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ORIGINAL RESEARCH

Faecal cortisol levels in a wild Iberian red deer population are best explained by prior weather conditions

A. Gort-Esteve[1,2], A. Carbajal[3], M. López[3], X. Manteca[1], J. Ruiz-Olmo[4] & J. L. Riera[5]

[1]Department of Animal and Food Science, Autonomous University of Barcelona, Bellaterra, Spain
[2]Research Unit of Biodiversity (UMIB, UO-CSIC-PA), Oviedo University, Campus Mieres, Miere, Spain
[3]Department of Animal Health and Anatomy, Autonomous University of Barcelona, Bellaterra, Spain
[4]Department of Climate Action, Food and Rural Agenda, Generalitat de Catalunya, Barcelona, Spain
[5]Department of Evolutionary Biology, Ecology and Environmental Sciences, University of Barcelona, Barcelona, Spain

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Correspondence
Araceli Gort-Esteve, Department of Animal and Food Science, Autonomous University of Barcelona, Bellaterra, 08193, Spain.
Email: celigort@gmail.com

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Abstract

The responsiveness of the hypothalamic–pituitary-adrenocortical axis to stressors is crucial for wild animals to survive and adapt to environmental changes without compromising individual welfare. We analysed the influence of prior weather conditions, seasonality, the influx of ecotourism, and nutrition on stress levels in a wild population of red deer in a Mediterranean hunting reserve in the Pre-Pyrenees, Spain. We used faecal cortisol metabolites as a proxy for physiological and psychological stress, and faecal triiodothyronine metabolites as an indicator of nutritional stress. Faecal analyses were chosen because it is a non-invasive technique that does not alter an individual's behaviour, and it is easy to conduct in a wild population, something which presents added challenges due to the lack of a controlled environment. Our results indicate that prior weather conditions, rather than just seasonality, best explained the variations in these hormones within seasons and among years. On the contrary, the results showed that high levels of the cortisol hormone did not necessarily correspond to low levels of the triiodothyronine hormone. This could be a sign of acute nutritional stress in this population. Finally, we did not find an effect of the ecotourism influx on cortisol levels, suggesting that this deer population tolerates the presence of tourists.

Introduction

Stress is defined as a state of threatened homeostasis that induces physiological and behavioural responses mediated by the release of glucocorticoid hormones (GC), and cortisol in most mammals (Cockrem, 2013; Greenberg et al., 2002). For this reason, GC is frequently used to study the stress response (Pacak & Palkovits, 2001). Stress evolved in vertebrates as an adaptive endocrine response that increases survival, helping individuals cope with noxious stimuli or stressors (Möstl & Palme, 2002; Pacak & Palkovits, 2001). Stressors activate the hypothalamic–-pituitary-adrenocortical axis (HPA), resulting in the release of GC into the bloodstream (Hadley & Levine, 2007; Norris, 2006). The main role of GC in mammals is the control of energy homeostasis (Roldan & Herzig, 2015). A high level of GC occupying the corticosteroid receptors located in the hippocampus regulates HPA GC release through negative feedback and restores stress disturbances to homeostasis (De Kloet et al., 1998; Shirazi et al., 2015). However, under chronic stress, the prolonged responsiveness of the HPA can reduce individual fitness by halting growth and development, suppressing the immune system, and inhibiting biological functions such as reproduction (Möstl & Palme, 2002; Sapolsky et al., 2000). Chronic stress reduces the number of receptors, resulting in a less efficient feedback signal (De Kloet & Reul, 1987). Therefore, the study of stress is crucial for the conservation of wild populations.

In wild populations, stressors include perceived threats such as adverse weather and predator encounters, as well as social interactions such as courtship and copulation (Broom & Johnson, 1993). Natural stressors are closely related to climatic conditions, including weather extremes such as snowstorms, heatwaves or cold spells (Wingfield, 2013). Many studies have analysed the stress response to climatological conditions in farm animals, often focusing on the effects of high temperatures (Huber et al., 2003; Millspaugh et al., 2001). Studies in wild populations, specifically in ungulates, are scarce and often limited to showing a seasonal pattern in response to climatic conditions (Alila-Johansson et al., 2003; Monfort et al., 1993), without further analysing links to specific weather conditions or within-season variability.

