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A field-friendly alternative to freeze-drying faeces for glucocorticoid metabolite quantification in animals of different feeding classes

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ABSTRACT

Monitoring stress-related faecal glucocorticoid metabolite (fGCM) concentrations is a reliable, popular and established approach for understanding wildlife responses to perceived stressors. To maintain fGCM integrity post-defaecation, faecal material must be promptly stored frozen, or dried to prevent continued suspected bacterial enzyme activity. We compare the effectiveness of freeze-drying with other field-friendly drying techniques (food dehydrator and homemade solar oven). We collected 10 fresh faecal samples each from nine species (giraffe, impala, blue wildebeest, plains zebra, African elephant, white rhino, cheetah, spotted hyena, and leopard) and monitored alterations in fGCM concentrations over time utilizing these different drying techniques. Our findings indicate that a homemade solar oven is as effective as freeze-drying faecal samples. A food dehydrator is also a suitable method for drying faecal samples for the carnivores monitored. Our findings provide field-friendly methods for researchers dealing with logistical constraints in remote field sites.

- For all species examined, a homemade solar oven offers a practical and affordable alternative to freeze-drying faeces for fGCM quantification.
- A food dehydrator provides an affordable alternative to freeze-drying faeces for fGCM analysis when monitoring carnivores.
- Different faecal sample drying techniques should not be utilized within a single study to ensure comparable analyses of fGCM values.

Specifications table

Subject area:	Agricultural and Biological Sciences
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Name of your method:	*Alternative drying methods for glucocorticoid metabolites quantification in animals of different feeding classes*
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Background

Steroid quantification is a widely used approach to investigate adrenocortical activity in wildlife [1]. Nowadays, this is often accomplished by using non-invasive techniques as this approach can be applied with minimal to no disturbance to animals. A non-invasive sampling approach would also be more convenient logistically for both the animal and person collecting, compared to e.g. blood or saliva sampling, and as animals are typically not handled during the sampling process, it can be considered as feedback-free [2]. Further, faecal hormone metabolite concentrations provide a more comprehensive signal of the frequently changing secretions and eliminations of hormones circulating in the blood [3]. This is mostly because faecal hormone metabolite concentrations are an accumulation of the varying hormone quantities present and eliminated from the bloodstream [4,5].

However, studies investigating alterations in steroid concentrations utilizing faeces as hormone matrix have to consider the stability of faecal hormone metabolite composition post-defaecation. Glucocorticoids, as one classical steroid class frequently investigated, are metabolized in the liver and gut before excretion and following defaecation, bacterial enzymes in the faeces are suspected to continue this activity [6]. To prevent this process from continuing, the collected faecal material usually gets frozen as soon as possible post-collection [7,8] and is then often dried prior to steroid extraction and subsequent analysis [3]. This drying process should be quick, and follow a standardized approach, with freeze-drying (lyophilization) currently regarded as the golden standard to do so while keeping samples effectively frozen during the moisture removal process [1,8,9].

Freezing of the faeces halts suspected bacterial enzymatic activity, as does the complete removal of moisture from the faecal samples (achieved by freeze-drying) [8]. This is, however, a comparatively expensive approach and requires stable access to electrical power. This can be a challenge, especially for field studies conducted in remote parts of the world with limited to no access to power [10,11]. The same logistical setting would be required for wet-faecal extraction [12], as non-dried samples would have to be stored frozen until analysis (to stop metabolic processes via bacterial enzymes [7]). Further, transporting frozen material to an analytical lab also adds to the costs for shipment, as samples would have to be sent in a manner that maintains a cold chain e.g., on dry ice.

To date, however, very few studies have investigated potential alternatives to freeze-drying of faecal samples for hormone monitoring. A study by Postiglione et al. (2022) on African wild dogs, found that a food dehydrator-based dehydration technique had a negligible-to-zero impact on the potential integrity of fGCMs compared to freeze-drying [8]. Results from a more recent investigation by Lacomme et al. (2023) indicated that drying African elephant faeces in a food dehydrator is feasible without affecting the integrity of the immunoreactive fGCM signal [1]. However, due to the distinct differences in dietary physiology between animal classes (e.g., herbivorous versus carnivorous), it is important to investigate if potential alternative methods can be applied across the board, or at least considered for specific groups, like feeding classes.

