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METHODS ARTICLE

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Instability of fecal glucocorticoid metabolites at 4°C: Time to freeze matters

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Abstract

The use of fecal corticosteroid metabolites (FCMs) has proven to be well suited to evaluate adrenocortical activity, a major component of the stress response, particularly in wildlife. As with any tools, confounding factors and drawbacks must be carefully considered. Among them, sample preservation and storage are of particular importance, as they can affect stability of FCMs and lead to biased results and interpretations. Arguably, immediate freezing of fecal samples upon collection is the best practice to preserve FCM integrity, however, for logistical reasons, this condition is rarely feasible in the field. It is generally argued that temporary storage of samples at low above-zero temperature is an acceptable way of preserving samples in the field before freezing them for long-term storage. However, to our knowledge, there is no empirical study that demonstrates the stability of fecal metabolites in samples stored at +4°C. In this study, we collected a fresh fecal sample from 20 captive roe deer, each of which was homogenized and split into three subsamples (60 subsamples in total) to investigate the effects on FCMs levels of temporary storage at +4°C for 24 h and 48 h before freezing versus immediate freezing at −20°C after feces collection. Compared to immediate freezing, mean FCMs levels decreased by 25% every 24 h when feces were stored at +4°C before freezing. The variance of FCMs levels followed the same pattern, leading to a clear reduction in the ability to detect biological effects. Minimizing the storage time at +4°C before freezing should therefore be seriously considered when establishing sampling and storage protocols for feces in the field for adequate hormonal profiling.

KEYWORDS

fecal cortisol metabolites, glucocorticoids, mammal, methodology, sample preservation

1 | INTRODUCTION

Over the past two decades, the assessment of stress level in domestic and wild animals has been an important focus of veterinary medicine, evolutionary ecology and conservation biology (Dantzer et al., 2014; Ganswindt et al., 2012; Palme, 2012). Indeed, one of the major coping mechanisms to environmental perturbations and life-threatening situations (e.g., human-induced disturbance, availability of space and food, social factor, translocation) is the stress response (Wingfield et al., 1998). The stress response can be defined as a suite of physiological and behavioral responses whose coordination aim at neutralizing the effects of stressors and restoring homeostasis (Romero et al., 2009; Wingfield et al., 1998). The most studied physiological stress response in vertebrates is the stimulation

of the hypothalamic-pituitary-adrenal (HPA) axis, or hypothalamic-pituitary-interrenal (HPI) axis in fish, amphibians, and reptiles (Reeder & Kramer, 2005), which results in the secretion of glucocorticoids such as cortisol and corticosterone. In the case of transient exposure to stressors, glucocorticoid levels return to baseline levels within few hours (deKloet et al., 2005) and maintain energy balance according to the individual's needs. On the opposite, when stressors are persistent or regularly repeated, glucocorticoids secretion remains chronically elevated, and several negative consequences are observed on physiology, which may affect individual fitness and ultimately population dynamics (Dulude-de Broin et al., 2020; Romero et al., 2009).

To evaluate the health status of organisms and their ability to efficiently respond to environmental perturbations, a wide range of tools have been developed to assess glucocorticoid levels in both domestic and wild animals. While historically glucocorticoids are measured in blood, proxies for glucocorticoid levels are now frequently quantified using noninvasive methods on various matrices such as saliva, feathers, urine, or feces. Several high-quality review articles compare the advantages and caveats of each matrix (Dantzer et al., 2014; Mormède et al., 2007; Sheriff et al., 2011). Among them, fecal cortisol or corticosterone metabolite (FCM) measurements have many advantages such as its noninvasiveness, ease of feces collection, the fact it reflects free plasma glucocorticoid levels, but is less impacted by acute events than glucocorticoids in saliva, urine or blood, or the possibility to use feces for other analyses (e.g., genetic studies). For these reasons, FCM measurement is presently the most commonly used means of noninvasively accessing the baseline or cumulative level of stress to which an individual has been exposed in its environment (Dantzer et al., 2014; Palme, 2019; Sheriff et al., 2011), and this in a variety of disciplines and on various taxa (Dantzer et al., 2010; Dehnhard et al., 2001; Ganswindt et al., 2003; Goymann et al., 2001; Sheriff et al., 2009; Touma et al., 2004).

