding soil and substrate. We collected approximately 150–250 g of faecal material per species, depending on the sample size. Collected samples were well-mixed by hand on-site, placed into a Ziplock bag, individually labelled, placed on ice for a maximum of 20–30 min and frozen at −20°C. The samples collected from Zinnshoek Private Game Reserve [white rhino (n = 10) and zebra (n = 9)] were frozen within 6 h of collection. All samples were stored until further preparation at the Endocrine Research Laboratory (Fig. 1).

Sample preparation and steroid extraction

The collected material was defrosted overnight at 4°C. The 10 samples per species were then thoroughly mixed by hand over a cool surface and divided into 27 equal sub-samples. The

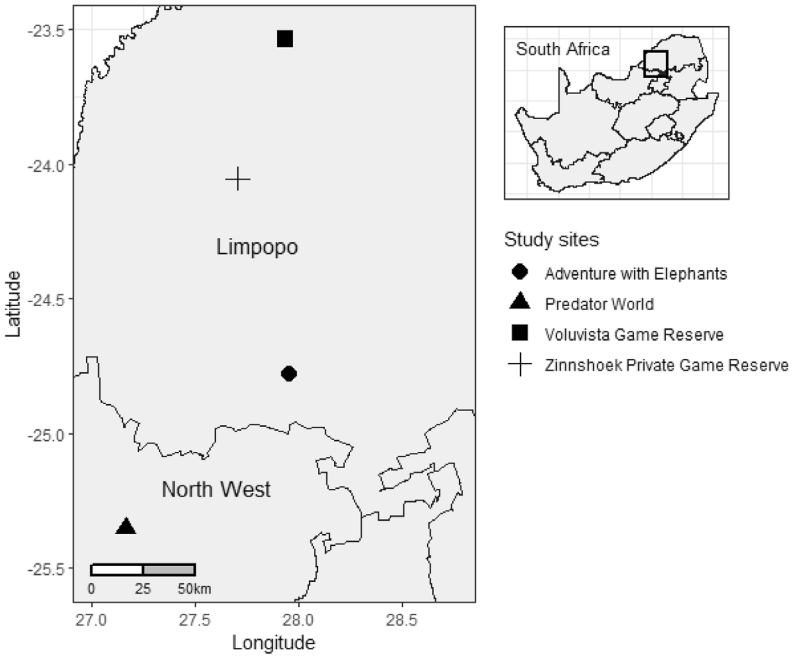

Figure 1 Map of the four South African study sites where faecal samples for all nine focal species were collected during 18 March–9 June 2022.

sub-samples were stored in containers exposed to open-air and laboratory room temperatures (which ranged from 12 to 22°C throughout the experiment). For each species, triplicates were then frozen at −20°C at intervals of: 0, 1, 3, 6, 12, 24, 48, 96 and 168 h respectively. This experimental design allowed for a prolonged drying of the faecal material as to what would be expected under what are usually much warmer conditions in the field. This allowed for an analysis which was able to encapsulate a longer timeframe, ultimately simulating particularly challenging field sampling conditions (Crossey et al., 2018).

All frozen faecal samples (n = 27 sub-samples per species) were lyophilized, pulverized and sifted through a wire-mesh strainer to separate any undigested material from the resultant faecal powder (Fieß et al., 1999). Steroids from faecal powder (0.050–0.055 g) for giraffe, elephant and cheetah and (0.100–0.110 g) for impala, blue wildebeest, white rhino, zebra, hyena and leopard, were extracted using 3 ml of 80% ethanol in water. The suspensions were vortexed for 15 min and centrifuged for 10 min at 1500x***g*** (Ganswindt et al., 2002). Supernatants were then transferred into microcentrifuge tubes and stored at −20°C until analysis.

Steroid analysis

All faecal steroid extracts were measured for immunoreactive fGCM concentrations using enzyme immunoassays (EIAs) previously established for the respective species in question (Table 1). Detailed assay characteristics for each EIA, including a full description of the assay components and cross-reactivities, are provided by Palme and Möstl (1997) for the 11-oxoaetiocholanolone I and corticosterone EIA; by Möstl et al. (2002) for the 11-oxoaetiocholanolone II EIA; by Touma et al. (2003) for the 5α-pregnane-3β,11β,21-triol-20-one EIA, and by Frigerio et al. (2004) for the 11β-hydroxyaetiocholanolone EIA. Serial dilutions of faecal extracts resulted in displacement curves that were parallel to the respective standard curves (relative variation of the slopes of respective trend lines <5% for the corticosterone EIA and <2% for the 11β-hydroxyaetiocholanolone EIA).