Therefore, the aim of this study was to evaluate alternative field-friendly drying methods to freeze-drying for the removal of moisture from faeces prior to fGCM quantification for nine species which comprise herbivorous (ruminants and hindgut fermenters) and carnivorous groups. More specifically, the objective of the study was to compare post-defaecation fGCM concentrations of samples from giraffe (*Giraffa camelopardalis*), impala (*Aepyceros melampus*), 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*) that were dried using either a food dehydrator, solar oven, or freeze-dryer.

Method details

Faecal sampling

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, namely; i) ruminants: a) impala (*Aepyceros melampus*), b) giraffe (*Giraffa camelopardalis*), c) blue wildebeest (*Connochaetes taurinus*); ii) hindgut fermenters: d) plains zebra (*Equus quagga*), d) African elephant (*Loxodonta Africana*), e) white rhino (*Ceratotherium simum)*; and iii) carnivores: f) cheetah (*Acinonyx jubatus*), g) spotted hyena (*Crocuta crocuta*), and h) leopard (*Panthera pardus*).

Sampling sites and sample collection

Fresh faecal samples were collected between 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 ($n = 10$), zebra ($n = 1$)) and from Zinnshoek Private Game Reserve (white rhino ($n = 10$), zebra ($n = 9$)), and semi-captive from Adventure with Elephants (elephant ($n = 10$) . Fresh faecal samples from captive carnivores were collected between 30 May – 09 June 2022 at Predator World, North-West province, South Africa (spotted hyena ($n = 10$), cheetah ($n = 10$), leopard ($n = 10$))

All 10 samples for each species were collected within 15 min after observing defaecation while wearing single-use gloves. The faecal material was collected from the center of a sample to avoid contamination with urine or surrounding soil and substrate. During sampling, no minimum or maximum weight was collected, as long as there was enough faecal material to fill the sampling container halfway. All samples were well-mixed by hand on-site, placed into Ziplock bags, individually labelled, placed on ice, and frozen at -20 ˚C within 6 h of collection until further preparation at the Endocrine Research Laboratory, University of Pretoria, South Africa.

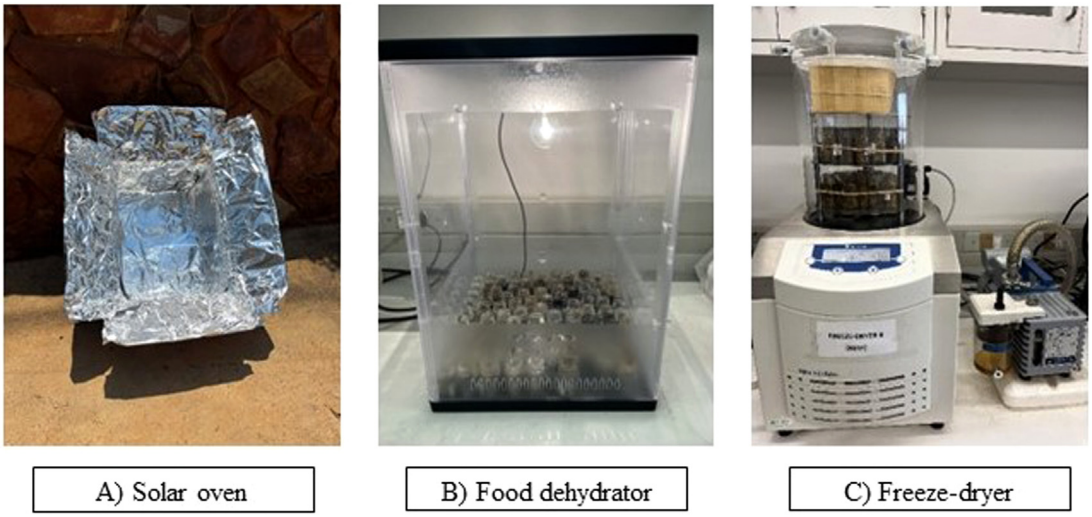

Fig. 1. The three drying devices used to remove moisture from faecal wildlife samples collected across the nine different species investigated.