However, a number of precautions should be taken into account to avoid measurement and interpretation errors. Of these, the protocol for sample preservation and storage has a particularly marked influence on the measured FCM levels (Millspaugh & Washburn, 2004; Palme, 2019; Terio et al., 2002). For field studies that may take place far from facilities, it is often not possible to freeze fecal samples immediately after collection, which exposes fecal samples to uncontrolled bacterial degradation and ultimately to erroneous FCM estimates (Palme, 2019). Several studies have pointed to the effect of temporary storage of fecal samples at ambient temperature and mostly found that stability of FCMs was impaired. For instance, increase in concentrations of FCMs after storage at ambient temperature have been reported in cattle, horses, pigs, deer or orangutans (Mostl et al., 1999; Millspaugh et al., 2003; Muehlenbein et al., 2012), while decrease has been observed in elk, gorillas and macaques (Gholib et al., 2018; Millspaugh et al., 2003; Shutt et al., 2012; Webber et al., 2018). It is worth to note that some studies have shown no marked effect of storage at ambient temperature on FCMs (e.g., see Dulude-de Broin et al., 2019; Nugraha et al., 2017), but the direction of change within a given species may also depend upon the immunoassay utilized for quantification (Lexen et al., 2008; Palme, 2019).

Research highlights

Fecal samples should be frozen as soon as possible when investigating fecal glucocorticoid metabolite levels, as 25% of the initial concentrations were lost every 24 h, even when kept at low above-zero temperatures (+4°C).

To prevent degradation of FCMs, fecal samples are commonly kept at low above-zero temperature on ice or in a cooler box in the field until returned to a facility with a freezer. However, while it is commonly assumed that FCMs should be stable when feces are stored at low temperature, to date, no empirical data are available to support this assumption (but see Thiel et al., 2005 for a study at +8°C). In the present study, we set up a protocol to evaluate the impact of temporary storage of roe deer fecal samples at +4°C for 24 and 48 h before freezing on FCM levels. Based on the general assumption that no degradation of FCMs should occur at low temperature, we did not expect significant differences in FCM levels between our control (immediate freezing) and the two experimental conditions (fecal samples from the same individual kept at +4°C for 24 and 48 h before freezing). We assessed the repeatability using the three samples issued from each individual, and we also considered individual variation by performing the same measurement on several individuals.

2 | MATERIALS AND METHODS

2.1 | Study site

This study was conducted on a captive population of roe deer living in the Gardouch research station (agreement for the ethical committee number B31210001), located in south-west of France. The station is owned and managed by the French Research Institute for Agriculture, Food and Environment (INRAE). It consists of 12 enclosures of 0.5 ha composed of meadow, trees and groves, each containing between one to six tamed roe deer. Individuals were supplemented with food pellets (600 g per individual per day). The current study involved a total of 20 roe deer, 18 females and 2 males, living in their enclosure for several years, aged between 3 and 14 years.

2.2 | Experimental design and data collection

The experimental procedure was carried out over 10 days in January 2019. To assess the effect of time before freezing the feces on FCMs, we visually monitored the 20 roe deer available at the research station at that time, and collected one fecal sample per individual.

Feces were collected immediately after the observations of defecation, which all took place between 11 a.m. and 1 p.m. Once collected, the total fecal sample from each individual was homogenized and separated into three equivalent portions and transferred into plastic bags. For each individual, we randomly assigned one of the three portions of feces to a treatment group (T0; T24; T48). In the T0 group, feces were immediately frozen at −20°C until FCM analysis. In the T24 group, feces were kept in their plastic bag and stored in a fridge at +4°C for 24 h before being frozen at −20°C. In the T48 group, feces were stored at +4°C for 48 h before being frozen −20°C until steroid analysis. Feces collection was performed by technicians during their usual daily check of the animals, without causing any additional disturbance.

