



## Little agreement among methodologies to determine fecal glucocorticoid metabolites in a mountain ungulate

Stefania Tampach<sup>a</sup>, Jorge Ramón López-Olvera<sup>a,\*</sup>, Rupert Palme<sup>b</sup>, Franz Schwarzenberger<sup>b</sup>, Anna Hillegonda Baauw<sup>a</sup>, Pia Anderwald<sup>c</sup>, Elena Albanell<sup>d,\*</sup>

<sup>a</sup> Wildlife Ecology & Health (WE&H) research group and Servei d'Ecopatologia de Fauna Salvatge (SEFaS), Departament de Medicina i Cirurgia Animals, Universitat Autònoma de Barcelona (UAB), Bellaterra, 08193 Barcelona, Spain

<sup>b</sup> Department of Biomedical Sciences, University of Veterinary Medicine, Veterinärplatz 1, 1210 Vienna, Austria

<sup>c</sup> Swiss National Park, Chastè Planta-Wildenberg, Zernez 7530, Switzerland

<sup>d</sup> Group of Research in Ruminants (G2R), Departament de Ciència Animal i dels Aliments, Universitat Autònoma de Barcelona (UAB), Bellaterra, 08193 Barcelona, Spain

### ARTICLE INFO

#### Keywords:

Enzyme immunoassay  
Faecal cortisol metabolites  
Near-infrared spectroscopy  
Radioimmunoassay  
*Rupicapra* spp.  
Stress

### ABSTRACT

Faecal glucocorticoid metabolites (FGMs) have gained relevance in ecological studies and population monitoring, allowing non-invasive remote sampling without the need to capture and handle animals. Enzyme immunoassays (EIAs) and radioimmunoassays (RIAs) are commonly used for FGM determination. Although these methods should be validated for each species, non-validated tests are still widely used to determine FGMs in wildlife. Near-infrared reflectance spectroscopy (NIRS) is a predictive method requiring calibration against reference methods used to assess FGMs in wildlife. EIAs and RIAs have been utilized to determine FGMs in chamois (*Rupicapra* spp.), a medium-sized mountain ungulate.

This study aims to assess the potential of NIRS to determine FGMs and to evaluate the correlation among analytical methods used to determine FGMs in chamois. Faecal samples from 125 Alpine chamois (*Rupicapra rupicapra rupicapra*) and 125 Pyrenean chamois (*R. pyrenaica pyrenaica*) were collected from the field, frozen at  $-20^{\circ}\text{C}$ , lyophilized, grounded, and scanned using a NIRSystems 5000 monochromator over a 1108–2492 nm wavelength. After this non-destructive NIRS analysis, FGMs were extracted and analysed using four immunoassays previously used in chamois studies: a 125-I-corticosterone RIA, a cortisol EIA, and two 11-oxoetiocholanolone EIAs (72a and 72T). Only the 11-oxoetiocholanolone 72T EIA has been validated for Alpine chamois. NIRS predictions were calibrated and cross-validated for each of the four immunoassays. The correlation among the four immunoassays was assessed using Spearman's rank.

The coefficient of determination for NIRS calibration ( $R^2$ ) values ranged from 0.37 to 0.75, and the ratio of performance to deviation values from 1.2 to 1.6. Therefore, NIRS could not predict FGM concentration in chamois faeces. This could be due to the complexity and variability of the FGM detected by the immunoassays as reference methods, and by potential interference of other compounds in the faecal matrix. The correlation among the immunoassays was low overall.

As a conclusion, NIRS cannot be recommended for measuring FGMs in chamois. The low correlation among the immunoassays used for FGM determination raises concern about the reliability of previous studies using non-validated methods in chamois. Only biologically

\* Corresponding authors.

E-mail addresses: [Jordi.Lopez.Olvera@uab.cat](mailto:Jordi.Lopez.Olvera@uab.cat) (J.R. López-Olvera), [Elena.Albanell@uab.cat](mailto:Elena.Albanell@uab.cat) (E. Albanell).

validated tests should be used to assess FGMs to avoid incorrect inferences about biological responses in physiological, conservational, and ecological studies or population monitoring. These conclusions are applicable beyond the species studied here.

## 1. Introduction

Stressful situations elicit behavioural, physiological and neuroendocrine responses in animals to help them cope with it (Moberg and Mench, 2000; Möstl and Palme, 2002). The endocrine stress response includes the hypothalamic-pituitary-adrenal (HPA) axis, whose effects are mediated mainly by glucocorticoid hormones (GCs), namely corticosterone (4-pregnene-11 $\beta$ ,21-diol-3,20-dione) and cortisol (4-pregnene-11 $\beta$ ,17 $\alpha$ ,21-triol-3,20-dione) (Selye, 1946; Mormède et al., 2007; Palme, 2019). Cortisol is the predominant GC in most mammals, although corticosterone is also present (Koren et al., 2012).

GCs and their metabolites have been used as biomarkers to monitor physiological stress response in animals (Palme, 2012; Palme, 2019). The concentration of cortisol in serum or plasma is considered a useful indicator of stress (Broom and Johnson, 1993; Terlouw et al., 1997). However, blood sampling itself elicits a stress response and consequently can interfere with cortisol determination (Möstl et al., 2002). Apart from being invasive, collecting blood samples in wildlife (either free-ranging or captive) usually implies capture, restraint, and handling, which, beyond interfering with stress assessment, is a logistic challenge and reduces the number of samples that can be obtained. Therefore, measurement of GCs and/or their metabolites in alternative biological samples not eliciting a stress response for collection, such as saliva, urine, faeces, hair and feathers has been investigated (for a detailed discussion of the pros and cons of the different matrices see: Mormède et al., 2007; Sheriff et al., 2011; Heimbürge et al., 2019).

The determination of faecal glucocorticoid metabolites (FGMs) has gained relevance in conservational and ecological studies and population monitoring, due to the advantages of non-invasive sampling without the need to capture and handle animals (Wikelski and Cooke, 2006; Busch and Hayward, 2009; Sheriff et al., 2011). Moreover, the concentration of FGMs reflects a dampened pattern of glucocorticoid secretion, providing an integrated measure of adrenocortical activity less affected than blood cortisol by circadian rhythms and acute stress (Palme et al., 1999; Palme, 2019). This has led to the vast and extensive study of FGMs in wildlife (Wasser et al., 2000; Palme et al., 2005; Schwarzenberger, 2007; Busch and Hayward, 2009; Sheriff et al., 2011; Fanson et al., 2017; Kumar and Umapathy, 2019; Palme, 2019).

However, glucocorticoids are metabolized primarily by the liver, excreted via bile predominantly as conjugates, and undergo further metabolism (e.g., hydrolysis) by intestinal bacteria, posing an analytical challenge as compared to other matrices (Taylor, 1971; Möstl and Palme, 2002; Palme et al., 2005; Palme, 2019). Consequently, FGMs are species-specific steroid mixtures, which are also influenced by intrinsic and extrinsic factors such as individual, sex, reproductive status, season, diet, and disease, among others (Palme et al., 2005; Dantzer et al., 2016; Coppes et al., 2018; Palme, 2019; Pérez et al., 2019). Additionally, sample freshness, transport and storage conditions and duration, and extraction methods also influence the measurement of FGM concentrations (Palme, 2019; Pérez et al., 2020).

Mass spectrometry techniques have allowed the characterization of the main FGMs in different species, immunoassays are more often utilized for their measurement due to their lower cost and easier handling (Murtagh et al., 2013). Both enzyme immunoassays (EIAs) and less frequently used radioimmunoassays (RIAs) face the challenge of reacting with the specific combination of metabolites for each species. Moreover, the antibodies used in the immunoassays cross-react in different percentages with each of the metabolites within the species-specific FGM combination, which influences concentrations measured with different immunoassays (Wudy et al., 2018; Palme, 2019). The so-called group-specific EIAs (designed to measure a group of metabolites) perform better than cortisol or corticosterone assays (Möstl et al., 2002; Palme, 2019). Nevertheless, whatever method is used, physiological and/or biological validation for each species, including experimental demonstration that biologically relevant increases in plasma GC concentrations indicating changes in HPA activity are reflected in FGM values, is a prerequisite for its successful application (Palme, 2019). However, in spite of the availability of fully validated tests, non-validated immunoassays are still widely used to determine FGMs in wildlife (Touma and Palme, 2005; Palme, 2019), which may result in incorrect inferences about biological responses, especially when the environmental stressors are complex and interactive (Hinchcliffe et al., 2021).

