Universidade de Aveiro Departamento de Biologia
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"*Vives rodeado de mistério e jamais o dominarás. Vive-o, respira-o.*
E dorme nele como no seio de uma floresta"

Vergílio Ferreira *in* 'Pensar'

o júri

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palavras-chave

Características individuais; *Cervus elaphus*; Ecofisiologia; Ecossistemas mediterrânicos; Gestão cinegética; Indicadores de condição; Nutrição; Otimização de metodologias; Stress; Variabilidade ambiental

resumo

O veado (*Cervus elaphus*) é, atualmente, uma das espécies de ungulados silvestres mais abundantes na Península Ibérica. É também uma espécie extremamente importante em termos ecológicos, por ser um elemento chave para o funcionamento dos ecossistemas, e socioeconómicos, por ser uma espécie de caça maior emblemática e altamente valorizada. Na Ibéria, as populações de veados estão sujeitas a diferentes práticas de gestão que podem afetar a condição física dos indivíduos de diferentes formas e, por conseguinte, determinar a sua dinâmica. Apesar da importância desta espécie, os estudos sobre os efeitos da gestão cinegética e das condições ambientais nos indicadores da sua condição física são ainda escassos nos ecossistemas mediterrânicos. Grande parte do conhecimento sobre a ecologia do veado e sobre o impacto da sua gestão cinegética é baseado em estudos realizados em regiões do norte e centro da Europa, onde as características climáticas e os sistemas de gestão diferem daqueles existentes nos ambientes mediterrânicos da Península Ibéria. Os estudos realizados a uma escala biogeográfica podem ser bastante úteis para compreender as relações das espécies com o ambiente, e podem contribuir para o desenvolvimento de práticas de gestão mais específicas e adequadas às necessidades de gestão. A otimização de procedimentos de amostragem e do uso de técnicas analíticas é também essencial para uma monitorização mais eficaz das populações silvestres em termos de tempo e custos associados e, portanto, é uma tema de grande interesse para gestores de fauna selvagem. Neste contexto, os principais objetivos desta tese foram: 1) otimizar os métodos usados para avaliar a condição física do veado; e 2) identificar os fatores de gestão cinegética e ambientais que afetam o estado nutricional e a fisiologia do stress do veado nos ecossistemas mediterrânicos da Ibéria, assim como investigar possíveis interações entre esses fatores. Dois estudos de cariz metodológico, apresentados na primeira parte desta tese, demonstraram que a condição física do veado pode ser avaliada usando procedimentos mais simples e rentáveis do que aqueles normalmente utilizados: i) demonstrou-se que o estado nutricional do veado pode ser avaliado usando apenas um rim e a sua gordura perirrenal; ii) provou-se, pela primeira vez, a viabilidade da espectroscopia no infravermelho próximo para estimar concentrações de metabólitos de hormonas de stress usando fezes de veado. Posteriormente, dois estudos observacionais, realizados em diferentes populações de veados representativas da região mediterrânica ibérica, evidenciaram a importância de considerar as variações ambientais sazonais e as variáveis de gestão cinegética para compreender a ecofisiologia e a ecologia nutricional do veado. Observou-se que as densidades populacionais elevadas afetaram de forma negativa o estado nutricional dos veados e estiveram também associadas a níveis de stress mais elevados em populações naturais sem alimentação suplementar. Para além disto, verificou-se que a realização de eventos de caça massivos com matilhas pode ser um fator de stress crónico nas populações de veados. Os resultados obtidos no âmbito desta tese têm diversas implicações no que diz respeito à monitorização e gestão das populações de veados nos ambientes mediterrânicos. Espera-se que os estudos aqui apresentados ajudem os gestores de fauna silvestre a implementar programas de monitorização populacional mais eficazes e práticas de gestão mais sustentáveis.

palabras clave

Características individuales; *Cervus elaphus*; Ecofisiología; Ecosistemas mediterráneos; Estrés; Gestión cinegética; Indicadores de condición; Nutrición; Optimización de metodologías; Variabilidad ambiental