2.3 | Extraction of FCMs

FCMs were extracted following a methanol-based procedure and assayed using a group-specific 11-oxoaetiocholanolone enzyme immunoassay (EIA), as previously described (Möstl & Palme, 2002) and validated for roe deer (Carbillet et al., 2020; Zbyryt et al., 2017). Briefly, each fecal sample was homogenized, and 0.5 ± 0.005 g of homogenate was transferred to a glass tube containing 5 mL of methanol-water 80% v/v. The suspended samples were vortexed at 1500 rpm for 30 min and centrifuged at 2500*g* for 15 min (Palme et al., 2013). An aliquot of the supernatant was further diluted (1:10) with assay buffer before EIA analysis. Measurements were carried out in duplicate (intra- and inter-assay coefficients of all samples were less than 10% and 15%, respectively) and the results expressed as nanograms per gram of wet feces (ng/g).

2.4 | Statistical analyses

2.4.1 | Variation in FCMs according to the number of days the samples were kept at +4°C before being frozen

To evaluate the effect of delay (in days) before freezing of feces on glucocorticoid metabolite measurements, we performed linear mixed-effects models with FCMs as the response variable and the number of days the samples were kept at +4°C before being frozen as a continuous fixed variable. Analyses were done based on 60 observations of 20 individuals (3 measures per individual). FCM values were log transformed to achieve normality of model residuals, sex and age were included as co-variables to account for their potential confounding effects, and individual identity was included as random effect on the intercept to avoid pseudo-replication issues (Hurlbert, 1984) and to control for unexplained variance due to among-individual differences. To select the best models of variation in FCM level, we used a model selection procedure based on the second-order Akaike Information Criterion (AICc, Burnham & Anderson, 2003). Models with a difference in AICc (ΔAICc) >2 units from the best model were considered to have less support, following Burnham and Anderson (2003). Normality of the residuals for the selected models was tested (Shapiro–Wilk test) and visually assessed with histograms. Goodness-of-fit was assessed by calculating conditional (i.e., total variance explained by the best supported model) and marginal (i.e., variance explained by fixed effects alone) R^2 values and standard residual plot techniques (Nakagawa & Schielzeth, 2013). In addition, and to test for individual differences in the kinetics of FCM degradation, we also fitted model equivalent to our best selected model, but that included a random effect on individuals' slopes in addition to the random effect on the intercept. We then used a likelihood ratio test to compare these two models. FCM degradation was calculated as the difference between the amount of FCM at T48 and T0 divided by 2 (the number of days before samples were frozen), and the percentage of FCM loss was calculated as (T48-T0)/T0*100. The predicted value of the percentage of FCM lost every 24 h was calculated using the model output values on log-transformed data. Predicted values were then back-transformed to the original scale using an exponential transformation to obtain a more biologically relevant percentage loss in ng/g. All analyses were carried out with R version 3.6.0 (R Core Team, 2016), using the lmer function from the lme4 package (Bates et al., 2014).

2.4.2 | Repeatability of FCM levels

Individual repeatability of FCM levels was estimated using mixed models (Nakagawa & Schielzeth, 2010). Repeatability was estimated as the ratio of among-individual variance to total variance with linear mixed-effects models (with individual identity as a random factor) using the restricted maximum likelihood (REML) method, using the "rptR" package (Stoffel et al., 2017) for Gaussian distributions. We calculated both "agreement repeatability" and "adjusted repeatability," the latter including the fixed effect of the number of days the samples were stored at +4°C before being frozen in the linear mixed models.

3 | RESULTS

According to the model selection procedure, the best model describing among-individual variations in FCMs included the effect of treatment applied to samples before being stored at −20°C, but also age and sex of individuals (Table 1). Precisely, when fecal samples were held at +4°C for 24 h before being frozen at −20°C, they exhibited on average 25% less FCMs than the same samples frozen directly after collection (Table 2). This decrease was 43% on average when fecal samples were held for 48 h at +4°C before being frozen. The relationship describing the decrease in FCMs (log-transformed values) over time is shown in Figure 1 (slope of the relationship: −0.281; CI = [−0.375; −0.193]; R^2m = 0.37 and R^2c = 0.82; Figure 1).

