The importance of human activities, environmental conditions and biological traits in modulating physiological stress levels in wild red deer

João P.V. Santos[1,2], Pelayo Acevedo[2], João Carvalho[1,3], Carlos Fonseca[1], João Queirós[2,4,5], Christian Gortázar[2], Miriam Villamuelas[3], Jorge Ramón López-Olvera[3], Joaquín Vicente[2]

[1]Departamento de Biologia & CESAM, Universidade de Aveiro, Campus Universitário de Santiago, 3810-193 Aveiro, Portugal
[2]Sanidad y Biotecnología (SaBio), Instituto de Investigación en Recursos Cinegéticos, IREC (CSIC-UCLM-JCCM), Ronda de Toledo s/n, 13071 Ciudad Real, Spain
[3]Servei d' Ecopatologia de Fauna Salvatge (SEFaS), Departament de Medicina i Cirurgia Animals, Universitat Autònoma de Barcelona (UAB), 08193 Bellaterra, Barcelona, Spain
[4]Centro de Investigação em Biodiversidade e Recursos Genéticos (CIBIO)/InBIO Laboratório Associado, Campus Agrário de Vairão, Rua Padre Armando Quintas, 4485-661 Vairão, Portugal
[5]Departamento de Biologia, Faculdade de Ciências da Universidade do Porto (FCUP), 4099-002 Porto, Portugal

Corresponding author:
João P.V. Santos
Departamento de Biologia & CESAM, Universidade de Aveiro, Campus Universitário de Santiago, 3810-193 Aveiro, Portugal
e-mail: joaovalente@ua.pt
Tel: +351 234370350; Fax: +351 234372587

Running title: Factors modulating stress levels in red deer

Word count:
Summary: 346; *Main text:* 6,543; *Acknowledgements:* 188; *References:* 2,999; *Figure legends:* 346; *Table legends:* 273
Number of figures: 5 ; **Number of tables:** 4
Number of references: 108

Summary

1. Identifying the ecological and anthropogenic processes that affect wildlife physiology and that may operate as chronic stressors, is of prime importance to implementing appropriate management and conservation strategies.

2. Although advances have been made in understanding the physiological ecology of wild ungulates, little is known of how multiple biological and ecological factors work, either independently or synergistically, to modulate their stress responses.

3. By using faecal glucocorticoid metabolites (FGM) as indicators of stress, a set of environmental and human determinants affecting the stress physiology of wild red deer (*Cervus elaphus*) was examined in the Mediterranean ecosystems of south-western Europe, where this species is subjected to contrasting weather regimes and hunting management systems. Variation-partitioning techniques were also used to estimate the comparative influence of factors related to an individual's biological characteristics, environmental conditions and management practices in shaping physiological stress levels.

4. Our results show that factors related to hunting management were the main drivers of FGM variation in red deer, followed by those related to the environmental conditions and individuals' traits, and their effects were closely associated to spatio-temporal variability. Holding massive hunting events involving the use of hounds, as well as high population densities, were related to more long-term stress levels in the populations studied. Evidence was also found that supplementary feeding practices may mitigate the negative effects of reduced food availability in overabundant deer populations. Weather conditions were also significant factors explaining variation in stress levels; accumulated precipitation and an increase in ambient temperatures during the coldest months were associated with a decrease in stress hormone levels. No differences in hormonal concentrations were found between males and females, but higher levels of hormone metabolites were detected in younger animals in both sexes.

5. ***Synthesis and applications***. Our findings provide an integrated perspective of how multiple factors impact on stress physiology in large wild herbivores and highlight the importance of considering management practices, as well as spatio-temporal variation, when assessing stress-inducing factors in wild populations. Given the broad implications of this study, it could also be an important basis to support wildlife management decisions.

Keywords: *Cervus elaphu*s, chronic stress, environmental variation, faecal glucocorticoid metabolites, hunting management, individual factors, Mediterranean habitats

Introduction

During their evolutionary history, organisms have developed different adaptive physiological and behavioural mechanisms to cope with adverse conditions or stressors (Bijlsma & Loeschcke 2005; Boonstra 2005). However, persistent exposure to harmful stimuli can seriously affect their physiology and overall condition (Romero & Butler 2007). Glucocorticoids (*i.e.*, cortisol and corticosterone) are steroid hormones that play a vital role in regulating a wide range of physiological processes, including the stress response (Randall, Burggren & French 2002). In mammals and other vertebrates, these hormones are synthesised and secreted by the adrenal cortex following activation of the hypothalamus-pituitary-adrenocortical (HPA) axis (Ulrich-Lai & Herman 2009). Stress can be divided into acute or chronic, depending on the duration of the exposure to the stressors. Of major relevance is chronic stress that can have detrimental effects on the animals' health, growth, reproductive performance (Boonstra 2005; Reeder & Kramer 2005; Wingfield & Sapolsky 2003), and ultimately can compromise their survival (Pride 2005). Due to its potentially negative effects on individuals' fitness, chronic stress has been proposed as having an important role in the dynamics of wild populations (*e.g.*, Bonier *et al.* 2009).

Recent advances in field endocrinology have made it possible to use new techniques for stress assessment in wildlife (reviewed by Sheriff *et al.* 2011). Among these, the quantification of glucocorticoid metabolites in faecal material has become a valuable tool for conservation and management issues (Millspaugh & Washburn 2004; Wikelski & Cooke 2006), as it permits the monitoring of the physiological status of both individuals and populations in a simple and non-invasive way (Keay *et al.* 2006). Moreover, because faecal glucocorticoid metabolite (FGM) levels represent

Comentado [u1]: Podrías quitar alguna referencia y poner e.g.?

pooled fractions of the amount of hormones that have been secreted and metabolised over a broad period of time (Wasser *et al.* 2000; Palme *et al.* 2005), they can provide a long-term and integrated profile of the animals' physiology (Sheriff, Krebs & Boonstra 2010). Owing to its great potential, FGM analysis has been increasingly applied to monitoring stress responses in a wide variety of wild animals (Wasser *et al.* 2000; Young *et al.* 2004; Chinnadurai *et al.* 2009).

Several factors may act as important sources of stress for mammals. Human activities such as hunting (Bateson & Bradshaw 1997; Burke *et al.* 2008), tourism (Zwijacz-Kozica *et al.* 2013; Rehnus, Wehrle & Palme 2014), or other recreational events (Creel *et al.* 2002) can lead to increased stress levels in their populations. Land use practices and the degree of human intervention in natural habitats can also affect species' physiology (Rimbach *et al.* 2013; Navarro-Castilla *et al.* 2014). Apart from human-induced stressful situations, social factors (Creel *et al.* 2013), an elevated risk of predation (Sheriff, Krebs & Boonstra 2009) as well as seasonal variations in the ambient temperature or in the availability of food resources (Dalmau *et al.* 2007; Beehner & McCann 2008) may also represent important environmental and ecological stressors. These extrinsic factors can affect, either independently or synergistically, individuals' physiological condition, and may also interact with intrinsic factors (*e.g.*, sex, life-history stage, reproductive status) in an integrated fashion (Crespi *et al.* 2013; Dantzer *et al.* 2014).

Comentado [u2]: Usas 7 referencias en esta frase; por qué no quitas alguna y sólo pones las referencias al final [aquí] con un e.g.?

Understanding how species cope with environmental or anthropogenic stressors and the physiological impact of such factors, is an increasingly relevant topic in ecological research and could be a very useful diagnostic tool for supporting wildlife management decisions. To date, little research has been conducted to investigate the factors that influence physiological stress in red deer (*e.g.*, Carragher, Ingram &

Matthews 1997; Ingram, Crockford & Matthews 1999; Huber, Palme & Arnold, 2003a), and even less was performed in the wild (*e.g.*, Sauwerwein *et al.* 2004; Corlatti *et al.* 2011). The aim of this study was to explore a set of environmental and human determinants that may potentially explain FGM concentrations in red deer populations under different environmental and management schemes throughout the Iberian Peninsula and that may be, therefore, indicative of chronic stress in those populations. We also used an integrated approach to explore the main drivers of FGM variability in this species. To our best knowledge, this is the first study addressing these issues in Mediterranean ecosystems, where climatological, phenological, and management regimes differ from those observed in central and northern Europe.

Comentado [u3]: Se puede quitar alguna?
Para ver cual quitar podrías ver el número de veces que se cita cada uno de los trabajos y así dejar sólo aquellos trabajos que más apoyo dan al texto

Materials and methods

Study areas

This study was performed at eight areas located in the Iberian Peninsula with contrasting environmental conditions and game management practices, where wild populations of red deer are present (Fig. 1): *Lombada* National Hunting Area and *Sierra de la Culebra* Regional Hunting Reserve (LSC); *Lousã* Mountain (LOU); *Cubeira* Tourist Hunting Area (CUB); *Negrita Norte* Tourist Hunting Area (NEG); *Doñana* National Park (DN); *Quintos de Mora* (QM); *Montes Universales* Hunting Reserve (RCMU); *Caspe-Fraga* Social Hunting Area (CF). All study areas share a Mediterranean type climate, but present distinct temperature or precipitation regimes (Fig. 1). Management practices also differ among the populations studied and depend largely on the administrative authorities' or gamekeepers' objectives. The characteristics of the study areas and red deer populations are summarised in Table 1.