In the strongly seasonal Mediterranean climate, food availability, and consequently nutritional stress, may also be expected to vary seasonally with the weather conditions. Periods of starvation can be brief and cause no apparent physiological impacts, or they can be moderate and affect reproduction, growth, or immune responses; in the extreme, they can be life-threatening (McCue et al., 2017). Besides its role in energy homeostasis, GC also plays a key role in the regulation of mammalian glucose, which is an essential response to starvation periods (Kuo et al., 2015). The major effect of GC on glucose homeostasis under stress is to preserve plasma glucose, which is necessary to reach maximal brain function (Kuo et al., 2015). Thus, one of the first responses following an immediate stressor is to mobilize glucose (Sapolsky et al., 2000). Another response to starvation periods implies changes in the levels of thyroid hormones. In the pituitary gland, the thyroid-stimulating hormone acts on the receptors in the thyroid gland to promote the synthesis and release of thyroxine (T4) and triiodothyronine (T3) hormones (Flier et al., 2000). Unlike its effect on cortisol, starvation rapidly suppresses T4 and T3 levels because thyroid hormones set the basal metabolic rate, and a drop in thyroid hormone levels should reduce the use of energy stores (Blake et al., 1991; Flier et al., 2000). Thus, T4 and T3 respond to nutritional deficits by lowering metabolism and allowing the body to conserve energy during periods with nutritional deficiencies (Silva, 2006; Wasser et al., 2010). Conversely, under periods of abundance, levels of thyroid hormones increase (Douyon & Schteingart, 2002; Kitaysky et al., 2005). Accordingly, thyroid hormones have been identified as a potentially viable index of body condition in ungulates due to their correlation with body fat (Bishop et al., 2009). Therefore, these hormones can help us discriminate between nutritional stress and other sources of stress.

Anthropogenic activities, such as ecotourism, trekking, hunting or nature photography, among others, may also be another cause of increased stress levels in wild populations. The effects range from short-term impacts, such as changes in the physiology and behaviour of individuals, to long-term effects, such as increased mortality or reduced breeding success of entire populations (Burns & Howard, 2003; Higginbottom & Scott, 2004). In ungulates, anthropogenic stress has been shown to cause behavioural changes, such as increasing the number of vigilant individuals, especially in species less tolerant of human presence (Borkowski, 2001; Jayakody et al., 2008; Pelletier, 2006; Wasser et al., 2011). Several studies, however, provide evidence that ungulates may tolerate or acclimate to the presence of humans (Sibbald et al., 2011).

Glucocorticoid stress responses and T3 nutritional stress can be studied through hormone levels in different body fluids or excreta. The analysis of GC in serum and saliva is useful for studying acute changes or sharp peaks of stress (Hernandez et al., 2014; Negrao et al., 2004), while long-term stress can be studied by measuring GC in hair (Salas et al., 2016; Tallo-Parra et al., 2015). GC, thyroid hormones, and their metabolites can also be accurately and reliably measured in faeces (Huber et al., 2003; Touma & Palme, 2005; Turpeinen & Hämäläinen, 2013; Wasser et al., 2010). The analysis of hormones in faeces informs about their blood levels 1 or 2 days before excretion, depending on the digestion time of the species (Möstl & Palme, 2002). This non-invasive method does not require individuals to be captured and handled, which can cause additional stress (Arroyo et al., 2013).

Here, we present a joint analysis of stress hormones and nutrition-related hormones in a free-ranging population of Iberian red deer (*Cervus elaphus hispanicus*). Our goal was to evaluate the influence of prior weather conditions, seasonality, nutrition, and the influx of ecotourism on stress levels. We expected to find a strong seasonal pattern, with higher levels of FCM in periods with high temperatures and low precipitation (summer) and in periods with low temperatures and extensive snow cover (winter), due to the direct physiological impacts of adverse weather conditions. We also expected to find high nutritional stress due to the reduced availability of high-quality food (producing low FT3M levels) in the same periods. As a consequence, we expected to find a negative correlation between FCM and FT3M, with high FCM levels associated with low FT3M levels in summer and winter. We further hypothesised that deer would have higher levels of stress in autumn, during the rutting period, due to mating-associated stress. Finally, we expected to find higher levels of stress in the high ecotourism location than in the low ecotourism location due to the added anthropogenic stress.

To our knowledge, this is the first time that cortisol and thyroid metabolites have been studied together in wild red deer populations using this non-invasive technique.

Materials and methods

Study area and sampling design

The National Game Reserve of Boumort, located in the Pre-Pyrenees (Fig. 1), covers an area of 13 097 hectares and has a maximum altitude of 2077 meters. Its climate is supramediterranean (Rivas & Gandullo, 1987), with hot and dry summers, cold winters with snow accumulation, and most rainfall concentrated in spring and autumn (Figure S1). It is inhabited by the Iberian red deer subspecies (Pérez-González et al., 2023). The population density of Iberian red deer is approximately 4 individuals per square kilometre (annual census data; Joan Curià, personal communication). Low-intensity hunting is allowed from September through March, mostly by stalking.

To evaluate the stress levels, we collected faecal samples that we analysed for cortisol metabolites (FCM) as a proxy for physiological and psychological stress, and T3/T4 metabolites (FT3M) as a proxy for nutritional stress.

We sampled in two zones during five consecutive years, coinciding with important events during the Iberian red deer annual cycle: summer, the hottest period (July of 2016 and 2017), autumn, the rutting period (September and October of 2016, 2017, and 2020), winter, the coldest period with snow (December to early March of 2016–2017 and 2020–2021), and spring, the period after the lowest temperatures (May of 2017 and 2018). Although our initial design was to sample each season in two different years, extensive snow cover over two winters and the recognition of high interannual variability (see results) prompted us to extend sample collection until the winter of 2020–2021. Weather data were obtained from the Climatological Station of

Talarn (42.1219° N – 0.05155° E, altitude: 807 m) run by the State Meteorological Agency of Spain (https://www.aemet.es/en/datos_abiertos/AEMET_OpenData), located 19.5 km from the sampling zones (Figure S1).