resumen

El ciervo rojo (*Cervus elaphus*) es actualmente uno de los ungulados silvestres más abundantes de la Península Ibérica. Es una especie con una elevada importancia ecológica, siendo un elemento clave en la dinámica de los ecosistemas mediterráneos, y presenta un gran valor socioeconómico, al ser una especie de caza mayor emblemática y muy apreciada. En el territorio peninsular, las poblaciones de ciervos están sometidas a diferentes sistemas de manejo que pueden modular la condición física de los individuos y, consecuentemente, determinar la dinámica de sus poblaciones. Pese a la relevancia de esta especie, aun son escasos los estudios sobre los efectos de la gestión cinegética y de las condiciones ambientales en los indicadores de su condición en los ecosistemas mediterráneos. La mayor parte del conocimiento sobre la ecología de esta especie y sobre el impacto de su gestión cinegética se basa en estudios realizados en regiones del norte y centro de Europa, donde las características climáticas y sistemas de manejo son diferentes a los presentes en los ambientes mediterráneos de la Península Ibérica. Los estudios realizados a una escala biogeográfica pueden ser muy útiles para comprender las relaciones de las especies con el ambiente, y pueden contribuir al desarrollo de prácticas de manejo más especificas y adecuadas a las necesidades de gestión. La optimización de procedimientos de muestreo y la puesta a punto de técnicas analíticas son también de gran importancia para una monitorización más eficaz de las poblaciones silvestres en términos de tiempo y costes asociados y, por consiguiente, son temas de gran interés para los gestores de la fauna silvestre. En este contexto, los objetivos principales de esta tesis doctoral fueron: 1) optimizar los métodos usados para evaluar la condición física del ciervo; y 2) identificar los factores de manejo y ambientales que afectan el estado nutricional y la fisiología del estrés del ciervo en los ecosistemas de la Iberia mediterránea, así como estudiar las posibles interacciones entre dichos factores. Dos estudios con un enfoque metodológico, presentados en la primera parte de esta tesis, demostraron que la condición física del ciervo se puede valorar usando procedimientos más sencillos y rentables que los usados tradicionalmente: i) se ha demostrado que el estado nutricional del ciervo se puede evaluar usando solamente un riñón y su grasa perirrenal; y ii) se comprobó, por primera vez, la viabilidad de la espectroscopia en el infrarrojo cercano para estimar las concentraciones de metabolitos de hormonas de estrés usando heces de ciervo. Posteriormente, dos estudios observacionales basados en poblaciones de ciervo representativas de la Iberia mediterránea evidenciaron la importancia de considerar las variaciones ambientales estacionales y las variables de manejo cinegético para comprender la ecofisiología y la ecología nutricional del ciervo. Se observó que las densidades poblacionales elevadas afectan de forma negativa al estado nutricional de los ciervos y éstas se asociaron con niveles de estrés más elevados en poblaciones naturales sin suplementación alimentaria. Además, se verificó que los eventos de caza masivos con rehalas pueden representar un factor de estrés crónico en las poblaciones de ciervos. Los resultados obtenidos en esta tesis tienen implicaciones en lo que respecta a la monitorización y gestión de las poblaciones de ciervos en ambientes mediterráneos, y se espera que los estudios aquí presentados ayuden a los gestores de fauna silvestre a implementar programas de monitorización más eficaces y prácticas de gestión más sostenibles.

keywords

Cervus elaphus; Condition indicators; Environmental variation; Game management; Individual attributes; Mediterranean ecosystems; Nutrition; Optimisation of methods; Physiological ecology; Stress

abstract

The red deer (*Cervus elaphus*) is currently one of the most widespread and abundant wild ungulates in the Iberian Peninsula and is extremely important both ecologically, as a key species for the functioning of the ecosystems, and economically, as a major game species. In Iberia, red deer populations are subjected to different management systems that may affect the physical condition of the individuals, with further consequences for population dynamics. Studies investigating the effects of management practices and environmental conditions on the performance of red deer are still rare regarding Mediterranean ecosystems. Much of the knowledge concerning the ecology of red deer and the impact of management on its physical condition is based on studies conducted in northern and central regions of Europe, where climatological features and management practices differ from those observed in the Mediterranean areas of Iberia. Studies on a biogeographical scale can provide important insights into the relationships between species and a particular environment and contribute to the development of more targeted and appropriate management practices. The optimisation of sampling procedures and the fine-tuning of pre-existing analytical techniques are also fundamental to a more cost-effective monitoring and, therefore, are of enormous value to wildlife managers. In this context, the main aims of this thesis were: 1) to optimise the procedures used to assess the physical condition of red deer; and 2) to identify relevant management and environmental factors affecting the nutritional condition and stress physiology of red deer in the Mediterranean ecosystems of Iberia, as well as any potential interactions between those factors. Two studies with a methodological focus, presented in the first part of the thesis, demonstrated that the physical condition of red deer can be evaluated more simply, using more cost- and time-effective procedures than those traditionally used: i) it was shown that only one kidney and its associated fat is enough to assess nutritional condition in red deer; and ii) the feasibility of using near infrared spectroscopy to predict the concentrations of stress hormone metabolites was demonstrated using faeces of red deer for the first time. Subsequently, two large-scale observational studies, conducted in representative red deer populations found in Mediterranean Iberia, highlighted the importance of considering seasonal environmental variations and variables related to hunting management practices to better understand the nutritional and physiological ecology of red deer. High population densities had adverse effects on the nutritional condition of the deer and were associated with increased stress levels in natural populations without supplementary feeding. Massive hunting events involving the use of hounds were also identified as a potential source of chronic stress in red deer. The research presented in this thesis has clear implications regarding the management and monitoring of red deer populations in Mediterranean environments and is intended to help wildlife managers to implement more effective monitoring programmes and sustainable management practices.

Declaro que esta tese é integralmente da minha autoria, estando devidamente referenciadas as fontes e obras consultadas, bem como identificadas de modo claro as citações dessas obras. Não contém, por isso, qualquer tipo de plágio quer de textos publicados, qualquer que seja o meio dessa publicação, incluindo meios eletrónicos, quer de trabalhos académicos.

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There are many hypotheses in science which are wrong. That's perfectly all right; they are the aperture to finding out what is right. Science is a self-correcting process. To be accepted, new ideas must survive the most rigorous standards of evidence and scrutiny.

Carl Edward Sagan

The understanding of life begins with the understanding of pattern.