Sampling and data collection

Sampling was carried out during three consecutive hunting seasons (September to February), from 2010-2011 to 2012-2013. Fresh faecal samples were collected directly from the rectum of 289 hunted red deer for FGM determination (Table 1). The collection, transportation and storage of samples were done according to Santos *et al.* (2014). Biological traits such as the sex and age class of each sampled animal were also recorded. Age determination was performed from the observation of tooth eruption patterns in younger animals (Sáenz de Buruaga, Lucio & Purroy 2001) or by counting incremental cementum layers on sectioned roots of the first incisors in individuals older than 2 (Hamlin *et al.* 2000). The sampled animals were subsequently grouped into four biologically relevant age categories as in previous studies with Iberian red deer (*e.g.*, Rodriguez-Hidalgo *et al.* 2010; Santos *et al.* 2013): calves (< 1 year old); yearlings (1 year old); sub-adults (2–3 years old); adults (≥ 4 years old).

FGM concentrations

Faecal pellets (approx. 15–20 g per sample) were dried in a conventional oven at 60 ºC for 48 h, ground in a cyclone-type mill to pass through a 1 mm screen, and then analysed by near infrared reflectance spectroscopy (NIRS) to estimate FGM concentrations. This analytical technique relies on the measurement of the amount of energy absorbed by a sample when irradiated with near infrared light (NIR; 750–2500 nm), thus making it possible to determine its chemical composition through spectral absorption bands. We used a subset of 78 samples (approx. 27% of the total sample size) to calibrate and validate NIRS technology for predicting stress hormone metabolite concentrations in red deer faeces (for details see Santos *et al.* 2014). Then, the calibration equation developed by Santos *et al.* (2014) for oven-dried faeces was used to convert the spectral information into quantitative data (expressed in ng/g of dry matter, DM). The data processing was done using WinISI III (v.1.6) software.

ENVIRONMENTAL FACTORS

Daily maximum, minimum and average air temperatures (ºC) and daily precipitation (mm) were obtained from the weather stations closest to each study area for the period from 2010 to 2013. The Spanish and Portuguese National Meteorology Institutes provided all the data. For analysis, 12 variables for temperature and 3 variables for precipitation were defined *a priori* (Table 2). Because the proximate factors that may influence FGM concentrations were the objective of our study, meteorological variables on a short-time scale were selected. Based on the delay time of approximately 24 h in FGM excretion reported for red deer (Huber *et al.* 2003b) and elk *Cervus elaphus canadensis* (Wasser *et al.* 2000), the temperatures for time *t* – 1 day in relation to the date on which animals were culled (*t*) were determined. Moreover, since FGM concentrations represent an integrated measure of adrenocortical activity over several hours or a few days (Wasser *et al.* 2000; Dantzer *et al.* 2014), the hypothesis that such concentrations might be indicative of the cumulative effects of environmental determinants on a more long-term basis was examined. Therefore, the average temperatures during the last *t* – 30, *t* – 60, and *t* – 90 days were also calculated. Accumulated precipitations were calculated within the same time intervals. Latitude and longitude of the study areas were used as a measure of the geographic position of the populations studied (Table 2). Finally, based on life-history events and on seasonal patterns in glucocorticoid secretion (Huber *et al.* 2003a; Corlatti *et al.* 2011), the sampling months were grouped in two categories: autumn (September to November) and winter (December to February) (Table 2).

Hunting management practices and other human factors

Data on the hunting management practices at the study site level were collected using questionnaires and/or personal interviews with game managers. Hunting events can have various physiological impacts at both individual and population levels (Bateson & Bradshaw 1997; Burke *et al.* 2008). In the Iberian Peninsula, red deer are typically hunted either by stalking, or through drive hunts or 'battues'. Such hunting methods involve different levels of culling intensity and disturbance for animals, with large drives with beaters and dogs having the highest expected impact on wild populations, whereas stalking has the lowest. Thus, to account for the levels of disturbance caused by the different hunting activities, our study areas were classified according to the presence or absence of massive hunting events (Tables 1 and 2). Additionally, three variables were used as relative measures of hunting pressure among the study sites: i) number of red deer harvested per hunting season, ii) number of deer harvested per surface area, and iii) proportion of the population harvested per hunting season (Tables 1 and 2).

Supplementary feeding is a commonly used practice in areas managed for hunting. In our study region, supplementary feeding is often associated with the maintenance of artificially high population densities above the ecological carrying capacity (Vicente *et al.* 2007a). However, the provision of supplementary food may also have adverse effects on deer physiology; *e.g.*, high densities and the aggregation of animals at artificial feeding sites can operate as stress factors (Forristal *et al.* 2012). To evaluate the effects of food provision on FGM concentrations, the study areas were categorised as having, or not, a supplementary feeding regime during the regular game seasons (Tables 1 and 2).

The extent of human-induced changes on natural habitats was also investigated as a source of anthropogenic disturbance. For each study area, an index that measures the potential land avoidance by wild ungulates in Mediterranean ecosystems was calculated (Table 2). The ‘Wild Ungulates Land Avoidance Index’ (WULAI; see Cassinello, Acevedo & Hortal 2006)) was determined by first assigning scores to land use categories on a CORINE Land Cover map for 2006 (100 x 100 m resolution; European Environment Agency, 2011). These scores are awarded to reflect the degree of avoidance of ungulates to a particular habitat, and range from 0 (no avoidance) to 100 (total avoidance; Table 1).

ESTIMATION OF RED DEER POPULATION DENSITY

High population densities have been identified as a source of stress in many mammalian species (Creel *et al.* 2013), including deer (Li *et al.* 2007; Forristal *et al.* 2012). To estimate red deer population density, line transect spotlight counts coupled with distance sampling methods were conducted annually in each study area (Acevedo *et al.* 2008). Field surveys were carried out during the rutting season (September and October), when deer are more easily detected. Distance sampling field measurements were analysed using *Distance 6.0 release 2* (Thomas *et al.* 2010). For each population studied, the data from annual surveys was pooled to generate a global detection function and the density estimates (expressed as deer/Km^2) were post-stratified by year. Due to the impossibility of carrying out field surveys in the NEG hunting area, we used the density data from a neighbouring area with similar characteristics, where the same survey methodology was applied (Lopes 2008).

Comentado [u4]: Deja sólo una cita

STATISTICAL ANALYSES

A preliminary exploration of the data (variable distribution, outlier detection, and collinearity diagnostics) was performed to obtain a better understanding of their characteristics, and to avoid violating assumptions of statistical procedures (Zuur, Ieno & Elphick 2010). Variance inflation factors (VIF) were checked to rule out multicollinearity between explanatory variables. As some of the predictors had multiple degrees of freedom (df), multicollinearity was assessed by checking adjusted generalised VIF values ($GVIF_{adj} = GVIF^{1/2*df}$, Fox & Weisberg, 2011). We used a $GVIF_{adj} = 2.5$ as a cut-off value, that is, the predictor with the highest $GVIF_{adj}$ value was dropped in a stepwise procedure until all the remaining predictors reached a $GVIF_{adj} \leq 2.5$ (Zuur *et al.* 2009).

Linear mixed models (LMM; McCulloch, Searle & Neuhaus 2008) were used to investigate the environmental and anthropogenic determinants affecting FGM levels in red deer. Because of the large number of potential explanatory variables, a forward stepwise AIC-based procedure (Akaike 1974) was run to find the most parsimonious model. For modelling purposes, the ‘study area’ was used as a random factor, while the ‘hunting season’ was included as a fixed factor due to the small number of levels available, which limits its use as a random factor (see Zuur, Ieno & Smith 2007). The sex and age class of animals sampled were also included in the analyses to account for potential differences in stress response and FGM excretion between males and females (López-Olvera *et al.* 2007; Goymann 2012), as well as between young and adult individuals (Millspaugh & Washburn 2004; Goymann 2012). Additionally, three interaction terms were constructed and tested because of their potential biological or ecological significance in red deer: sex*age class (*e.g.*, Vicente, Pérez-Rodríguez & Gortázar 2007b), season*temperature at time $t - 1$ day (*e.g.*, Huber *et al.* 2003a; Corlatti *et al.* 2011), and supplementary feeding*population density (*e.g.*, Vicente *et*

Comentado [u5]: Deja sólo una cita

Comentado [u6]: Deja sólo una cita

al. 2007a). The data exploration and statistical analyses were conducted with R statistical software, version 3.1.2 (R Core Team, 2014). The LMM were fit using the 'lme4' package, version 1.1-7 (Bates *et al.* 2014). Type III F-tests with Satterthwaite's approximation for degrees of freedom were used to assess significance level of predictors using the function 'anova' available in the 'lmerTest' package, version 2.0-20 (Kuznetsova, Brockhoff & Christensen 2014) for R. Statistical significance was set at $P \leq 0.05$.

As a final step, variation-partitioning techniques (Borcard, Legendre & Drapeau 1992) were employed to assess the comparative influence of biological and ecological components on modulating FGM levels in red deer. To this end, the explanatory variables retained in the final model were grouped into four main components (*i.e.*, sets of related predictors): individual, environmental, human and spatio-temporal ('study area' and 'hunting season')(see Tables 3 and 4)Partitioning was carried out in a two-step procedure as follows:

1) How much variation of the final model was explained independently (pure effects) and jointly (combined effects) by the 'spatio-temporal' component in relation to the remaining set of variables was determined. In this step, two partial models were developed (*i.e.*, models adjusted independently with the variables related to each component. Spatio-temporal: 'SpaTemp', and three main components: 'Main') and the amount of variation of the final model (SpaTemp+Main), explained by each partial model, was calculated in terms of conditional R^2 (see below). Subsequently, the R^2 values obtained for each model were subjected to subtraction operations in order to separate out the different fractions of the variation explained by the final model.