While Figure 2 shows that the rate of FCMs disappearance varies among individuals, with some individuals (2 out of 20) for whom

TABLE 1 Performance of the subset of candidate linear mixed-effect models within a ΔAICc lower than the ΔAICc of the null model fitted to investigate variation in FCM levels in the roe deer population of Gardouch in relation to the storage method of fecal samples.

Models	K	AICc	ΔAICc	AICw
Treatment + sex + sge	**6**	**72.1**	**0.00**	**0.604**
Treatment + sex	7	74.7	2.61	0.164
Treatment	6	75.0	2.91	0.141
Treatment + age	7	75.8	3.79	0.091
Sex + age	6	97.9	25.83	0.00
Sex	5	100.6	28.52	0.00
Null model	3	101.0	28.91	0.00

Note: The model in bold was used for estimation of parameters. Our set of candidate models was composed of all simpler models that included the variables treatment, individual age and sex. Individual identity and sample collection date were included as random effects. AICc is the value of the corrected Akaike's Information Criterion and K is the number of estimated parameters for each model. The ranking of the models is based on the differences in the values for ΔAICc and on the Akaike weights (AICw).

Abbreviation: FCM, faecal corticosteroid metabolites.

TABLE 2 Characteristics of the selected linear mixed-effect models for explaining variation in FCM levels in the roe deer population of Gardouch in relation to storage method of fecal samples.

Parameter	Estimate	Lower CI	Upper CI
(R^2m: 0.37; R^2c: 0.82)			
Intercept	6.204	5.728	6.675
Treatment (per day)	−0.281	−0.375	−0.193
Age (per year)	0.090	0.017	0.165
Sex (males)	−1.010	−1.784	−0.321

Note: The effect of the treatment applied to samples (samples immediately stored at −20°C; stored at +4°C for 24 h before being stored at −20°C; or stored at +4°C for 48 h before being stored at −20°C), age and sex were fitted. Models included individual identity and date of sample collection as random effects. R^2m and R^2c are the marginal and conditional explained variance of the model, respectively. CI stands for confidence interval. See text for definition of model sets.

Abbreviation: FCM, faecal corticosteroid metabolites.

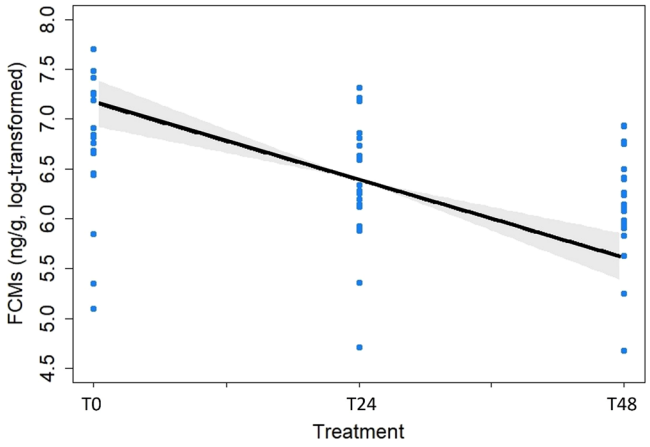

FIGURE 1 Relationship between faecal corticosteroid metabolites level (log-transformed) and time held at +4°C before being frozen. T0 stands for control samples immediately stored at −20°C; T24 for samples stored at +4°C for 24 h before being stored at −20°C; and T48 for samples stored at +4°C for 48 h before being stored at −20°C. Points represent observed values, lines represent model predictions and gray area represents the 95% confidence interval.