Fritjof Capra

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List of Abbreviations and Acronyms

A	**ADF**	acid detergent fibre
	ADL	acid detergent lignin
	AIC	Akaike’s information criterion
	ANCOVA	analysis of covariance
	ANOVA	analysis of variance
D	**DF**	degrees of freedom
	DM	dry matter
	DT	detrend
E	**EIA**	enzyme immunoassay
F	**FCP**	faecal crude protein
	FGM	faecal glucocorticoid metabolites
	FN	faecal nitrogen
	FW	fat weight
G	**GLMM**	generalised linear mixed models
	$GVIF_{adj}$	adjusted generalised variance inflation factor
H	**HPA**	hypothalamus-pituitary-adrenal
K	**KFI**	kidney fat index
	KW	kidney weight

L	**L1**	first-stage larvae
	L3	third-stage larvae
	LMM	linear mixed models
	LPG	larvae per gram
	LRT	likelihood ratio test
M	**ML**	maximum likelihood
	MODIS	moderate resolution imaging spectroradiometer
	MPLS	modified partial least squares
	MSC	multiplicative scatter correction
N	**NDF**	neutral detergent fibre
	NDVI	normalised difference vegetation index
	NIRS	near infrared spectroscopy
P	**PSM**	plant secondary metabolites
R	**REML**	restricted maximum likelihood
	RER	range error ratio
	RIA	radioimmunoassay
	RPD	ratio of performance deviation
S	**SE**	standard error
	SEC	standard error of calibration
	SECV	standard error of cross-validation
	SNV	standard normal variate
V	**VIF**	variance inflation factor
W	**WULAI**	wild ungulate land avoidance index

Chapter 1

Introduction

1.1. General introduction

Wildlife populations are naturally regulated by intrinsic demographic processes, as well as by biological and physical components of the environment (Begon et al., 2006). However, human activities are increasingly significant forces driving the dynamics of natural populations by affecting the rate and nature of species being lost and gained (Wardle et al., 2011). Nowadays, more than ever, it can be argued that there is no animal population or ecosystem on the planet free from human influence.

Wild ungulates, particularly large herbivores, have a wide global distribution and occupy a great variety of habitats on Earth (Danell et al., 2006; Geist, 1998). They are key species for the functioning of ecosystems because of their contribution to shaping the composition, structure and dynamics of plant communities and to the recycling and redistribution of nutrients in the ecosystems they inhabit (Hester et al., 2006; Pastor et al., 2006; Smit and Putman, 2011). Ungulates are also ecologically important as the main prey species for large carnivores and are, therefore, crucial elements for the conservation of these endangered populations (Boitani et al. 2015; Linnell et al., 2008), thus helping to maintain the trophic networks and the integrity of ecosystems (Jędrzejewska and Jędrzejewski, 2005; Ripple and Beschta, 2012). From a socio-economic perspective, they constitute an important source of income through sport hunting and/or ecotourism in rural areas (Apollonio et al., 2010; Gordon et al., 2004).

The current worldwide situation of wild ungulate populations is heterogeneous. While in some Asian and African regions they have been decreasing and there is an urgent need for the implementation of conservation measures (*e.g.*, Mallon and Jiang, 2009; Ripple et al., 2015; Stoner et al., 2007), in Europe and North America, the populations of wild ungulates have expanded considerably over the last decades, which has led to an increasing concern about their appropriate management (Apollonio et al., 2010; Gordon et al., 2004). Particularly in Europe, such expansion has been driven by several factors, including the rapid socio-economic and demographic changes experienced in many countries, which has altered land-use and cover patterns, contributing, in numerous situations, to the re-naturalization of habitats. The decline in the populations of natural predators, the reintroduction and restocking of wild ungulate populations for hunting and conservation purposes, as well as factors associated with their reproductive potential and dispersal capacity have also played a crucial role (Apollonio et al., 2010; Gordon et al., 2004; Putman et al., 2011). If it is true that, on the one hand, the increase of ungulate populations can generate economic benefits, on the other hand, the inadequate management and the excessive growth of those populations also has many associated problems. These include negative impacts on the performance and health of individuals (*e.g.*, Gortázar et al., 2006; Stewart et al., 2005), environmental problems (*e.g.*, Carpio et al., 2014; Fuller and Gill, 2001) and economic losses in agriculture and forestry (*e.g.*, Putman and Moore, 1998).

The red deer (*Cervus elaphus* Linnaeus, 1758) is a paradigmatic case of the recent expansion of ungulates in Europe. The species is, nowadays, one of the most widespread and abundant and also one of the most hunted big game species. The management of red deer populations varies considerably among European countries, mainly as a result of cultural differences and environmental characteristics. In some circumstances, the management policies can also differ between regions within the same country (Apollonio et al., 2010; Milner et al., 2006). In the Iberian Peninsula, the red deer is also currently abundant, but populations are irregularly distributed throughout this region (Fig. 1.1), which constitutes the westernmost limit of the species' distribution area in Europe. Knowledge of the past demographic history of red deer is limited, although the fossil records indicate that its presence in Iberia extends, at least, back to the late Pleistocene, when the peninsula served as a refugium during the glacial events of the Quaternary (Meiri et al., 2013; Sommer et al., 2008; also Queirós et al., submmitted). Medieval literature suggests that the red deer was a common species both in Portugal and Spain (*e.g.*, books of *Montaria/Montería* by *D. João I* and *D. Alfonso XI;* see Mendonça, 2003; Valverde, 2009). Later, between the second half of the nineteenth century and the early twentieth century, its populations experienced a significant decline, mainly due to excessive hunting and loss of favourable habitat conditions, having completely disappeared in many Iberian regions (Gortázar et al., 2000; Salazar, 2009; Vingada et al., 2010). The recovery of red deer populations started mainly in the decades of 1960-70 in Spain with the creation of game reserves and through the restocking and reintroduction programmes carried out by administrative authorities, accompanied by natural expansion phenomena (Gortázar et al., 2000; Artigot and Martínez, 2006; Carranza, 2010). In Portugal, the turnaround occurred just a few years later, principally in the early 1980s. The increase in deer numbers and geographical range was particularly marked in those areas adjacent to the border with Spain, from where numerous animals dispersed naturally into the Portuguese territory. In addition, a number of reintroductions have taken place since then throughout the country, mainly for hunting purposes (Salazar, 2009; Vingada et al., 2010). The red deer has been, undoubtedly, the most promoted large game species in the Iberian Peninsula during the last decades. Although there are no reliable estimates of its abundance for the entire peninsula, deer numbers may now exceed 900,000 (Carranza, 2010; Vingada et al., 2010). Hunting statistics indicate that more than 3,000 red deer have been harvested each year in Portugal since 2009 (Autoridade Florestal Nacional (AFN), unpublished data), while, in Spain, the number of animals hunted is above 80,000/year, and shows a tendency to increase (Herruzo and Martínez-Jauregui, 2013).