2) The amount of variation that was explained independently and simultaneously by the three main components was estimated. Here, six partial models were built, *i.e.*, one for each main component (individual: Ind, environmental: Env, and human: Hum), and also for each pair of components (Ind+Env, Ind+Hum, and Env+Hum), and the amount of variation explained by each of them was calculated in terms of marginal R^2 (see below). As in step 1, subtraction rules between the R^2 values obtained for each partial model were applied in order to split the variation explained by each component into pure and combined fractions (see Fig. S1 in Supporting Information for details on procedures; see also Acevedo et al. 2010; Legendre & Legendre 2012).

The proportion of variation explained (R^2) by the abovementioned models was calculated according to Nakagawa & Schielzeth (2013). The marginal R^2 refers to the proportion of variation explained only by the fixed factors in a model, while the conditional R^2 describes the proportion of variation explained by both the fixed and random effects. The 'varPart' function (Barbosa *et al.* 2014) was used to generate Venn diagrams in the R package showing the single and shared contributions of the various components in the final model.

Results

An average of 36.13 ± 4.40 (SE) faecal samples of red deer were collected per population studied over a three-year sampling period: 156 males and 133 females. Of these, 23 were calves, 52 yearlings, 42 sub-adults, and 172 adults. Throughout this study, FGM concentrations varied among study areas (Fig. 2). During data exploration, two FGM measurements were identified as extreme outliers (*i.e.*, more than 3 times outside the interquartile range) and therefore were excluded from subsequent analyses. Hence, the remaining 287 measurements were used for analytical purposes.

Thirteen variables of weather, three variables of hunting pressure and one variable of geographic location that could explain FGM levels in red deer were excluded from the initial set of predictors, to avoid multicollinearity (see Table 2). The final model (Tables 3 and 4) included 10 predictors and three interaction terms (see Table S1 in the Supporting Information for results of the stepwise model selection analysis). Briefly, no significant differences were found in FGM concentrations between males and females, but such concentrations differed significantly among age classes, with younger animals showing higher levels of hormone metabolites when compared to adult animals (Table 4). The interaction sex*age class had no significant effect on FGM concentrations. On the other hand, no significant differences were observed between the autumn and winter period, but a significant effect of season was found when controlling for variation in the average temperature recorded one day before sampling, as revealed by the significant interaction between these two predictors (Fig. 3). The precipitation accumulated one month before sampling was negatively associated with FGM concentrations), whereas the latitude of the study areas had a positive significant effect on the hormonal levels in red deer (Table 4). Supplementary feeding and population density alone had no significant effects on FGM levels . However, the interaction between these variables significantly affected FGM concentrations in red deer. A negative association between FGM levels and population density was observed in study areas where supplementary food is provided, whereas an inverse pattern was found in areas where supplementary feeding practices do not exist (Fig. 4). The presence or absence of big hunting events also significantly affected FGM concentrations, with such concentrations being higher in areas where hunting methods involve the use of hounds and beaters (Table 4). In addition, a statistically negative relationship was found between hormone levels and WULAI .

The final model explained 51.2% of the total variation in FGM concentrations in red deer (Fig. 5a). The results from variation-partitioning analysis showed that almost 42% of that variation was explained by the combined effect of the spatio-temporal component with the remaining set of variables of the main components analysed, while only a small fraction was explained by their pure effects (Fig. 5a). Regarding the variation attributable only to the three main components, it was found that the pure effects of the anthropogenic factors explained the highest percentage of that variation (almost 90%) in FGM levels, followed by the pure effects of the environmental (70.8%) and individual (51.5%) components (Fig. 5b).

Discussion

As far as we know, this is the first study providing data on FGM concentrations in free-ranging populations of red deer in Mediterranean ecosystems and therefore we do not have directly comparable quantitative data for this species under such conditions. However, an overall concordance was found between our FGM values and the levels of hormone metabolites determined in other studies that have been conducted in different environmental, management or experimental contexts with red deer/elk (Huber *et al.* 2003a; Corlatti *et al.* 2011; Azorit *et al.* 2012; Forristal *et al.* 2012), roe deer *Capreolus capreolus* (Dehnhard *et al.* 2001), fallow deer *Dama dama* (Konjević *et al.* 2011), white-tailed deer *Odocoileus virginianus* (Millspaugh *et al.* 2002), Père

Comentado [u7]: Usa e.g. y quita referencias

David's deer *Elaphurus davidianus* (Li *et al.* 2007), and Pampas deer *Ozotoceros bezoarticus* (Pereira, Duarte & Negrão 2006).

Identifying the ecological and anthropogenic processes that act as sources of physiological stress in wildlife is of prime importance to implementing appropriate management and conservation strategies (Dantzer *et al.* 2014). Taken together, our results demonstrate how multiple factors can work at different levels, either independently or simultaneously, in modulating the FGM concentrations and physiological stress response in red deer. Of the various factors analysed, those related to anthropogenic activities explained most of the variation in FGM concentrations. The factors associated to environmental change and to individuals' biological traits were also relevant in modulating stress levels, with the former contributing more than the latter to explaining those levels. Moreover, it was found that the effects of those factors were not fully independent from spatio-temporal variability. The results of this research have also allowed us to identify some potential sources of chronic stress for red deer in the Mediterranean habitats.

Biological traits and FGM concentrations in red deer

Intrinsic factors such as sex and age are described as significant factors affecting glucocorticoid levels, but sex- and age-related differences in glucocorticoid secretion have not always been consistently demonstrated among vertebrates (Sands & Creel 2004; Wada *et al.* 2006; Crespi *et al.* 2013). In the present study, no differences were found in the FGM concentrations between sexes in red deer. Similar results were obtained for this species by Huber *et al.* (2003a) and also by Millspaugh *et al.* (2001) for elk. Although our research focused exclusively on the autumn and winter periods, those authors found similarities in FGM levels between both sexes among all the seasons. Despite this, the absence of sex-specific differences in hormonal levels is, *a priori*, unexpected, especially in a highly sexually dimorphic species such as red deer, in which distinct physiological and energy requirements are predictable between males and females (Clutton-Brock, Guinness & Albon 1982). However, a plausible explanation for these results can be provided from an energetic viewpoint. Glucocorticoids have important metabolic functions, helping animals to adjust the way energy is used in their bodies (Randall *et al.* 2002). As suggested by Key & Ross (1999), in species displaying a highly developed sexual dimorphism, the energy costs of gestation and lactation for the females can be equivalent to those of maintaining a large body size for the males. Thus, energy trade-offs for maintaining different physiological functions can help explain the similar results obtained in FGM levels for males and females in our study.

An analogous reasoning might be valid for explaining the differences observed in FGM levels among age classes, which were found to be higher in younger than in adult animals in both sexes. Such results may reflect age-related differences in the basal metabolic rate (*i.e.*, amount of energy required to maintain basic cellular functions), which is inversely related to body size (Randall *et al.* 2002). Moreover, the extra energy demands associated with body growth and maintenance in younger animals may impose increased physiological stress (Santos *et al.* 2013), leading to increased levels of glucocorticoids. In contrast to our results, Sauwerwein *et al.* (2004) did not find differences among age groups in glucocorticoid metabolite concentrations measured in ileal digesta of red deer. However, a trend towards higher hormone metabolite levels in young deer was observed in their study.

At the level of the individual, several other factors not analysed in this study, such as diet and gut microbiota variability, may affect the relative composition of the hormone metabolites formed and, consequently, their concentrations (reviewed by Goymann 2012). Inter-individual variability on the HPA axis response to stressful events is also an important factor modulating glucocorticoid secretion (Federenko *et al.* 2004). As red deer are social and gregarious animals (Putman 1988), probably multiple psychosocial stressors affect the stress physiology in this species (Creel *et al.* 2013). The way animals cope with previous experiences has also been suggested as being critical in determining different stress responses among individuals (Burke *et al.* 2008).

Environmental factors and FGM concentrations in red deer

The modulation of basal and stress-induced glucocorticoid levels to environmental challenges is relatively well documented in terrestrial vertebrates, and is often associated with the energy costs of seasonal weather conditions and resource limitations (Reeder & Kramer 2005; Bonier *et al.* 2009). Here, we observed a more pronounced decrease in FGM concentrations with increasing ambient temperature during the winter in relation to autumn. These results are coherent with bio-energetic processes, since any increment in ambient temperature is expected to have a greater proportional negative effect on metabolic rate during the winter, when the temperatures are generally lower (Randall *et al.* 2002). Accordingly, during cold weather conditions, basal glucocorticoid secretion tends to be higher to stimulate catabolic pathways, thus compensating for the effects of the increased energy demands to maintain body homeostasis (Huber *et al.* 2003a; Corlatti *et al.* 2011; see also Dalmau *et al.* 2007). Other studies have reported peaks of FGM concentrations in the summer in North American elk (Millspaugh *et al.* 2001), or during the spring, summer, and autumn in bighorn sheep *Ovis canadensis*, with the lowest values in the winter (Goldstein *et al.* 2005), which may reflect the influence of specific local conditions.