The sensitivities of the assays, intra- and inter-assay coefficients of variation (CV), determined by repeated measurements of high and low-quality controls are found in Table 1. Assay procedures followed published protocols by Ganswindt et al. (2002) and were conducted in the Endocrine Research Laboratory, University of Pretoria, South Africa.

Statistical analyses

The R program (R Development Core Team 4.2.1) with the use of the R Studio interface was used for statistical analyses. To determine alterations of fGCM concentrations post-defaecation, each species-specific median fGCM concentration determined at time = 0 h (100% initial concentration) was used as the baseline concentration from which alterations in fGCM concentration for each time interval were calculated (based on median alterations in fGCM concentration for each triplicate sample set). Analysis of a priori model residuals indicated fGCM concentration percentages for 8 of the 9 species followed a normal distribution. However, giraffe fGCM

Table 1 Previously validated enzyme immunoassays (EIAs) for fGCM quantification for all nine focal species; with information on assay sensitivity, intra- and inter-assay coefficients of variation (CV) and reference of the validation study

Species	EIA	Reference	Sensitivity (ng g^{-1} faecal dry mass)	Intra-assay CVs	Inter-assay CVs
Impala	11-oxoaetiocholanolone II (lab code: 72 T, measuring fGCMs with a 5β-3α-ol-11-one structure)	Chizzola et al. (2018)	0.75	5.5% 6.5%	6.6% 14.7%
Giraffe	11-oxoaetiocholanolone II (lab code: 72 T, measuring fGCMs with a 5β-3α-ol-11-one structure	Bashaw et al. (2016)	1.5	5.5% 6.5%	6.6% 14.7%
Blue Wildebeest	11-oxoaetiocholanolone I (lab code: 72a measuring fGCMs with a 11, 17-dioxandrostanes structure)	Wolf et al. (2021)	1.2	4.0% 4.8%	11.6% 11.9%
Plains Zebra	11β-hydroxyaetiocholanolone (lab code: 69a, measuring fGCMs with a 5β,3α,11β-diol structure	Seeber et al. (2018)	2.4	7.8% 8.0%	12.3% 14.2%
White Rhino	5α-pregnane-3β,11β,21-triol-20-one (lab code: 37e, measuring fGCMs with a 5β,3α,11β-diol structure)	Badenhorst et al. (2016)	2.4	4.7% 5.7%	7.0% 8.2%
African Elephant	11-oxoaetiocholanolone II (lab code: 72 T, measuring fGCMs with a 5β-3α-ol-11-one structure)	Ganswindt et al. (2002)	1.5	5.5% 6.5%	6.6% 14.7%
Spotted Hyena	Corticosterone	Goymann et al. (1999)	2.4	2.8% 6.3%	8.3% 9.5%
Cheetah	Corticosterone	Ludwig et al. (2012)	4.8	2.8% 6.3%	8.3% 9.5%
Leopard	5α-pregnane-3β,11β, 21-triol-20-one (lab code: 37e, measuring fGCMs with a 5β,3α,11β-diol structure)	Webster et al. (2018)	2.4	7.8% 8.0%	12.3% 14.2%

concentration percentages exhibited a negatively skewed distribution. As such, these data were log10-transformed prior to statistical analysis in order to meet the required assumptions for parametric statistical testing.

For each species, differences in relative alteration rate between samples stored at $t = 0$ h and 1–168 h post-thawing were examined by one-way repeated measures analysis of variance (ANOVA), followed by pairwise *t*-tests *post hoc* to identify which time intervals significant differences in fGCM concentrations compared to $t = 0$ h. In all pairwise multiple comparison procedures, the α-level was adjusted by applying a Bonferroni correction. Statistical significance for all tests was set at alpha (α) = 0.05 and inferred at $P < 0.05$.

Results

For seven of the nine species examined, there was a significant change in fGCM concentrations during the monitored seven-day post-defaecation period (Table 2).

Faecal GCM concentrations in ruminant herbivores, determined using pre-established assays, remain statistically comparable until 6 h post-thawing for impala before fGCM concentrations declined significantly ($P = 0.046$) by 16% after 6 h. The giraffe and blue wildebeest fGCM concentrations remained stable until 48 h post-thawing, after which they showed significant decreases by 49% ($P < 0.001$) and 59% ($P < 0.001$) (Fig. 2).