We selected these two sampling zones within the study area to assess the effects of ecotourism on FCM levels, one easily accessible with a high influx of ecotourists, and the other more isolated with a low influx of ecotourists. The influx of visitors was higher in summer and extremely high in autumn, during the rut (Figure S2). These two study zones were more than 2.2 km (linear distance) apart and had similar habitats (Fig. 1). The analysis of hormones in faeces informs on their blood levels 1 or 2 days before excretion, depending on the digestion time of the species (Möstl & Palme, 2002). This implies that the individual had to be in the sampling zone for a minimum of 2 days to detect the stress caused by ecotourism. The monthly home range of females in the study area is smaller than 1.5 km^2 (data non-published). In the case of males, the home range is larger, but the mean distances made during 2 days were 126 m. and 151 m., respectively (data non-published). Therefore, we considered impossible that the individuals sampled in the high ecotourism zone could be the same as the ones sampled in the low ecotourism zone.

Samples were collected along a fixed 150 m transect at each of the two zones. Sample pellets were collected in separate pellet groups, with each group assumed to belong to a different individual, ensuring maximum independence between sampled individuals. We collected a total of 147 samples. Each sample contained at least 5 pellets. Until the winter of 2016–2017, we collected 8 samples seasonally, 4 per zone. Afterwards, we proceeded to collect 8 samples per zone to maximize the power of the statistical analyses. An exception was the winter of 2020–2021 when we obtained 14 samples in the high ecotourism zone and 13 in the low ecotourism zone from the early and late winter to ensure there were no differences between them. We took samples from recently defecated faeces, which were moist and warm at the time of collection, implying the samples were no more than an hour old. Immediately after collection, the samples were kept on ice until we reached the laboratory (2 h), where the samples were frozen at −20°C until analysis. We followed the same protocol used by other authors such as Palme (2005), Millspaugh and Washburn (2004) and Shutt et al. (2012).

Steroid extraction and hormone analysis

A methanol-based extraction method was used for hormone extraction following methods previously published by our group (Tallo-Parra et al., 2015). Briefly, samples were put into an oven at 60°C to evaporate the water (48 h, faeces smaller than 2 cm, and 72 h, faeces larger than 2 cm). Dried faeces were mechanically ground with a mixer mill and 300 mg of the powdered sample was introduced into a conical tube. Subsequently, 5.5 mL of a 55% methanol solution was added to each sample and vortexed for 30 min. Samples were then centrifuged and the supernatant was transferred into a new microtube and stored at −20°C until analysis.

ELISA kits were validated for the analysis of cortisol and T3 in the sample and species of interest (Table S1). Ten different extracts were pooled for assay validation. The precision of the test was assessed by calculating the intra-assay coefficient of variation from all duplicated samples analysed. The precision was measured with the dilution test, determined by serially diluting the pool with EIA buffer. Finally, the accuracy

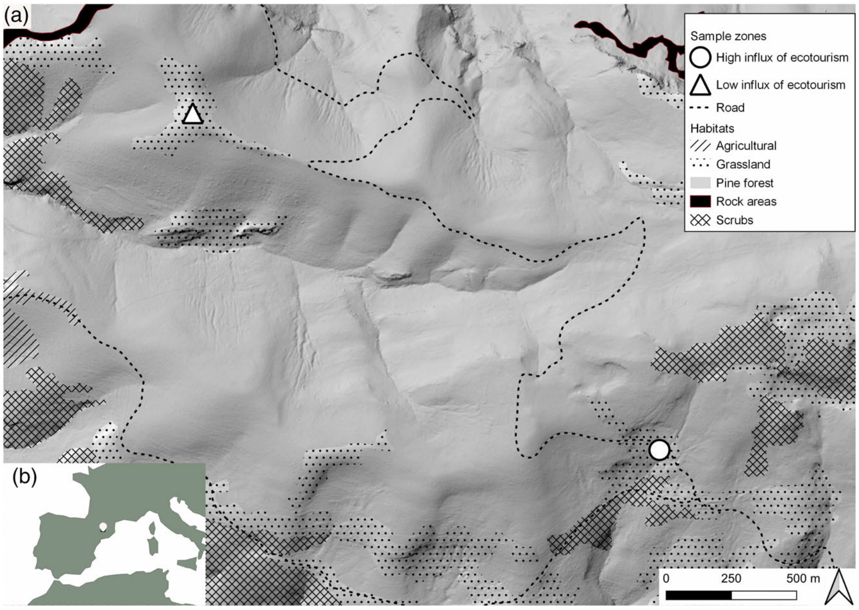

Figure 1 (a) Map of the sample zones (Base map: MET 2×2 of the Cartographic and Geological Institute of Catalonia (ICGC), licence CC BY 4.0; Habitat's map: Carreras & Ferré, 2014). (b) Study area in relation to Europe (Land map, version 4.1.0. Made in Natural Earth).

was assessed through the spike-and-recovery test, calculated by mixing different volumes of the pool with different volumes of pure standard cortisol solution.