In environments where temperatures remain relatively constant throughout the year (*e.g.*, subtropical regions), seasonal fluctuations of FGM levels are mostly influenced by precipitation regimes, which dictate the availability of food resources (Pereira *et al.* 2006; Chinnadurai *et al.* 2009). In this study, a significantly negative relationship between the accumulated rainfall one month before sampling and the FGM concentrations was found. Although we have no FGM data for the summer months, it is thought that this result may be associated to the particular climatic conditions found in our study region during that period. In Mediterranean areas, the summer drought is often long and intense, thus limiting plant growth and survival, which imposes nutritional constraints on herbivores, including red deer (Bugalho & Milne 2003). For this reason, the amount of precipitation that falls during the autumn and winter is critical for plant regeneration (especially for herbaceous vegetation) and increased food availability in these seasons (Bugalho & Milne 2003; Rodriguez-Hidalgo *et al.* 2010).

Finally, we obtained a significantly positive correlation between the latitude and the FGM levels. Comparative studies exploring the influence of large-scale geographic factors on glucocorticoid secretion patterns are not widely available, especially for mammals. Most of the research addressing these issues has been conducted with birds, amphibians and reptiles and, despite physiological differences among groups, the results obtained to date have shown positive associations between latitudinal variation and the basal levels of glucocorticoids (see Eikenaar *et al.* 2012). Some hypotheses have been advanced to elucidate possible mechanisms underlying physiological responses to macro-environmental conditions. An interesting and detailed discussion on this topic can be found in a recent paper by Eikenaar *et al.* (2012). Here, we suggest that the results obtained in this study could be explained by the 'preparative hypothesis' (*sensu* Romero 2002), which posits that levels of glucocorticoid secretion are seasonally modulated to respond to predictable stressful situations. Thus, considering that animal populations at higher latitudes are exposed to harsher weather conditions during the coldest months, one should expect to find higher levels of glucocorticoids in those populations.

Anthropogenic factors and FGM concentrations in red deer

Significantly higher levels of FGM were detected in red deer populations subjected to hunting methods involving drive hunts with beaters and hounds, in comparison to those where hunting is only performed selectively by stalking. This result makes sense since drive hunts produce higher levels of disturbance for wildlife, with proportionally larger effects when repeated throughout the hunting seasons. Behavioural studies have already demonstrated that ungulates can perceive hunting activities as a threat similar to that caused by natural predators (Stankowich 2008; Grignolio *et al.* 2011). Other studies have provided evidence on the short- and long-term physiological effects of hunting activities on wild herbivores. For example, Bateson & Bradshaw (1997) found marked differences in the post-mortem physiological status of red deer hunted with hounds as compared to undisturbed deer that have been shot by a single hunter. Cortisol levels and other physiological measures were significantly higher in deer hunted with the aid of dogs, and further increased with the increasing extent and duration of the hunts. Implicitly, these results also suggest that physiological and psychological stress caused by hunting may persist in the remaining population for some time after hunting events (Burke *et al.* 2008).

By contrast, other studies did not find any correlation between hunting activity and FGM concentrations in elk (Millspaugh *et al.* 2001), and in Pyrenean chamois (Dalmau *et al.* 2007). However, we suspect that the hunting methods applied and the duration of hunting seasons may explain those results.

A very interesting finding, from the point of view of management, was that deer population density and the presence of supplementary feeding interacted to explain FGM levels: in non-supplemented populations the stress hormone concentrations increased with increasing population density, whereas an inverse pattern was found in food-supplemented populations. High population densities can strongly affect adrenocortical activity, as they lead to increased intraspecific competition for resources and/or promote aggregation and social interactions (reviewed by Creel *et al.* 2013). Under natural conditions, a reduction in food availability *per capita*, as a consequence of increased density, leads to a depletion of body reserves and increased nutritional stress more easily. On the other hand, the negative effects of high densities on an animal's physiological condition are frequently mitigated by artificially providing food (Vicente *et al.* 2007a). The expected negative effect of increasing aggregation mediated by social stress, as population density increases, was not observed (Saltz & White 1991). Perhaps the positive effects of high food availability counteracted the increased social stress caused by higher population densities. As contrasting situations are reported in the literature (see Forristal *et al.* 2012), this balance may depend on specific local conditions and the strength of the effects.

The WULAI scores obtained for our study areas covered a small range of values, and evidenced low levels of human-altered landscapes. Even so, there was a significant negative correlation between the WULAI and the FGM concentrations. This relationship might reflect different responses to habitat quality and/or landscape structure by populations. However, future research including a wider range of WULAI values, as well as other indicators of landscape alteration, may further elucidate the real meaning of this relationship.

MAJOR FINDINGS, CONCLUSIONS AND IMPLICATIONS FOR RED DEER MANAGEMENT

In terms of the methodological approach, the usefulness of collecting fresh faeces from hunted deer to monitor their physiological condition was demonstrated. This procedure brings some advantages over faeces collected from the ground, as it makes it possible to obtain information on the sex and age class of individuals *in situ*, without additional costs. On the other hand, two of the major drawbacks are that this form of monitoring is limited to game species and restricted to hunting seasons.

From this research, it was possible to derive meaningful information of how individual and ecological processes affect stress hormone levels in wild red deer, and estimate their relative contribution in shaping those levels. Anthropogenic factors, especially those related to hunting management, were identified as the main source of variation in FGM concentrations, although environmental conditions and intrinsic factors were also important drivers of FGM variation. Regarding anthropogenic factors, two results, which may be considered of particular interest for red deer management, stand out, because of their relevance as potential sources of chronic stress. Firstly, the impacts of large hunts with hounds on deer physiology should be noted and more studies targeted to evaluate their cumulative effects on individuals' performance and population dynamics are recommended. Animal welfare concerns are becoming increasingly relevant nowadays and they also apply to the context of managing wild ungulates (see Ohl & Putman 2014), and should serve as a basis for major improvements in management plans and policies. Secondly, this study may serve as a warning concerning the negative effects of overabundant populations on the stress levels of red deer. Although our results showed that the provision of supplementary food may help minimize nutritional stress in dense populations, this practice is not devoid of associated risks, *e.g.*, disease transmission (Gortázar *et al.* 2006). For management assessment, FGM values *per se* were not indicative of the management system, but our results indicate that it is fundamental to consider management variables to interpret them, especially in open populations, whereas other physiological indicators should be implemented in artificially fed deer populations, at least in our study latitudes. At the level of the individual, it would be appropriate to include, in further studies, other measures of physical condition (*e.g.*, fat reserves, parasite burdens, diet quality, reproductive status) and investigate their relationship with stress hormone concentrations.

Finally, our findings strongly support evidence that the factors affecting physiological stress response in wild populations are dynamic, varying in space and time. Thus, on a large scale, it is crucial to consider geographic and temporal variation to correctly interpret the physiological responses of wildlife to anthropogenic and environmental stressors.

Acknowledgements

The authors are grateful to Javier Marco, Javier Ferreres and Marco Escudero (Ebronatura S.L.) for providing us with some data and for their logistic support during fieldwork. We would also like to thank to Elena Albanell and Raquel Pato González (*Universitat Autònoma de Barcelona*, UAB) and Carmen (*Laboratorio de Quintos de Mora*, OAPN) for their support in the laboratory. We acknowledge all the support in the field and their collaboration in the interviews on the part of all the Directors, game managers and rangers from Game Services. We also thank AEMET (Spanish Meteorological Agency) for providing us with weather data for free (request No. 990130948). J.P.V. Santos was supported by a PhD Grant (SFRH/BD/65880/2009) from *Fundação para a Ciência e a Tecnologia* (FCT) co-financed by the European Social Fund POPH-QREN programme. P. Acevedo is supported by the Spanish *Ministerio de Economía y Competitividad* (MINECO) and *Universidad de Castilla-La Mancha* (UCLM) through a 'Ramón y Cajal' contract (RYC-2012-11970). J. Carvalho and J. Queirós were both supported by PhD Grants (SFRH/BD/98387/2013 and SFRH/BD/73732/2010, respectively) from FCT. Jon Nesbit (Faculty of Letters, University of Coimbra, Portugal) kindly reviewed the English text.

Data Accessibility

The authors intend to archive the data associated with this publication at Dryad (datadryad.org/). Data will be made publicly available and the location disclosed upon publication of this study.

References

Acevedo, P., Farfán, M.A., Márquez, A.L., Delibes-Mateos, M., Real, R. & Vargas, J.M. (2011) Past, present and future of wild ungulates in relation to changes in land use. *Landscape Ecology*, **26**, 19-31.

Acevedo, P., Ruiz-Fons, F., Estrada, R., Márquez, A.L., Miranda, M.A., Gortázar, C. & Lucientes, J. (2010) A broad assessment of factors determining *Culicoides imicola* abundance: Modelling the present and forecasting its future in climate change scenarios. *PLoS ONE*, **5**, 12, e14236.

Acevedo, P., Ruiz-Fons, F., Vicente, J., Reyes-García, A.R., Alzaga, V. & Gortázar, C. (2008) Estimating red deer abundance in a wide range of management situations in Mediterranean habitats. *Journal of Zoology, London*, **276**, 37-47.

Akaike, H. (1974) A new look at the statistical model identification. *IEEE Transactions on Automatic Control*, **19**, 716-723.

Apollonio, M., Andersen, R. & Putman, R. (2010) *European Ungulates and Their Management in the 21st Century*. Cambridge University Press, Cambridge.

Azorit, C., Castro, J.M., 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. *Animal Production Science*, **52**,756–760.