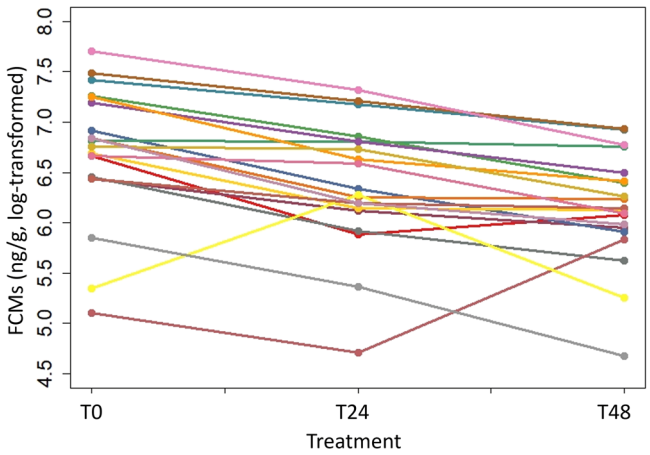

FIGURE 2 Individual patterns of faecal corticosteroid metabolites concentrations in fecal samples stored at +4°C for 24 (T24) or 48 (T48) hours compared to immediate freezing (T0). Each line and color represent one of the 20 individuals included in the study.

concentration increases with the time the samples have spent at +4°C, individual differences in degradation of FCMs over time were not significant. Indeed, adding a random effect on the individuals' slopes did not improve the model fit compared to a model with a random effect on the intercept only ($\chi^2 = 3.37$; $p = 0.19$). However, the rank of FCM data obtained after immediate freezing of fecal samples was different from that observed when the fecal samples were stored for 24 or 48 h at 4°C. For example, the individuals with the highest and lowest FCMs values at T0 are no longer those with the most extreme values at T48 (Figure 2).

Figure 3a shows that the rate of treatment-related FCM loss is highly correlated with the initial FCM levels measured at T0 (i.e., when the feces samples were immediately frozen after collection). Precisely, the rate of FCM degradation increased with higher initial amount of FCMs (slope of the relationship: −0.298; CI = [−0.362; −0.234]). Figure 3b shows that the percentage of FCM loss was also higher with higher initial amount of FCMs (slope of the relationship: −0.032; CI = [−0.064; −0.001]). However, this relationship was highly dependent on one individual (the leftmost point in the graph Figure 3b), which was the only one that showed a gain in FCM with