Near-infrared reflectance spectroscopy (NIRS) has been widely and successfully applied in agriculture, for the evaluation of food quality, in soil science, and physiological studies, among others (Williams and Norris, 2001; Roberts et al., 2004; Cen and He, 2007; Vendrame et al., 2012). NIRS is a predictive (indirect) method which requires calibration models against appropriate standard laboratory methods. Hence, the performance of NIRS as a procedure for quantitative analysis will depend on the accuracy and precision of the reference methods (Cen and He, 2007). Faecal material has been analysed by NIRS to assess nutrition, physiology and ecology of domestic and free-ranging herbivores (Dixon and Coates, 2009; Dryden, 2003; Foley et al., 1998; Gálvez-Cerón et al., 2013; Kho et al., 2023; Lyons and Stuth, 1992; Morgan et al., 2021). However, few studies have addressed hormonal analysis in urine (Kinoshita et al., 2012) or faeces (Litman, 2016; Santos et al., 2014; Talló-Parra et al., 2015a) using NIRS. A study by Santos et al. (2014) evaluated faecal NIRS prediction models of FGM in red deer (*Cervus elaphus*), using lyophilised vs oven dried faeces. The FGM concentrations measured by RIA were used as the reference data of partial least square (PLS) regression and good accuracies were obtained using data from both drying procedures (lyophilised:  $R^2 = 0.90$ ; oven-dried:  $R^2 = 0.88$ ), which allowed to forecast a potential use of NIRS to determine FGM in other wildlife ruminant species. NIRS has the advantage of being a non-destructive and reagent-free technique that provides a rapid analysis of complex samples containing a wide range of components (Williams, 2001). In addition, the samples require little or no preparation before analysis and can be reused for other analyses afterwards (Roggo et al., 2007; Siesler et al., 2002).

However, despite such advantages and the exploratory studies performed, biological or laboratory NIRS validation to determine FGMs has not been attempted and the accuracy of prediction models among reference laboratory methods (RIA and EIA) has not been compared yet.

Chamois (*Rupicapra* spp.) are medium-sized mountain ungulates originally distributed in mountain massifs of Europe and Western Asia and introduced in different countries such as New Zealand (Catusse et al., 1996). The current scientific consensus recognizes two species, Northern chamois (*Rupicapra rupicapra*), with seven subspecies, and Southern chamois (*Rupicapra pyrenaica*), with three subspecies. Although both species are considered as Least Concern by the International Union for the Conservation of Nature, some subspecies are declining and/or have a Vulnerable status, diseases being currently among the most important threats (Herrero et al., 2020; Anderwald et al., 2021a; Garrido-Amaro et al., 2023). While reference values for serum cortisol concentration and its variations due to stress have been reported for physically and chemically-captured Southern chamois (López-Olvera et al., 2006a, 2006b, 2007, 2009; Angelucci et al., 2023), such information is not available for Northern chamois. Instead, FGMs have been determined in both species (Table 1), using either EIAs or RIAs (Schwarzenberger et al., 2000; Thaller et al., 2004; Hoby et al., 2006; Dalmau et al., 2007; Thaller, 2007; Corlatti et al., 2012; Zwijacz-Kozica et al., 2013; Corlatti et al., 2014; Hadinger et al., 2015; Corlatti, 2018; Fattorini, et al., 2018a, 2018b; Formenti et al., 2018; Anderwald et al., 2021b). However, only one (the 72T EIA) of the diverse tests used in the previously published studies determining FGMs has been physiologically validated (Anderwald et al., 2021b) as recommended (Touma and Palme, 2005; Palme, 2019).

The objectives of this study are (1) assessing the potential of NIRS to determine FGM concentration in both chamois species, and (2) evaluating the correlation among the different analytical methodologies previously used to determine FGM in chamois. This will demonstrate whether NIRS, a non-destructive, non-pollutant, environmentally-friendly, cheaper and more sustainable analytical methodology than the currently used laboratory methods, can be used to determine FGMs in these species. Moreover, these objectives will also allow to assess the consistency and reliability of the immunoassays used for FGM determination, not only for chamois but for any other species where non-validated methods have been used in previously published studies.

## 2. Materials and methods

### 2.1. Samples

Fresh faecal samples were collected from 125 Alpine chamois (*Rupicapra rupicapra rupicapra*, a subspecies of Northern chamois) in the Il Fuorn area (46.67°N, 10.16°E, altitude 1650 m above sea level -m.a.s.l-) in the Swiss National Park in south-eastern Switzerland; and from 125 Pyrenean chamois (*Rupicapra pyrenaica pyrenaica*, a subspecies of Southern chamois) in the National Game Reserve of Freser-Setcases (42°22'N, 2°09'E, altitude ranging from 1200 to 2910 m.a.s.l.), located on the southern side of the eastern Pyrenees in north-eastern Spain. The freshness of the faecal samples was assessed as previously described (Hibert et al., 2011). The faecal samples were stored individually in labelled zip-lock plastic bags in a cooler box with freeze blocks and transported to the laboratory (Palme et al., 2005; Sheriff et al., 2011), where they were stored in a freezer at - 20 °C (Millsbaugh and Washburn, 2004) until further processing and laboratory analyses.

### 2.2. Sample preparation and NIRS analysis

The samples were homogenized and split into two aliquots. One aliquot was used as duplicate to determine dry matter by drying at 103 °C. The second aliquot of each sample was first lyophilized at - 20 °C for 96 h until reaching constant dry weight, and then ground

**Table 1**  
Previous studies determining faecal glucocorticoid metabolites in chamois.

Methodology	Standard	References	Species and subspecies
Enzyme immunoassay	11-oxoetiocholanolone 72a EIA measuring 11,17-dioxoandrostanes	Schwarzenberger et al. (2000) Thaller et al. (2004) Hoby et al. (2006) Hadinger et al. (2015)	<i>Rupicapra rupicapra rupicapra</i>
	11-oxoetiocholanolone 72T EIA* measuring FGMs with a 5β-3α-ol-11-one structure	Corlatti et al. (2012) Corlatti et al. (2014) Corlatti (2018) Corlatti et al. (2019) Anderwald et al. (2021b) Donini et al. (2022) Formenti et al. (2018)	<i>Rupicapra rupicapra rupicapra</i>
	Cortisol	Zwijacz-Kozica et al. (2013) Fattorini et al. (2018a), (2018b)	<i>Rupicapra pyrenaica ornata</i> <i>Rupicapra rupicapra tatrica</i> <i>Rupicapra pyrenaica ornata</i>
Radioimmunoassay	Cortisol	Dalmau et al. (2007)	<i>Rupicapra pyrenaica pyrenaica</i>

\*The 72T EIA is the only assay that has been biologically validated for chamois to date (Palme, 2019; Anderwald et al., 2021b).

in a cyclone-type mill (Cyclotec 1093, FOSS Tecator, Höganäs, Sweden) to pass a 1 mm screen. The resulting dried and ground faecal samples were packed into 35 mm diameter closed ring cup cells with quartz glass windows and scanned using an NIRSystems 5000 scanning monochromator (FOSS, Hillerød, Denmark) over a wavelength range of 1108–2492 nm. Reflectance was recorded at 2 nm intervals as  $\log 1/R$ , where R represents the reflected energy, resulting in 692 data points per sample. Each sample was scanned twice by manually rotating the sample cup approximately 180° relative to the previous scan.

### 2.3. Immunoassays

After the non-destructive analysis by NIRS, FGMs were extracted from the dried and ground faecal samples by mixing in a test tube  $0.5 \pm 0.005$  g of dry faeces with 4 mL of 100 % methanol and 1 mL of distilled water. The mixture was subsequently shaken in a vortex for 1–2 min, followed by an orbital shaker at 230 rpm for 45 min, and finally centrifuged at 3000 rpm for 20 min (Palme et al., 2013). The resulting supernatant containing the FGMs was collected and stored at  $-20^\circ\text{C}$  until analysis through one radioimmunoassay and three enzyme immunoassays.

FGM concentration was determined in both the Alpine and Pyrenean chamois samples using a 125-I-Cortisterone RIA kit (#07-120103, MP Biomedicals, LLC, Orangeburg, NY), validated for a variety of mammalian species (Wasser et al., 2000) and previously used to analyse FGM in Pyrenean chamois faeces (Dalmau et al., 2007). The manufacturer's protocol was followed, diluting the faecal extracts 1:10 in the kit buffer. The FGM concentrations were expressed in ng/g of dry matter (DM). Cross-reactivities for the antiserum, according to the manufacturer, are 100 % for corticosterone, 0.34 % for deoxycorticosterone, 0.10 % for testosterone, 0.05 % for cortisol, 0.03 % for aldosterone, 0.02 % progesterone, 0.01 % androstenedione, 0.01 % for 5-dihydrotestosterone and  $< 0.01$  % for other steroids.

A cortisol EIA detection kit (Neogen® Corporation Europe, Ayr, UK) was used according to the manufacturer's protocol (Talló-Parra et al., 2015b) to detect FGM also in both the Alpine and Pyrenean chamois samples). This enzyme immunoassay has a sensitivity of 0.73 ng/g dry faeces. According to the manufacturer, cross-reactivities of the antibody with other steroids are 47.4 % for prednisolone, 15.7 % for cortisone, 15.0 % for 11-deoxycortisol, 7.83 % for prednisone, 4.81 % for corticosterone, 1.37 % for 6 $\beta$ -hydroxycortisol, 1.36 % for 17-hydroxyprogesterone, 0.94 % for deoxycorticosterone and  $< 0.06$  % for other steroids.