The type of management carried out in hunting grounds, game reserves, or any other area, may affect the performance of the individuals, with consequences for population dynamics (*e.g.*, Clutton-Brock et al., 1989; Rodriguez-Hidalgo et al., 2010). In Iberia, there are two broad scenarios regarding red deer management. In general, the populations that occupy the northern part of the peninsula, characterised or influenced by an Atlantic climate, occur at lower relative densities and are subjected to lower levels of human intervention. Those populations depend mostly on the plant resources naturally available in the environment and many of them are regulated by wolf predation (Iberian wolf, *Canis lupus signatus* Cabrera, 1907), apart from the hunting activities. This scenario contrasts with that of many Mediterranean areas in central and southern Iberia, where red deer populations are usually more intensively managed and are present at higher densities (Acevedo et al., 2008), often above the ecological carrying capacity. In many situations, these populations are maintained by supplementary feeding and water resources are also frequently manipulated, increasing the risk of transmission of pathogens (Gortázar et al., 2006; Vicente et al., 2006, 2007a) and affecting individuals' condition (Vicente et al., 2007b). In addition to these general differences, the number of red deer hunted and the type of hunting methods used may also vary.

Comparative studies investigating the effects of management practices on the performance and demography of wild red deer are still rare in the Iberian Peninsula (but see Rodriguez-Hidalgo et al., 2010; Torres-Porras et al., 2014). In fact, given the importance of the species in the ecological and socio-economic contexts, the relatively little research conducted to date addressing these issues is quite surprising. In spite of being one of the most well studied wild ungulate species in the world, much of the knowledge concerning their ecology and behaviour, as well as the impact of management on population parameters, is based on studies conducted in northern and central regions of Europe (*e.g.*, Clutton-Brock et al., 1989; Mysterud, 2014), where climatological features and management practices differ from those observed in the Mediterranean areas of Iberia. Since the factors affecting the condition and performance of red deer vary according to the geographical context (*e.g.*, Martínez-Jauregui et al., 2009), studies on a biogeographical scale can provide important insights into the relationships between species and a particular environment and contribute to the development of more targeted and appropriate management practices.