Cortisol and T3 metabolites from faecal extracts and all the validation tests were determined with EIA detection kits of cortisol (Neogen® Corporation Europe, Ayr, UK) and T3 (IBL International®, Hamburg, Germany).

Statistical analysis

FCM and FT3M data were modelled using linear mixed-effects models. Weather effects were incorporated in two ways: (1) by including the season as a fixed factor to assess a seasonal pattern, with the year as a random effect on the intercept to account for interannual variability, and (2) by introducing weather-related variables to examine direct responses to prior weather conditions (as detailed below), with the sample collection date as a random effect on the intercept. The candidate weather variables considered were solar irradiance, minimum, average, and maximum daily temperatures, and the daily precipitation aggregated over a specific period as averages or, in the case of precipitation, the sum over that period. Ecotourism (high vs. low) was included in all models as a fixed effect. Additionally, FT3M was included as a candidate predictor for FCM, assuming that nutritional stress also contributes to an increase in cortisol levels (i.e. contributes to physiological stress as measured by FCM). After assessing model residuals, cortisol metabolite values were cube-root transformed to meet the assumptions of normality and homoscedasticity.

To select the most appropriate set of weather predictors and the aggregation period preceding sampling dates, we explored model fits for aggregation periods ranging from 1 to 31 days. For each of these periods, we constructed the most parsimonious model using a stepwise backward selection approach, beginning with a full model that included all main fixed terms and their pairwise interactions. Model selection was based on the Akaike Information Criterion (AIC), which measures model fit while accounting for model complexity. The final models were built using the aggregation period that resulted in the lowest AIC.

To assess the significance of model terms in the final selected models, we conducted likelihood ratio tests for marginal tests (i.e. comparing models with and without the target variable plus all other variables in the model). To visualize the model fit to explanatory variables, we calculated model predictions for focal variables while holding non-focal variables at their mean values and factors at their reference level. To visualize model predictions based on weather variables over the study period's duration, we calculated predictions as described previously, using the selected weather variables. To facilitate comparisons between observations and model predictions, we constructed 95% confidence intervals around population predictions (accounting only for fixed effects) using code provided by Ben Bolker (http://bbolker.github.io/mixedmodels-misc/glmmFAQ.html). The goodness of fit was assessed using marginal (explained by fixed factors alone) and conditional (explained by both fixed and random factors) pseudo-*R*-squared values, calculated using the *r*.squared generalized linear mixed models function in the R package MuMin (Barton, 2022). All statistical analyses were conducted using the R statistical package, version 4.2.1, and the mixed models were fitted using the lme function in the nlme package.

Results

Mixed models for FCM, including season, and models with weather-related variables showed similar explanatory power as assessed by AIC (model with season: −79.287, model with weather variables: −88.099). However, the model with season and year as random effects concentrated variability in the random effect, leaving the fixed factor season as non-significant. This strongly suggests that whatever seasonal pattern was in the data was obscured by high interannual and intraseasonal variability. Thus, the model carried little information regarding the drivers of FCM levels and was disregarded in favour of the models with weather-related variables.

In the process of selecting the most adequate antecedent period to integrate weather variables, the best model (as per AIC) was obtained for a period of 13 days prior to sample collection (Figure S3). This model included solar irradiance, average minimum daily temperature, and FT3M. In all models (ranging from 1 to 31 days prior to sample collection), FT3M and solar irradiance were consistently the best predictors. For clarity of exposition, we refitted this model to include all our hypothesised target factors, that is, including tourism, which were not significant (Table 1). This model showed high goodness-of-fit (marginal R^2: 0.59, conditional R^2: 0.66) to mean levels across days and experimental conditions (as defined by fixed effects) while still showing high within-day variability among individuals under the same conditions (Figs 2 and 3).

In agreement with our hypothesis that the highest stress levels would occur in periods of adverse weather conditions, we found that levels of FCM increased with both higher solar irradiance (Fig. 2a) and lower temperatures (Fig. 2b). In contrast to our hypothesis, FCM levels increased with FT3M levels, although with a small effect size (Fig. 3). Throughout the entire study period, the model's predictions clearly demonstrate that a straightforward seasonal pattern, represented in this model by minimum temperature, falls short in capturing the pronounced year-to-year fluctuations evident in the data. Incorporating solar irradiance into the model proves instrumental in accurately forecasting average FCM levels, which exhibit substantial year-to-year variations. This can be observed, for instance, in the significant disparities in observed FCM levels toward the conclusion of the winter seasons in 2019 and 2021 (Fig. 4).

The best FT3M model included mean climatological variables over 2 days prior to sample collection (Figure S4). The selected predictors included the interaction between solar irradiance and average minimum daily temperature, plus daily precipitation (Table 2). In this case, also in agreement with our hypothesis that the highest nutritional stress levels would occur in periods of adverse weather conditions, we found that levels of FT3M decreased with lower temperatures (Fig. 5a) and lower precipitation (Fig. 5b). As in the case of FCM, we did not find significant differences between the high and low influx of ecotourism zones (Table 2). Model goodness of fit, as measured by pseudo-R^2, was lower for FT3M than for FCM (marginal R^2: 0.23, conditional R^2: 0.35) (Fig. 6).

