

# Non-invasive monitoring of adrenocortical activity in European badgers (*Meles meles*) and effects of sample collection and storage on faecal cortisol metabolite concentrations

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## Abstract

Due to the increasing demand for methods to quantify adrenal activity in response to stressors in wild animals, we evaluated whether stimulation with adrenocorticotropic hormone (ACTH) increases faecal cortisol metabolite levels in European badgers. We also conducted several experiments to evaluate the effect of storage time and collection methods on cortisol metabolite levels of faecal samples. Faecal samples were collected from five captive European badgers (*Meles meles*) and cortisol metabolites were quantified by an enzyme immunoassay. Faecal cortisol metabolite levels increased 1–2 days after ACTH injection and dropped to pre-treatment levels 3 days after ACTH stimulation. We found that cortisol metabolite levels were not affected by the 8-hour time lapse from collection in the field until freezing of faecal samples, environmental exposure (temperature  $25.66 \pm 2.55^\circ\text{C}$ ; range 21.20–32.20) or mean relative humidity  $62.77 \pm 9.90\%$  (range 42.00–79.00). Cortisol metabolites were not homogeneously distributed in the scats. Cortisol metabolite levels were significantly lower when faecal extracts and samples were frozen for long periods of time (14 weeks or more). Thus, the measurement of faecal cortisol metabolites is a suitable method for the non-invasive evaluation of adrenocortical activity in European badgers. However, some aspects related to the collection and storage of faecal samples should be considered in future studies conducted with this species.

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**Keywords**

ACTH challenge; enzyme immunoassay; European badger; faecal cortisol metabolites; sample collection; sample storage

**Introduction**

In carnivores, diverse factors such as inclement weather, capture, predators, social conflicts, and human disturbances, disrupt homeostasis and cause chronic stress (Boonstra et al., 1998; Frigerio et al., 2004; Sands and Creel, 2004; Schütz et al., 2006; Barja et al., 2007, 2008). A major adaptation to these noxious stimuli is the hypothalamic-pituitary-adrenal (HPA) axis, which releases glucocorticoid hormones in response to stressors (Axelrod and Reisine, 1984; Stewart, 2003). When these stressors act in the short-term, glucocorticoids are released, activating glucose metabolism to help the organism cope with the stressor. However, glucocorticoids remain chronically above basal levels in response to long-term stressors (Boonstra, 2005). In such cases, glucocorticoids can have deleterious physiological consequences, such as immunosuppression, reproductive suppression, and tissue atrophy (Sapolsky, 1992; Sapolsky et al., 2000; Stewart, 2003). These consequences increase animals' vulnerability and may compromise their survival and reproductive capacity (Lochmiller and Deerenberg, 2000).

The emerging discipline of conservation physiology addresses the physiological responses of organisms to human alteration of the environment (Wikelski and Cooke, 2006). For conservation strategies to be successful, it is necessary to understand these physiological responses (Wingfield, 2008). New physiological techniques allow the rapid assessment of the causes of conservation problems and the consequences of conservation actions (Wikelski and Cooke, 2006). Because different forms of stress can affect the fitness of individuals, glucocorticoid measurement is a valuable tool in conservation biology, evolutionary ecology, and animal welfare studies. It provides information on human-wildlife interactions, which can be of great value when making management decisions to ensure long-term species conservation (Creel et al., 2002). These methodologies are also useful tools for understanding the conservation status of species (e.g., African elephant: Foley et al., 2001) and can contribute to *ex-situ* conservation programs of threatened and endangered species (e.g. the Iberian lynx: Jewgenow et al., 2006; Vargas et al., 2008).

Traditionally, glucocorticoids have been measured in blood plasma. However, capture and blood sampling procedures are usually stressful and thus increase peripheral glucocorticoid concentrations within minutes (Terlouw, 1997; Young et al., 2004; Romero and Reed, 2005). Individuals of threatened and endangered species should not be subjected to stressors such as capture and handling, as such stress can worsen their conservation state. Capturing carnivores under natural conditions

is also difficult, as many species are elusive or mainly nocturnal, have large territories, and often live in low densities. Furthermore, plasma glucocorticoid levels can vary circadianly and have pulsatile secretory patterns, and a blood sample represents plasma glucocorticoid levels within a short period of time (Monfort et al., 1993). For these reasons, the use of blood plasma samples has many limitations.

