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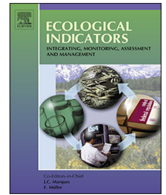

Short Note

Fecal cortisol metabolites under anonymized sampling: Robust estimates despite significant individual heterogeneity

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ABSTRACT

The levels of fecal cortisol metabolites (FCMs) are widely used as bioindicators of stress in wildlife studies. Although FCMs allow to assess the impact of ecological and physiological stressors through anonymous scat sampling, recent studies have highlighted the importance of accounting for individual heterogeneity to deal with pseudoreplication and obtain robust parameter estimates. It has been suggested that repeatable among-individual differences in the levels of FCMs may be the result of selective pressures. If so, species-specific variations in the level of heterogeneity in glucocorticoid secretion may occur, possibly bearing contrasting consequences on parameter estimates when fecal sampling is carried out anonymously. I used data collected on individually identified male chamois *Rupicapra rupicapra* in Gran Paradiso National Park (Western Italian Alps) in 2011 and 2012, to study the importance of individual heterogeneity on the variation of FCM levels in response to several biotic and abiotic stressors over different periods, and to evaluate the robustness of parameter estimates under anonymized (pseudoreplicated) data collection. The importance of individual heterogeneity varied conspicuously with life history stages, with the highest individual variation in FCM values occurring in the mating season. Despite widespread significant individual heterogeneity, parameter estimates under different sampling designs were consistently similar, suggesting that the lack of individual information does not always preclude the possibility to obtain valuable indications of the impact of ecological stressors on populations using FCM levels. The consequences of neglecting pseudoreplication when evaluating FCM levels may thus vary with species- or study-specific features. While identifiable sampling is always desirable to obtain robust estimates of stressors' impacts through FCMs, researchers should carefully evaluate the costs and benefits of retrieving individual information, possibly based on individual sampling frequency, variance explained by biotic and abiotic predictors, target species and period of sampling.

1. Introduction

The secretion of glucocorticoids by the neuroendocrine system enables individuals to cope with biotic and abiotic stressful events, including environmental changes and social and physiological traits (Sapolsky et al., 2000). Consequently, variations in glucocorticoid levels are often used as bioindicators of the impact of ecological changes on animal populations (Boonstra, 2004). The physiological response of animals to different stressors may be assessed non-invasively through the analysis of the levels of cortisol metabolites present in feces (Möstl et al., 2002; Thiel et al., 2005). This method proved effective in studying potential drivers of stress in several species, e.g. temperature variations in red deer *Cervus elaphus* (Huber et al., 2003), recreational activities in wolf *Canis lupus* (Creel et al., 2002) and in chamois *Rupicapra rupicapra* (Zwijacz-Kozica et al., 2013), mating competition in bison *Bison bison* (Mooring et al., 2006). Notably, fecal cortisol metabolites (FCMs) offer the advantage of being feedback-free – i.e. they are not influenced by the researcher's activities – and of removing the need to physically capture animals (Sheriff et al., 2009; Goymann, 2012). Consequently, measures of FCM levels to study the impact of stressors can be obtained using either identifiable or anonymous fecal sampling strategies (i.e. each fecal sample may be either linked or unlinked to individual animals, *cf.* Creel et al., 2002). Recent studies, however, have brought to the attention of researchers the importance of considering individual heterogeneity when evaluating the variations in the levels of glucocorticoids in wildlife studies (Schoenemann and Bonier, 2018; Taff et al., 2018).

Neglecting the individual effect may indeed have profound consequences on FCM data analysis. Recent studies on mountain hare *Lepus timidus* (Rehnus and Palme, 2017) and capercaillie *Tetrao urogallus* (Coppes et al., 2018), showed that by anonymizing previously identified fecal samples, the analysis may generate biased and inaccurate

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results and thus cause misleading interpretations of the effects of biotic and abiotic stressors on the target species. These issues may arise because by treating repeated FCM measures of the same individuals as if they were independent data (pseudoreplication), the potential to obtain spurious effects might increase (Hurlbert, 1984). The correlation among repeated measures of the same clusters is generally referred to as intra-class correlation (ICC), or repeatability. ICC is calculated as $\sigma_\alpha^2/(\sigma_\alpha^2+\sigma_\varepsilon^2)$, where σ_α represents the variability between clusters and σ_ε the variability within clusters (Nakagawa and Schielzeth, 2010). In this case, ICC indicates the degree of similarity of the measures of FCMs within each individual, relative to the levels measured among individuals: in other words, it measures the effect of individual clustering in FCM measures, where ICC = 0 indicates no clustering (independence) and ICC = 1 complete clustering (full dependence, i.e. no variability within individuals). Notably, Smith et al. (2012) suggested that repeatable inter-individual differences in FCMs might be a potential target of selection in natural populations: if so, the values of ICC in FCM secretion might vary with species or life history strategies – although it is possible that, owing to the adaptive plasticity of hormone concentrations, FCM levels may not respond to selective pressures (Schoenemann and Bonier, 2018). Given the potential evolutionary component underlying the value of repeatability, exploring the importance of individual clustering when analyzing FCM levels in different *taxa* may have important practical implications for wildlife studies.

When some individual clustering occurs in the data (i.e. ICC > 0), mixed-effect models may be used to obtain robust estimates. By fitting individual identity as a random term, models can deal with pseudoreplication and effectively resolve the non-independence that stems from having multiple measures by the same individual (Zuur and Ieno, 2016). However, costs associated with the collection of individual information (through captures or DNA analysis) may be important. No hard rule exists on how large the value of ICC should be to proclaim consequential lack of independence, hence justify the use of a multi-level modeling approach. Thus, comparing the performance of models with and without individual random effect across different species and life history stages may help optimize the trade-offs between costs and benefits of identifiable *vs*. anonymous sampling strategies.