Discussion

FCM levels depend on weather prior conditions, rather than on a clear seasonal pattern

Numerous studies have shown a clear seasonal pattern in FCM levels in a wide variety of species (Alila-Johansson et al., 2003; Huber et al., 2003; Monfort et al., 1993), including deer (Bubenik & Brown, 1989; Nilssen et al., 1985). Millspaugh et al. (2001) found a relationship between FCM and the seasonal metabolic rhythms in elk (*C. canadensis*) population and hypothesised that further studies might show these changes to be related to human activities or high temperatures. Ingram et al. (1999) also demonstrated a seasonal rhythm in cortisol secretion in red deer (*C. elaphus*) and related it to its annual biological cycle. However, our data on a wild Iberian red deer population show a more complex response, with strong interannual variability that obscured any clear pattern when using the season as a categorical variable. Only by directly including prior weather conditions as continuous variables and at the right temporal scale could we adequately model FCM level variation within and among years.

Our best model includes two weather variables: average daily minimum temperature and solar irradiance. Both vary seasonally, with solar irradiance varying more irregularly among years and in the short term (Figure S1). Accordingly, FCM levels in the modelled data appear to increase in the summer months and decrease in the winter months (Fig. 4), partly supporting our expectation of higher stress levels under the harsh conditions of the summer. Yet, there is substantial between-year and within-season variability that appears to be related to short-term (2 weeks) prior weather conditions. Wingfield (2013) already points to the importance of short-term disturbances (what he terms abiotic direct labile perturbation factors) as triggers of GC secretion, presumably as an adaptive response to prime the organism to cope with changing conditions or to accompany behavioural responses. Our climatological data are limited, and the selected variables probably function here as a proxy for a more complex set of weather conditions with direct impacts on organisms. Still, solar radiation is directly linked to perceived temperature under the sun, and thus consistent with the control of energy homeostasis, which is the main role of GC in mammals (Roldan & Herzig, 2015).

Similarly, minimum temperature enters the model with a negative coefficient. This means that high FCM values tend to be higher either at low temperatures with relatively high solar irradiance (i.e. on cold, sunny days in the winter or early

Table 1 Mixed effects models for faecal cortisol metabolites in Iberian red deer (*Cervus elaphus hispanicus*) from Boumort Game Reserve

Term	Estimate	Std. error	d.f.	*t* value	*P*-value
Intercept	0.365	0.102	42.451	3.562	0.00092
Solar irradiance	0.129	0.013	25.423	9.691	<0.00001
Min. temperature	−0.023	0.005	25.225	−4.756	0.00007
FT3M	0.021	0.005	141.621	3.769	0.00024
Ecotourism influx	0.004	0.013	127.742	0.374	0.708

Mixed effects models for faecal cortisol metabolites as a function of climate variables and the *a priori* fixed effects (i.e. tourism influx and pellet size). Column "d.f." gives degrees of freedom with the Satterthwaite correction.