Finally, FGM concentrations were determined in Alpine and Pyrenean chamois using two different 11-oxoetiocholanolone EIAs, one for each species. The Alpine chamois samples were analysed using the 11-oxoetiocholanolone EIA lab code 72T (antibodies raised in rabbits against 11-oxoetiocholanolone-17-CMO:BSA), which measures FGMs with a 5 $\beta$ -3 $\alpha$ -ol-11-one structure. This test was developed for sheep (Möstl et al., 2002) and has been used (Corlatti et al., 2012, 2014; Corlatti, 2018; Formenti et al., 2018; Corlatti et al., 2019; Donini et al., 2022) and validated (Anderwald et al., 2021b) for Alpine chamois (Table 1). The cross-reactions of this 72T EIA are 100 % for 5 $\beta$ -androstane-3 $\alpha$ -ol-11,17-dione, 37 % for 5 $\beta$ -pregnane-3 $\alpha$ -ol-11,20-dione, 3.3 % for 5 $\beta$ -androstane-3 $\alpha$ ,11 $\beta$ -diol-17-one, and 1.2 % for 5 $\beta$ -androstane-3,11,17-trione. All the other steroids tested (11-ketoandrosterone, etiocholanolone, pregnanediol, tetrahydrocortisol, 5 $\beta$ -dihydrocortisol, cortisol, 5 $\beta$ -pregnane-3 $\alpha$ ,11 $\beta$ ,21-triol-20-one, 5 $\beta$ -pregnane-3 $\alpha$ ,11 $\beta$ ,17 $\alpha$ ,20 $\alpha$ ,21-pentol, 5 $\beta$ -pregnane-3 $\beta$ -ol-11,20-dione and 5 $\beta$ -pregnane-3 $\alpha$ ,11 $\beta$ -diol-20-one) have cross-reactions below 1 %. The Pyrenean chamois samples were analysed with the lab code 72a test, which uses 11-oxoetiocholanolone-3-HS:BSA as immunogen and measures 11, 17-dioxoandrostanes. This test was also developed for sheep (Palme and Möstl, 1997) and has been previously used for Alpine chamois faeces (Schwarzenberger et al., 2000; Thaller et al., 2004; Hoby et al., 2006; Thaller, 2007; Hadinger et al., 2015; Table 1). The reported cross-reactivities for this assay are 100 % for 5 $\beta$ -Androstane-3 $\alpha$ -ol-11,17-dione, 84 % for 5 $\beta$ -Androstane-3,11,17-trione, 14.7 % for 5 $\alpha$ -Androstane-3,11,17-trione, 6.7 % for 5 $\alpha$ -Androstane-3 $\beta$ -ol-11,17-dione, 5.7 % for 5 $\alpha$ -Androstane-3 $\alpha$ -ol-11,17-dione, 0.6 % for 5 $\beta$ -Androstane-3 $\alpha$ ,11 $\beta$ -diol-17-one and  $< 0.01$  % for other steroids (Palme and Möstl, 1997).

### 2.4. NIRS analysis

In order to obtain better accuracy in calibration, the mean of the two scans of each sample (average spectral data) was used for calibration and further analyses. The WinISI 4.10 (Infrasoft International, Port Matilda, PA, USA) software program was employed for the spectral data analysis and the development of chemometric models. Prior to calibration, the  $\log(1/R)$  spectra were corrected for the effects of scatter using the standard normal variate (SNV) and detrend (D) algorithms, and by multiplicative scatter correction (MSC), in order to reduce the effects of the particle size, improve signal-to-noise ratio and, therefore, maximize the signal intensity for the analytes of interest (Heise and Winzen, 2002). The prediction models were performed by the modified partial least squares regression (MPLS) and combinations of scattering correction (SNV, D, SNV + D, MSC). Sixteen derivative mathematical treatments were tested (1,4,4,1; 1,5,5,1; 1,8,8,1; 1,10,10,1; 1,4,4,2; 1,5,5,2; 1,8,8,2; 1,10,10,2; 2,4,4,1; 2,5,5,1; 2,8,8,1; 2,10,10,1; 2,4,4,2; 2,5,5,2; 2,8,8,2; and 2,10,10,2), where the first digit is the number of the derivative, the second is the gap over which the derivative is calculated, the third is the number of data points in the first smoothing, and the fourth is the number of data points in the second smoothing. Hence, 64 regression equations were developed for each one of the four laboratory techniques of FGM determination by combining sixteen spectral derivative math treatments and four scatter correction methods.

Cross-validation was applied to optimize calibration models, determine the optimal number of terms for the calibration equation, and to identify chemical and spectral outliers. In addition to cross-validation, an external validation was performed using a set of 20 % of the total samples. The samples in the validation set were randomly selected from the total matrix and were balanced according to the two species (Alpine and Pyrenean chamois) to include the wider range of values for the four techniques. The samples in the validation set were not used for calibration and vice versa, and the range of the validation set was always included within the range of the calibration set.

The optimum model was selected according to the following statistics: minimum standard error of calibration (SEC), minimum standard error of prediction (SEP), greatest coefficient of determination for calibration ( $R_{CAL}^2$ ), greatest coefficient of determination for validation ( $R_{VAL}^2$ ), and the ratio of performance to deviation (RPD, defined as the ratio of standard deviation for the validation samples to the value of SEP). A calibration with an  $RPD \leq 1.9$  is not considered adequate; RPD values between 2.0 and 2.4 are considered poor and only adequate for rough screening purposes; RPD values between 2.5 and 2.9 provide a fair prediction that can be used for screening; and RPD values  $\geq 3.0$  indicate good prediction and can be used for quantitative analysis (Shenk and Westerhaus, 1996; Williams, 2014; Williams and Sobering, 1996).

### 2.5. Correlation among immunoassays

A Shapiro-Wilk normality test was conducted for the output of all four immunoassays (Zuur et al., 2010), detecting that their distribution departed significantly from normality ( $p$ -value  $< 0.05$ ). Therefore, the correlation between all the possible pairs of variables was assessed using the Spearman's rank correlation coefficient for each species (Alpine chamois and Pyrenean chamois) separately, as well as for both species altogether. According to this coefficient, correlations can be considered very weak (from 0.00 to 0.19), weak (from 0.20 to 0.39), moderate (from 0.40 to 0.69), strong (from 0.70 to 0.89), or very strong (from 0.90 to 1.00) (Fowler et al., 2009), as well as either negative or positive. To explore the goodness of fit and explain the variation between each pair of variables, generalized linear models were developed to calculate  $R^2$  values (Zhang, 2017). Data residuals distribution was explored using the DHARMA package, including Kolmogorov-Smirnov and dispersion tests. All the analyses were performed using the R software (Version 4.1.3; R Development Core Team, 2022).

## 3. Results

Table 2 shows the compositional data of the chamois faecal samples used to develop the calibration and validation models in this study. All the variables were well represented in both calibration and validation matrices covering similar ranges and a relatively broad range as recommended (Næs et al., 2002). Table 3 shows the calibration and validation statistics of the prediction models to determine FGM concentration in the Pyrenean and Alpine chamois faecal samples using NIRS. Calibrations by MPLS regression were performed using the average spectral of the duplicate, and different pre-treatments of spectral data were tested for their ability to remove or reduce disturbing effects not related to the chemical absorption of light. The scatter correction method that fitted the best results was the MSC, whereas the mathematical treatment differed among the four laboratory techniques. The models obtained (Table 3) had  $R_{CAL}^2$  values ranging from 0.37 to 0.75, and the accuracy of the calibration was evaluated based on the RPD statistics ranging from 1.2 to 1.6.

Table 4 and Fig. 1 show the agreement among all four laboratory methods for the determination of FGM in chamois used in this study separately for each species and for both species altogether. The correlation values among the laboratory techniques were overall low, both for each species separately and for the two species together (Table 4). The distribution of the correlation data residuals and the results of the Kolmogorov-Smirnov and dispersion tests are shown in Supplementary Material Fig. 1 to 7 and Supplementary Material Table 1. While all the residuals were uniformly distributed, they significantly departed from normality for some of the correlations (Supplementary Material Table 1).

## 4. Discussion

This study attempted to calibrate NIRS for predicting FGMs in chamois faeces against four immunoassays and assessed the correlation among these methodologies, widely used to determine FGM concentration for physiological, population, conservation, and

**Table 2**

Glucocorticoid metabolite concentrations in the faecal samples of Pyrenean and Alpine chamois determined by immunoassays and used in the NIRS calibration and validation data sets.