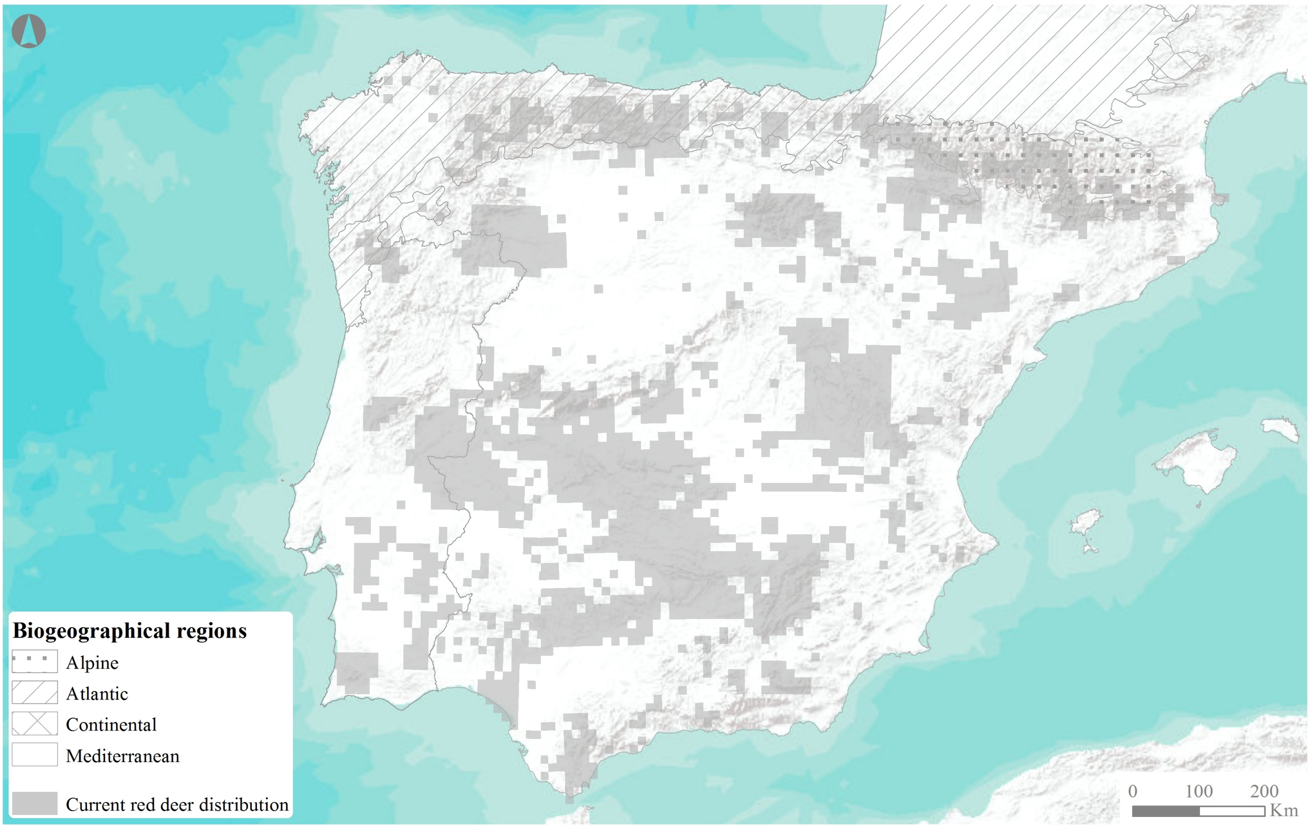

Fig. 1.1. Current distribution of red deer (*Cervus elaphus*) in the Iberian Peninsula (depicted in grey), from Carranza (2007) and Salazar (2009). Biogeographic regions are also shown (from European Environment Agency, 2011).

Sustainable and effective management of wild ungulate populations, and particularly of red deer, requires a monitoring of population trends and knowledge of the biology and ecology of the species. Ideally, monitoring should be as multidisciplinary as possible and, therefore, should not be restricted to a mere assessment of deer numbers present in a given area. As stated long ago by Aldo Leopold (1933), '*the composition and condition of the stock is often quite as important as its numbers, and may have a bearing on all the steps of the management process*'. Since estimates of the population size, *per se*, are unable to provide information on the species-environment relationships, it is extremely important that such data can be complemented by an evaluation of the condition and health of individuals (Kie, 1988; Marco and Gortázar, 2002). At this point, both researchers and wildlife managers must consider the use of indicators that may reflect environmental changes, whether natural or human-induced. Such indicators are commonly referred to as ‘*ecological indicators*’ (Dale and Beyeler, 2001) or ‘*indicators of ecological change*’, and their use has been recently proposed in the context of the management of large wild herbivores (see Morellet et al., 2007). They include, among others, several indicators of animal performance, such as: measurements of body mass, individual growth, and reproductive success (*e.g.*, Bonenfant et al., 2002; Rodriguez-Hidalgo et al., 2010; Stewart et al., 2005; Toïgo et al., 2006). Also of great interest, and extremely useful, are those indicators that reflect fluctuations in the nutritional or energy status (*e.g.*, Cook et al., 2004, 2005; Leslie et al., 2008) or the physiological responses of individuals to environmental challenges (Keay et al., 2006; Möstl and Palme, 2002), as they allow the assessment of the animals’ physical condition and welfare. Welfare issues are increasingly important within the framework of wildlife management and conservation and this also applies to wild ungulates (Ohl and Putman, 2014). This is mainly because there is an increased awareness that the internal state of animals has significant impacts on many aspects of their life history, and may influence the dynamics and fate of populations.

Substantial effort has also been made over the past years to integrate the theoretical principles and practical knowledge obtained from the disciplines of animal ecology, physiology, and nutrition, in order to better manage and conserve wildlife populations (*e.g.*, Barboza et al., 2009; Bradshaw, 2003; Raubenheimer et al., 2009). As a result, some new research areas have emerged (*e.g.*, Cooke et al., 2013) and also new tools and/or analytical approaches have been developed (*e.g.*, Bradshaw, 2003; Dixon and Coates, 2009; Sheriff et al., 2011). The optimisation of sampling procedures and the development of new analytical methods (or simply the fine-tuning of pre-existing techniques) are fundamental to a more cost-effective monitoring and, therefore, are of enormous value for wildlife managers as well as for researchers in ecology and related areas.

1.2. Aims

Nutritional condition and physiological balance are interdependent key components of an animal's performance (Barboza et al., 2009; Randall et al., 2002). Since human impacts on wildlife vary in magnitude and direction, it is of central importance to understand how management practices affect the performance of wild ungulates and how those practices interact with natural environmental factors and the species' biological attributes. Such knowledge, apart from improving our understanding of a species' ecology, may provide an important scientific basis to support management decisions. Successful management also depends on the availability of tools that can help both wildlife managers and researchers to monitor the physical condition of wild animals in general, and of game species in particular, more effectively. The main aims of this thesis are:

1) To explore, develop, and validate alternative and cost-effective procedures used to assess the physical condition and performance of red deer in the Mediterranean areas of the Iberian Peninsula;

2) To identify relevant management and environmental factors affecting the nutritional condition and stress physiology of red deer in the Mediterranean ecosystems of the Iberian Peninsula, as well as any potential interactions between those factors. Because the red deer is a highly sexually dimorphic species (Clutton-Brock et al., 1982) and because nutritional and energy requirements, as well as overall physiology, are likely to vary according to life-history stage (*e.g.*, Barboza et al., 2009), variations in the nutritional status and stress levels, with respect to sex and age, are also analysed and described in the context of this thesis.