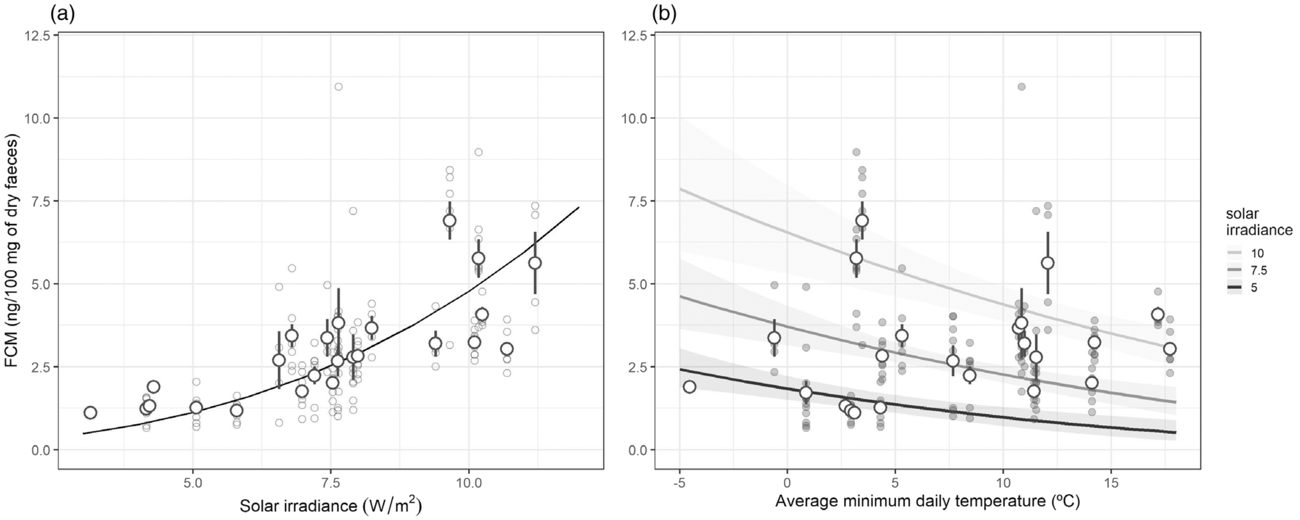

Figure 2 Observed faecal cortisol metabolites values (FCM) levels and mixed model fit against (a) solar irradiance, and (b) average minimum daily temperature. Lines are mean expected values from model (a) in Table 1. Grey tones in lines in (b) indicate varying levels of solar irradiance. Ribbons show 95% confidence intervals around mean expected values. Grey dots are individual sample values; white dots are mean daily sample values, with error bars indicating 95% confidence intervals.

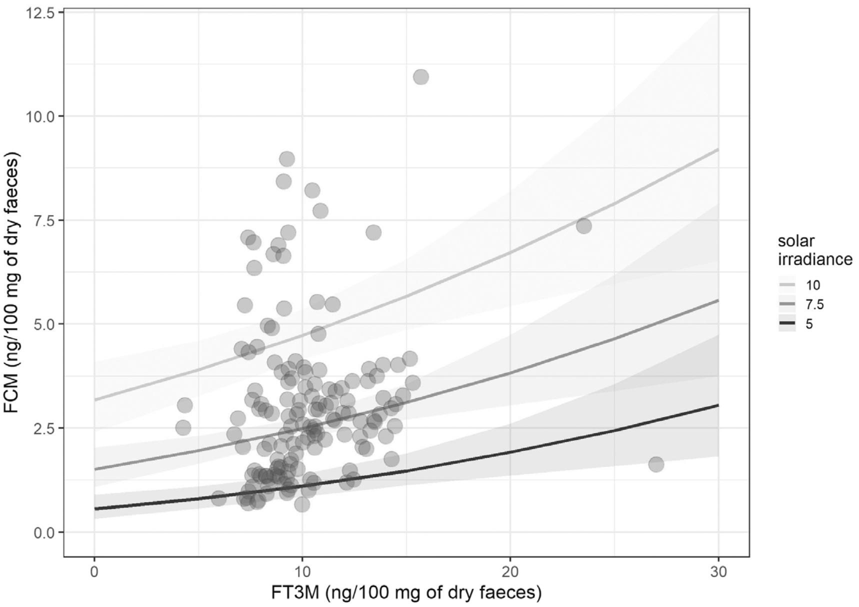

Figure 3 Observed faecal cortisol metabolites values (FCM) levels and mixed model fit against faecal triiodothyronine metabolites (FT3M) levels. Lines are mean expected values from model (a) in Table 1. Grey tones in lines indicate varying levels of solar irradiance. Ribbons show 95% confidence intervals around mean expected values. Error bars in dots indicate 95% confidence intervals.

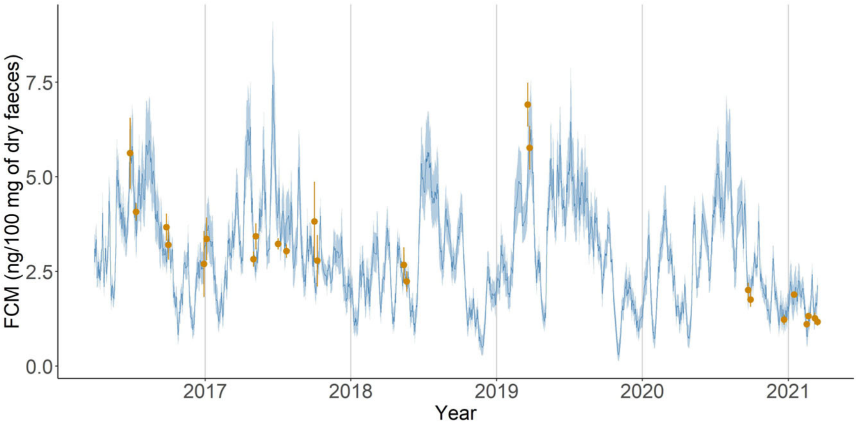

Figure 4 Model predictions and observed faecal cortisol metabolites values (FCM). The solid line shows the predicted mean levels based on a moving average of the climatological variables over the prior 13 days. The ribbon shows 95% confidence intervals around the expected mean. Solid symbols with error bars are mean daily observed values with 95% confidence intervals.

spring) or at high solar irradiance in the summer. Huber et al. (2003) also attributed an increase in stress levels to harsh environmental conditions and, as in our population, also found a significant negative relationship between minimum ambient temperature and FCM. It is noteworthy that precipitation was not selected as a predictor in the model. Other weather-related measures might provide a more direct explanation for the link between abiotic conditions and FCM levels. For example, periods of combined low solar irradiance, low temperatures, and high wind might be especially harsh.