Barbosa, A.M., Brown, J.A., Jiménez-Valverde, A. & Real, R. (2014) modEvA: an R package for model evaluation and analysis. R package, version 0.5.1. http://modtools.wordpress.com/packages/modeva/

Bates, D., Maechler, M., Bolker, B. & Walker, S. (2014) lme4: Linear mixed-effects models using Eigen and S4. R package, version 1.1-7. http://cran.r-project.org/web/packages/lme4/

Bateson, P. & Bradshaw, E.L. (1997) Physiological effects of hunting red deer (*Cervus elaphus*). *Proceedings of the Royal Society of London B*, **264**, 1707-1714.

Beehner, J.C. & McCann, C. (2008) Seasonal and altitudinal effects on glucocorticoid metabolites in a wild primate (*Theropithecus gelada*). *Physiology & Behavior*, **95**, 508-514.

Benhaiem, S., Delon, M., Lourtet, B., Cargnelutti, B., Aulagnier, S., Hewison, A.J.M., Morellet, N. & Verheyden, H. (2008) Hunting increases vigilance levels in roe deer and modifies feeding site selection. *Animal Behaviour*, **76**, 611-618.

Bijlsma, R. & Loeschcke, V. (2005) Environmental stress, adaptation and evolution: an overview. *Journal of Evolutionary Biology*, **18**, 744-749.

Bonier, F., Martin, P.R., Moore, I.T. & Wingfield, J.C. (2009) Do baseline glucocorticoids predict fitness? *Trends in Ecology and Evolution*, **24**, 634-642.

Boonstra, R. (2005) Equipped for life: the adaptive role of the stress axis in male mammals. *Journal of Mammalogy*, **86**, 236-247.

Borcard, D., Legendre, P. & Drapeau, P. (1992) Partialling out the spatial component of ecological variation. *Ecology*, **73**, 1045-1055.

Brown, R.D. & Cooper, S.M. (2006) The nutritional, ecological, and ethical arguments against baiting and feeding white-tailed deer. *Wildlife Society Bulletin*, **34**, 519-524.

Buckland, S.T., Anderson, D.R., Burnham, K.P., Laake, J.L., Borchers, D.L. & Thomas, L. (2001) *Introduction to Distance Sampling*. Oxford University Press, Oxford.

Bugalho, M.N. & Milne, J.A. (2003) The composition of the diet of red deer (*Cervus elaphus*) in a Mediterranean environment: a case of summer nutritional constraint? *Forest Ecology and Management*, **181**, 23-29.

Burke, T., Page, B., Van Dyk, G., Millspaugh, J. & Slotow, R. (2008) Risk and ethical concerns of hunting male elephant: behavioural and physiological assays of the remaining elephants. *PLoS ONE*, **3**, 6, e2417.

Burnham, K.P. & Anderson, D.R. (2002) *Model Selection and Multi-Model Inference*. Springer, New York.

Carragher, J.F., Ingram, J.R. & Matthews, L.R. (1997) Effects of yarding and handling procedures on stress responses of red deer stags (*Cervus elaphus*). *Applied Animal Behaviour Science*, **51**, 143-158.

Carranza, J. (2010) Ungulates and their management in Spain. *European Ungulates and Their Management in the 21st Century* (eds M. Apollonio, R. Andersen & R. Putman), pp. 419-440. Cambridge University Press, Cambridge.

Cassinello, J., Acevedo, P. & Hortal, J. (2006) Prospects for population expansion of the exotic aoudad (*Ammotragus lervia*; Bovidae) in the Iberian Peninsula: clues from habitat suitability modelling. *Diversity and Distributions*, **12**, 666-678.

Chinnadurai, S.K., Millspaugh, J.J., Matthews, W.S., Canter, K., Slotow, R., Washburn, B.E. & Woods, R.J. (2009) Validation of fecal glucocorticoid metabolite assays for South African herbivores. *The Journal of Wildlife Management*, **73**, 1014-1020.

Clutton-Brock, T.H., Guinness, F.E. & Albon, S.D. (1982) *Red Deer: Behaviour and Ecology of Two Sexes*. University of Chicago Press, Chicago.

Corlatti, L., Palme, R., Frey-Roos, F. & Hackländer, K. (2011) Climatic cues and glucocorticoids in a free-ranging riparian population of red deer (*Cervus elaphus*). *Folia Zoologica*, **60**, 176-180.

Creel, S., Dantzer, B., Goymann, W. & Rubenstein, D.R. (2013) The ecology of stress: effects of the social environment. *Functional Ecology*, **27**, 66-80.

Creel, S., Fox, J.E., Hardy, A., Sands. J., Garrott. B. & Peterson, R.O. (2002) Snowmobile activity and glucocorticoid stress responses in wolves and elk. *Conservation Biology*, **16**, 809-814.

Crespi, E.J., Williams, T.D., Jessop, T.S. & Delehanty, B. (2013) Life history and the ecology of stress: how do glucocorticoid hormones influence life-history variation in animals? *Functional Ecology*, **27**, 93-106.

Dalmau, A., Ferret, A., Chacon, G. & Manteca, X. (2007) Seasonal changes in faecal cortisol metabolites in Pyrenean chamois. *The Journal of Wildlife Management*, **71**, 190-194.

Dantzer, B., Fletcher, Q.E., Boonstra, R. & Sheriff, M.J. (2014) Measures of physiological stress: a transparent or opaque window into the status, management and conservation of species? *Conservation Physiology*, **2**, 1-18.

Dehnhard, M., Clauss, M., Lechner-Doll, M., Meyer, H.H.D. & Palme, R. (2001) Noninvasive monitoring of adrenocortical activity in roe deer (*Capreolus capreolus*) by measurement of faecal cortisol metabolites. *General and Comparative Endocrinology*, **123**, 111-120.

Eikenaar, C., Husak, J., Escallón, C. & Moore, I.T. (2012) Variation in testosterone and corticosterone in amphibians and reptiles: relationships with latitude, elevation, and breeding season length. *The American Naturalist*, **180**, 642-654.

Ellis, R.D., McWhorter, T.J. & Maron, M. (2012). Integrating landscape ecology and conservation physiology. *Landscape Ecology*, **27**, 1-12.

European Environment Agency, EEA (2011) Corine Land Cover raster data. Available at: http://www.eea.europa.eu/data-and-maps/data/corine-land-cover-2006-raster-3

Federenko, I.S., Nagamine, M., Hellhammer, D.H., Wadhwa, P.D. & Wüst, S. (2004) The heritability of hypothalamus pituitary adrenal axis responses to psychosocial stress is context dependent. *The Journal of Clinical Endocrinology & Metabolism*, **89**, 6244-6250.

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.

Forristal, V.E., Creel, S., Taper, M.L., Scurlock, B.M. & Cross, P.C. (2012) Effects of supplemental feeding and aggregation on fecal glucocorticoid metabolite concentrations in elk. *The Journal of Wildlife Management*, **76**, 694-702.

Fox, J. & Weisberg, S. (2011) *An R Companion to Applied Regression*. 2nd edn. SAGE Publications, London.

Goldstein, E.J., Millspaugh, J.J., Washburn, B.E., Brundige, G.C. & Raedeke, K.J. (2005) Relationships among fecal lungworm loads, fecal glucocorticoid metabolites, and lamb recruitment in free-ranging Rocky Mountain bighorn sheep. *Journal of Wildlife Diseases*, **41**, 416-425.

Gordon, I.J., Hester, A.J. & Festa-Bianchet, M. (2004) The management of wild large herbivores to meet economic, conservation and environmental objectives. *Journal of Applied Ecology*, **41**, 1021-1031.

Gortázar, C., Acevedo, P., Ruiz-Fons, F. & Vicente, J. (2006) Disease risk and overabundance of game species. *European Journal of Wildlife Research*, **52**, 81-87.

Goymann, W. (2012) On the use of non-invasive hormone research in uncontrolled, natural environments: the problem with sex, diet, metabolic rate and the individual. *Trends in Ecology and Evolution*, **3**, 757-765.

Grignolio, S., Merli, E., Bongi, P., Ciuti, S. & Apollonio, M. (2011) Effects of hunting with hounds on a non-target species living on the edge of a protected area. *Biological Conservation*, **144**, 641-649.

Hamlin, K.L., Pac, D.F., Sime, C.A., Desimone, R.M. & Dusek, G.L. (2000) Evaluating the accuracy of ages obtained by two methods for Montana ungulates. *The Journal of Wildlife Management*, **64**, 441-449.

Hijmans, R.J., Cameron, S.E., Parra, J.L., Jones, P.G. & Jarvis, A. (2005) Very high resolution interpolated climate surfaces for global land areas. *International Journal of Climatology*, **25**, 1965-1978.

Huber, S., Palme, R. & Arnold, W. (2003a) Effects of season, sex and sample collection on concentrations of fecal cortisol metabolites in red deer (*Cervus elaphus*). *General and Comparative Endocrinology*, **130**, 48-54.

Huber, S., Palme, R., Zenker, W. & Möstl, E. (2003b) Non-invasive monitoring of the adrenocortical response in red deer. *The Journal of Wildlife Management*, **67**, 258-266.

Ingram, J.R., Crockford, J.N. & Matthews, L.R. (1999) Ultradian, circadian and seasonal rhythms in cortisol secretion and adrenal responsiveness to ACTH and yarding in unrestrained red deer (*Cervus elaphus*) stags. *Journal of Endocrinology*, **162**, 289-300.