Non-invasive methods, such as measuring glucocorticoid metabolites in faeces, have been reported in several vertebrate species as a useful alternative to blood sampling in wild fauna (Graham and Brown, 1996; Dehnhard et al., 2001; Schatz and Palme, 2001; Touma and Palme, 2005; Wielebnowski and Watters, 2007; Santymire and Armstrong, 2010). Using these methods, animals do not need to be captured, several samples can be obtained from a single individual and faecal glucocorticoid metabolites represent pooled fractions of plasma glucocorticoids because fluctuations due to secretory patterns are attenuated in faeces (Harper and Austad, 2000; Sheriff et al., 2011).

Despite these advantages, the measurement of glucocorticoid metabolites in carnivore faeces has been limited, and few researchers have applied these non-invasive techniques to free-ranging species (but see research on cats and dogs: Schatz and Palme, 2001; spotted hyenas: Goymann et al., 2001; Van Meter et al., 2009; bears: Ohe et al., 2004; wolves: Creel et al., 2002; Sands and Creel, 2004; Barja et al., 2006; Barja et al., 2008; European pine martens: Silván et al., 2006; Barja et al., 2007, 2011; and badgers: Schütz et al., 2006). Before such methods are applied to a particular species, the assays used to measure faecal glucocorticoid metabolites must be validated, and the technique should be evaluated to test whether it is appropriate for monitoring adrenocortical activity (Touma and Palme, 2005). Furthermore, the factors associated with the storage and collection of faecal samples could bias results, as they remain poorly understood in carnivores. Therefore, additional research is needed to understand the effects of such factors on faecal glucocorticoid metabolite measurements over a broader range of conditions and species. The apparent species-specific responses to these factors limit the potential for generalising results from one species to another (Millsbaugh and Washburn, 2004).

The aims of this study were to determine whether the measurement of faecal cortisol metabolites is a suitable technique for monitoring adrenocortical activity in European badgers and to evaluate the effect of collection and storage time of faecal samples on cortisol metabolite levels. With regard to the collection of samples, we aimed to analyse the effects of: a) time of exposure (time lapse between collection and freezing); b) exposure to environmental conditions (temperature and humidity); and c) the portion of scat analysed. Finally, we tested the effects of storage time of frozen faecal samples and extracts.

## Materials and methods

### *Study animals*

This study was performed in July 2008 on five captive European badgers at two different fauna centres: two pairs of badgers in Collserola Natural Park (Barcelona, Spain) and one solitary individual in the Cañada Real Open Centre (Madrid, Spain). In Collserola Natural Park, faecal samples were collected from two males (14 kg each) and two females (8 and 10 kg, respectively). These individuals were 6–8 years old, and each pair was housed in a different enclosure. Each enclosure had an artificial sett that consisted of a glass tunnel (6 m<sup>2</sup>) and a 16-m<sup>2</sup> exterior area with a sandbank and drinking trough. Both enclosures were located in a mixed Mediterranean forest mainly comprised of deciduous oak (*Quercus cerruoides*) and Aleppo pine (*Pinus halepensis*). In the Cañada Real Open Centre, one adult female European badger (10 kg and two years old) was housed in a 40-m<sup>2</sup> enclosure with a natural sett.

### *ACTH challenge*

To stimulate adrenocortical activity, ACTH was administered to the two pairs of badgers housed at the Collserola Natural Park by intramuscular injections (Nuvachten Depot, Laboratorios Padro, S.A.). Males and females were administered 0.04 mg/kg and 0.03 mg/kg, respectively. Food and water were not withheld before anaesthesia. The badgers were captured in the glass tunnels and transferred to holding cages. They were then immobilised and anaesthetised with a combination of dexmedetomidine (DEX) (Dexdomitor; Orion Pharma, Espoo, Finland) and ketamine (KTM) (Ketolar 500; Parke-Davis-Pfizer, Madrid, Spain) (KTM: DEX; 10:0.02 mg/kg) administered intramuscularly prior to the administration of ACTH. Following other ACTH challenge experiments (Goymann et al., 1999; Terio et al., 1999; Wasser et al., 2000), fresh faecal samples were collected prior to ACTH administration to determine baseline faecal glucocorticoid metabolite levels. Fresh faeces were defined as faeces with a moist layer of mucus, a strong smell and no signs of dehydration. ACTH was administered in the morning, and at that time, all scats present in the enclosure were removed. From that moment onwards, all scats deposited by the badgers were collected until the end of the experiment (five days, 120 h). Depositions occurred during the night, as enclosures were monitored periodically throughout the day (8:00 am to 20:30 pm) and new scats were only found in the first revision of the day at 8.00 am. This agrees with this species' behaviour which shows peak activity at dawn and during the night (Neal and Cheeseman, 1991). We did not monitor enclosures during the night in order to obtain results that could be compared to those obtained in field conditions. As nocturnal faeces collection is both challenging and time-consuming, sampling efforts can be optimized by arriving at sampling sites in the field at sunrise.