Method (units)	Calibration set				Validation set			
	N	Range	Mean	SD	n	Range	Mean	SD
125-I-Corticosterone RIA (ng/g) <sup>1</sup>	200	29.5-468.6	131.9	74.7	50	35.9-418.4	138.9	76.3
Cortisol EIA (ng/g) <sup>2</sup>	199	0.8-9.1	2.8	1.4	50	0.9-6.3	2.8	1.3
11-oxoetiocholanolone (72a) EIA (ng/g) measuring 11,17-dioxoandrostanes <sup>3*</sup>	88	0.1-26.8	11.1	7.3	20	2.3-21.4	13.5	6.8
11-oxoetiocholanolone (72T) EIA (ng/g) measuring FGMs with a 5 $\beta$ -3 $\alpha$ -ol-11-one structure <sup>4**</sup>	101	0.7-16.7	5.1	3.8	25	1.1-14.3	4.7	3.5

N = number of samples for calibration; n = number of samples for external validation; Range = interval between the maximum and minimum value of data set; SD = standard deviation.

<sup>1</sup>Dalmay et al. (2007); Wassar et al. (2000).

<sup>2</sup>Talló-Parra et al. (2015b).

<sup>3</sup>Palme and Möstl (1997); Hadinger et al. (2015).

<sup>4</sup>Möstl et al. (2002); Anderwald et al. (2021b).

\*Only Pyrenean chamois samples.

\*\* Only Alpine chamois samples.

**Table 3**

Calibration and validation statistics of prediction models used to determine glucocorticoid metabolite concentrations in Pyrenean and Alpine chamois faecal samples by near infrared reflectance spectroscopy analysis.

	Calibration				Validation				
	<sup>a</sup> Math treatment	<sup>b</sup> Scatter correction	R <sup>2</sup> <sub>CAL</sub>	SEC	R <sup>2</sup> <sub>VAL</sub>	SEP	Bias	Slope	RPD
125-I-Corticosterone RIA	1,4,4,1	MSC	0.39	41.05	0.25	59.32	3.87	0.40	1.5
Cortisol EIA	2,4,4,2	MSC	0.52	0.75	0.48	0.87	0.01	0.88	1.6
11-oxoetiocholanolone 72a EIA measuring 11,17-dioxoandrostanes*	1,5,5,1	MSC	0.37	2.85	0.30	2.93	0.02	0.97	1.2
11-oxoetiocholanolone 72T EIA measuring FGMs with 5β-3α-ol-11-one**	1,10,10,2	MSC	0.75	3.77	0.36	5.78	0.54	0.78	1.2

<sup>a</sup>Math treatment: derivative order, subtraction gap, first smoothing, second smoothing.

<sup>b</sup>MSC multiple scatter correction.

R<sup>2</sup><sub>CAL</sub> coefficient of determination for calibration, SEC standard error of calibration, R<sup>2</sup><sub>VAL</sub> coefficient of determination for validation, SEP standard error of validation, RPD ratio of performance to deviation (SD/SEP).

\*Only Pyrenean chamois sample.

\*\*Only Alpine chamois samples.

**Table 4**

Spearman's correlation coefficient (rho) and R-squared (R<sup>2</sup>) values of the four immunoassays used in this study to measure faecal glucocorticoid metabolites in Alpine chamois, Pyrenean chamois, and both chamois species altogether. The statistically significant correlations are indicated in bold type.

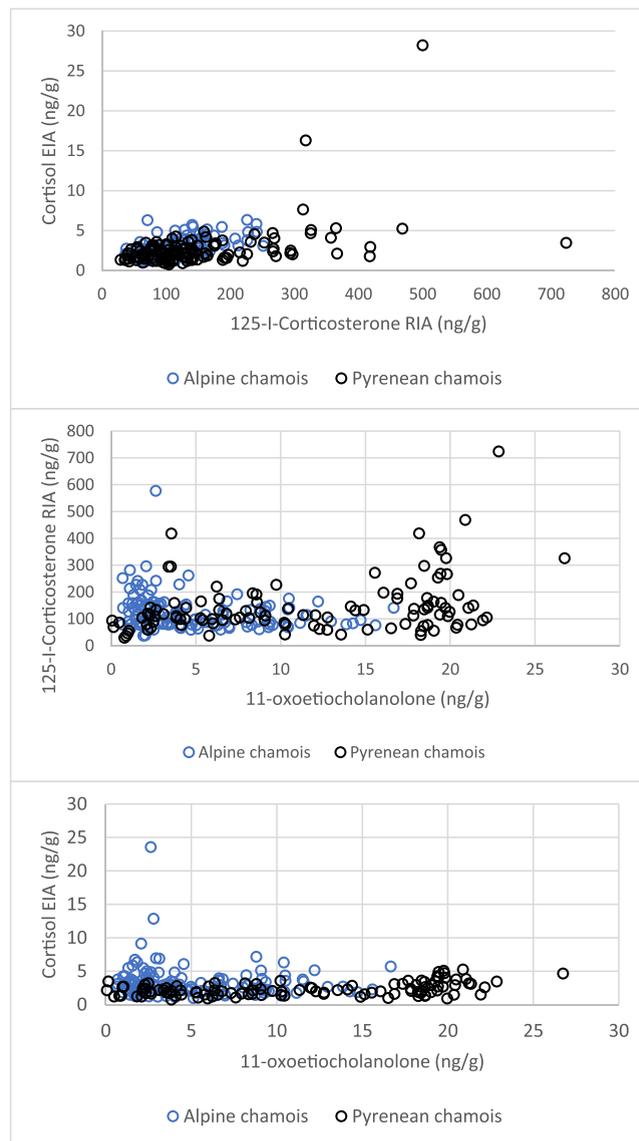
		Alpine chamois*		Pyrenean chamois**		Both species	
		rho	R <sup>2</sup>	rho	R <sup>2</sup>	rho	R <sup>2</sup>
125-I-Corticosterone RIA vs. Cortisol EIA		<b>0.665</b>	<b>0.5834</b>	<b>0.390</b>	<b>0.212</b>	<b>0.470</b>	<b>0.278</b>
11-oxoetiocholanolone EIA vs. 125-I-Corticosterone RIA	<b>p-value</b>	<b>&lt; 0.001</b>	<b>&lt; 0.001</b>	<b>&lt; 0.001</b>	<b>&lt; 0.001</b>	<b>&lt; 0.001</b>	<b>&lt; 0.001</b>
11-oxoetiocholanolone EIA vs. Cortisol EIA		<b>-0.296</b>	<b>0.060</b>	<b>0.317</b>	<b>0.111</b>		
	<b>p-value</b>	<b>&lt; 0.001</b>	<b>0.006</b>	<b>&lt; 0.001</b>	<b>&lt; 0.001</b>		
		-0.135	0.010	<b>0.367</b>	<b>0.182</b>		
	<b>p-value</b>	0.1308	0.2559	<b>&lt; 0.001</b>	<b>&lt; 0.001</b>		

RIA: radioimmunoassay; EIA: enzyme immunoassay. \*11-oxoetiocholanolone 72T EIA measuring FGMs with 5β-3α-ol-11-one; \*\*11-oxoetiocholanolone 72a EIA measuring 11,17-dioxoandrostanes.

ecological studies and monitoring in chamois species (Schwarzenberger et al., 2000; Thaller et al., 2004; Hoby et al., 2006; Dalmau et al., 2007; Thaller, 2007; Corlatti et al., 2012; Zwijacz-Kozica et al., 2013; Corlatti et al., 2014; Hadinger et al., 2015; Corlatti, 2018; Fattorini et al., 2018a, 2018b; Formenti et al., 2018; Anderwald et al., 2021b). NIRS failed to consistently predict the FGM concentrations predicted by the reference laboratory methods used for calibration and validation. Moreover, the FGM concentrations determined by the four immunoassays tested did not correlate. While these results do not allow to recommend NIRS for the study of FGM in chamois, they also raise concern about the use of non-validated laboratory methods to analyse FGM in this and other species, generating doubts on the conclusions of previous studies.

The NIRS calibration required different spectral models to compare each immunoassay for FGM analysis, rather than a single model for all four techniques. The first derivative treatment performed best for most techniques, except for the cortisol EIA. The first and second derivatives are the most common forms in which spectra of agricultural products are displayed; third-order derivatives are possible but are rarely used to interpret spectra (Shenk and Westerhaus, 1996). The RPD values obtained for the NIRS validation of the four laboratory techniques for FGM analysis, ranging from 1.2 to 1.6, confirmed the low precision of the models developed (Table 3). For complex matrices, a prediction model with an RPD ≤ 1.9 is considered unsuitable and unable to provide precise predictions (Williams, 2014). These results agree with the low R<sup>2</sup> value (0.152 and 0.489) found when trying to validate NIRS for measuring FGM in chimpanzee (*Pan troglodytes*) faeces (Litman, 2016), conversely to a previous study suggesting the feasibility of NIRS to predict FGM in faeces of red deer (Santos et al., 2014). According to the low RPD values and the low correlation with the FGM concentrations obtained with the biologically validated 72T EIA, NIRS could not predict FGM concentration in chamois faeces.

