Evaluation of long-term 11-oxoetiocholanolone stability in red deer faecal samples under different storage conditions

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Abstract

Faecal cortisol metabolite (FCM) analysis is a reliable non-invasive method used in field endocrinology studies to assess levels of stress in animals. It is known that weather and, above all humidity, can affect FCM concentrations in faeces. As well, the prolonged storage of samples and delay in their analysis may increase or decrease metabolite concentrations. Intrinsic factors such as the heterogeneous distribution of FCMs within scats may likewise cause intra-sample variation. All of these sources of variation in FCM concentrations need to be addressed if we are to interpret results correctly. The aim of this study was to assess the effects of lyophilisation and storage temperature on the long-term stability of 11-oxoetiocholanolone (11-o) in red deer (*Cervus elaphus*) faecal samples. After pre-cleaning with hexane and extraction with methanol, 11-o levels were calculated using high-performance liquid chromatography coupled with tandem mass spectrometry HPLC-MS/MS at 1, 2, 4, 6, 8, 16 and 32 weeks post-collection. We used linear mixed models to explore the effects of temperature and storage time on concentrations of faecal 11-o in wet and dry samples. Our results showed significant variations in 11-o concentrations in wet faecal samples over time and at different storage temperatures. However, after lyophilisation, storage time was found not to affect observed 11-o concentrations. By contrast, the 11-o values of dry samples were more stable in terms of storage temperatures, with values for samples kept at -80 ºC being highest at the beginning and at the end of the study period. Lyophilising red deer faecal samples and storage at -80ºC guarantees the stability of 11-o for several months.

Keywords: *Cervus elaphus*, faecal 11-o, HPLC-MS/MS, non-invasive monitoring, storage conditions, stability, stress index

Introduction

The welfare of wild animal species is of increasing concern in research involving disciplines that use them as experimental models. This affects both free-ranging animals and those kept in captivity. Thus, measuring stress is crucial for monitoring the welfare of experimental animals and complying with current legislation (Terio et al. 2002; Lexen et al. 2008).

Cortisol is the main stress biomarker in ruminants (Touma and Palme 2005) but after being metabolized by liver and gut microbiota it is practically absent and therefore undetectable in faeces (Palme and Möstl 1997; Dehnhard et al. 2001). Currently, the non-invasive characterization of stress is increasingly being based on the analysis of faecal cortisol metabolites (FCM) (Palme 2019). Of these FCMs, 11- and 17-dioxoandrostanes (in particular, 11-oxoetiocholanolone) (in the bibliography its former name of 11-ketoetiocholanolone is still found) are regarded as major faecal cortisol metabolites in ruminants (Palme and Möstl 1997; Palme et al. 1999; Bahr et al. 2000; Keay et al. 2006; Hadinger et al. 2015; Allwin et al. 2016). Several studies focussed on wild ungulates have revealed evidence of a clear seasonal pattern in FCM excretion (Huber et al. 2003; Dalmau et al. 2007; Allwin et al. 2016).

When working with non-fresh faecal samples, environmental conditions (above all, humidity) influence microbial activity and so alter FCM measurements (Washburn and Millspaugh 2002). Aerial oxidation of the glucocorticoid side-chain may also occur (Edmonds et al. 2006). Furthermore, circumstances occurring after the collection of biological samples (changes occurring *ex vivo*: e.g. the storage method) can influence the measurement of hormone concentrations, leading to analytical and pre-analytical variability hindering the correct interpretation of data (Bielohuby et al. 2012). The designs of field endocrinology studies thus need to quantify the effects of intra-sample variation if they are to (a) identify sources of error correctly (Millspaugh and Washburn 2003; Descovich et al. 2012) and (b) detect individual and sex-specific variations (Pérez et al. 2019) and the potentially uneven distribution of metabolites in faeces (Hadinger et al. 2015).

From a logistical point of view, the lack of specific laboratory equipment (e.g. an ultra-freezer) or the remoteness of a study area may affect the study design, the selection of the most appropriate methods for sample storage, the quantification of the stress, and/or the timing of the sample analysis (Gholib et al. 2018). Yet, the possibility of storing samples over a period of time does allow us to optimize comparative analyses. The aim of our study was thus to assess the effect of different methods of

long-term storage on the stability of 11-oxoetiocholanolone (used as a stress index) in red deer (*Cervus elaphus*) faecal samples.