### *Environmental exposure*

As previous studies indicate that glucocorticoid metabolite levels vary due to bacterial activity and environmental conditions (Millsbaugh and Washburn, 2004), we performed an experiment to test whether the time lapse between the collection and freezing of faecal samples affects cortisol metabolite levels of faeces exposed to natural environmental conditions. Eight fresh scats were collected from the two pairs of badgers housed at Collserola. Every time that a badger deposited an scat, we homogenized them because steroids are not evenly distributed throughout scats (Brown et al., 1994) and later we picked up a portion of the same, which was immediately frozen at  $-20^{\circ}\text{C}$ . Therefore, the first sample gathered at 8:00 am acted as control because it had lapsed little time from the defecation. After homogenisation and collection in the enclosure (8:00 am), scats were maintained in natural environmental conditions, and faecal samples were collected from each scat every 30 min from 8 to 9 h, every 15 min from 9 h to 10 h, every 20 min from 10 h to 11 h, every 30 min from 11 h to 14 h, and every 60 min from 14 to 16 h. Thus, the entire experimental period lasted eight hours, and a total of 18 faecal samples were collected from each scat. Each time a sample was collected, ambient temperature and relative humidity were recorded (weather station WS-9152), as these parameters can influence levels of glucocorticoid metabolites obtained from faeces collected in natural conditions (Millsbaugh and Washburn, 2004).

### *Portion of scat analysed*

Three samples were taken from each fresh scat ( $n = 8$ ) deposited by one female adult European badger housed in Cañada Real. One sample was removed from the surface of the faecal mass, one from the interior, and one from the thoroughly mixed faecal mass (henceforth homogenised). These samples were used to estimate within-sample variation of cortisol metabolites.

### *Storage time of faecal samples and extracts*

To assess the effects of storage time on the samples collected from the four badgers in Collserola Natural Park, we removed three replicate samples from each homogenised scat ( $n = 12$ ). The three replicate samples were placed in a freezer at  $-20^{\circ}\text{C}$  for four, fourteen and twenty-six weeks, respectively, until hormonal analysis. Another storage experiment was conducted with a similar design for faecal extracts, but these three replicate samples were analysed after one, fourteen and twenty-six weeks, respectively. Thus, we collected one faecal sample from each of 18 scats.

### *Extraction and enzyme immunoassay*

Cortisol metabolites were extracted from faecal samples according to the modified methods of Shideler et al. (1993). Frozen faecal samples were dried (until constant

weight), and then the dry samples (0.7 g) were placed in assay tubes with 2.5 ml of phosphate buffer and 2.5 ml of methanol. The tubes were then shaken for 16 h. The supernatant was centrifuged at 4000 g for 30 min, and then the pellet was discarded and the faecal extracts were stored at  $-20^{\circ}\text{C}$  until assayed.

A commercial cortisol enzyme immunoassay (EIA; DRG Instruments GMBH, 211 Marbug, Germany) was used to analyse faecal samples. Three tests must be done to validate any EIA: parallelism, accuracy and precision (Goymann et al., 1999; Young et al., 2004). A parallelism test was performed with one quantified faecal sample for cortisol with serial dilutions (1:32, 1:16, 1:8, 1:4, 1:2, and 1:1) resulting in a curve for the badgers parallel to standard curves ( $P > 0.05$ ). Recovery of added cortisol was  $127.61 \pm 3.40\%$  ( $n = 6$ ). The intra- and inter-assay coefficients of variation (precision) for three biological samples were  $5.60 \pm 1.64$  and  $6.90 \pm 0.50\%$ , respectively. In each assay, we ran a standard included in the DRG kits, whose cortisol metabolite concentration is known. When standard cortisol metabolite concentrations deviated more than 10% from the expected value, the assay was rejected and the samples were re-analysed.