In consonance with these objectives, the following general hypotheses guided this research:

I. *The physiological status and physical condition of wild red deer can be evaluated more simply and using more cost-effective approaches;*

II. *The assessment of the physiological and nutritional status of red deer is an asset to understanding its ecology and is essential for making informed decisions regarding the management of wild populations.*

1.3. Organisation of the Thesis

The abovementioned objectives have been addressed and are set out in the following independent but related chapters:

Chapter 2. Methodological approaches for monitoring the physical condition of red deer
Chapter 3. Nutritional ecology of red deer in Mediterranean ecosystems
Chapter 4. Stress physiology of red deer in Mediterranean environments
Chapter 5. Summary and conclusions

Chapter 2 has a methodological focus and is aimed at exploring alternative and cost-effective procedures to assess indicators of performance in wild red deer. It is comprised of two parts: firstly, the possibility of using one kidney only, with its associated fat, to evaluate the nutritional status of red deer is tested. Secondly, the feasibility of using near infrared spectroscopy (NIRS) to predict stress hormone metabolite concentrations in the faeces of red deer is examined. In **Chapters 3 and 4**

the effects of management, population density, weather conditions, and individual traits on the nutritional condition and stress physiology of red deer are analysed. Here, the procedures validated in Chapter 2 were applied to determine the indicators of condition and performance analysed in those studies. At the end of each chapter, the major findings and implications for red deer management are discussed. Finally, in **Chapter 5**, the key findings and main conclusions are summarised. Supplementary material is also provided at the end of the thesis to complement the studies conducted in Chapters 3 and 4.

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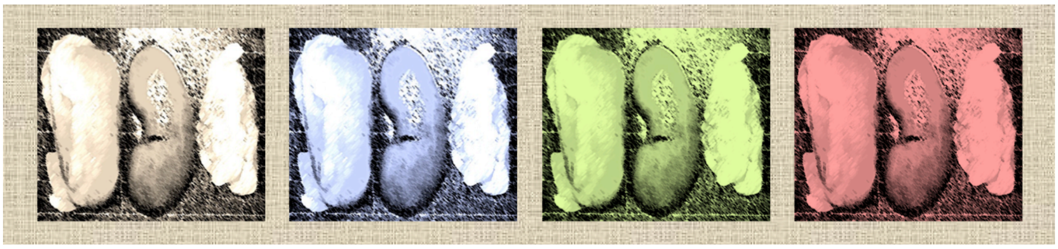
Chapter 2

Methodological Approaches for Monitoring the Physical Condition of Red Deer



Part 1 Optimising the sampling effort to evaluate body condition in ungulates: a case study on red deer

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Summary

Body condition is a useful measure of the nutritional status and performance in ungulates. The most widely used indicators of body condition are based on fat reserves, mainly those surrounding the kidneys. To estimate the body condition in large herbivores, researchers often collect and pool the data from both kidneys and their perirenal fat in order to compensate for potential differences between the body condition indicators obtained from the left and right kidneys. Since these differences do not seem to be properly established in wildlife, we checked for the potential differences between the body condition indicators, derived from both kidneys, in wild ungulates. In the present study, we used red deer *Cervus elaphus*, both under experimental and field conditions, as a model to analyse the patterns of response of the body condition indicators, calculated from left and right kidneys, to supplementary feeding, sex, age class and season. By considering two analytical approaches – kidney fat index and fat weight (including kidney weight as a covariate) – we consistently obtained similar statistical results and the same ecological interpretations by using left, right and average indicators for all the factors tested. In the experimental study, supplementary feeding had a significant effect on all the body condition indicators of red deer hinds. Under field circumstances, the condition indicators were statistically higher in females than in males and they increased with age. Sex related differences were significant in the winter. Taking into account our results, we hypothesise that information from only one kidney will be enough to assess the body condition in red deer and probably in other wild ungulates. However, a standardised sample (*i.e.*, always using the same kidney and associated fat) should be used, while a better understanding regarding possible asymmetries in the amount of the energy reserves on each side of the animals' bodies has not been reached. Our study has clear implications concerning wildlife monitoring since it represents a considerable reduction of time and effort, both in the field (collecting the samples) and in the laboratory (processing and weighing the samples) when evaluating the body condition of individuals and populations.

Keywords: analytical approaches, body condition, *Cervus elaphus*, kidney fat index, perirenal fat, sampling effort

2.1.1. Introduction

Monitoring body condition is an essential tool in wildlife management to evaluate the nutritional status of both individuals and populations. It can also provide a proxy for habitat quality (Taillon et al., 2011) and population dynamics (Morellet et al., 2007). Moreover, it is a useful means to diagnose potential situations of species overabundance (Caughley, 1981). Body condition can be defined as the amount of animals' energy reserves (Hanks, 1981) and is determined by the net balance between nutritional intake and physiological demands (Hickman et al., 1997; Parker et al., 2009; Schmidt-Nielsen, 1975). Several factors may affect the body condition of large herbivores, which in turn can have effects on survival, performance and reproduction (Cook et al., 2004; Parker et al., 2009). For instance, in seasonally changing environments, the availability and quality of food resources vary within a year (Moen et al., 2006), leading to changes in the seasonal cycle of food intake (Illius, 2006) and inducing nutritional constraints (Parker et al., 2009). Additionally, climatic variation and harsh weather conditions (*e.g.*, intense and prolonged rainfall, variation in temperature, severe winds) increase the animals' energy demands to maintain thermal homeostasis (Clutton-Brock and Albon, 1989; Garroway and Broders, 2005; Serrano et al., 2011). Hence, the body condition could be greatly influenced by intra- and inter-annual environmental fluctuations. Body condition also depends on population density (Couturier et al., 2009; Gaidet and Gaillard, 2008; Stewart et al., 2005) and may be highly variable depending on individual traits such as sex and age class (Mitchell et al., 1976) and reproductive status (Carrión et al., 2008; Yoccoz et al., 2002). Finally, parasite burdens can negatively affect individual body condition (Irvine et al., 2006; Mulvey et al., 1994; Vicente et al., 2004) and supplementary feeding practices – which are also related to diseases and parasite transmission (Gortázar et al., 2006; Vicente et al., 2007) – may help to maintain or enhance the animals' nutritional status (Putman and Staines, 2004).