Materials and methods

Sample collection, pooling and subsample storage

Fresh faecal samples were collected manually from the rectums of 20 adult female red deer shot during a *montería* (a traditional collective hunting method common in southern Spain) in Hornos de Segura (Sierra de Cazorla Segura y Las Villas Natural Park, S Spain) (38º13'00''N – 2º43'09''W). Samples were placed in individual hermetic plastic bags, labelled, stored in carbonic ice, and then transported to the laboratory, where they were immediately mixed and homogenized to obtain a faecal powder. Subsequently, the pool was divided into two parts, one of which was lyophilised (dry fraction) and the other not (wet fraction). Then, each of the two parts was divided into four sub-subsamples: the first was stored at -80º C, the second at -20º C, the third at 4º C and the fourth at room (ambient) temperature.

Determination of 11-o concentration

One of the main cortisol metabolites, 11-oxoetiocholanolone (11-o), was quantified in these faecal samples using the analytical method described by Azorit et al. (2012) and optimized by Molina-García et al. (2018). Briefly, this method consists of pre-cleaning with hexane, the extraction of faecal glucocorticoid metabolites (FGCM) with methanol, the purification and pre-concentration of the FGCM extracts, and finally analysis with high-performance liquid chromatography coupled with tandem mass spectrometry HPLC-MS/MS. This process allows for the unequivocal determination of 11-o concentrations. This methodology was used to measure 11-o concentrations in each of the eight subsamples (four wet and four dry) at 1, 2, 4, 6, 8, 16 and 32 weeks post-collection. We carried out two 11-o determinations for each subsample and average values were used for further comparisons. The analytical validation of this method, its precision and linearity and the limit of its detection indicators have been determined and reported by Molina García et al. (2018).

Statistical analysis

In order to perform a descriptive analysis of the data, we drew time-series plots of the dependent variables at each temperature and for the two sample states (dry and wet) using the *ggplot* function in the R package *ggplot2* (Wickham 2016). We set up two separate datasets to analyse the data: dry and wet samples. For both datasets, we explored the analysis of variance (ANOVA) using a linear mixed model (LMMs) procedure for longitudinal data analysis (McCullagh and Nelder 1989; Zuur et al. 2009).

We tested the effects of treatments (temperature and time) on the response variable (11-o concentrations) in wet and dry samples separately. For the covariance structure of the two linear mixed models, we selected the Compound Symmetry covariance structure for repeated measures matrix from several correlation structures using the *gls function* in the *nlme* package in R (Pinheiro et al. 2013). The covariance structure was selected using an automatic selection procedure based on Akaike's Information Criterion using the *anova.lme* function in the *nlme* package.

To study the effects of the interaction between temperature and time on 11-o concentrations, we tested the significance of each effect at each level of the interacting effect. For the significance of each pair-wise comparison with interaction effects, we used Tukey's Honesty Significant Difference adjustment for the whole pair-wise comparisons using the *glht* function with the *multcomp* package in R (Hothorn et al. 2008).

We used R software 3.3.2 (R Development Core Team, 2017) to conduct all the statistical analyses. The significance level was set to $\alpha = 0.05$.

Results

The mean, standard deviation and coefficient of variation obtained for intra- and inter-assay analyses of the high and low concentration pools are shown in Table 1.

The dynamics of 11-o values in the different subsamples over time can be seen in Figure 1. In all cases except wet samples stored at room temperature, there was an initial decrease (during the two first weeks), followed by an increase (at 6–8 weeks), another decrease (up to 16 weeks), and a final slight increase in the 11-o values. Wet samples maintained at room temperature had higher 11-o values at the

end of the experiment, while dry samples kept at -80ºC had very similar 11-o values at the beginning and the end of the study (Fig. 1).

The values for the 11-o concentrations obtained for each subsample fitted a normal distribution (Table 1). Intra-sample variation was lower in dry samples regardless of the storage temperature (Table 1, Fig. 1). ANOVA results for comparisons of 11-o concentrations in dry samples only showed significant effects for storage temperature ($p < 0.001$) (Table 2). We did not find any significant differences in 11-o concentrations over time ($p = 0.445$) or when considering the interaction between temperature and time ($p = 0.902$). Tukey's pair-wise comparisons between different categories of temperature indicate that the only significant differences in 11-o concentrations appeared between samples kept at -80º C and those stored at room temperature ($p < 0.001$) (Table 2).