When considering hindgut fermenters, concentrations of fGCMs increased significantly for African elephant (27%; $P = 0.007$) at 6 h and for zebra (14%; $P = 0.032$) at 12 h post-thawing but remained stable for white rhino until 24 h post-thawing. All three species then showed decreases in fGCM concentrations that are significantly different from fGCM concentrations at $t = 0$ ($P < 0.001$) at 24 h post-thawing for zebra (39%) and at 48 h for African elephant (62%) and white rhino (46%).

Table 2 *P*-value, adjusted R^2 and *F*-Statistic from the overall ANOVA investigating if there is a difference in fGCM concentrations between time intervals across nine different species. The time interval indicates the earliest significant changes (*P*-value <0.05) from baseline fGCM concentrations were obtained from the *post hoc* pairwise *t*-test

Species	*P*-value	Adjusted R^2	*F*-statistic	Time interval (h)
Impala	<0.01	0.97	$122.40_{(8,18)}$	6
Giraffe	<0.01	0.19	$38.01_{(8,18)}$	48
BW	<0.01	0.95	$76.86_{(8,18)}$	48
Zebra	<0.01	0.98	$177.80_{(8,18)}$	12
White Rhino	<0.01	0.94	$62.13_{(8,18)}$	48
African Elephant	<0.01	0.95	$73.46_{(8,18)}$	48
Cheetah	0.22	0.13	$1.51_{(8,18)}$	—
Spotted Hyena	<0.01	0.76	$11.40_{(8,18)}$	24
Leopard	0.04	0.35	$2.75_{(8,18)}$	168