Finally, it is worth noting that weather variables appear to be good predictors of mean FCM levels but leave unexplained very substantial variability among individuals on any particular day. This variability may be related to differences in age, condition, and possibly behavioural modes in the days prior to sample collection, suggesting the need for further analysis of differences in FCM levels at the individual level.

Higher nutritional stress was related to FCM levels and weather

The elevated levels of GC might be most functional if associated with elevated T3, so the resultant elevated metabolism can make the best use of the increased availability of glucose

Table 2 Mixed effects models for faecal triiodothyronine metabolites Iberian in red deer (*Cervus elaphus hispanicus*) from Boumort Game Reserve

Term	Estimate	Std. error	d.f.	*t* value	*P*-value
Intercept	4.897	1.464	22.346	3.345	0.003
Solar irradiance	0.488	0.179	21.312	2.719	0.013
Min. temperature	0.602	0.164	19.376	3.676	0.001
Precipitation	0.157	0.056	17.221	2.818	0.011
Ecotourism influx	−0.086	0.190	124.097	−0.453	0.651
Min. temperature × solar irradiance	- 0.057	0.019	19.743	−2.922	0.008

Mixed effects model for faecal triiodothyronine metabolites as a function of climate variables and the a priori fixed effects (i.e. tourism influx and pellet size). Column "d.f." gives degrees of freedom with the Satterthwaite correction.

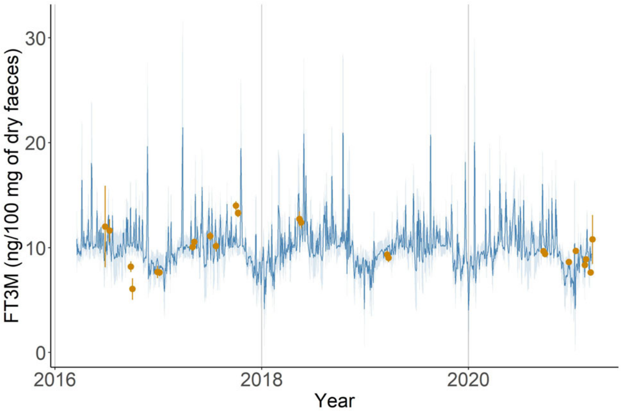

Figure 6 Model predictions and observed faecal triiodothyronine metabolites values (FT3M). The solid line shows the predicted mean levels based on a moving average of the climatological variables over the prior 2 days. The ribbon shows 95% confidence intervals around the expected mean. Solid symbols with error bars are mean daily observed values with 95% confidence intervals.

(Mondol et al., 2020; Wasser et al., 2017). This may explain why, contrary to our hypothesis, we found a statistically significant positive relation between FCM and FT3M (Fig. 3). On the other hand, T3 function is crucial to survive starvation. During long periods of food deprivation, levels of GC increase while T3 hormones decrease (Douyon & Schteingart, 2002; Kitaysky et al., 2005). Chronically elevated GC might deplete internal reserves, making it important to reduce T3 and associated metabolism to prevent the body from using all its remaining reserves (Mondol et al., 2020; Wasser et al., 2017). This suggests that the Iberian deer population in this study did not endure sustained periods of chronic nutritional stress.

The decrease of thyroid hormones in periods of food deprivation has been shown experimentally by Bishop et al. (2009) in two populations of mule deer (*Odocoileus hemionus*), one being the control and the other receiving food supplementation in winter. In that study, the population without the food supplementation had lower levels of thyroid hormones and a weaker physical condition with less body fat. In our study, FT3M levels decreased as temperatures decreased, which suggests higher nutritional stress during the snow period (Fig. 4a, b). The snow cover prevents deer from feeding because it hinders foraging. In addition, the quality of the red deer diet potentially decreases in both summer and winter as they increase consumption of woody plants due to high temperatures and dry conditions in the summer and snow cover in winter (Bugalho & Milne, 2003; Garin et al., 2001). A study of red deer (*C. elaphus*) in New Zealand found a seasonal pattern in thyroid hormones, decreasing in winter and increasing in spring and summer (Shi & Barrell, 1992). In contrast, in white-tailed deer (*Odocoileus virginianus*) in southern Texas, lower concentrations of the thyroid hormone were found in