### *Data analyses*

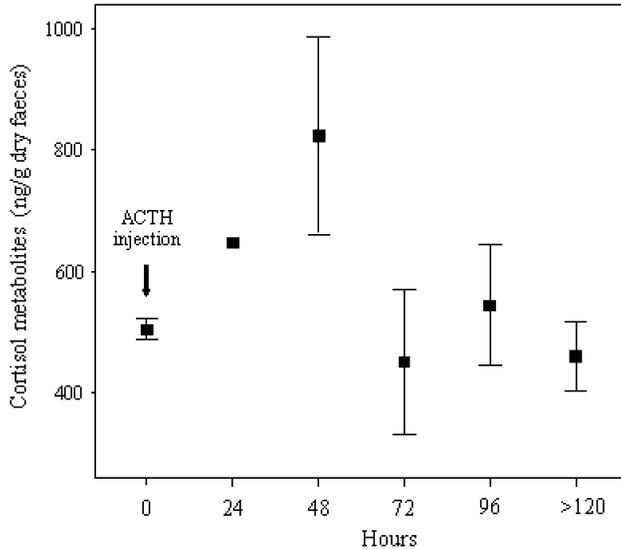
We performed a logarithmic transformation of quantitative variables prior to analyses to ensure homoscedasticity. Comparisons among faecal cortisol metabolite levels in relation to the time from the ACTH injection (0, 24, 48, 72, 96, and  $>120$  hours) were performed using one-way ANOVA. Repeated measure ANOVA was applied to test for significant differences between faecal cortisol metabolite levels (response variables) and the time faecal samples (4, 14 and 26 weeks) and sample extracts (1, 14 and 26 weeks) were stored. We used Duncan tests for post-hoc testing. A generalized linear mixed model (GLMM) was performed to determine the effects of the time lapse from collection to the freezing of samples (fixed factor), scats (random factor) and temperature and humidity (covariates) on faecal cortisol metabolite levels and all possible interactions between fixed factors and covariates. A GLMM was also used to analyse the effect of the portion of scat analysed (fixed factor) on mean concentrations of cortisol metabolites with the identity of particular scats as controls (random factor).

The data are presented as means  $\pm$  SE. The level of significance for all statistical tests was  $P < 0.05$ . All statistical analyses were performed in STATISTICA 7.0 (StatSoft Inc., USA), except the GLMM model, which was conducted using SAS System 9.2 by means of GLIMMIX macro (Little et al., 1992).

## **Results**

### *ACTH challenge*

Faecal cortisol metabolites increased following the administration of the ACTH injection. Faecal cortisol metabolite concentrations reached a peak after 24–48 h and



**Figure 1.** Faecal cortisol metabolites in badgers before and after administration of ACTH to stimulate adrenal activity.

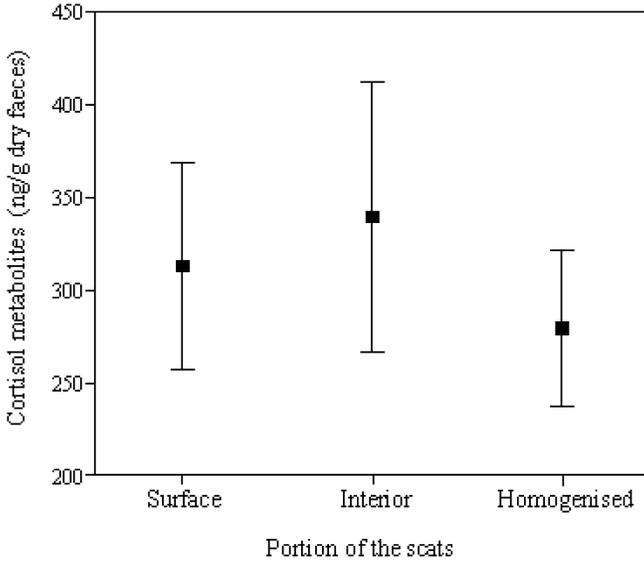
returned to below basal levels within 72 to 120 h after treatment. These differences were significant (ANOVA:  $F_{5,12} = 6.49$ ,  $P = 0.003$ ; fig. 1; post-hoc Duncan test,  $P < 0.05$  between 48 h and times 0, 72, 96, 120 h,  $P > 0.05$  between 48 and 24 h). The highest levels were obtained 48 h after ACTH administration.