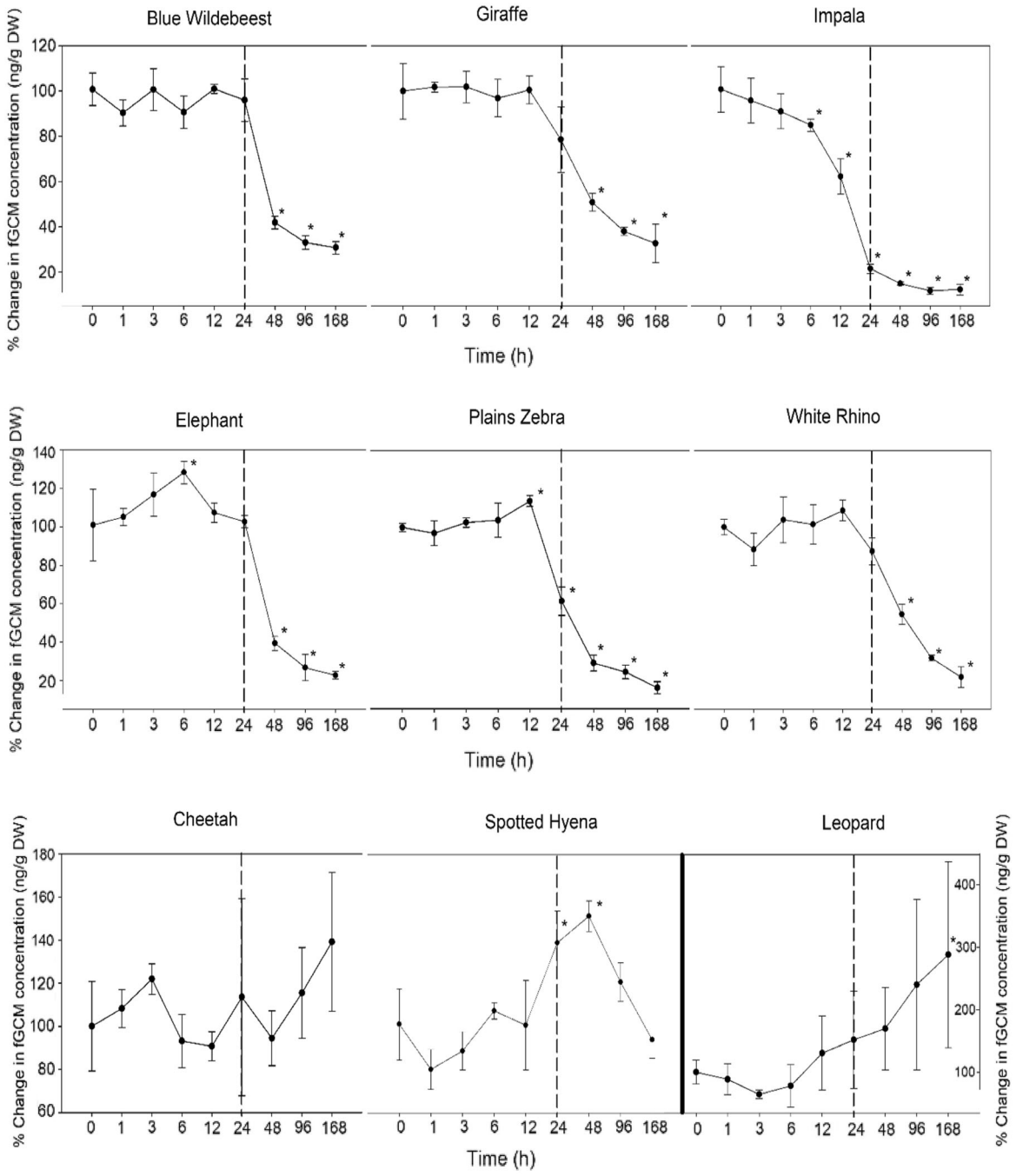

Figure 2 Percentage change in fGCM concentrations for nine different species (Blue Wildebeest, Giraffe, Impala, African Elephant, Plains Zebra, White Rhino, Cheetah, Leopard and Spotted Hyena) post-defaecation (0–168 h). The points represent the mean per cent fGCM concentrations of the respective sample triplicate, and the whiskers show the SD. An asterisk (*) indicates statistically significant differences in % change of fGCM concentration for time intervals compared to $t = 0$. Time in (h) post-defaecation.

Overall, fGCM concentrations for the three investigated carnivore species showed no significant alteration in fGCM concentrations until 12 h post-thawing, followed by a non-significant increase until 24 h post-thawing for cheetah and leopard ($P > 0.05$), and a significant increase ($P = 0.010$) of 38% for the spotted hyena. Faecal GCM concentrations for the leopard remained statistically comparable until 168 h post-thawing followed by a significant increase ($P = 0.009$) in fGCM concentrations of 88% compared to respective levels at $t = 0$. However, the variability in fGCM concentrations of

analysed triplicates distinctively increased from 12 h post-thawing (Fig. 2). For cheetah no significant alteration in fGCM concentrations was found throughout the period monitored ($P = 0.221$), but there was a comparatively higher variability of 200% in triplicate fGCM concentrations at 24 h.

Discussion

Our findings indicated that fGCM concentrations in all monitored herbivores remain stable for a species-specific duration before beginning to decline. This pattern aligns with previous research on blue wildebeest (Wolf et al., 2021), African elephant (Webber et al., 2018) white rhino (Nhleko et al., 2022) and other herbivorous species, such as African buffalo (Ganswindt et al., 2012) and sheep (Lexen et al., 2008). Our results for the three investigated carnivore species showed a trend of significant increase in hyena fGCMs, which is consistent with trends observed in other carnivore species, with a study by Crossey et al. (2018) showing that African wild dog fGCM concentrations remain stable for up to 24 h before significantly increasing and then dropping back to comparable levels with $t = 0$ at 48 h post-defaecation.

To better place the results of our study into context, it is important to note that when evaluating the outcomes for a particular species or feeding class, one needs to consider not only the species-specific GC metabolite composition but also the specificity of the antibody utilized in a particular EIA. This was demonstrated by Lexen et al. (2008) in a study on sheep, where an 11-oxoaetiocholanolone EIA I detecting 11,17-dioxandrostanes (Palme & Möstl, 1997) showed an increase in fGCM concentrations post-defaecation, while an 11-oxoaetiocholanolone EIA II targeting fGCMs with a 5β-3α-ol-11-one structure (Möstl et al., 2002) revealed a decrease in the corresponding hormone metabolite concentrations post-defaecation. The utilization of different EIA can also yield comparative patterns, as described by Webster et al., 2018. When using the same 11-oxoaetiocholanolone EIA in the present study (detecting fGCMs with a 5β-3αol-11-one structure), a similar trend of decrease was observed in impala. Despite our study using 4 different EIAs in total for quantifying fGCMs in the six herbivorous species, all showed a similar trend of decreasing fGCM values over time post-defaecation.

When comparing trends of fGCM concentrations post-defaecation for species already investigated in the past, the results obtained in this study for blue wildebeest can be better contextualized when considering those of Wolf et al. (2021). These authors examined the stability of fGCM concentrations post-defaecation by utilizing the same 11-oxoaetiocholanolone EIA and showed a distinct decrease of about 30% between 8 and 16 h post-defaecation, concluding that collecting faecal samples older than 8 h after defaecation might not give a reliable indication of the fGCM concentrations for the species. This could be due to individual differences in microbiome, whereby the resulting faecal samples may be subject to different levels of bacterial enzymatic activity, and which could result in pronounced differences in metabolite stability when individual animals are compared (Möstl et al., 1999). In our study, fGCM concentrations in blue wildebeest seem comparable until 24 h post-defaecation. Pinpointing exactly where this significant change may occur between 8 and 48 h will require additional studies which incorporate an investigation of a greater frequency of time intervals post-defaecation.