I use data collected on the Alpine chamois *R. r. rupicapra*, a species with a more conservative life history than mountain hare and capercaillie, to study different metrics used to describe the importance of individual heterogeneity on the variation of FCM levels (ICC, variance) over different time periods. I then compare parameter estimates in models with and without individual effect, thus simulating identifiable and anonymous sampling regimes, and discuss practical implications of my results on wildlife studies based on the use of FCMs.

2. Materials and methods

The study was conducted in 2011 and 2012 in the upper Orco Valley, a 10 km^2 protected area within the Gran Paradiso National Park (GPNP, Western Italian Alps, 45°26′30″ N, 7°08′30″ E). The climate is Alpine-continental, with strong seasonal variations in temperature and precipitation. The chamois population in GPNP has been protected since 1922, and at the time of sampling the area was free of predators. Twenty-two adult male chamois were captured by the Park personnel and individually marked with a GPS (Global Positioning System) collar. Between January 2011 and December 2012, I tracked every individual on a monthly basis and collected 1 fresh faecal sample/animal, for as many marked chamois as possible within a given month, thus adopting an identifiable sampling strategy (see Corlatti et al., 2014 for more details). Scats were collected immediately after deposition, to avoid degradation or washing out effects. Over the 2 years, I collected 393 samples, fairly evenly distributed across months and individuals (individual sample size varied between 6 and 24 over 2 years, mean ± SD = 17.8 ± 6.1). Scats were frozen at −20 °C until analysis, when 0.5 g of each sample was extracted with 5 ml aqueous methanol (80%; Palme et al., 2013) and analyzed in duplicate. FCM levels were measured with an 11-oxoaetiocholanolone enzyme immunoassay (EIA, detecting FCM with a 5ß-3α-hydroxy-11-oxo structure, Möstl et al., 2002). Because in temperate environments ecological and social stressors undergo strong seasonal changes, to investigate variations in FCMs several ethological (mating behavior [territorial – T and non-territorial – NT males], age) and ecological (minimum temperature, precipitation, snow depth) variables were associated to each individual scat (*cf*. Corlatti et al., 2014). Assuming an approximate excretion time lag of 18 h (Huber et al., 2003), only weather data registered the day before feces deposition were included in the dataset. To correctly interpret environmentally and socially induced stress responses, data analysis was conducted over different temporal scales: year, December-March, April-May, June-October, November (*cf*. Corlatti et al., 2014).

To investigate the importance of individual heterogeneity on FCM level variation in chamois, within each time period I fitted 2 linear models. First, a global Gaussian linear model with identity link function was fitted for each temporal scale (Eq. (1)), including all predictors considered relevant to explain FCM variation within different periods, and the animal identity as a random intercept to incorporate the dependency among FCM levels of the same individual, thus reflecting the adopted identifiable sampling strategy.

$$\begin{aligned}
&\mathrm{FCM}_{ij} \sim N(\mu_{ij}, \sigma^2)\\
&\mathrm{E}(\mathrm{FCM}_{ij}) = \mu_{ij} \text{ and } \mathrm{var}(\mathrm{FCM}_{ij}) = \sigma^2\\
&\mu_{ij} = \mathrm{X1}_{ij}+\ldots+Xn_{ij} + \mathrm{Individual}_i\\
&\mathrm{Individual}_i \sim N(0, \sigma^2_{Individual})
\end{aligned} \tag{1}$$

where FCM_{ij} is the $\log_{10}$-transformed value of cortisol metabolites for measure j at individual i, with i varying from 1 to 22 over the year, in December-March and June-October, from 1 to 20 in April-May, and from 1 to 18 in November. $\mathrm{Individual}_i$ is the random intercept, which is assumed to be normally distributed with mean 0 and variance $\sigma^2_{Individual}$, whereas$\mathrm{X1}_{ij} + \ldots + \mathrm{Xn}_{ij}$ represent the fixed covariates used within each period (see below). For all global models I calculated different metrics: a) the conditional and marginal R^2 statistics developed by Nakagawa and Schielzeth (2013) – where the difference between the two represents the variance explained by the individual random factor; b) the value of repeatability estimated as the intra-class correlation coefficient (ICC, i.e. the within-individual correlation among FCM measurements, which represents the proportion of the variance of the random effect to the total variance) following Wolak et al. (2012), and as the adjusted repeatability (i.e. the repeatability obtained after accounting for fixed effects) following Stoffel et al. (2017).

Then, a naïve Gaussian linear model with identity link function was fitted for each temporal scale (Eq. (2)), including the same set of predictors of the corresponding global models, but without individual random intercept (cf. Coppes et al., 2018), thus simulating anonymous sampling strategy and analysis of pseudoreplicated data.

$$\begin{aligned}
&\mathrm{FCM}_{ij} \sim N(\mu_i, \sigma^2)\\
&\mathrm{E}(\mathrm{FCM}_i) = \mu_i \text{ and } \mathrm{var}(\mathrm{FCM}_i) = \sigma^2\\
&\mu_i = \mathrm{X1}_i+\ldots+Xn_i
\end{aligned} \tag{2}$$

where FCM_i is the $\log_{10}$-transformed value of cortisol metabolites for measure i and$\mathrm{X1}_i + \ldots + \mathrm{Xn}_i$ represent the fixed covariates used within each period (see below). To study the significance of the random intercept within different periods I ran likelihood ratio tests between the global models (refitted using maximum likelihood instead of restricted maximum likelihood) and the corresponding naïve models. Finally, I checked for consistency in parameter estimates between global and naïve models within periods to study the consequences of anonymizing the sampling regime.

Prior to analysis, continuous predictors were standardized to return comparable coefficients. Not all predictive variables were considered in

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