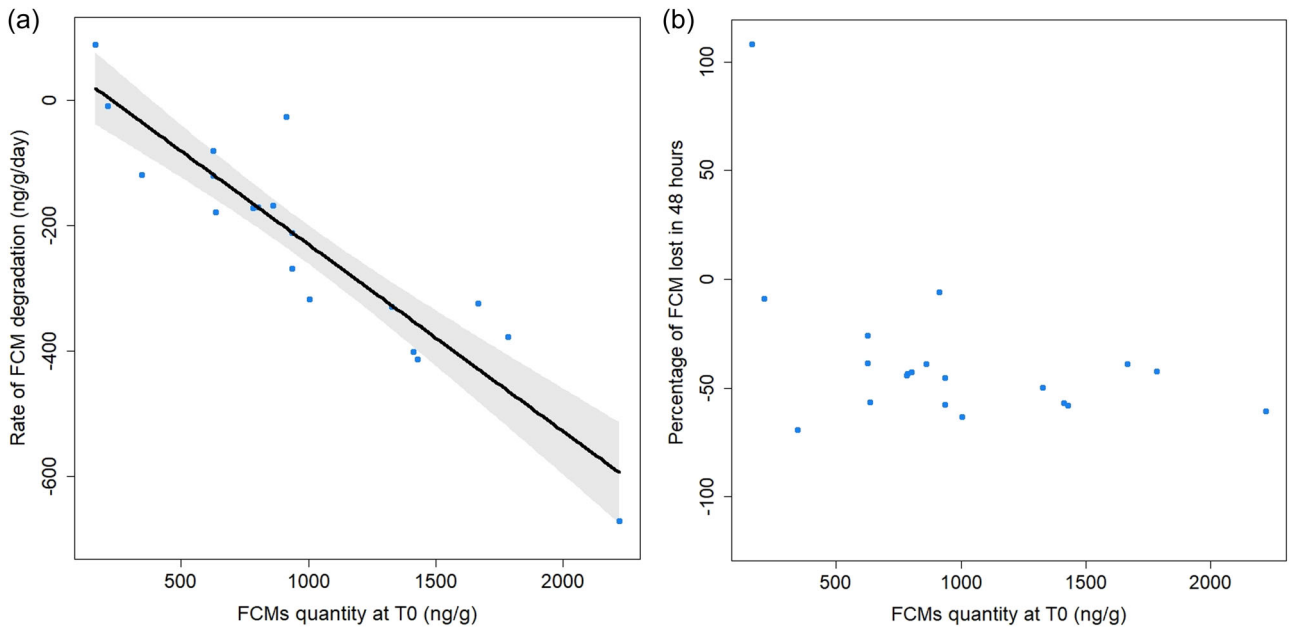

FIGURE 3 (a) Rate of FCMs degradation and (b) Percentage of FCM loss when samples were held at +4°C for 48 h compared to their initial concentration (immediately stored at −20°C). Points represent observed values, lines represent model predictions and gray area represents the 95% confidence interval. FCMs, faecal corticosteroid metabolites.

treatment. The relationship was not holding when this point was removed (slope of the relationship: −0.010; CI = [−0.026; 0.006]).

Finally, repeatability analyses showed that FCM levels were highly repeatable ($R = 0.639$, 95% confidence interval = [0.379, 0.804]), with even higher repeatability when the time spent by the samples at 4°C before freezing was taken into account ($R = 0.79$, 95% confidence interval = [0.594, 0.893]).

4 | DISCUSSION

Our results provide one of the first experimental evidence that temporary storage of fecal samples at low above-zero temperatures (+4°C) for 24 and 48 h compromised the stability of FCMs, resulting in a marked decrease in detected FCM levels. Possible explanations and practical implications of these results for studies aiming at quantifying FCM levels in domestic and wild animals are discussed below.

Focusing on our main results, our data add to previous observations that inadequate storage and preservation of feces can adversely affect the stability of FCMs (Lexen et al., 2008; Millspaugh & Washburn, 2004; Palme, 2019). While almost no studies have investigated the effect of temporary storage of fecal sample at low above-zero temperatures, previous work has examined the effect of storage at ambient temperature. There is no general consensus emerging from the literature regarding the direction of the storage effect, however, the majority of studies have found a decrease in FCMs with increasing time spent at ambient temperature, as in our study (Gholib et al., 2018; Lexen et al., 2008; Shutt et al., 2012). The most common explanation for the degradation of FCMs in feces relies on the metabolic activity of the gut microbiome (Khan et al., 2002; Mostl et al., 1999; Shutt et al., 2012). Bacterial activity in feces can chemically alter steroid metabolites and their binding affinity shortly after feces deposition, resulting in altered (increased or decreased) immunoreactivity with primary antibodies during the EIA protocol (Lexen et al., 2008; Mostl et al., 1999). The nature of steroid metabolites may differ in a species-specific manner (Palme et al., 2005) depending on diet and the microbial activity of the gut, and different immunoassays may pick up the newly formed metabolites to a different degree (better/lesser), which may explain the lack of general pattern in the storage effect of feces on FCMs. Chemical alterations of glucocorticoid metabolites usually consist of oxidation/reduction or deconjugation. Our FCM analyses were performed using a group-specific 11-oxoaetiocholanolone EIA. Hence, from a biochemical point of view, the most plausible explanation to the observed decrease in FCM values is a bacterial conversion of the hydroxyl group at position 3 of the steroid molecules, as hypothesized in a previous study using the same group-specific assay (Lexen et al., 2008). Such a conversion would lead to a decreased ability of the antibody to bind with cortisol metabolites (Möstl et al., 2005), and consequently to an apparent decrease in the FCMs content. However, the fact that this bacterial conversion was maintained while the samples were cooled and maintained at low above-zero temperature was not expected at first sight. Nevertheless, a previous study on modeling of *Escherichia coli* population proliferation indicates that the sensitivity of gut bacteria to decreased temperature is actually low (Martinez et al., 2013). With an estimated temperature coefficient (Q_{10}) of 1.5 (for every 10°C decrease in temperature, the bacteria reactions rates would be divided by 1.5), the activity of bacteria when held at 4°C would actually represent

about 25% of that at 37°C in the gut environment. With these considerations, and contrary to what is generally assumed, it is thus not surprising that degradation of the FCMs occurs in feces samples kept at 4°C, which is confirmed by our results.