The NIRS technique is based on the light absorption by the R-H groups (O-H, C-H, N-H, S-H, and so on) of organic molecules in the near-infrared range (750 to 2600 nm; Murray and Williams, 1987; Shenk and Westerhaus, 1996). The resulting spectra must be calibrated and validated in two independent sample subsets against reference laboratory methods (Font et al., 2007). Therefore, the more complex and variable the molecules detected by the reference methods are, the more variable, less reliable, and poorly adjusted the NIRS calibration and validation are. FGMs are molecularly variable after bile excretion and bacterial metabolism, and such variability combined with the relative cross-reactivity of the antibodies utilized in the immunoassays means that all the methodologies determine a mix of 'immunoreactive cortisol metabolites' (Möstl and Palme, 2002; Möstl et al., 2002; Pecorella et al., 2016). Differences in the FGM assemblage of the molecular groups identified by each immunoassay (e.g., 5β-3α-ol-11-one structure in the case of the 72T assay) and the O-H and C-H bonds detected by NIRS could also explain the lack of correlation between the four immunoassays and the NIRS predictions. Subtle differences, such as the direction or position of a specific chemical structures (e.g., 3α- or 3β-OH; or a



**Fig. 1.** Scatterplots among the faecal glucocorticoid metabolite laboratory tests used in this study for both Alpine (*Rupicapra rupicapra rupicapra*) and Pyrenean (*Rupicapra pyrenaica pyrenaica*) chamois. The Alpine chamois samples were analysed with the 11-oxoetiocholanolone 72T EIA, measuring FGMs with 5 $\beta$ -3 $\alpha$ -ol-11-one, whereas the Pyrenean chamois samples were analysed with the 11-oxoetiocholanolone 72a EIA, measuring 11,17-dioxoandrostanones.

5 $\alpha$ - or 5 $\beta$  steroid metabolite) may lead to significant differences in the values provided by each method (Palme, 2019). Furthermore, the presence of other compounds, such as fibres in the faecal matrix could significantly interfere with the NIRS predictions.

The gradient found in the  $R^2$  values of the NIRS validation for the four laboratory techniques assessed in this study agreed with the variability and potential cross-reactions of each method (Tables 2 and 3). Thus, the methods non-validated in chamois (125-I-Corticosterone RIA, cortisol EIA, and 11-oxoetiocholanolone 72a EIA) had lower  $R^2$  values than the 11-oxoetiocholanolone 72T EIA, which detects FGMs with a 5 $\beta$ -3 $\alpha$ -hydroxy-11-oxo structure (Möstl et al., 2002) and is the only method biologically validated for Alpine chamois (Palme, 2019; Anderwald et al., 2021a). This further supports the need to use validated tests when analysing FGMs (Palme, 2019). Nevertheless, the performance of NIRS validation was low even for the validated test, which could be explained by the variability mentioned above in the FGM assemblage, the differences in the molecular groups detected by each technique (Möstl et al., 2002; Palme, 2019), and the cross-reaction of each immunoreactive cortisol metabolite with each test (Möstl and Palme, 2002; Möstl et al., 2002; Pecorella et al., 2016). Moreover, the best-performing assay to detect FGMs may vary even between closely related species (Fanson et al., 2017), and FGM assemblages are not only species-specific but may also depend on individual, sex, reproductive status, season, and diet (Palme et al., 2005; Dantzer et al., 2016; Coppes et al., 2018; Palme, 2019), expanding on the idea of measuring FGMs as an art (Wudy et al., 2018). Conversely to Alpine chamois, no immunoassay has been biologically validated for Pyrenean chamois up

to date.

Previous reports on the agreement among different laboratory techniques used for FGM analyses are scarce and controversial (Pahuja and Narayan, 2023a, 2023b; Santamaria et al., 2023). The little correlation and the lack of normality of the residuals found in this study (Table 4, Fig. 1, Supplementary Material Fig. 1 to 7, and Supplementary Material Table 1) among four immunoassays previously used for physiological, population, conservation, and ecological studies and population monitoring in chamois (Schwarzenberger et al., 2000; Thaller et al., 2004; Hoby et al., 2006; Dalmau et al., 2007; Corlatti et al., 2012; Zwijacz-Kozica et al., 2013; Corlatti et al., 2014; Hadinger et al., 2015; Corlatti, 2018; Fattorini et al., 2018a, 2018b; Formenti et al., 2018; Anderwald et al., 2021b), with the same sample yielding either high or low values depending on immunoassay used, mean that the results and conclusions of these previous studies could depend on the analytical technique used. This raises concern about the reliability of the results and conclusions obtained by these studies, particularly those that used laboratory methods that have not been validated (Palme, 2019). The importance of assay selection has also been reported in other species (Hinchcliffe et al., 2021). Such conclusions and the recommendation for using validated methods to measure FGMs are applicable beyond the case-study species used for this experiment and raise concerns about the reliability and conclusions drawn from non-validated FGM determination techniques not only in chamois but in a wide range of species.

## 5. Conclusion

The determination of FGM concentration in the same faecal samples using different widely used laboratory techniques, and the attempted calibration and validation for the predictive NIRS, have unveiled inconsistencies in the results and values rendered by each technique. The inconsistencies are probably related to the different antibodies used in each assay, and the different cross-reactivities of these antibodies with the assemblage of faecal immunoreactive cortisol metabolites. These results do not allow to recommend NIRS for FGM measurement in chamois faeces. Biologically validated tests are recommended to assess FGMs concentrations (Palme, 2019). However, while methods that do not produce reliable results when physiologically and/or biologically validated should be discarded, methods where validation has not even been attempted yet remain in a grey zone until biological validation tests confirm or refute their potential reliability. Extensive comparative assessment, cross-calibration, and validation of protocols and methodologies in different species, populations, conditions, sample collection, and management are essential to select the most suitable method for FGM determination in each wildlife species.

## 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.

## Data availability

Data will be made available on request.

## Acknowledgements

The authors are indebted to Patrick Schleppe for lyophilizing the Alpine chamois samples at his lab at WSL Birmensdorf, Julia Paterno, Claudio Schorta for conducting the Alpine chamois extractions, and Edith Klobetz-Rassam for EIA analyses of the Alpine chamois. Ruedi Haller helped at all stages of the project and numerous trained interns assisted with sample collection in the field at the Swiss National Park. The authors are also grateful to the Servei de Bioquímica Clínica Veterinària and the Endocrinology, Reproductive Physiology and Animal Welfare Research Group at the Veterinary School in the Universitat Autònoma de Barcelona for their collaboration in the analysis of faecal glucocorticoid metabolites in the Pyrenean chamois samples. Our beloved and missed friends Miriam Villamuelas and Blas Sánchez significantly contributed in the field and laboratory work before sadly passing away during the development of this study. A long list of collaborators and volunteers at the Servei d'Ecopatologia de Fauna Salvatge (SEFaS), the Wildlife Ecology & Health (WE&H) research group, and the Group of Research in Ruminants (G2R), participated in the field collection and NIRS analyses. Their essential contribution is deeply acknowledged.

## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.gecco.2024.e02832](https://doi.org/10.1016/j.gecco.2024.e02832).

## References

- Anderwald, P., Ambarli, H., Avramov, S., Ciach, M., Corlatti, L., Farkas, A., Jovanovic, M., Papaioannou, H., Peters, W., Sarasa, M., Šprem, N., Weinberg, P., Willisch, C., 2021a. *Rupicapra rupicapra* (amended version of 2020 assessment). The IUCN Red List of Threatened Species 2021: e.T39255A195863093. <https://dx.doi.org/10.2305/IUCN.UK.2021-1.RLTS.T39255A195863093.en>. (Accessed on 14 January 2022).