#### *Effects of environmental exposure*

Mean temperature registered during the experiment was  $25.66 \pm 2.55^\circ\text{C}$  (range 21.20–32.20) and mean relative humidity was  $62.77 \pm 9.90\%$  (range 42.00–79.00). The results of the GLMM indicated that the considered variables (time lapse between the collection and freezing of faecal samples, temperature and humidity) did not have a significant effect on faecal cortisol metabolite levels (factor time,  $F_{16,69} = 0.65$ ,  $P = 0.827$ ; factor temperature,  $F_{1,69} = 0.43$ ,  $P = 0.516$ ; factor humidity,  $F_{1,69} = 2.13$ ,  $P = 0.149$ ; factor time  $\times$  humidity,  $F_{16,69} = 0.80$ ,  $P = 0.667$ ; factor time  $\times$  temperature,  $F_{16,69} = 0.81$ ,  $P = 0.671$ ).

#### *Effects of the portion of scat analysed*

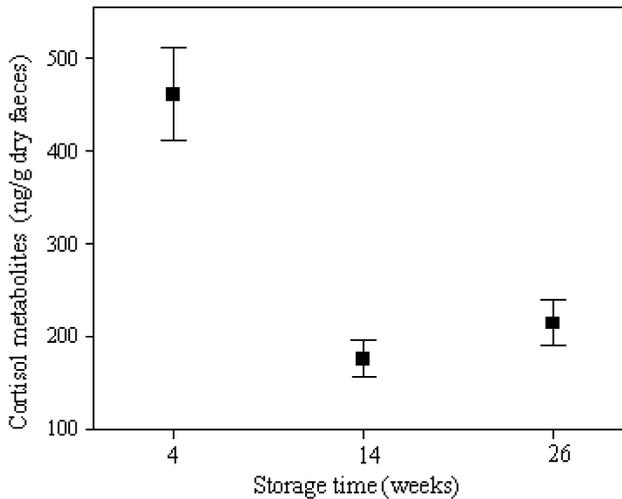
The portion of scat analysed affected mean levels of faecal cortisol metabolites ( $F_{2,14} = 4.39$ ,  $P = 0.033$ ) (fig. 2). Interior samples showed higher levels than homogenised samples (post-hoc Duncan test:  $P < 0.05$  for interior samples and homogenised samples, and  $P > 0.05$  for comparisons between the other samples). Faecal cortisol metabolite levels of homogenised samples (190–379 ng/g dry faeces) were less variable than those of interior samples (160–482 ng/g dry faeces) and surface samples (150–352 ng/g dry faeces).



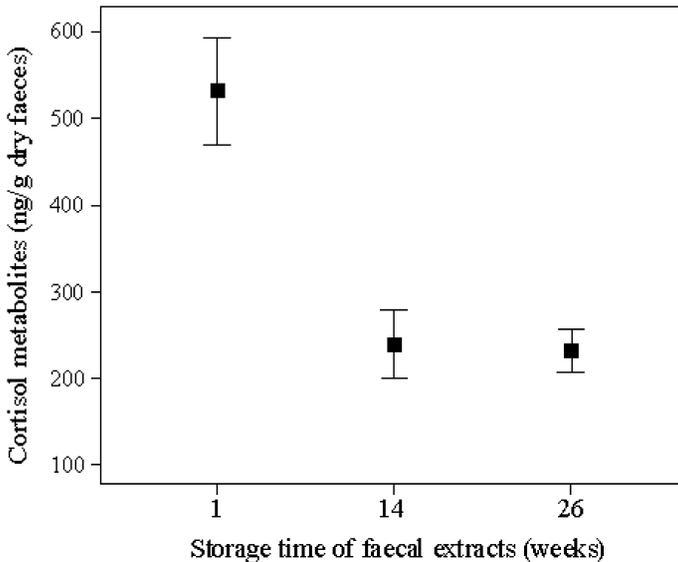
**Figure 2.** Cortisol metabolites from samples collected from different portions of the scat. One sample was collected from the surface and one from the interior. After removing these two samples, the remaining faecal mass was homogenised and one additional sample was removed.

#### *Effects of storage time on faecal samples and extracts*

Storage time had a significant effect on the faecal cortisol metabolite concentration of faecal samples and extracts. Samples frozen for 4 weeks had significantly higher concentrations of faecal cortisol metabolites than those frozen for 14 or 26 weeks



**Figure 3.** Variation of cortisol metabolites in relation to the length of time that faecal samples were stored at  $-20^{\circ}\text{C}$ .



**Figure 4.** Variation of cortisol metabolites according to storage time of faecal extracts.

(ANOVA:  $F_{2,22} = 138.86$ ,  $P < 0.001$ ; fig. 3; Duncan test,  $P < 0.001$  between 4 weeks and 14 and 26 weeks,  $P > 0.05$  between 14 and 26 weeks). Furthermore, levels of faecal cortisol metabolites in extracts were significantly higher after 1 week of storage compared to 14 or 26 weeks of storage (ANOVA:  $F_{2,34} = 97.04$ ,  $P < 0.001$ ; fig. 4; post-hoc Duncan test,  $P < 0.05$  between 1 week and 14 and 26 weeks,  $P > 0.05$  between 14 and 26 weeks).