Our article also has its limitations. First, for methodological reasons, the pellets from each individual were homogenized and then separated into three subsamples to reduce intrasample variability issues. However, it cannot be excluded that breaking the integrity of the fecal pellets may expose more surface to the environment and affect the rate of bacterial degradation compared to unmanipulated samples. Unfortunately, this effect, if it exists, is not testable. Second, our experimental design (adult individuals only with highly skewed sex ratio) does not allow us to formally conclude and discuss whether the age or sex of individuals affects the rate of FCM degradation at 4°C, even though these factors were selected in the model selection procedure. However, given that gut microbial communities may significantly differ between males and females, or between different age classes, especially in cervids (Beaumelle et al., 2021; Minich et al., 2021), these questions are definitely of interest and would require future works to elucidate to which extent FCM degradation may be impacted by these two factors. Finally, our results were obtained under controlled conditions of constant +4°C temperature, but little information can be inferred about the rate of FCM degradation in feces held in natural conditions when exposed to other environmental factors. These can include the effects of wind, sunlight or variations in air and soil moisture on the hydration level of feces, but also the degree of exposure to UV radiation or variations in ambient temperature, all of which can affect the level of bacterial activity in an uncontrolled way.

Nevertheless, the main question that emerges from our results is to what extent the degradation of FCM would compromise studies that require sampling of feces in the field away from laboratory facilities or quick access to a freezer. Our results show that FCM measures obtained from feces that have been temporarily stored at +4°, cannot be considered as absolute values, as stability of FCMs was not guaranteed at this temperature. In the first place, this can be seen as a minor problem since most studies in evolutionary ecology, conservation biology or animal welfare sciences rarely seek to determine absolute values, but rather to compare relative FCM levels among individuals (Formenti et al., 2018; Jachowski et al., 2015; Rehnus et al., 2014). But a closer look at these results shows that this problem of FCM degradation must be reconsidered carefully when feces are stored at +4°C before being frozen. Indeed, our results clearly demonstrated that the degradation occurring at +4°C was time-dependent, so that degradation of FCMs continued as long as the samples were kept at +4°C, at least for the first 48 h. If there is a large variation in the storage time of samples at +4°C, it is tempting to avoid this problem by taking a "time effect" into account in the statistical analyses. In our study, the degradation of FCMs in feces maintained at +4°C was more pronounced when the initial level of FCMs in the feces was high (Figure 3a), suggesting that the percentage of FCMs loss was similar between samples. When removing the only individual that showed an increase of FCMs over time, which was also the individual with the lowest FCM level, we did not observe any significant relationship between initial FCM level and the percentage of loss (Figure 3b). Moreover, the relatively strong repeatability of FCM values tend to support the idea that adjustment of FCM values according to the time samples spent at +4°C may be possible, at least in the case of roe deer. Thus, in roe deer, taking into account the delay before freezing to correct FCM values may be applied when initial FCM values are not too low (>200 ng/g).

More generally, this approach may be an acceptable alternative, but only when it has been previously validated (i) that the effect of delay makes it possible to strongly predicts the observed FCM values at time t ($R^2 > 0.80$) and (ii) that the percentage of FCM loss is independent of the initial FCM concentration. In addition, the reduction in the variance among treatments observed with raw FCM data can be accounted for by using log-transformed data for FCM analyses.

Based on our results on roe deer, we calculate that 4% of the log-transformed FCM concentrations, or 25% of the initial FCM concentration are lost every 24 h feces have spent at +4°C. We suggest that the effects of temporary storage at +4°C should also be carried out on a finer time scale to more accurately assess the kinetics of FCMs degradation. Our findings advocate and confirm that the best practice to avoid alteration of FCMs is to freeze fecal samples immediately upon collection, or alternatively, to lyophilize the samples on the field (Postiglione et al., 2022). Where this condition is not possible, similar storage experiments need to be performed on the species of interest (Palme, 2019) to access the rate of FCMs degradation at low above freezing temperatures and to improve sample preservation practices in FCMs studies.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request. All data and code used in this manuscript are available upon request to the authors.

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