In the ANOVA results for the measurements of 11-o in wet samples there were significant effects on 11-o concentrations for temperature, as well as for interactions between time and temperature (Table 2). In all of the Tukey's pair-wise comparisons we found significant differences in 11-o concentrations between time and different temperature categories, and in interactions between time and temperature categories (Table 2).

Discussion

In field studies focussed on assessing the physiological status of wildlife, non-invasive approaches (Keay et al. 2006; Pauli et al. 2009) coupled with analytical methods that offer specificity, sensitivity and precision constitute a potentially powerful tool. Nevertheless, such methods need to be validated and calibrated for target species, which involves sample handling and specific storage protocols (Khan et al. 2002; Herring and Gawlik 2009; Parnell et al. 2015). Our method was first calibrated for the target species (Azorit et al. 2012), then optimized and validated for Iberian ibex (*Capra pyrenaica*) (Molina-García et al. 2018) and, finally, used to address inter-individual differences in 11-o concentrations in ibex (Pérez et al. 2019). However, methods of sample storage may potentially affect the quality of FGM measurements (Keay et al. 2006).

Glucocorticoid metabolites are not always evenly distributed in ungulate faecal samples (Millspaugh and Washburn 2003) or even in pellets from the same animal (Hadinger et al. 2015), a fact

that may generate great intra-sample variability. According to Millspaugh and Washburn (2003), faecal glucocorticoid metabolite measurements from mixed samples were less variable than those realized using pellet groups from the same faecal mass. These authors recommend collecting the entire faecal mass and mixing it thoroughly before selecting a sub-sample for analysis. In our case, the homogenization of pooled samples not only helped gain precision in the 11-o determination but also enabled us to generate enough subsamples for our study.

Results from previous studies demonstrate that greater relative humidity (i.e. due to rainfall) and heat accelerate the bacterial degradation of samples, leading to changes in FGM concentrations (Terio et al. 2002; Washburn and Millspaugh 2002). In fact, Möstl et al. (1999) reported significant increases in FCM concentrations in bovine, equine and porcine faeces stored at room temperature for up to 24 hours. In our study, this was evident when observing the dynamics of 11-o concentrations in wet samples over time (Fig. 1). Only in the samples stored at room temperature were the 11-o values at the end of the experimental period higher than those obtained at the beginning. Although this type of increase in FCM concentrations were explained – at least partially – in previous studies by alterations in the affinity for the antibody of certain metabolites, our results suggest that the metabolization of residual molecules of cortisol in faeces occurs.

We only found significant differences when comparing 11-o concentrations between dry samples kept at -80ºC and at room temperature. Other potential causes of variations in 11-o concentrations include aerial oxidation and the hydroxylation of FGMs (Möstl et al. 1999; Edmonds et al. 2006). All these studies highlight the importance of collecting faecal samples that are as fresh as possible to prevent *ex-vivo* sources of variation in 11-o concentrations.

Variations in faecal glucocorticoid concentrations that depend on the number of days of sample storage – be it at room temperature or at -20ºC – have been reported from samples from baboons (*Papio cynocephalus*) fixed in 95% ethanol (Khan et al. 2002). Other *in situ* (e.g. just after collection) treatments for preserving faecal samples include autoclaving, oven drying or fixation in other preservatives such as 10% formalin, 2% acetic acid or 2% sodium hydroxide (Millspaugh et al. 2003; Keay et al. 2006). On the other hand, the use of reagents such as ethanol may complicate sample transportation.

Further studies are needed to address the effects of sample fixation procedures on the stability of FGMs, the usefulness of *in-situ* extraction and the dynamics of FGM concentrations (particularly 11-o)

during the first hours and days after sample collection. Such studies will allow us to standardize protocols for sample collection, transport and storage.

De Clercq et al. (2014) recommend lyophilising bovine faecal samples and storing them at -80ºC under aerobic conditions to ensure the stability of natural and synthetic faecal glucocorticoids for up to 10 weeks. Our results support this recommendation and suggest that, under less favourable conditions (e.g. higher storage temperature), this period could even be extended.

Author contributions

JMP, MB and CA conceived and designed the study; JMP and JE obtained the samples used in this study; LM-G analysed samples; AJLM performed the statistical analysis of the data; all co-authors contributed in the writing of the manuscript and gave their final approval for its publication.

Declaration of interest

The authors declare that they have no financial/personal interest or belief that could affect their objectivity.