Drying treatments

All samples were defrosted and thoroughly mixed by hand over a cool surface. One sample from each individual in each species investigated was thoroughly mixed and subsequently subdivided into 30 sub-samples. Ten sub-samples were then dried using either i) a homemade solar oven (a cardboard box covered in tin foil; Fig. 1a); ii) a food dehydrator (Model: BK002, Mellerware Biltong King, Johannesburg, South Africa) which makes use of a Philips Classic halogen 70 w lamp in conjunction with a circulating fan to provide continuous airflow (Fig. 1b); or iii) a freeze-dryer (Model: ALPHA 1–2 LD plus, Christ, Osterode, Germany; Fig. 1c).

Faecal samples allocated for freeze-drying were lyophilized for 5 days at −56 ˚C. Samples dried using the homemade solar oven or food dehydrator were weighed and rotated every four hours from 07h00 to 19h00 and were deemed dry when their subsequent masses varied by <0.01 g. During each weighing period, the temperature inside each device was also recorded. Readings in the solar oven ranged between 5 and 38 ˚C, and between 30 and 35 ˚C inside the food dehydrator. Once completely dried, all samples were stored at room temperature (20–25 ˚C) until further processing.

Hormone extraction and analysis

All dried faecal samples (n = 30 per species) were pulverized and sifted through a wire-mesh strainer to separate any undigested material from the resultant faecal powder [13]. 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 *1500xg* [14]. Supernatants were then transferred into microcentrifuge test tubes and stored at - 20˚C until 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-oxoetiocholanolone I and Corticosterone EIA [15]; by Möstl et al. (2002) for the 11-oxoetiocholanolone II EIA [16]; by Touma et al. (2003) for the 5α-pregnane3β,11β,21-triol-20-one EIA [17], and by Frigerio et al. (2004) for the 11β-hydroxyetiocholanolone EIA [18]. 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 [14].

Statistical analyses

Statistical analyses were performed utilizing R (R Development Core Team 4.2.1) algorithms with the aid of the R Studio interface. To determine the differences in fGCM concentrations for each species across alternative drying treatments compared to freeze-drying, first, a one-way Kruskal-Wallis (ANOVA) was used to compare fGCM concentrations between all drying treatments. Thereafter, Pairwise t-tests were conducted *post hoc* in order to identify if the significant differences in fGCM concentrations were between freeze-drying compared to the food dehydrator and the solar oven, respectively. Data sets were tested for normality using a histogram, q-q plot, and lastly, a Shapiro-Wilk test. Data from three of the species (blue wildebeest, spotted hyena, and leopard) had to be log-transformed before a linear model could be applied to meet the assumptions of normality. In cases of all pair-wise multiple comparison procedures, the α-level was adjusted by applying the Bonferroni correction. Lastly, to find out if the different drying techniques reveal comparable patterns in fGCM concentrations for samples of different concentrations, a Pearson correlation was used to evaluate fGCM

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

Species	EIA's	References	Sensitivity (ng/g faecal dry mass)	Intra-assay CVs	Inter-assay CVs
Impala	11-oxoaetiocholanolone (measuring fGCMs with a 5β−3α-ol-11-one structure)	Chizzola et al. 2018 [19]	0.75	5.5 % 6.5 %	6.6 % 14.7 %
Giraffe	11-oxoaetiocholanolone 2 (measuring fGCMs with a 5β−3α-ol-11-one structure)	Bashaw et al. 2016 [20]	1.5	5.5 % 6.5 %	6.6 % 14.7 %
Blue Wildebeest	11-oxoaetiocholanolone 2 (measuring fGCMs with a 11, 17-dioxandrostanes structure)	Wolf et al. 2021 [21]	1.2	4.0 % 4.8 %	11.6 % 11.9 %
Plains Zebra	11β-hydroxyaetiocholanolone 1 (measuring fGCMs with a 5β,3α,11β-diol structure)	Seeber et al. 2018 [22]	2.4	7.8 % 8.0 %	12.3 % 14.2 %
White Rhino	5α-pregnane-3β,11β,21-triol-20-one (measuring fGCMs with a 5β,3α,11β-diol structure)	Badenhorst et al. 2016 [23]	2.4	4.7 % 5.7 %	7.0 % 8.2 %
African Elephant	11-oxoaetiocholanolone 2 (measuring fGCMs with a 5β−3α-ol-11-one structure)	Ganswindt et al. 2003 [24]	1.5	5.5 % 6.5 %	6.6 % 14.7 %
Spotted Hyena	Corticosterone	Goymann et al. 1999 [25]	2.4	2.8 % 6.3 %	8.3 % 9.5 %
Cheetah	Corticosterone	Ludwig et al. 2012 [26]	4.8	2.8 % 6.3 %	8.3 % 9.5 %
Leopard	5α-pregnane-3β,11β, 21-triol-20-one (measuring fGCMs with a 5β,3α,11β-diol structure)	Webster et al. 2018 [27]	2.4	7.8 % 8.0 %	12.3 % 14.2 %