A study by Webber et al. (2018) demonstrated that African elephant fGCM concentrations remained constant for 48 for males, and 72 h for females, before showing a significant decline. Throughout our study, however, fGCM concentrations for the African elephant remained comparable for only 24 h post-defaecation before respective hormone metabolite levels started to decline. It is important to note here that Webber et al. (2018) used a different sample drying approach (air-drying under natural conditions), which likely resulted in shorter drying times compared to the ones in our study (air-drying under a constant temperature in a laboratory setting).

A similar principle relating to differences in drying times could also account for the differences seen in white rhino results in our study compared to those of Nhleko et al. (2022). The study by Nhleko et al. (2022) showed no differences in fGCM concentration over the course of 7 days, whereas our study fGCM concentrations remained comparable for only 24 h post-defaecation. In the experiment by Nhleko et al. (2022), however, samples were dried in a natural setting, likely drying far more quickly than the worst-case-scenario laboratory setting implemented in our study. Thus, it is possible that field-dried samples, which might dry more quickly, will not experience the same alterations as those observed after 48 h in our study. In addition to drying the sample under full sun in a natural environment, it is crucial to evaluate the potential effects of precipitation in a natural setting on fGCMs (Mesa-Cruz et al., 2014).

According to our study, fGCM concentrations in carnivore faeces seem to be comparable for a longer period of time than those measured in herbivore samples. This could be due to a number of factors including diet and gut passage time. When investigating wild bears, Stetz et al. (2013) discovered that diet had a significantly greater impact on fGCM concentrations than digestion transit time. Bears are omnivores and a diet with higher dietary fibre compared with a more meat-based diet results in faster digestive transit time causing a larger effect on fGCM concentrations. Among carnivores, we observed increased variability in the triplicates, likely due to inadequate manual homogenisation of the sample. To mitigate this concern, thorough homogenisation in a standardized laboratory setting, allowing the sample to thaw slightly longer might be a potential solution.

Every new species undergoing fGCM monitoring should be examined up front with respect to alteration in steroid metabolite concentrations post-defaecation. This is a logistical challenge affecting many researchers when conceptualizing their experimental design protocols. As such, the results of our study provide new knowledge into the potential general trends of fGCM concentration changes across feeding groups, assisting researchers in evaluating suitable sampling strategies for future validation and contextualized studies. It is crucial, however, to highlight that the generalizability of these results requires more exploration. Future studies should consider

experimental settings that more closely mimic those likely to be found in the field. This will help take into account research conducted in areas with major climate differences and seasonal effects (e.g. seasonal freezing). In addition to the size, structure and water content of the sample, the length of time it takes for faeces to dry completely relies largely on weather patterns, particularly the number of hours the material is exposed to direct sunlight and no rainfall (Webber et al., 2018). Therefore, a longer drying period could give bacteria (which are suspected to be the chief cause of fGCM alteration post-defaecation) more opportunity to alter the steroid composition of the faecal matrix (Galama et al., 2004).

Conclusion

The results of this study indicate that fGCM concentrations in carnivores were comparable until at least 12 h with no predictable trend afterwards. In herbivores, fGCM concentrations remained stable in most cases for 12–24 h, then would always decline irrespective of species-assay combination. However, exceptions, such as the 6 h time window for collection in impala underline the necessity to investigate every new species to ensure accurate and comparable results. It is highly recommended to test the real-world scenario for each species, analytical method and experimental setup by collecting samples as soon as possible after defaecation (Palme, 2019). Further research is needed to investigate how factors such as sample size, sex, individual differences in microbiomes, temperature, humidity and drying methods affect fGCM measurements post-defaecation. Provided that logistical considerations allow for faecal collection, non-invasive hormone monitoring remains a promising tool for gaining insights into the endocrine status of various species.

Acknowledgements

The authors would like to thank Nicole Hagenah-Schrader and Stefanie Ganswindt from the Endocrine Research Laboratory, University of Pretoria, for their technical assistance. We want to express our appreciation to the field assistants, as well as to the owners and management of the study sites, for their invaluable support during the project.

Author contributions

KO, BC and AG conceptualized the study; KO designed the methodology, validation and investigation, collected the data and prepared the original draft; KO, BC and TM worked on the visualization. BC and TM analysed the data; AG and BC supervised the study; AG provided the resources and funding acquisition. All authors contributed critically to the drafts and gave final approval for publication.

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