Jayakody, S., Sibbald, A.M., Gordon, I.J. & Lambin, X. (2008) Red deer *Cervus elaphus* vigilance behaviour differs with habitat and type of human disturbance. *Wildlife Biology*, **14**, 81-91.

Keay, J.M., Singh, J., Gaunt., M.C. & Kaur, T. (2006) Fecal glucocorticoids and their metabolites as indicators of stress in various mammalian species: a literature review. *Journal of Zoo and Wildlife Medicine*, **37**, 234-244.

Key, C. & Ross, C. (1999) Sex differences in energy expenditure in non–human primates. *Proceedings of the Royal Society of London B*, **266**, 2479-2485.

Konjević, D., Janicki, Z., Slavica, A., Severin, K., Krapinec, K., Božić, F. & Palme, R. (2011) Non-invasive monitoring of adrenocortical activity in free-ranging fallow deer (*Dama dama* L.). *European Journal of Wildlife Research*, **57**, 77-81.

Kottek, M., Grieser, J., Beck, C., Rudolf, B. & Rubel, F. (2006) World Map of the Köppen-Geiger climate classification updated. *Meteorologische Zeitschrift*, **15**, 259-263.

Kuznetsova, A., Brockhoff, P.B. & Christensen, R.H.B. (2014) lmerTest: Tests for random and fixed effects for linear mixed effect models (lmer objects of lme4 package). R package, version 2.0-20. http://cran.at.r-project.org/web/packages/lmerTest/

Legendre, P. & Legendre, L. (2012) *Numerical Ecology*. 3rd English edn. Elsevier, Amsterdam.

Li, C., Jiang, Z., Tang, S. & Zeng, Y. (2007) Influence of enclosure size and animal density on fecal cortisol concentration and aggression in Père Davids deer stags. *General and Comparative Endocrinology*, **151**, 202-209.

Lopes, F.V. (2008) *O Veado na Zona de Caça Nacional do Perímetro Florestal da Contenda. Monitorização da população*. Autoridade Florestal Nacional.

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. *Canadian Journal of Veterinary Research*, **71**, 41-51.

McCulloch, C.E., Searle, S.R. & Neuhaus, J.M. (2008) *Generalized, Linear, and Mixed Models*. 2nd edn. John Wiley and Sons, New Jersey.

Millspaugh, J.J., Brundige, G.C., Gitzen, R.A. & Raedeke, K.J. (2000) Elk and hunter space-use sharing in South Dakota. *The Journal of Wildlife Management*, **64**, 994-1003.

Millspaugh, J.J. & Washburn, B.E. (2004) Use of fecal glucocorticoid metabolite measures in conservation biology research: considerations for application and interpretation. *General and Comparative Endocrinology*, **138**, 189-199.

Millspaugh, J.J., Washburn, B.E., Milanick, M.A., Beringer, J., Hansen, L.P. & Meyer, T.M. (2002) Non-invasive techniques for stress assessment in white-tailed deer. *Wildlife Society Bulletin*, **30**, 899-907.

Millspaugh, J.J., Woods, R.J., Hunt, K.E., Raedeke, K.J., Brundige, G.C., Washburn, B.E. & Wasser, S.K. (2001) Fecal glucocorticoid assays and the physiological stress response in elk. *Wildlife Society Bulletin*, **29**, 899-907.

Mysterud, A. & Sæther, B.-E. (2011) Climate change and implications for the future distribution and management of ungulates in Europe. *Ungulate Management in Europe: Problems and Practices* (eds R. Putman, M. Apollonio & R. Andersen), pp. 349-375. Cambridge University Press, Cambridge.

Nakagawa, S. & Schielzeth, H. (2013) A general and simple method for obtaining R^2 from generalized linear mixed-effects models. *Methods in Ecology and Evolution*, **4**, 133-142.

Navarro-Castilla, A., Barja, I., Olea, P.P., Piñeiro, A., Mateo-Tomás, P., Silván, G. & Illera, J.C. (2014) Are degraded habitats from agricultural crops associated with elevated faecal glucocorticoids in a wild population of common vole (*Microtus arvalis*)? *Mammalian Biology*, **79**, 36-43.

Ohl, F. & Putman, R. (2014) Welfare issues in the management of wild ungulates. *Behaviour and Management of European Ungulates* (eds R. Putman & M. Apollonio), pp. 236-266. Whittles Publishing, Dunbeath.

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. *Annals of the New York Academy of Sciences*, **1040**, 162-171.

Perea, R., Girardello, M. & San Miguel, A. (2014) Big game or big loss? High deer densities are threatening woody plant diversity and vegetation dynamics. *Biodiversity and Conservation*, **23**, 1303-1318.

Pride, R.E. (2005) High faecal glucocorticoid levels predict mortality in ring-tailed lemurs (*Lemur catta*). *Biology Letters*, **1**, 60-63.

Putman, R.J. (1988) *The Natural History of Deer*. Comstock Publishing Associates, Cornell University Press, New York.

Putman, R.J. & Staines, B.W. (2004) Supplementary winter feeding of wild red deer *Cervus elaphus* in Europe and North America: justifications, feeding practice and effectiveness. *Mammal Review*, **34**, 285-306.

Queirós, J., Vicente, J., Boadella, M., Gortázar, C. & Alves, P.C. (2014) The impact of management practices and past demographic history on the genetic diversity of red deer (*Cervus elaphus*): an assessment of population and individual fitness. *Biological Journal of the Linnean Society*, **111**, 209–223.

R Core Team (2014) *R: A language and environment for statistical computing*. R Foundation for Statistical Computing, Vienna, Austria. http://www.R-project.org/

Randall, D., Burggren, W. & French, K. (2002) *Eckert. Animal physiology: mechanisms and adaptations*. 5th edn. W.H. Freeman and Company, New York.

Reeder, D.M. & Kramer, K.M. (2005) Stress in free-ranging mammals: Integrating physiology, ecology, and natural history. *Journal of Mammalogy*, **86**, 225-235.

Rehnus, M., Wehrle, M. & Palme, R. (2013) Mountain hares *Lepus timidus* and tourism: stress events and reactions. *Journal of Applied Ecology*, **51**, 6-12.

Rimbach, R., Link, A., Heistermann, M., Gómez-Posada, C., Galvis, N. & Heymann, E.W. (2013) Effects of logging, hunting, and forest fragment size on physiological stress levels of two sympatric ateline primates in Colombia. *Conservation Physiology*, 1, 1-11.

Rodriguez-Hidalgo, P., Gortázar, C., Tortosa, F.S., Rodriguez-Vigal, C., Fierro, Y. & Vicente, J. (2010) Effects of density, climate, and supplementary forage on body mass and pregnancy rates of female red deer in Spain. *Oecologia*, **164**, 389–398.

Romero, M.L. (2002) Seasonal changes in plasma glucocorticoid concentrations in free-living vertebrates. *General and Comparative Endocrinology*, **128**, 1-24.

Romero, L.M., Butler, L.K. (2007) Endocrinology of stress. *International Journal of Comparative Psychology*, **20**, 89-95.

Sáenz de Buruaga, M., Lucio, A.J. & Purroy, F.J. (2001) *Reconocimiento de Sexo y Edad en Especies Cinegéticas*. Edilesa, León.

Saltz, D. & White, G.C. (1991) Urinary cortisol and urea nitrogen responses to winter stress in mule deer. *The Journal of Wildlife Management*, **55**, 1-16.

Sands, J. & Creel, S. (2004) Social dominance, aggression and fecal glucocorticoid levels in a wild population of wolves, *Canis lupus*. *Animal Behaviour*, **67**, 387-396.

Santos, J.P.V., Fernández-de-Mera, I.G., Acevedo, P., Boadella, M., Fierro, Y., Vicente, J. & Gortázar, C. (2013) Optimizing the sampling effort to evaluate the body condition in ungulates: a case study on red deer. *Ecological Indicators*, **30**, 65-71.

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. *Ecological Indicators*, **45**, 522-528.

Sauerwein, H., Müller, U., Brüssel, H., Lutz, W. & Möstl, E. (2004) Establishing baseline values of parameters pontentially indicative of chronic stress in red deer (*Cervus elaphus*) from different habitats in western Germany. *European Journal of Wildlife Research*, **50**, 168-172.

Sheriff, M.J., Krebs, C.J. & Boonstra, R. (2009) The sensitive hare: sublethal effects of predator stress on reproduction in snowshoe hares. *Journal of Animal Ecology*, **78**, 1249-1258.

Sheriff, M.J., Krebs, C.J. & Boonstra, R. (2010) Assessing stress in animal populations: do fecal and plasma glucocorticoids tell the same story? *General and Comparative Endocrinology*, **166**, 614-619.

Sheriff, M.J., Dantzer, B., Delehanty, B., Palme, R. & Boonstra, R. (2011) Measuring stress in wildlife: techniques for quantifying glucocorticoids. *Oecologia*, **166**, 869-887.

Siesler, H.W., Ozaki, Y., Kawata, S. & Heise, H.M. (2002) *Near-Infrared Spectroscopy: Principles, Instruments, Applications*. Wiley-VCH, Weinheim.

Sönnichsen, L., Bokje, M., Marchal, J., Hofer, H., Jędrzejewska, B., Kramer-Schadt, S. & Ortmann, S. (2013) Behavioural responses of European roe deer to temporal variation in predation risk. *Ethology*, **119**, 233-243.