- Anderwald, P., Campell Andri, S., Palme, R., 2021. Reflections of ecological differences? Stress responses of sympatric Alpine chamois and red deer to weather, forage quality, and human disturbance. *Ecol. Evol.* 11 (22), 15740–15753. <https://doi.org/10.1002/ece3.8235>.
- Angelucci, S., Antonucci, A., Di Tana, F., Innocenti, M., Di Domenico, G., Madonna, L., Smoglica, C., Di Francesco, C.E., López-Olvera, J.R., 2023. Welfare and clinical assessment on physical captures followed by anesthesia in Apennine chamois (*Rupicapra pyrenaica ornata*). *Animals* 13, 460. <https://doi.org/10.3390/ani13030460>.
- Broom, D.M., Johnson, K.G., 1993. *Stress and animal welfare*. Chapman and Hall, London, United Kingdom, p. 174.
- Busch, D.S., Hayward, L.S., 2009. Stress in a conservation context: a discussion of glucocorticoid actions and how levels change with conservation-relevant variables. *Biol. Conserv.* 142, 2844–2853. <https://doi.org/10.1016/j.biocon.2009.08.013>.
- Catusse, M., Corti, R., Cugnase, J.M., Dubray, D., Gibert, P., Michellet, J., 1996. Les chamois et les isards. In: *Le grande faune de montagne*. Haitier Littérature Générale, Paris, France, pp. 17–69.
- Cen, H., He, Y., 2007. Theory and application of near infrared reflectance spectroscopy in determination of food quality. *Trends Food Sci. Tech.* 18, 72–83. <https://doi.org/10.1016/j.tifs.2006.09.003>.
- Coppes, J., Kämmerle, J.L., Willert, M., Kohnen, A., Palme, R., Braunisch, V., 2018. The importance of individual heterogeneity for interpreting faecal glucocorticoid metabolite levels in wildlife studies. *J. Appl. Ecol.* 55, 2043–2054. <https://doi.org/10.1111/1365-2664.13140>.
- Corlatti, L., 2018. Fecal cortisol metabolites under anonymized sampling: Robust estimates despite significant individual heterogeneity. *Ecol. Indic.* 95 (1), 775–780. <https://doi.org/10.1016/j.ecolind.2018.08.028>.
- Corlatti, L., Béthaz, S., von Hardenberg, A., Bassano, B., Palme, R., Lovari, S., 2012. Hormones, parasites and male mating tactics in Alpine chamois: identifying the mechanisms of life history trade-offs. *Anim. Behav.* 84 (4), 1061–1070. <https://doi.org/10.1016/j.anbehav.2012.08.005>.
- Corlatti, L., Palme, R., Lovari, S., 2014. Physiological response to etho-ecological stressors in male Alpine chamois: timescale matters! *Naturwissenschaften* 101, 577–586. <https://doi.org/10.1007/s00114-014-1195-x>.
- Corlatti, L., Lorenzetti, C., Bassano, B., 2019. Parasitism and alternative reproductive tactics in Northern chamois. *Ecol. Evol.* 9, 8749–8758. <https://doi.org/10.1002/ece3.5427>.
- Dalmay, A., Ferret, A., Chacón, G., Manteca, X., 2007. Seasonal changes in fecal cortisol metabolites in Pyrenean chamois. *J. Wildl. Manag.* 71 (1), 190–194. <https://doi.org/10.2193/2005-492>.
- Dantzer, B., Santicchia, F., van Kesteren, F., Palme, R., Martinoli, A., Wauters, L.A., 2016. Measurement of fecal glucocorticoid metabolite levels in Eurasian red squirrels (*Sciurus vulgaris*): effects of captivity, sex, reproductive condition, and season. *J. Mammal.* 97 (5), 1385–1398. <https://doi.org/10.1093/jmammal/gyw095>.
- Dixon, R., Coates, D., 2009. Near infrared spectroscopy of faeces to evaluate the nutrition and physiology of herbivores. *J. Infrared Spec.* 17, 1–31. <https://doi.org/10.1255/jnirs.822>.
- Dryden, G.M., 2003. Near infrared reflectance spectroscopy: applications in deer nutrition. Rural Industries Research and Development Corporation (RIRDC), Kingston, Australia.
- Donini, V., Iacona, E., Pedrotti, L., Macho-Maschler, S., Palme, R., Corlatti, L., 2022. Temporal stability of fecal cortisol metabolites in mountain-dwelling ungulates. *Naturwissenschaften* 109, 20. <https://doi.org/10.1007/s00114-022-01792-y>.
- Fanson, K.V., Best, E.C., Bunce, A., Fanson, B.G., Hogan, L.A., Keeley, T., Narayan, E.J., Palme, R., Parrotti, M.L., Sharp, T.M., Skogvold, K., Tuthill, L., Webster, K.N., Bashaw, M., 2017. One size does not fit all: Monitoring faecal glucocorticoid metabolites in marsupials. *Gen. Comp. Endocrinol.* 244, 146–156. <https://doi.org/10.1016/j.ygcen.2015.10.011>.
- Fattorini, N., Brunetti, C., Baruzzi, C., Macchi, E., Pagliarello, M.C., Pallari, N., Lovari, S., Ferretti, F., 2018. Being “hangry”: food depletion and its cascading effects on social behaviour. *Biol. J. Linn. Soc.* 125, 640–656.
- Fattorini, N., Lovari, S., Brunetti, C., Baruzzi, C., Cotza, A., Macchi, E., Pagliarella, M.C., Ferretti, F., 2018. Age, seasonality, and correlates of aggression in female Apennine chamois. *Behav. Ecol. Sociobiol.* 72, 171. <https://doi.org/10.1007/s00265-018-2584-5>.
- Foley, W.J., McIlwee, A., Lawler, I., Aragones, L., Woolnough, A.P., Berding, N., 1998. Ecological applications of near infrared reflectance spectroscopy – a tool for rapid, cost-effective prediction of plant and animal tissues and aspects of animal performance. *Oecologia* 116, 293–305. <https://doi.org/10.1007/s004420050591>.
- Font, R., Río-Celestino, M., Haro-Bailón, A., 2007. Near-Infrared Reflectance Spectroscopy: methodology and potential for predicting trace elements in plants. In: Willey, N. (Ed.), *Phytomediation. Methods and Reviews*. Series Methods in Biotechnology, 23. Humana Press Inc., Totowa, New Jersey, USA, pp. 205–210. [https://doi.org/10.1007/978-1-59745-098-0\\_17](https://doi.org/10.1007/978-1-59745-098-0_17).
- Formenti, N., Viganò, R., Fraquelli, C., Trogu, T., Bonfanti, M., Lanfranchi, P., Palme, R., Ferrari, N., 2018. Increased hormonal stress response of Apennine chamois induced by interspecific interactions and anthropogenic disturbance. *Eur. J. Wildl. Res.* 64, 68. <https://doi.org/10.1007/s10344-018-1228-4>.
- Fowler, J., Cohen, L., Jarvis, P., 2009. *Practical statistics for field biology*. John Wiley & Sons, Chichester, United Kingdom, p. 132.
- Gálvez-Cerón, A., Serrano, E., Bartolomé, J., Mentaberre, G., Fernández-Aguilar, X., Fernández-Sirera, L., Navarro-González, N., Gassó, D., López-Olvera, J.R., Lavín, S., Marco, I., Albanell, E., 2013. Predicting seasonal and spatial variations in diet quality of Pyrenean chamois (*Rupicapra pyrenaica pyrenaica*) using near infrared reflectance spectroscopy. *Eur. J. Wildl. Res.* 59, 115–121. <https://doi.org/10.1007/s10344-012-0672-9>.
- Garrido-Amaro, C., Jolles, A., Velarde, R., López-Olvera, J.R., Serrano, E., 2023. The synzootic potential of common epidemics in chamois populations. *Eur. J. Wildl. Res.* 69 (4), 79. <https://doi.org/10.1007/s10344-023-01700-3>.
- Hadinger, U., Haymerle, A., Knauer, F., Schwarzenberger, F., Walzer, C., 2015. Faecal cortisol metabolites to assess stress in wildlife: evaluation of a field method in free-ranging chamois. *Methods Ecol. Evol.* 6 (11), 1349–1357. <https://doi.org/10.1111/2041-210X.12422>.
- Heimbürge, S., Kanitz, E., Otten, W., 2019. The use of hair cortisol for the assessment of stress in animals. *Gen. Comp. Endocrinol.* 270, 10–17. <https://doi.org/10.1016/j.ygcen.2018.09.016>.
- Heise, H.M., Winzen, R., 2002. Fundamental chemometric methods. In: Siesler, H.W., Ozaki, Y., Kawata, S., Heise, H.M. (Eds.), *Near-Infrared Spectroscopy: Principles, Instruments, Applications*. Wiley-VCH, Weinheim, Germany, pp. 125–162.
- Herrero, J., Lovari, S., Nores, C., Toigo, C., 2020. *Rupicapra pyrenaica*. The IUCN Red List of Threatened Species 2020: e.T19771A171131310. <https://dx.doi.org/10.2305/IUCN.UK.2020-2.RLTS.T19771A171131310.en>. (Accessed on 14 January 2022).
- Hibert, F., Maillard, D., Fritz, H., Garell, M., Abdou, H.N., Winterton, P., 2011. Ageing of ungulate pellets in semi-arid landscapes: how the shade of colour can refine pellet-group counts. *Eur. J. Wildl. Res.* 57, 495–503. <https://doi.org/10.1007/s10344-010-0458-x>.
- Hinchcliffe, D.L., Lea, J.M.D., Palme, R., Shultz, S., 2021. Faecal glucocorticoid metabolites as biomarkers in equids: assay choice matters. *J. Wildl. Manag.* 85, 1175–1186. <https://doi.org/10.1002/jwmv.22066>.
- Hoby, S., Schwarzenberger, F., Dohr, M.G., Robert, N., Walzer, C., 2006. Steroid hormone related male biased parasitism in chamois *Rupicapra rupicapra rupicapra*. *Vet. Parasitol.* 138, 337–348. <https://doi.org/10.1016/j.vetpar.2006.01.028>.
- Kho, E.A., Fernandes, J.N., Tilbrook, A.J., Fox, G.P., Sikulu-Lord, M.T., Kotze, A.C., Beasley, A.M., James, P.J., Tolleson, D.R., Cozzolino, D., 2023. State of the art and the future of fecal analysis using infrared spectroscopy. *Appl. Spectrosc. Rev.* 58, 755–785. <https://doi.org/10.1080/05704928.2022.2143795>.
- Kinoshita, K., Miyazaki, M., Morita, H., Vassileva, M., Tang, C., Li, D., Ishikawa, O., Kusunoki, H., Tsenkova, R., 2012. Spectral pattern of urinary water as a biomarker of estrus in the giant panda. *Sci. Rep.* 2, 856. <https://doi.org/10.1038/srep00856>.
- Koren, L., Whiteside, D., Fahlman, A., Ruckstuhl, K., Kutz, S., Checkley, S., Dumond, M., Wynne-Edwards, K., 2012. Cortisol and corticosterone independence in cortisol-dominant wildlife. *Gen. Comp. Endocrinol.* 177, 113–119. <https://doi.org/10.1016/j.ygcen.2012.02.020>.
- Kumar, V., Umaphathy, G., 2019. Non-invasive monitoring of steroid hormones in wildlife for conservation and management of endangered species – A review. *Indian J. Exp. Biol.* 57, 307–314. <http://nopr.niscares.in/handle/123456789/47171>.
- Litman, B., 2016. Rapid analysis of fecal glucocorticoid metabolites: testing an alternative method for measuring stress markers in chimpanzees. Master Thesis, Florida Atlantic University. [https://fau.digital.flvc.org/islandora/object/fau\\_%3A333697/datastream/OBJ/view/Rapid\\_Analysis\\_of\\_Fecal\\_Glucocorticoid\\_Metabolites\\_Testing\\_an\\_Alternative\\_Method\\_for\\_Analyzing\\_Stress\\_Markers\\_in\\_Chimpanzees.pdf](https://fau.digital.flvc.org/islandora/object/fau_%3A333697/datastream/OBJ/view/Rapid_Analysis_of_Fecal_Glucocorticoid_Metabolites_Testing_an_Alternative_Method_for_Analyzing_Stress_Markers_in_Chimpanzees.pdf).