Several indicators have been developed to assess body condition in wildlife (for reviews see Franzmann et al., 1995; Kirkpatrick, 1980) and in deer species in particular (see Cook et al., 2005; Kie, 1988). The most commonly used indicators are based on fat reserves and, among them, the kidney fat index (KFI; Riney, 1955) has been the most widely used to estimate ungulates' body condition over the last decades (reviewed by Serrano et al., 2008). Originally, the KFI was proposed to evaluate the nutritional status of wild deer and it was defined as the weight of perirenal fat in relation to the weight of the kidneys, as a percentage (Riney, 1955). The KFI correlates positively with total body fat (Finger et al., 1981) and changes in perirenal fat accompany those in body weight (Mitchell et al., 1977). Despite this evidence regarding its physiological significance, the KFI has been questioned as a measure of condition across seasons due to variations in kidney weight that could affect the index values (Batcheler and Clarke, 1970; Dauphiné, 1975). Instead of KFI, Warren and Kirkpatrick (1982) recommended the use of absolute perirenal fat to assess nutritional status, however Waid and Warren (1984) showed a strong correlation between perirenal fat and KFI. Moreover, these authors argued that KFI can be as accurate and reliable as absolute perirenal fat to determine nutritional condition. More recently, Serrano et al. (2008) pointed out some potential problems associated with the use of KFI – a ratio – when performing analysis of variance (ANOVA) and they suggested the analysis of the perirenal fat weight (with kidney weight as a covariate; named here as KCOV) as an alternative method to analyse the variations in the body condition of wild ungulates.

Independently of the indicator or analytical technique used, it is a common procedure to use both kidneys and their associated fat to evaluate ungulates' body condition. According to Anderson et al. (1972), there are important differences between KFI values – both kidney weights and perirenal fat – derived from left and right kidneys, therefore comparisons between them must be avoided. In order to balance these differences, researchers frequently pool the data from the left and right kidneys to perform their analyses. Thus, to date, the use of the two kidneys is the most widely used form of quantifying body condition (*e.g.*, Cook et al., 2005; Dauphiné, 1975; Serrano et al., 2008), but differences between kidneys are not well established in wildlife and deserve further investigation.

The optimisation of sampling procedures to monitor ungulates is an important issue for wildlife ecologists and managers (see Tayce et al., 2008). In this context, the purpose of this paper is to revisit the topic started by Anderson et al. (1972) on the use of one or two kidneys to evaluate body condition in large herbivores. Here, by using red deer *Cervus elaphus* as a model, we investigate the potential differences between body condition indicators derived from left and right kidneys, by focusing our attention on the response of those indicators to a series of ecological and biological factors that could affect the nutritional condition of the species. Specifically, we aimed at analysing the sensitivity (*i.e.*, ability to detect changes) of body condition indicators derived from left and right kidneys to supplementary feeding, sex, age class and season. In this sense, we simultaneously assessed two approaches – KFI (Riney, 1955) and KCOV (Serrano et al., 2008) – to verify whether there are differences in the outcomes of the analyses and subsequent interpretations when quantifying body condition by using the left and right kidneys separately, and their perirenal fat, in relation to pooling the information from both kidneys.

2.1.2. Materials and methods

Study area

Our study was carried out in a 900 ha fenced private hunting estate located in the province of Ciudad Real (south-central Spain, UTM 30S 387400 E - 4308561 N; 600-850 m.a.s.l.). This area is characterized by a Mediterranean climate, with hot and dry summers and mild winters, and a mesomediterranean bioclimate (Rivas-Martínez, 1987). During 2000-2008, the average annual rainfall was 469 ± 47 mm and the average maximum and minimum temperatures were 34 °C and –1 °C, respectively. The vegetation is mainly composed of evergreen oak

(*Quercus ilex*) scrubland, but large patches of savannah-like grasslands (*dehesas*) – mostly formed by pastures and scattered trees – are also found throughout the hunting estate. Red deer in the study area live in sympatry with Eurasian wild boar *Sus scrofa* and Barbary sheep *Ammotragus lervia* and Mouflon *Ovis aries*, the latter two species are rarely present. Red deer density is medium-to-high for this region (38 ± 2 deer/km^2 between 2000 and 2008; for a range of densities in the study region see Acevedo et al., 2008) and is maintained by supplementary feeding throughout the whole year, particularly at the end of summer when natural food resources are limited. Under these circumstances, density-dependent effects on body condition are less evident than in natural conditions (Rodriguez-Hidalgo et al., 2010).