concentrations from the freeze dryer compared to the food dehydrator and solar oven respectively. Statistical significance for all tests was set at alpha (α) = 0.05 and inferred at $p < 0.05$, and results are reported as means ± standard deviation.

Method validation

Alternatives to lyophilization

The results of the present study suggest that there are feasible, field-friendly alternatives to the freeze-drying of faecal samples for steroid quantification for all nine species assessed. For ruminants, there were no significant differences in fGCM concentrations across all drying treatments for giraffe ($F_{2,27} = 1.53$; $n = 10$; $P = 0.233$, Fig. 2a). Faecal GCM concentrations differed for the impala dependent on drying techniques ($F_{2,27} = 12.31$; $n = 10$; $P < 0.001$, Fig. 2a) with fGCM concentrations resulting from samples dried with the food dehydrator being significantly lower ($P < 0.010$) compared to those involving the freeze dryer. Similarly, fGCM concentrations differed for blue wildebeest ($F_{2,27} = 8.57$; $n = 10$; $P < 0.001$, Fig. 2a), with fGCM concentrations resulting from food dehydrator samples being significantly lower ($P < 0.010$) compared to those involving the freeze dryer.

There were no significant differences amongst hindgut fermenters in fGCM concentrations across all drying treatments for white rhino ($F_{2,27} = 3.33$; $n = 10$; $P = 0.050$, Fig. 2b). There was a significant difference between at least two drying techniques for both zebra ($F_{2,27} = 6.56$; $n = 10$; $P < 0.005$, Fig. 2b) and African elephant ($F_{2,27} = 29.30$; $n = 10$; $P < 0.001$, Fig. 2b), with fGCM concentrations being significantly lower in samples dried using a food dehydrator (zebra: $P = 0.003$; African elephant: $P < 0.001$) for both species.

There were no significant differences in fGCM concentrations across all drying treatments for the three carnivorous species investigated: spotted hyena ($F_{2,27} = 0.289$; $n = 10$; $P = 0.751$, Fig. 2c), cheetah ($F_{2,27} = 0.409$; $n = 10$; $P = 0.668$, Fig. 2c), and leopard ($F_{2,27} = 0.030$; $n = 10$; $P = 0.971$, Fig. 2c).

A strong positive correlation was found between fGCM concentrations determined from freeze-dried faecal samples and faecal samples dried in a solar oven from all investigated species (correlation coefficient=0.990; $t = 66.13$; df = 88; $P < 0.001$, Fig. 3). Similarly, there was a strong positive correlation between fGCM concentrations determined from freeze-dried faeces and faeces dried using a food dehydrator from all investigated species (correlation coefficient=0.977; $t = 42.98$; df = 88; $P < 0.001$, Fig. 3). This indicates that irrespective of the alternative drying technique used the pattern revealed is comparable to that resulting when freeze-drying samples. And although comparable for some species (Fig. 2), absolute values should not be compared between data sets generated using different sample drying techniques [28]. As the pattern remains consistent for all drying methods, each approach would be suitable when addressing questions related to responses to stressors.

For all the species investigated, the use of a solar oven offers a cost-friendly alternative to freeze-drying that would be preferred when no power is available (with samples being completely dry within 24–48 h, compared to 96–144 h using the food dehydrator). The concentrations of fGCMs measured across all species in the present study were not significantly different when samples were