Stankowich, T. (2008) Ungulate flight responses to human disturbance: A review and meta-analysis. *Biological Conservation*, **141**, 2159-2173.

Thomas, L., Buckland, S.T., Rexstad, E.A., Laake, J.L., Strindberg, S., Hedley, S.L., Bishop, J.R.B., Marques, T.A. & Burnham, K.P. (2010) Distance software: design and analysis of distance sampling surveys for estimating population size. *Journal of Applied Ecology*, **47**, 5-14.

Torres-Porras, J., Carranza, J., Pérez-González, J., Mateos, C. & Alarcos, S. (2014) The tragedy of the commons: unsustainable population structure of Iberian red deer in hunting estates. *European Journal of Wildlife Research*, **60**, 351-357.

Ulrich-Lai, Y.M. & Herman, J.P. (2009) Neural regulation of endocrine and autonomic stress responses. *Nature Reviews Neuroscience*, **10**, 397-409.

Vicente, J., Höfle, U., Fernández de Mera, I.G. & Gortázar, C. (2007a) The importance of parasite life history and host density in predicting the impact of infections in red deer. *Oecologia*, **152**, 655-664.

Vicente, J., Pérez-Rodríguez, L. & Gortázar, C. (2007b) Sex, age, spleen size, and kidney fat of red deer relative to infection intensities of the lungworm *Elaphostrongylus cervi*. *Naturwissenschaften*, **94**, 581–587.

Vingada, J., Fonseca, C., Cancela, J., Ferreira, J. & Eira, C. (2010) Ungulates and their management in Portugal. *European Ungulates and Their Management in the 21st Century* (eds M. Apollonio, R. Andersen & R. Putman), pp. 392-418. Cambridge University Press, Cambridge.

Wada, H., Moore, I.T., Breuner, C.W. & Wingfield, J.C. (2006) Stress responses in tropical sparrows: comparing tropical and temperate *Zonotrichia*. *Physiological and Biochemical Zoology*, **79**, 784-792.

Wasser, S.K., Hunt, K.E., Brown, J.L., Cooper, K., Crockett, 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 non-domestic mammalian and avian species. *General and Comparative Endocrinology,* **120**, 260-275.

Wikelski, M. & Cooke, S.J. (2006) Conservation physiology. *Trends in Ecology and Evolution*, **21**, 38-46.

Wingfield, J.C. & Sapolsky, R.M. (2003) Reproduction and resistance to stress: when and how. *Journal of Neuroendocrinology*, **15**, 711-724.

Young, K.M., Walker, S.L., Lanthier, C., Waddell, W.T., Monfort, S.L. & Brown, J.L. (2004) Noninvasive monitoring of adrenocortical activity in carnivores by fecal glucocorticoid analyses. *General and Comparative Endocrinology*, **137**, 148-165.

Zuur, A.F., Ieno, E.N. & Elphick, C.S. (2010) A protocol for data exploration to avoid common statistical problems. *Methods in Ecology and Evolution*, **1**, 3-14.

Zuur, A.F., Ieno, E.N. & Smith, G.M. (2007) *Analysing Ecological Data*. Springer, New York.

Zuur, A.F., Ieno, E.N., Walker, N.J., Saveliev, A.A. & Smith, G.M. (2009) *Mixed Effects Models and Extensions in Ecology with R*. Springer, New York.

Table 1. Main characteristics of study areas regarding red deer *Cervus elaphus* management practices and landscape alteration levels. Measures of hunting pressure and red deer population densities are shown as average values (± SE) for the entire sampling period (game seasons from 2010–2011 to 2012–2013). Study areas correspond with those in Fig. 1 and sample size (n) is also indicated. For abbreviations, see materials and methods section.

Study area (n)	**Surface (ha)**	**Type of management**	**Supplementary feeding**	**Massive hunting events**	**Harvested deer (N ± SE)**	**Harvested deer per surface area (N ± SE)**	**Population harvested (% ± SE)**	**Population density (deer/Km^2 ± SE)**	**WULAI**
LSC (16)	48,740	Public	Yes	No	40 ± 5.04	0.1 ± 0.01	2.7 ± 0.34	3.1 ± 0.08	0.85
LOU (44)	32,517	Public/Private	Yes	Yes	41 ± 3.48	0.1 ± 0.01	2.0 ± 0.20	6.3 ± 0.33	1.56
CUB (32)	1,561	Private	Yes	Yes	104 ± 5.21	6.6 ± 0.33	17.5 ± 1.86	36.5 ± 2.39	0.87
NEG (47)	1,722	Private	Yes	Yes	72 ± 12.66	4.2 ± 0.74	26.7 ± 4.69	15.7 ± 1.20*	1.02
DN (19)	50,720	Public	No	No	40 ± 0.00	0.1 ± 0.00	0.8 ± 0.07	10.1 ± 0.85	1.21
QM (41)	6,864	Public	No	Yes	419 ± 66.36	6.1 ± 0.97	21.9 ± 3.26	27.9 ± 1.85	0.79
RCMU (43)	49,778	Public	No	Yes	754 ± 28.02	1.5 ± 0.06	20.7 ± 0.44	7.3 ± 0.24	1.33
CF (47)	31,327	Private	No	Yes	517 ± 45.84	1.6 ± 0.15	26.3 ± 1.59	6.4 ± 0.89	7.75

* Data obtained from Lopes (2008). See the text for details.

Table 2. Explanatory variables used for modelling faecal glucocorticoid metabolite concentrations in red deer *Cervus elaphus* in the Iberian Peninsula during three consecutive hunting seasons (2010–11 to 2012–13). Temperature and precipitation variables were calculated in relation to the date (*t*) on which animals were culled. Variables excluded due to multicollinearity filters are indicated by $^{(*)}$.

Variables/Codes	Description
Weather and climate	
TEMPAvg_24h	Average temperature (ºC) at time *t* – 1 day
TEMPAvg_m1 $^{(*)}$	Average temperature (ºC) of the last 30 days
TEMPAvg_m2 $^{(*)}$	Average temperature (ºC) of the last 60 days
TEMPAvg_m3 $^{(*)}$	Average temperature (ºC) of the last 90 days
TEMPMax_24h $^{(*)}$	Maximum temperature (ºC) at time *t* – 1 day
TEMPMax_m1 $^{(*)}$	Average maximum temperature (ºC) of the last 30 days
TEMPMax_m2 $^{(*)}$	Average maximum temperature (ºC) of the last 60 days
TEMPMax_m3 $^{(*)}$	Average maximum temperature (ºC) of the last 90 days
TEMPMin_24h $^{(*)}$	Minimum temperature (ºC) at time *t* – 1 day
TEMPMin_m1 $^{(*)}$	Average minimum temperature (ºC) of the last 30 days
TEMPMin_m2 $^{(*)}$	Average minimum temperature (ºC) of the last 60 days
TEMPMin_m3 $^{(*)}$	Average minimum temperature (ºC) of the last 90 days
PRECIP_m1	Accumulated precipitation (mm) in the last 30 days
PRECIP_m2 $^{(*)}$	Accumulated precipitation (mm) in the last 60 days
PRECIP_m3 $^{(*)}$	Accumulated precipitation (mm) in the last 90 days
Seasonality	
SEASON	Autumn = (Sep, Oct, Nov); Winter = (Dec, Jan, Feb)
Geographic	
LAT	Latitude (decimal degrees)
LONG $^{(*)}$	Longitude (decimal degrees)
Demographic	
D_deer	Population density (red deer/Km^2)
Game management	
SUPPL	Supplementary feeding (0 = absence; 1 = presence)
MASS_HUNT	Massive hunting events (0 = absence; 1 = presence)
N_HARV $^{(*)}$	No. of harvested deer per hunting season
N_HARV_KM2 $^{(*)}$	No. of harvested deer per surface area per hunting season
PERC_HARV_POP $^{(*)}$	Harvested population per hunting season (%)
Landscape alteration	
WULAI	Wild Ungulates Land Avoidance Index

Comentado [u8]: Los codes no se usan en el texto ni en las tablas (sólo WULAI), por qué no los quitas?

Table 3. Results of tests of fixed effects and their interactions in a linear mixed model explaining variation in faecal glucocorticoid metabolite (FGM) concentrations in red deer *Cervus elaphus*. The effects of variables related to individual traits, as well as environmental conditions and human activities, on FGM levels were analysed, with 'hunting season' as a fixed factor and 'study area' as a random factor. Significant results are highlighted in bold.