References

Allwin B, Gokarn NS, Pandian SS, Vedamanickam S, Gopal S, Manoj KM, Jothi B (2016) Assessment of faecal cortisol levels in free-ranging Nilgiri tahrs (*Nilgiritragus hylocrius*) in correlation with meteorological parameters: a non-Invasive Study. J Climatol Weather Forecasting 4:175. DOI: 10.4172/2332-2594.1000175

Azorit C, Castro JM, Carrasco R, Tellado S, Orpez R, Moro J (2012) Faecal 11-ketoetiocholanolone measurement in Iberian red deer (*Cervus elaphus hispanicus*): validation of methodology using HPLC-MS/MS. Anim Prod Sci 52:756-760. http://dx.doi.org/10.1071/AN12022

Bahr NI, Palme R, Möhle U, Hodges JK, Heistermann M (2000) Comparative aspects of the metabolism and excretion of cortisol in three individual nonhuman primates. Gen Comp Endocrinol 117:427-438. doi:10.1006/gcen.1999.7431

Bielohuby M, Popp S, Bidlingmaier M (2012) A guide for measurement of circulating metabolic hormones in rodents: pitfalls during the pre-analytical phase. Mol Metab 1:47–60. https://doi.org/10.1016/j.molmet.2012.07.004

Dalmau A, Ferret A, Chacon G, Manteca X (2007) Seasonal changes in fecal cortisol metabolites in Pyrenean chamois. J Wildll Manage 71:190-194. DOI: 10.2193.2005-492

De Clercq N, Vanden Bussche J, Croubels S, Delahaut P, Vanhaecke L (2014) Development and validation of a high-resolution mass-spectrometry–based method to study the long-term stability of natural and synthetic glucocorticoids in faeces. J Chromatograph A 1336:76–86. http://dx.doi.org/10.1016/j.chroma.2014.02.033

Dehnhard M, Clauss M, Lechner-Doll M, Meyer HH, Palme R (2001) Non-invasive monitoring of adrenocortical activity in roe deer (*Capreolus capreolus*) by measuring fecal cortisol metabolites. Gen Comp Endocrinol 123:111–120. https://doi.org/10.1006/gcen.2001.7656

Descovich KA, Lisle AT, Johnston S, Keeley T, Phillips CJ (2012) Intrasample variation and the effect of storage delay on faecal metabolite concentrations in the southern hairy-nosed wombat (*Lasiorhinus latifrons*). Aust Mammal 34:217–222. DOI: 10.1071/AM11047

Edmonds JS, Morita M, Turner P, Skelton BW, White AH (2006) Aerial oxidation of the glucocorticoid side-chain under pH control. Steroids 71:34-41. doi:10.1016/j.steroids.2005.08.003

Gholib G, Heistermann M, Agil M, Supriatna I, Purwantara B, Nugraha TP, Engelhardt A (2018) Comparison of fecal preservation and extraction methods for steroid hormone metabolite analysis in wild crested macaques. Primates 59:281-292. https://doi.org/10.1007/s10329-018-0653-z

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 6:1349-1357. doi: 10.1111/2041-210X.12422

Herring G, Gawlik DE (2009) Stability of avian fecal corticosterone metabolite levels in frozen avian feces. J Wildl Manage 73:1010-1013. DOI: 10.2193/2008-398

Hothorn T, Bretz F, Westfall P (2008) Simultaneous inference in general parametric models. Biom J 50:346–363. doi: 10.1002/bimj.200810425

Huber S, Palme R, Arnold W (2003) Effects of season, sex, and sample collection on concentrations of fecal cortisol metabolites in red deer (*Cervus elaphus*). Gen Comp Endocrinol 130:48-54. https://doi.org/10.1016/S0016-6480(02)00535-X

Keay JM, Singh J, Gaunt MC, Kaur T (2006) Faecal glucocorticoids and their metabolites as indicators of stress in various mammalian species: a literature review. J Zoo Wildl Med 37:234–244. DOI: 10.1638/05-050.1

Khan MZ, Altmann J, Isani SS, Yu J (2002) A matter of time: evaluating the storage of fecal samples for steroid analysis. Gen Comp Endocrinol 128:57–64. https://doi.org/10.1016/S0016-6480(02)00063-1

Lexen E, El-Bahr SM, Sommerfeld-Stur I, Palme R, Möstl E (2008) Monitoring the adrenocortical response to disturbances in sheep by measuring glucocorticoid metabolites in the faeces. Wien Tierärztl Mschr 95:64 – 71.