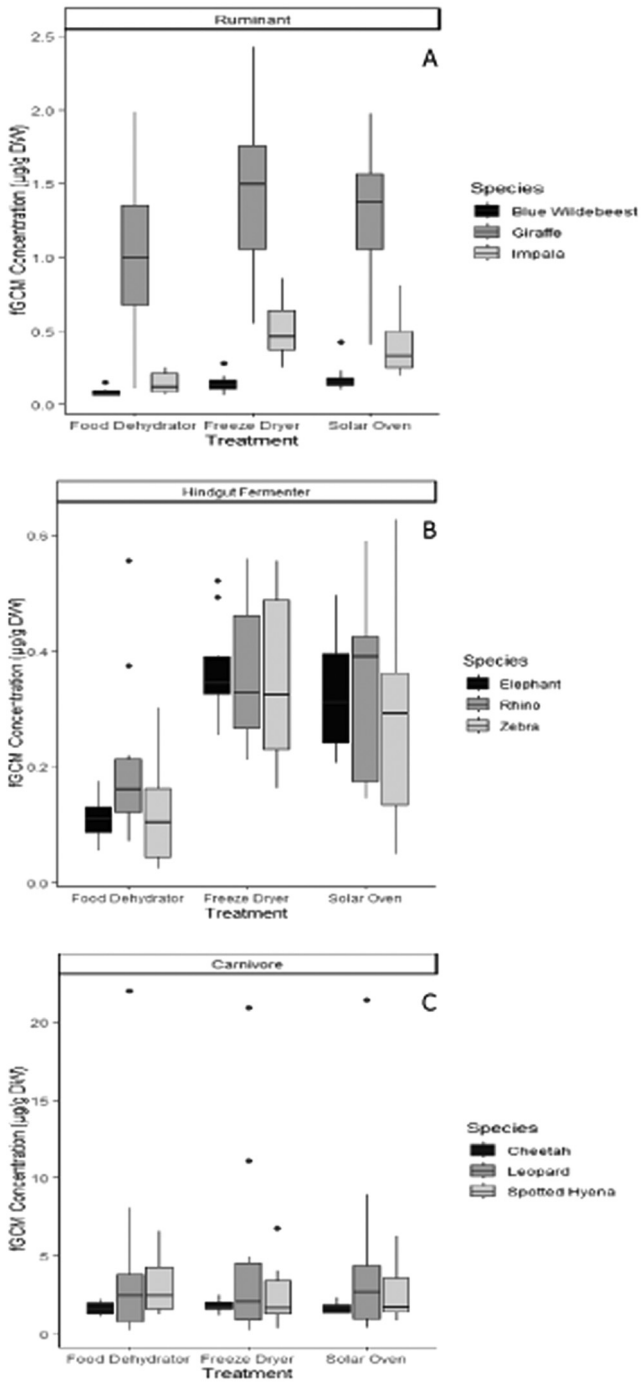

Fig. 2. Comparison of multiple species (ruminants: blue wildebeest, giraffe, impala; hindgut fermenters: African elephant, white rhino, zebra; carnivores: cheetah, leopard, and spotted hyena) faecal glucocorticoid metabolite (fGCM) concentrations (µg/g DW) determined from differently dried faeces (food dehydrator, freeze dryer, and solar oven). The boxes represent fGCM concentrations (µg/g DW) median, lower and upper quartiles, whiskers signify the range, and closed dots indicate outlier data points**.** Different colours represent investigated species in each feeding group.