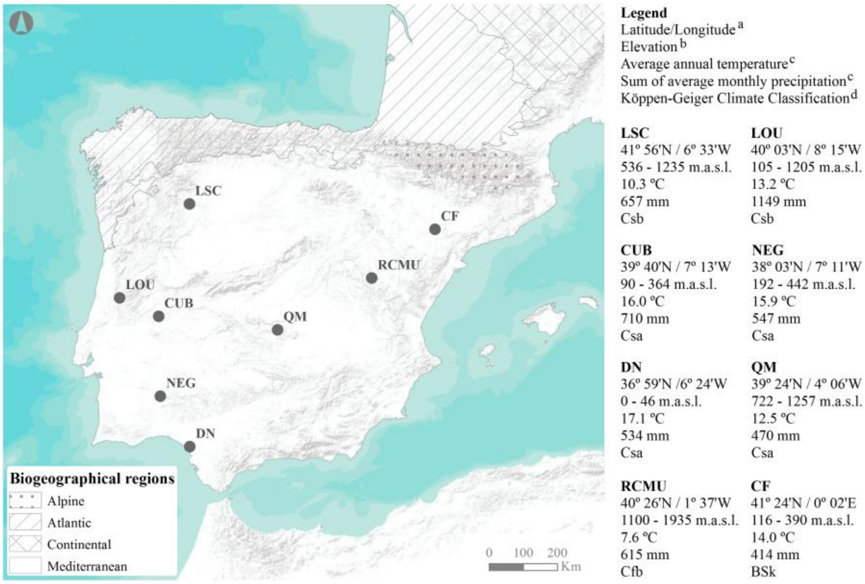
Component	**Predictors**	**Num, Den df**	***F* value**	**P value**
	Hunting season	2, 267	1.935	0.146
	Sex	1, 267	0.018	0.893
Individual	Age class	3, 267	5.410	**0.001**
	Sex * Age class	3, 267	0.494	0.686
	Season	1, 267	0.068	0.794
	Average temperature on *t* – 1 day	1, 267	8.819	**0.003**
Environmental	Accumulated precipitation in last 30 days	1, 267	5.514	**0.020**
	Latitude	1, 267	35.944	**<0.0001**
	Season * Average temperature on *t* – 1 day	1, 267	4.048	**0.045**
	WULAI	1, 267	32.013	**<0.0001**
	Supplementary feeding	1, 267	2.719	0.100
Human	Red deer density	1, 267	0.061	0.805
	Massive hunting events	1, 267	4.158	**0.042**
	Supplementary feeding * Red deer density	1, 267	10.356	**0.001**

Table 4. Results of parameter estimates* of the linear mixed-effect model explaining the influence of variables related to individual traits, as well as environmental conditions and human activities, on the variation of faecal glucocorticoid metabolite concentrations (expressed as ng/g of dry matter) in red deer *Cervus elaphus*. The variable 'hunting season' was used in the model as a fixed factor and the variable 'study area' was included as a random factor. Significant results are highlighted in bold.

Component	**Predictors**	**Estimate**	**Std. Error**	***t* value**	**P value**
	Intercept	-927.66	189.85	-4.886	**<0.0001**
	Hunting season (2011-2012)	-12.00	9.09	-1.320	0.188
	Hunting season (2012-2013)	12.25	20.10	0.610	0.543
	Sex (Females)	-14.42	28.36	-0.508	0.612
	Age class (Yearlings)	26.81	19.10	1.403	0.162
	Age class (Sub-adults)	32.58	20.07	1.623	0.106
Individual	Age class (Adults)	8.49	16.44	0.517	0.606
	Sex (Females) * Age class (Yearlings)	11.60	33.20	0.349	0.727
	Sex (Females) * Age class (Sub-adults)	32.13	34.08	0.943	0.347
	Sex (Females) * Age class (Adults)	8.66	29.87	0.290	0.772
	Season (Winter)	-8.51	32.51	-0.262	0.794
	Average temperature on *t* – 1 day	2.09	2.13	0.984	0.326
Environmental	Accumulated precipitation in last 30 days	-0.25	0.10	-2.348	**0.020**
	Latitude	27.19	4.54	5.995	**<0.0001**
	Season (Winter) * Average temperature on *t* – 1 day	6.24	3.10	2.012	**0.045**
	WULAI	-25.02	4.42	-5.658	**<0.0001**
	Supplementary feeding (Yes)	-39.47	23.94	-1.649	0.100
Human	Red deer density	2.05	1.13	1.822	**0.070**
	Massive hunting events (Yes)	48.48	23.78	2.039	**0.042**
	Supplementary feeding (Yes) * Red deer density	-3.80	1.18	-3.218	**0.001**

* Parameter estimates for the levels of fixed factors were computed by considering a reference value of 0 for: level '2010-2011' for hunting season; level 'Males' for sex; level 'Calves' for age class; level 'Autumn' for season; level 'No' for supplementary feeding; and level 'No' for massive hunting events.

Fig. 1. Location of the study areas in the Iberian Peninsula, within the Mediterranean biogeographic region (after European Environment Agency, 2011). Climatic and topographical features of each sampling site are also shown (right panel).



[a] Centroid geographic coordinates (degrees, minutes).

[b] Elevation ranges (expressed as meters above sea level, m.a.s.l.) were derived either from the 25 x 25 m resolution Digital Elevation Model (DEM) produced by the Spanish Geographical National Institute (CNIG) or from the 1:25,000 topographic maps (series M888) from the Portuguese Army Geographical Institute (IGeoE).

[c] Local temperature (ºC) and precipitation (mm) regimes were obtained from WorldClim (v.1.4. release 3; Hijmans *et al.* 2005).

[d] Köppen-Geiger climate classification (Kottek *et al.* 2006).

Fig. 2. Average concentrations of faecal glucocorticoid metabolites (FGM, expressed as ng/g of dry matter, DM) measured in the faeces of red deer *Cervus elaphus* during three consecutive game seasons in eight study areas located in the Iberian Peninsula. Deer populations are ordered by latitude, from south to north. Bars indicate standard errors (SE).

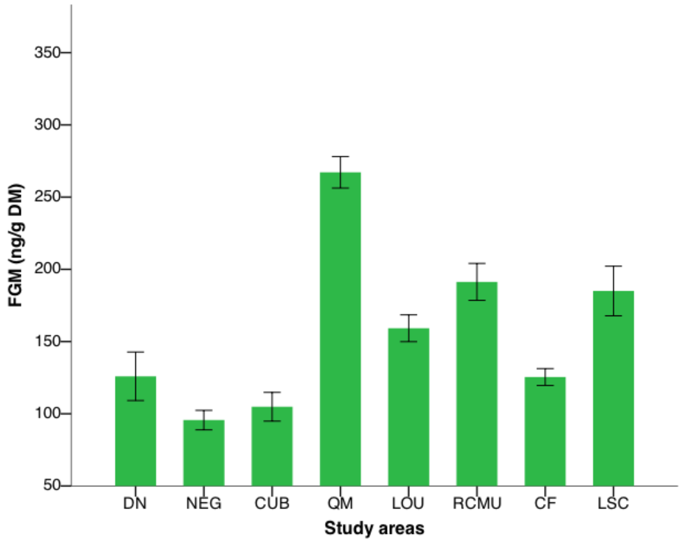


Fig. 3. Relationship between the average daily temperature (ºC) and the concentrations of faecal glucocorticoid metabolites (FGM, expressed as ng/g of dry matter, DM) in red deer *Cervus elaphus* in the autumn and winter. The average daily temperature refers to the average temperature recorded one day before the date on which the animals were culled.

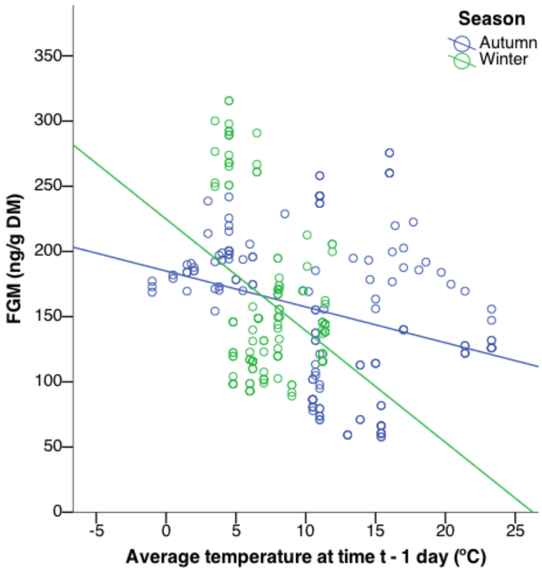


Fig. 4. Relationship between population density (calculated as deer/Km^2) and the concentrations of faecal glucocorticoid metabolites (FGM, expressed as ng/g of dry matter, DM) in food-supplemented and non-food-supplemented populations of red deer *Cervus elaphus*.

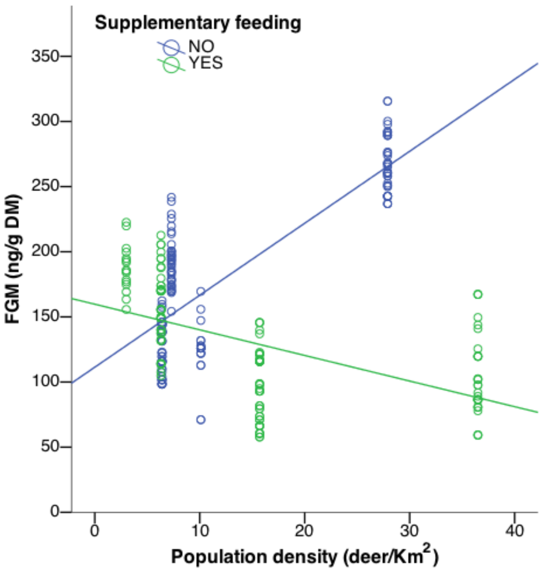


Fig. 5. Venn diagrams showing (a) the percentage of variation explained by the spatio-temporal factors versus the remaining set of predictors included in the final model, as well as their overlaid effects; and (b) the percentage of variation explained by the individual (Ind), environmental (Env) and human (Hum) components, and their intersections, on the variation of faecal glucocorticoid metabolite concentrations in red deer *Cervus elaphus*. See Fig. S1 in Supporting Information for details of the procedures used to separate out the explained variation into their pure and shared effects.