McCullagh P, Nelder JA (1989) Generalized linear models. Chapman and Hall, London – New York

Millspaugh JJ, Washburn BE (2003) Within-sample variation of fecal glucocorticoid measurements. Gen Comp Endocrinol 132:21-26. doi:10.1016/S0016-6480(03)00061-3

Millspaugh JJ, Washburn BE, Milanick MA, Slotow R, van Dyk G (2003) Effectso f heat and chemical treatments on fecal glucocorticoid measurements: implications for sample transport. Wildl Soc Bull 31:399-406. DOI: 10.2307/3784319

Molina-García L, Pérez JM, Sarasa M, Ureña-Gutiérrez B, Espinosa J, Azorit C (2018) HPLC-QTOF method for quantifying 11-ketoetiocholanolone, a cortisol metabolite, in ruminants' feces: optimization and validation. Ecol Evol 8:9218–9228. DOI: 10.1002/ece3.4285

Möstl E, Messmann S, Bagu E, Robia C, Palme R (1999) Measurement of glucocorticoid metabolite concentrations in faeces of domestic livestock. J Vet Med 46:621-631

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. Int J Mammal Biol 62 (Suppl. II):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 Mschr 86:237-241

Parnell T, Narayan EJ, Nicolson V, Martin-Vegue P, Mucci A, Hero JM (2015) Maximizing the reliability of non-invasive endocrine sampling in the tiger (*Panthera tigris*): environmental decay and intra-sample variation in faecal glucocorticoid metabolites. Conserv Physiol 3. doi:10.1093/conphys/cov053

Pauli JN, Whiteman JP, Riley MD, Middleton AD (2009) Defining noninvasive approaches for sampling of vertebrates. Conserv Biol 24:349-352. https://doi.org/10.1111/j.1523-1739.2009.01298.x

Pérez JM, Molina L, Ureña-Gutiérrez B, Espinosa J, López-Montoya AJ, Boos M, Granados JE, Cano-Manuel FJ, 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

Pinheiro J, Bates D, DebRoy S, Sarkar D, the R Development Core Team (2013) nlme: linear and nonlinear mixed effects models. R package version 3.1-108

R Development Core Team (2017) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. http://www.R-project.org

Terio KA, Brown JL, Moreland R, Munson L (2002) Comparison of different drying and storage methods on quantifiable concentrations of fecal steroids in the cheetah. Zoo Biol 21:215-222. DOI: 10.1002/zoo.10036

Touma C, Palme R (2005) Measuring fecal glucocorticoid metabolites in mammals and birds: the importance of validation. Ann N Y Acad Sci 1046:54–74. https://doi.org/10.1196/annals.1343.006

Washburn BE, Millspaugh JJ (2002) Effects of simulated environmental conditions on glucocorticoid metabolite measurements in white-tailed deer feces. Gen Comp Endocrinol 127:217-222. https://doi.org/10.1016/S0016-6480(02)00056-4

Wickham H (2016) ggplot2: elegant graphics for data analysis. Springer-Verlag, New York

Zuur AF, Ieno EN, Walker NJ, Saveliev AA, Smith GM (2009) Mixed effects models and extensions in Ecology with R. Springer, New York.