## Discussion

The results of this study show that faecal cortisol metabolite levels reached a peak within 24–48 h after the badgers received the ACTH injection. Other studies in carnivore species found that ACTH stimulation increased faecal glucocorticoid metabolite concentrations after 1–2 days (Monfort et al., 1998; Young et al., 2004). This suggests that ACTH stimulation in European badgers results in increased adrenocortical activity. Hence, the measurement of cortisol metabolites in faeces is a useful tool for monitoring adrenocortical activity in captive and free-ranging badgers. Furthermore, it is a practical alternative to blood sampling, as it does not require the handling of badgers. Animal manipulation (capture, anaesthesia) can affect cortisol metabolite levels differently in each control individual. These effects might cause bias in the results regarding the interval between ACTH administration and the increase of faecal cortisol metabolite levels. Unfortunately, this type of information was not available in this study. However, this aspect should be considered in future studies.

The most feasible sampling option for elusive carnivores involves collecting faecal samples that are potentially several days old (Millspaugh and Warhburn, 2004).

However, some studies have reported that the time lapse between faecal deposition and the freezing of samples may alter glucocorticoid metabolite levels (Palme, 2005). This is because faeces contain large numbers of bacteria that produce enzymes that can alter the structural integrity of glucocorticoid metabolites after defecation (Millsaugh and Warhburn, 2004). To minimise these impacts, faeces should be collected as soon as possible (Schwartz and Monfort, 2008). Nevertheless, the faecal cortisol metabolite levels obtained in this study did not vary during the eight hours from collection until the freezing of samples (see also Mashburn and Atkinson, 2004). In domestic fowl, a delay of up to 24 h between defecation and the freezing of a dropping did not affect faecal levels of oestradiol and testosterone metabolites (Cockrem and Rounce, 1994). Abáigar et al. (2010) found similar results for Iberian lynx, where sexual steroid metabolite levels measured in faeces did not change after one week. Studies that aim to address carnivore conservation questions may rely on these data, but as faecal bacteria can vary among species, the alterations that affect faecal metabolites may be species-dependent. Thus, further studies should be carried out with other carnivore species. Species in which glucocorticoid levels do not change with long delays from the time of deposition until the freezing of faecal samples are the most favourable for studies under natural conditions. In these cases, the use of non-invasive sampling techniques to monitor stress levels in natural populations should be greatly encouraged.

The non-significant differences in cortisol metabolite levels found between the interior and surface may be due to the difficulty of discriminating between these two areas of the scat, or to insufficient sampling, as we only collected one subsample for each category (surface, interior and homogenised). However, higher values were found for the interior part of the scat than for the homogenised samples, indicating that faecal cortisol metabolites were not homogeneously distributed. Brown et al. (1994) reported that estrogens and progestins are not evenly distributed in individual faecal samples for some felids. Hence, considering our results and the difficulties of accurately sampling different parts of a carnivore scat, we suggest that researchers homogenise the entire faecal mass before removing a sample of faecal material to standardize sampling. This method has also been suggested for other mammals (e.i. elk: Millsaugh et al., 2001; white-tailed deer: Millsaugh and Warhburn, 2003).

Faecal cortisol metabolite levels of faecal samples and extracts stored at  $-20^{\circ}\text{C}$  for long periods (fourteen to twenty-six weeks) were significantly lower than those of samples and extracts stored for shorter periods (one or four weeks). This demonstrates that badger faecal samples and extracts do not provide an accurate assessment of cortisol metabolite levels after storage at  $-20^{\circ}\text{C}$  for long time periods. Therefore, in order to obtain comparable results it is crucial to store all samples and extracts for the same period of time, from 1 to 16 weeks. Khan et al. (2002) also reported that faecal glucocorticoid metabolite levels varied according to the number of days that samples were maintained at  $-20^{\circ}\text{C}$ , although, levels increased in their study. In contrast, faecal corticosterone metabolite levels in birds did not

change when samples were frozen for long periods of time (Herring and Gawlik, 2009). Because results appear to be species-specific, we suggest that researchers exert caution when dealing with this factor in absence of experimental data and include the comparison of samples with different storage times in their experimental protocol.

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