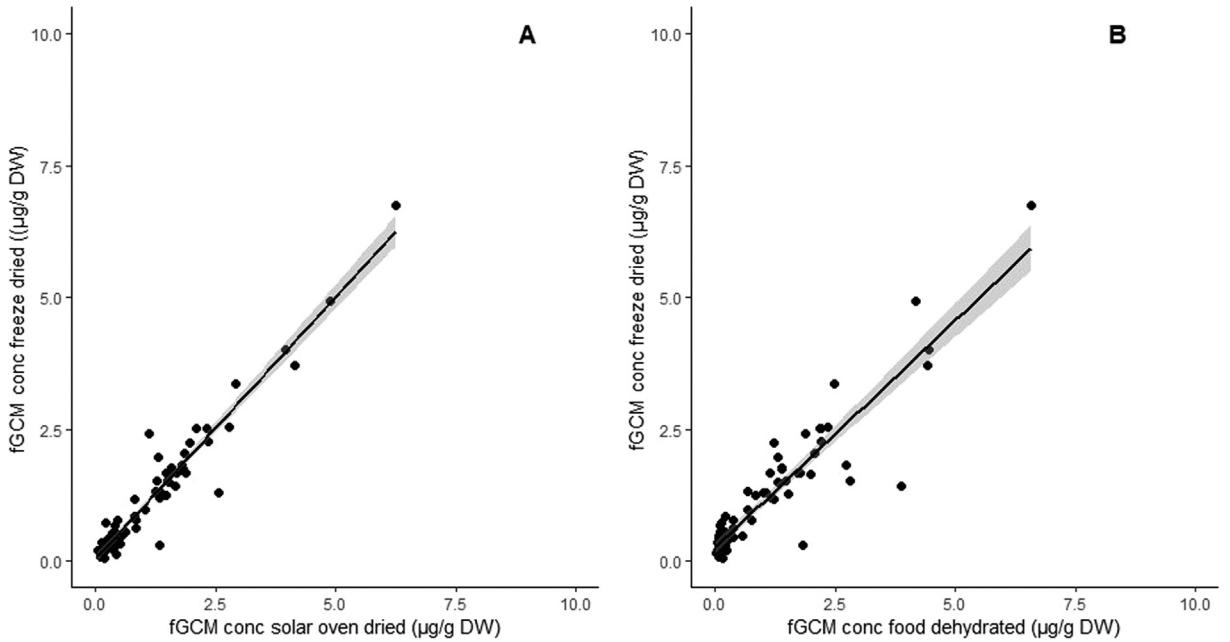

Fig. 3. Relationship between fGCM concentrations (µg/g DW) measured in (carnivores: cheetah, leopard, spotted hyena; ruminants: blue wildebeest, giraffe, impala; hindgut fermenters: African elephant, white rhino, and zebra) using alternative drying techniques to that of a freeze-dryer. The X-axis represents fGCM concentrations obtained by utilizing a freeze-drier; the Y-axis represents fGCM concentrations obtained by utilizing a solar oven (**A**) or food dehydrated (**B**). The black shade represents the confidence interval (0.95). The black line represents the straight line ($y = x$).

dried in the solar oven compared to those from the freeze-dryer, indicating an equally fast and effective drying process. Based on personal observations, however, we would advise against the use of plastic sampling containers while using this method since the solar oven can reach temperatures exceeding 38 °C, causing the tubes to melt. There were no other limitations observed.

A food dehydrator appears to be a viable alternative tool for drying faecal samples of carnivorous species. Despite needing some electricity, one can control the environmental conditions to a greater extent, and this can be conducted indoors where investigative animals, like monkeys, are less likely to examine the drying material. However, with regards to ruminants (e.g., impala and blue wildebeest) and hindgut fermenter species (e.g., zebra and African elephant), we observe overall lower absolute values compared to fGCM concentrations generated by using the freeze-dryer. Underlining once more the absolute values determined using different sample drying techniques cannot be compared. The findings obtained from the carnivores in this study were consistent with those of Postiglione et al. (2022), who investigated similar approaches for the alternative drying of the faeces of African wild dogs (*Lycaon pictus*) [8]. The comparability is likely due to carnivores having similar processes and anatomy. A carnivore's digestive tract is short and one of the least complicated amongst mammals, this could cause for the speculation that the differences in comparison to the herbivores could be based mainly on diet and processing of the samples in the gut [29]. Herbivore samples likely contain higher levels of moisture, suspected bacteria, and less undigested material when excreted. For example, out of all the nine species, the African elephant samples took the longest to dry, and the species is known for having a comparatively high moisture level in the faeces and they excrete 60 % of undigested plant material [30,31].

Conclusion

For the nine species examined in the present study, the use of a homemade solar oven offers a comparable and affordable alternative to freeze-drying faeces for fGCM quantification. The food dehydrator would also work as a comparable alternative for carnivorous species, but not herbivorous species. Instead of using the expensive and often logistically challenging freeze-drying process, these alternative drying approaches allow researchers to incorporate fGCM analysis more readily into their studies. Ultimately, encouraging the use of non-invasive hormone monitoring as a more field-friendly tool in conservation and physiological studies.

Limitations

N/A.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Kayla Rae Osburn: Conceptualization, Methodology, Validation, Investigation, Data curation, Writing – original draft, Visualization. **Bruce Crossey:** Conceptualization, Software, Formal analysis, Supervision, Writing – review & editing, Visualization. **Tshepiso L Majelantle:** Software, Formal analysis, Writing – review & editing, Visualization. **Andre Ganswindt:** Conceptualization, Resources, Supervision, Writing – review & editing, Funding acquisition.

Data availability

Data will be made available on request.

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