- López-Olvera, J.R., Marco, I., Montané, J., Lavín, S., 2006. Haematological and serum biochemical values of Southern chamois (*Rupicapra pyrenaica*) captured by drive-net. *Vet. Rec.* 158 (14), 479–484. <https://doi.org/10.1136/vr.158.14.479>.
- López-Olvera, J.R., Marco, I., Montané, J., Lavín, S., 2006. Transport stress in Southern chamois (*Rupicapra pyrenaica*) and its modulation by acepromazine. *Vet. J.* 172 (2), 347–355. <https://doi.org/10.1016/j.tvjl.2005.06.007>.
- López-Olvera, J.R., Marco, I., Montané, J., Casas-Díaz, E., Lavín, S., 2007. Effects of acepromazine on the stress response in Southern chamois (*Rupicapra pyrenaica*) captured by means of drive-nets. *Can. J. Vet. Res.* 71 (1), 41–51.
- López-Olvera, J.R., Marco, I., Montané, J., Casas-Díaz, E., Mentaberre, G., Lavín, S., 2009. Comparative evaluation of effort, capture and handling effects of drive nets to capture roe deer (*Capreolus capreolus*), Southern chamois (*Rupicapra pyrenaica*) and Spanish ibex (*Capra pyrenaica*). *Eur. J. Wildl. Res.* 55, 193–202. <https://doi.org/10.1007/s10344-008-0232-5>.
- Lyons, R.K., Stuth, J.W., 1992. Fecal NIRS equations for predicting diet quality of free-ranging cattle. *J. Range Manag.* 45, 238–244. <https://doi.org/10.2307/4002970>.
- Millspaugh, J.J., Washburn, B.E., 2004. Use of fecal glucocorticoid metabolite measures in conservation biology research: considerations for application and interpretation. *Gen. Comp. Endocrinol.* 138 (3), 189–199. <https://doi.org/10.1016/j.ygcen.2004.07.002>.
- Moberg, G.P., Mench, J.A. (Eds.), 2000. *The biology of animal stress: basic principles and implications for animal welfare*. CABI Publishing, Wallingford, New York, USA, p. 377.
- Morgan, L.R., Marsh, K.J., Tolleson, D.R., Youngentob, K.N., 2021. The application of NIRS to determine animal physiological traits for wildlife management and conservation. *Remote Sens.* 13, 3699. <https://doi.org/10.3390/rs13183699>.
- Mormède, P., Andanson, S., Aupérin, B., Beerda, B., Guémené, D., Malmkvist, J., Manteca, X., Manteuffel, G., Prunet, P., van Reenen, C.G., Richard, S., Veissier, I., 2007. Exploration of the hypothalamic-pituitary-adrenal function as a tool to evaluate animal welfare. *Physiol. Behav.* 92 (3), 317–339. <https://doi.org/10.1016/j.physbeh.2006.12.003>.
- Möstl, E., Palme, R., 2002. Hormones as indicators of stress. *Domest. Anim. Endocrin.* 23, 67–74. [https://doi.org/10.1016/S0739-7240\(02\)00146-7](https://doi.org/10.1016/S0739-7240(02)00146-7).
- Möstl, E., Maggs, J.L., Schrötter, G., Besenfelder, U., Palme, R., 2002. Measurement of cortisol metabolites in faeces of ruminants. *Vet. Res. Commun.* 26 (2), 27–39. <https://doi.org/10.1023/a:1014095618125>.
- Murray, I., Williams, P.C., 1987. *Chemical principles of near infra-red technology*. In: Williams, P.C., Norris, K. (Eds.), *Near-infrared technology in the agricultural and food industries*. American Association of Cereal Chemists Inc, St. Paul, MN, USA, pp. 17–34.
- Murtagh, R., Behringer, V., Deschner, T., 2013. LC-MS as a method for non-invasive measurement of steroid hormones and their metabolites in urine and faeces of animals. *Wien. Tierärztl. Monat. – Vet. Med. Austria* 100, 247–254.
- Næs, T., Isaksson, T., Fearn, T., Davies, T., 2002. *A user-friendly guide to multivariate calibration and classification*. NIR Publications, Chichester, United Kingdom, p. 344.
- Pahuja, H., Narayan, E., 2023. Discussion paper on "Comparing the agreement of a commercial cortisol kit with a biologically validated assay in evaluating faecal cortisol metabolite levels in koala joeys". *Comp. Biochem. Phys. A* 280, 111417. <https://doi.org/10.1016/j.cbpa.2023.111417>.
- Pahuja, H.K., Narayan, E.J., 2023. Comparing the agreement of a commercial cortisol kit with a biologically validated assay in evaluating faecal cortisol metabolite levels in koala joeys. *Comp. Biochem. Phys. A* 276, 111353. <https://doi.org/10.1016/j.cbpa.2022.111353>.
- Palme, R., 2012. Monitoring stress hormone metabolites as a useful, non-invasive tool for welfare assessment in farm animals. *Anim. Welf.* 21, 331–337. <https://doi.org/10.1016/09627286.21.3.331>.
- Palme, R., 2019. Non-invasive measurement of glucocorticoids: advances and problems. *Physiol. Behav.* 199, 229–243. <https://doi.org/10.1016/j.physbeh.2018.11.021>.
- Palme, R., Möstl, E., 1997. Measurement of cortisol metabolites in faeces of sheep as a parameter of cortisol concentration in blood. *Z. Säugetierkd.* 62(Suppl 2), pp. 192–197.
- Palme, R., Robia, C., Messmann, S., Hofer, J., Möstl, E., 1999. Measurement of faecal cortisol metabolites in ruminants: A non-invasive parameter of adrenocortical function. *Wien. Tierärztl. Monat.* 86, pp. 237–241.
- Palme, R., Rettenbacher, S., Touma, C., El-Bahr, S.M., Möstl, E., 2005. Stress hormones in mammals and birds: comparative aspects regarding metabolism, excretion and noninvasive measurement in fecal samples. *Ann. Ny. Acad. Sci.* 1040, 162–171. <https://doi.org/10.1196/annals.1327.021>.
- Palme, R., Touma, C., Arias, N., Dominchin, M.F., Lepschy, M., 2013. Steroid extraction: Get the best out of faecal samples. *Wien. Tierärztl. Monat. – Vet. Med. Austria* 100, 238–246.
- Pecorella, I., Ferretti, F., Sforzi, A., Macchi, E., 2016. Effects of culling on vigilance behaviour and endogenous stress response of female fallow deer. *Wildl. Res* 43 (3), 189–196. <https://doi.org/10.1071/WR15118>.
- Pérez, J.M., Molina, L., Ureña-Gutiérrez, B., Espinosa, J., López-Montoya, A.J., Boos, M., Granados, J.E., Cano-Manuel, F.J., Azorit, C., 2019. Individual stress responses to *Sarcoptes scabiei* infestation in Iberian ibex, *Capra pyrenaica*. *Gen. Comp. Endocrinol.* 281, 1–6. <https://doi.org/10.1016/j.ygcen.2019.05.007>.
- Pérez, J.M., Espinosa, J., Boos, M., Azorit, C., Molina, L., López-Montoya, A.J., 2020. Evaluation of long-term 11-oxo-androsterone stability in red deer faecal samples under different storage conditions. *Eur. J. Wildl. Res.* 66, 56. <https://doi.org/10.1007/s10344-020-01399-6>.
- R Development Core Team 4.1.3, 2022. R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. <http://www.R-project.org/>.
- Roberts, C.A., Workman Jr., J., Reeves, J.B., 2004. Near-infrared spectroscopy in agriculture. American Society of Agronomy, Inc./Crop Science Society of America, Inc./Soil Science Society of America, Inc., Madison, WI, USA, p. 822.
- Roggo, Y., Chaluz, P., Maurer, L., Lema-Martinez, C., Edmond, A., Jent, N., 2007. A review of near infrared spectroscopy and chemometrics in pharmaceutical technologies. *J. Pharm. Biomed.* 44, 683–700. <https://doi.org/10.1016/j.jpba.2007.03.023>.
- Santamaria, F., Palme, R., Schlagloth, R., 2023. Discussion paper on "Comparing the agreement of a commercial cortisol kit with a biologically validated assay in evaluating faecal cortisol metabolite levels in koala joeys". *Comp. Biochem. Phys. A* 280, 111416. <https://doi.org/10.1016/j.cbpa.2023.111416>.
- Santos, J.P.V., Vicente, J., Villamuelas, M., Albanell, E., Serrano, E., Carvalho, J., Fonseca, C., Gortázar, C., López-Olvera, J.R., 2014. Near infrared reflectance spectroscopy (NIRS) for predicting glucocorticoid metabolites in lyophilised and oven-dried faeces of red deer. *Ecol. Indic.* 45, 522–528. <https://doi.org/10.1016/j.ecolind.2014.05.021>.
- Schwarzenberger, F., 2007. The many uses of non-invasive faecal steroid monitoring in zoo and wildlife species. *Int. Zoo. Yearb.* 41 (1), 52–74. <https://doi.org/10.1111/j.1748-1090.2007.00017.x>.
- Schwarzenberger, F., Walzer, C., Weiss, R., Pucher, H.E., Leus, K., Bircher, P., Moran, J.F., 2000. Faecal cortisol metabolites in response to management practices and disturbance in different herbivores and carnivores (*Ceratotherium simum*, *Diceros bicornis*, *Elephas maximus*, *Loxodonta africana*, *Okapia johnstoni*, *Rupicapra rupicapra* and *Lutra lutra*). In: Proceedings of the 3rd scientific meeting of the European Association of Zoo and Wildlife Veterinarians (EAZWV), 31 May–4 June 2000, Paris, France, pp. 201–204.
- Selye, H., 1946. The general adaptation syndrome and the diseases of adaptation. *J. Clin. Endocrinol.* 6, 117–230. <https://doi.org/10.1210/jcem-6-2-117>.
- Shenk, J.S., Westerhaus, M.O., 1996. Calibration the ISI way. In: Davies, A.M.C., Williams, P.C., (Eds.), *Near Infrared Spectroscopy: the future waves*. NIR Publications, Chichester, United Kingdom, pp. 198–202.
- Sheriff, M.J., Dantzer, B., Delehanty, B., Palme, R., Boonstra, R., 2011. Measuring stress in wildlife: techniques for quantifying glucocorticoids. *Oecologia* 166, 869–887. <https://doi.org/10.1007/s00442-011-1943-y>.
- Siesler, H.W., Ozaki, Y., Kawata, S., Heise, H.M., 2002. *Near-Infrared Spectroscopy: principles, instruments, applications*. Wiley-VCH, Weinheim, Germany, p. 361.
- Talló-Parra, O., Albanell, E., Carbajal, A., Monclús, L., Sabés-Alsina, M., Riba, C., Martin, M., Abelló, M., López-Béjar, M., 2015a. Prediction of faecal cortisol metabolites from Western lowland gorilla (*Gorilla gorilla gorilla*) by near infrared reflectance spectroscopy (NIRS). In Proceedings of the 5th ISWE Conference, Berlin, Germany, p. 61.
- Talló-Parra, O., Manteca, X., Sabés-Alsina, M., Carbajal, A., López-Béjar, M., 2015. Hair cortisol detection in dairy cattle by using EIA: protocol validation and correlation with faecal cortisol metabolites. *Animal* 9 (6), 1059–1064. <https://doi.org/10.1017/S1751731115000294>.