Figure 1

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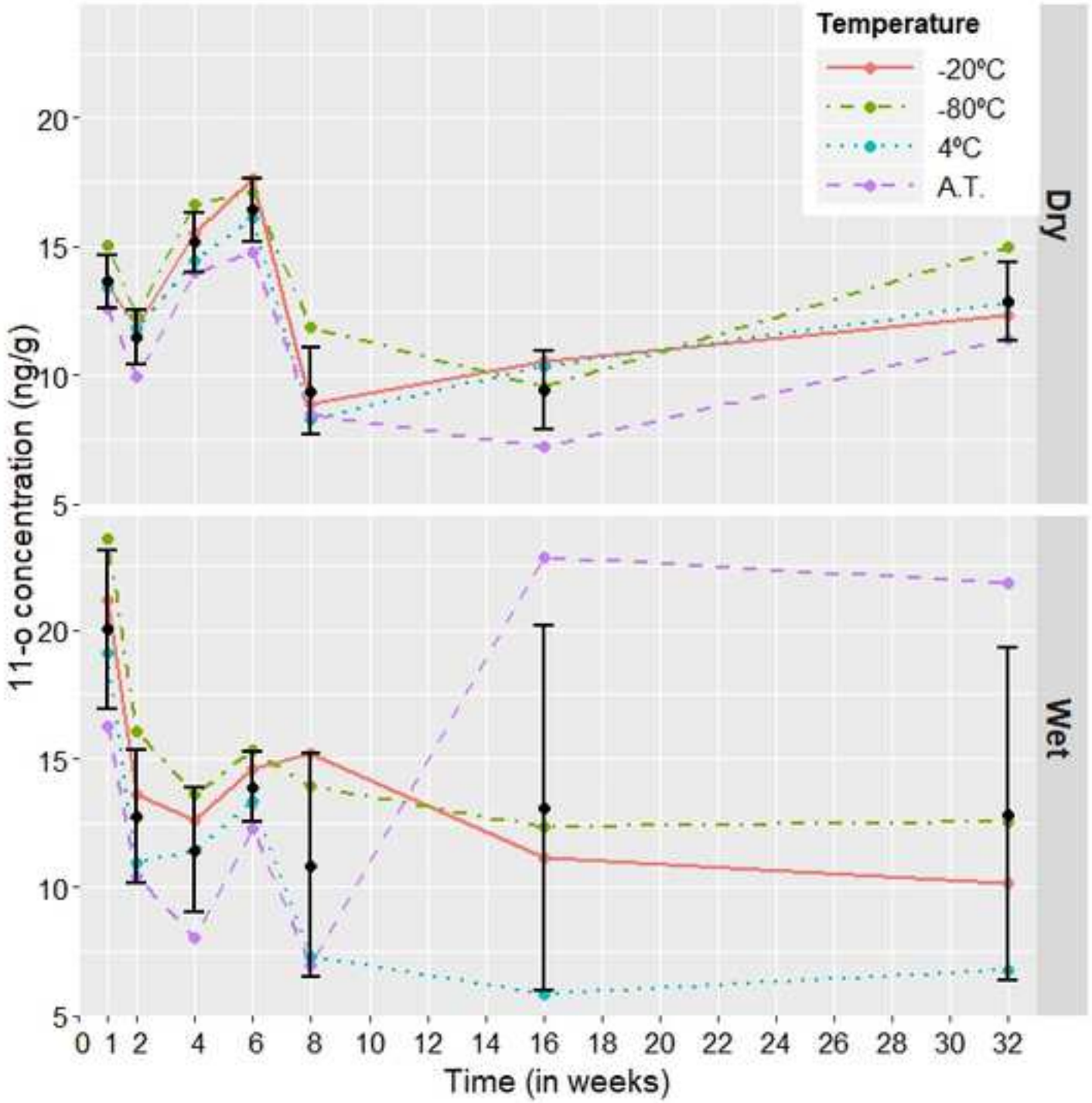

Table 1

Sample Treatment	Temperature	11-o concentration (ng/g) Mean ± SD	Shapiro-Wilk p-value	Intra CV (%)	Inter CV (%)
Dry	- 80 ºC	13.94 ± 2.75[a]	0.5984	9.53	14.32
	- 20 ºC	12.90 ± 2.96[ab]	0.9663	15.75	
	4 ºC	12.46 ± 2.59[ab]	0.9952	10.41	
	A.T.	11.22 ± 2.83[b]	0.8326	12.47	
Wet	- 80 ºC	15.33 ± 3.86[c]	0.0175	5,62	38.64
	- 20 ºC	14.07 ± 3.59[c]	0.3191	7.39	
	4 ºC	10.70 ± 4.63[c]	0.4010	15.79	
	A.T.	14.10 ± 6.40[d]	0.3565	8.70	

Table 2

Sample treatment	Variables	Num DF	Den DF	F value	*p*-value
Dry	Time	1	5	0.688	0.445
	Temperature	3	15	13.009	< 0.001
	Time*Temperature	3	15	0.189	0.902
Wet	Time	1	5	3.8817	0.106
	Temperature	3	15	20.1506	< 0.001
	Time*Temperature	3	15	7.1563	0.003

Table 2. Results of repeated measures ANOVA test for samples treatment (dry *vs* wet), time, temperature, and time*temperature interaction effects on 11-k concentration. Num DF = Numerator degree of freedom; Den DF=Denominator degree of freedom.