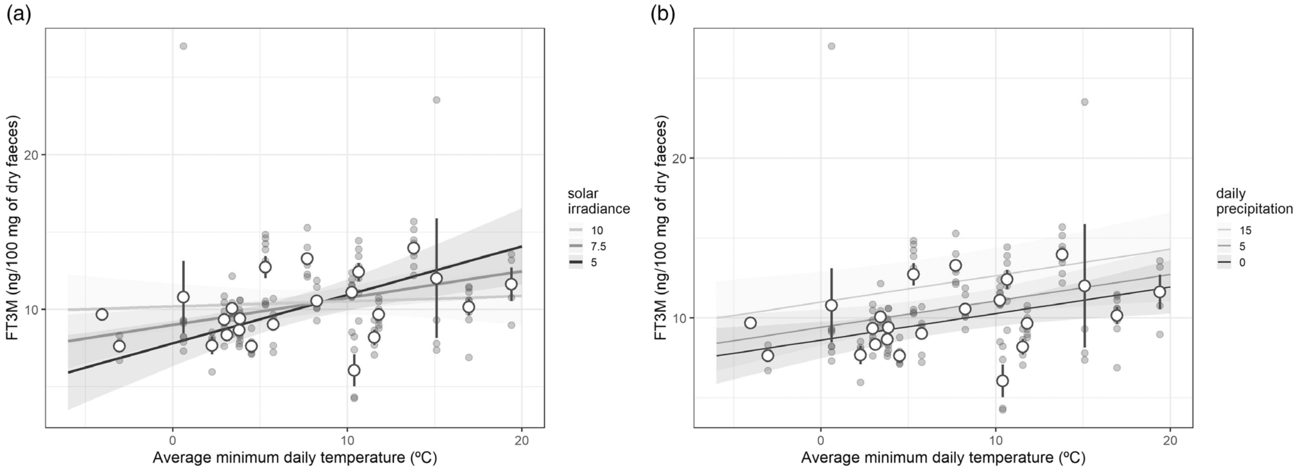

Figure 5 Observed faecal triiodothyronine metabolites (FT3M) levels and mixed model fit against (a) the minimum daily average temperature, and (b) the daily precipitation. Lines are mean expected values from the model in Table 2. Grey tones in lines in (a) indicate varying levels of solar irradiance and in (b) varying levels of daily precipitation. Ribbons show 95% confidence intervals around mean expected values. Grey dots are individual sample values; white dots are mean daily sample values, with error bars indicating 95% confidence intervals.

summer, which appears to be the harshest feeding period under this hot, arid climate (Chin & Brown, 1984). Yet, contrary to our expectations, we did not find high FCM levels or low FT3M coinciding with the dry summer period, only with low temperatures. This suggests that harsh winters could lead to chronic stress in this population.

No evidence of anthropogenic stress

Over the last decades, ecotourism has become ever more popular in Catalonia, with rural tourism increasing from 176 600 travellers in 2003 to 485 200 in 2018, a growth of over 270% in 15 years (Statistical Institute of Catalonia, 2020; www.idescat.cat). The study area also attracts ecotourists, in particular, to watch Iberian red deer during the rut (Figure S2). Yet, contrary to our expectations, ecotourism did not appear to be significantly associated with increased levels of FCM. Concentrations of FCM did not differ between the high and low ecotourism zones, nor were they significantly higher in the months with high influx of tourists, that is, August, and the rut period, September and October. A possible explanation is that animals have become habituated to the presence of watchers or take refuge in the zones with less ecotourism influx. This agrees with the apparent lack of response to the disturbance caused by hill walkers in red deer (*C. elaphus*) (Sibbald et al., 2011), and with the tolerant behaviour to ecotourism by the Sika deer (*C. nippon*) in the Tanzawa Mountains, Japan, where the human population is extremely dense (Borkowski, 2001). In contrast, Jayakody et al. (2008) found differences in red deer behaviour in two zones with contrasting numbers of visitors. In the recreational and hunting season in the Scottish Highlands, they observed a higher frequency of individuals with vigilant behaviour, which implies a decrease in feeding time, in the zone with more visitors and the hunting season. In our study area, stalking hunting was practised during the hunting period and did not coincide with the previous days of the sampling periods.

Conclusion

This study of a wild population gives us a new perspective on how cortisol varies throughout the year. It shows that this hormone did not vary according to a simple seasonal pattern but also responded to short-term (around 2 weeks) changes in weather conditions. If Iberian red deer respond strongly to weather extremes, then climate change might lead to increased periods of stress unless deer can show plasticity in the physiological response or modify their behavioural patterns. In our case, T3 did not decrease when cortisol levels increased, showing no evidence of chronic nutritional stress. Contrary to our expectations, the cortisol hormone did not vary with the different influx of ecotourism.

Author contributions

AGE, JRO and JLLR conceived the ideas; AGE, ML and XM designed the methodology; AGE collected the data; AC and ML did the steroid extraction and hormone analysis; AGE and JLLR analysed the data; all the authors led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

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Conflict of interest

There are no actual or perceived conflicts of interest for all authors.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Assay validation of the EIA kit; results show the precision (intra-assay coefficient of variation (CV)), specificity (dilution test), accuracy (spike-and recovery test) and sensitivity of the assay.

Figure S1. Mean daily average temperature, solar irradiance and precipitation for the study period (2016–2021). The light grey ribbon for temperature depicts the maximum and the minimum daily temperature. Data from the State Meteorological Agency of Spain, Climatological Station of Talarn (421219 N – 005155 E, altitude: 807 m).

Figure S2. Mean daily number of cars that entered Boumort Hunting Reserve per month in 2017 and 2018, showing peak affluence during the rut season (shown by a grey rectangle). The top row gives the maximum daily number of cars per month and year.

Figure S3. AIC of the faecal glucocorticoid metabolites (FCM) model for aggregation periods from 1 to 31 days.

Figure S4. AIC of the faecal triiodothyronine metabolites (FT3M) model for aggregation periods from 1 to 31 days.