- Taylor, W., 1971. The excretion of steroid hormone metabolites in bile and feces. *Vitam. Horm.* 29, 201–285. [https://doi.org/10.1016/S0083-6729\(08\)60050-3](https://doi.org/10.1016/S0083-6729(08)60050-3).
- Terlouw, E.M., Schouen, W.G.P., Ladewig, J., 1997. Physiology. In: Appleby, M.C., Hughes, B.O. (Eds.), *Animal welfare*. CAB International. University Press, Cambridge, United Kingdom, pp. 143–158.
- Thaller, D., 2007. Jahreszeitliche Anpassungsmechanismen und Mögliche Auswirkungen Anthropogener Störreize bei Gämsen (*Rupicapra r. rupicapra*). PhD Thesis, Veterinärmedizinischen Universität Wien, Wien, Austria.
- Thaller, D., Schwarzenberger, F., Bögel, R., Slotta-Bachmayr, L., Walzer, C., Arnold, W., 2004. Seasonal variation in cortisol secretion in chamois (*Rupicapra rupicapra*). In: Proceedings of the 5th scientific meeting of the European Association of Zoo and Wildlife Veterinarians (EAZVV). 19–23 May 2004, Ebeltoft, Denmark, pp. 283–284.
- Touma, C., Palme, R., 2005. Measuring fecal glucocorticoid metabolites in mammals and birds: the importance of validation. *Ann. Ny. Acad. Sci.* 1046, 54–74. <https://doi.org/10.1196/annals.1343.006>.
- Vendrame, P.R.S., Marchão, R.L., Brunet, D., Becquer, T., 2012. The potential of NIR spectroscopy to predict soil texture and mineralogy in Cerrado Latosols. *Eur. J. Soil Sci.* 63, 743–753. <https://doi.org/10.1111/j.1365-2389.2012.01483.x>.
- Wasser, S.K., Hunt, K.E., Brown, J.L., Cooper, K., Crocket, C.M., Bechert, U., Millspaugh, J.J., Larson, S., Monfort, S.L., 2000. A generalized fecal glucocorticoid assay for use in a diverse array of nondomestic mammalian and avian species. *Gen. Comp. Endocrinol.* 120 (3), 260–275. <https://doi.org/10.1006/gcen.2000.7557>.
- Wikelski, M., Cooke, S.J., 2006. Conservation physiology. *Trends Ecol. Evol.* 21, 38–46. <https://doi.org/10.1016/j.tree.2005.10.018>.
- Williams, P., 2014. Tutorial: the RPD statistic: a tutorial note. *NIR N.* 25 (1), 22. <https://doi.org/10.1255/nirn.1419>.
- Williams, P.C., 2001. Implementation of near infrared technology. In: Williams, P.C., Norris, K.H., (Eds.), *Near-infrared technology in the agricultural and food industries*. American Association of Cereal Chemists, St. Paul, MN, USA, pp. 145–169.
- Williams, P.C., Norris, K.H., 2001. *Near-infrared technology in the agricultural and food industries*, second ed. American Association of Cereal Chemists, St. Paul, MN, USA.
- Williams, P.C., Sobering, D.C., 1996. How do we do it?: a brief summary of the methods we use in developing near infrared calibrations. In: Davies, A.M.C., Williams, P.C. (Eds.), *Near Infrared Spectroscopy: The Future Waves*. NIR Publications, Chichester, West Sussex, United Kingdom, pp. 185–188.
- Wudy, S.A., Schuler, G., Sánchez-Guijo, A., Hartmann, M.F., 2018. The art of measuring steroids: principles and practice of current hormonal steroid analysis. *J. Steroid Biochem.* 179, 88–103. <https://doi.org/10.1016/j.jsbmb.2017.09.003>.
- Zhang, D., 2017. A coefficient of determination for generalized linear models. *Am. Stat.* 71 (4), 310–316. <https://doi.org/10.1080/00031305.2016.1256839>.
- Zuur, A.F., Ieno, E.N., Elphick, C., 2010. A protocol for data exploration to avoid common statistical problems. *Methods Ecol. Evol.* 1, 3–14. <https://doi.org/10.1111/j.2041-210X.2009.00001.x>.
- Zwijacz-Kozica, T., Selva, N., Barja, I., Silván, G., Martínez-Fernández, L., Illera, J.C., Jodłowski, M., 2013. Concentration of fecal cortisol metabolites in chamois in relation to tourist pressure in Tatra National Park (South Poland). *Acta Theriol.* 58, 227–235. <https://doi.org/10.1007/s13364-012-0108-7>.