Study design

The analyses of the factors affecting the body condition indicators were carried out using two complementary studies. On the one hand, the effect of the supplementary feeding was assessed in two experimental homogeneous groups of hinds. One group had supplementary food – non-commercial pellets – whereas the other group only had naturally available food sources, obviously also available for the former. After four years in contrasted nutritional conditions, their body condition was assessed. On the other hand, the effects of sex, age class and season were evaluated in a food-supplemented population that is managed for hunting, using the data available from nine years of monitoring.

Experimental study

To investigate the effect of supplementary feeding on the body condition of red deer the hunting estate managers performed a controlled experiment between September 2003 and November 2007. Thirty-six females were divided into two matched age groups and placed into two 14 ha contiguous experimental fenced areas with similar habitat composition, each comprising 1/3 of Mediterranean scrubland and 2/3 of *dehesa* (Fig. 2.1.1), and without other ungulate species. All animals used in the experiment came from a single wild non-supplemented population about 50 km from our study area. Prior to release in the experimental fenced area, blood and faecal samples were collected to monitor their physiological and parasitological status. In addition, they were weighed (± 0.5 kg) and total body length was measured (± 0.5 cm). No differences between supplemented and non-supplemented hinds for the recorded parameters were found before release (Fernández-de-Mera, 2007), thus supporting the assumption of the homogeneity of the groups prior to the experiment.

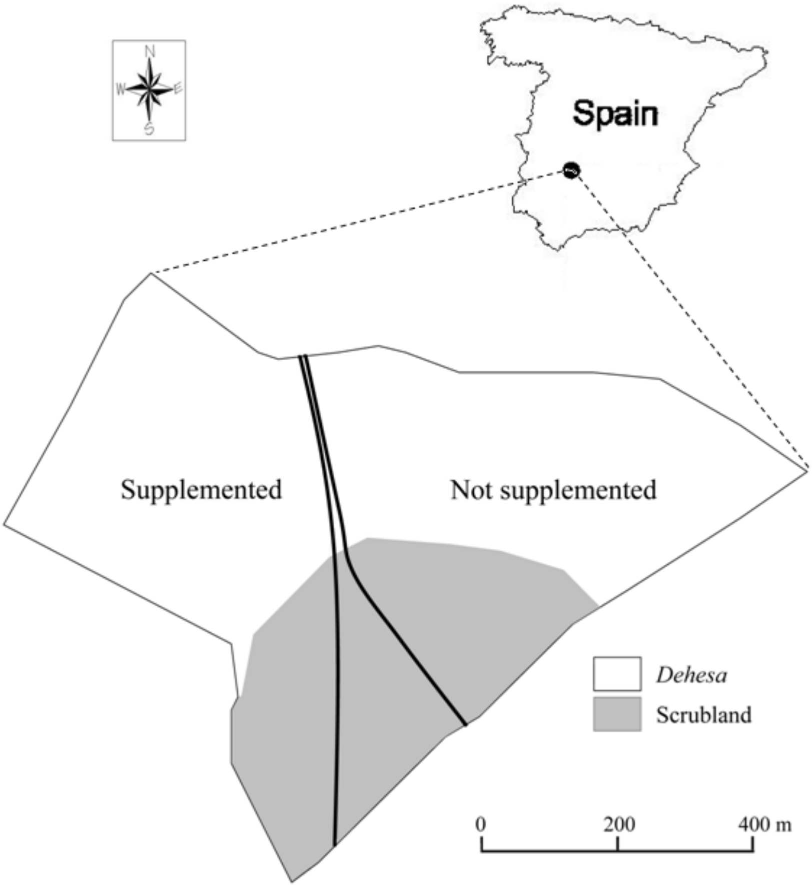

Fig. 2.1.1. Map of Spain showing the location of the study area (above; black dot) and detail of the experimental area (below).

During the experiment, the non-supplemented group (n = 18) only had access to naturally available food sources, whereas the other group (n = 18) was also supplemented with 10 kg/day of a protein-rich pelleted feed (27.6% protein). At the end of the experiment, all hinds were killed and necropsied and both kidneys with attached perirenal fat were collected to assess the body condition. In the laboratory, the amount of fat attached to both apical ends of the kidneys was trimmed and discarded, according to Riney (1955). Left and right kidneys were then weighed separately with their remaining perirenal fat (± 0.1 g). Then, the kidneys were weighed individually once again after being stripped of all fat. The KFI was independently calculated for the left (KFI_L) and right (KFI_R) kidneys and their attached fat as follows: $KFI = (FW / KW) \times 100$, where FW is the weight of the perirenal fat and KW the weight of the kidney. The average KFI value for both kidneys was also calculated by $KFI_{AVG} = (KFI_L + KFI_R) / 2$.

Food-supplemented population: a case study

During a nine-year sampling period – from 2000 to 2008 – samples from 362 red deer (205 males and 157 females) harvested in the remaining 872 ha of the estate (which is managed for hunting purposes) were used to evaluate the effect of sex, age and seasonal factors on the body condition under food-supplemented conditions. The collection of the samples was restricted to September-February, which is the period when red deer hunting usually takes place. After culling, each deer was taken to the estate's necropsy facility. Sex was assessed by visual inspection. For younger animals, age was determined by tooth eruption and replacement patterns (Sáenz de Buruaga et al., 2001), whereas histologically prepared sections of the first incisors were used for age determination in individuals older than 2 at the Matson's laboratory (Montana, USA) (Hamlin et al., 2000). Both kidneys with attached fat were collected and weighed to assess the body condition variables by following the same procedures as described previously for the experimental study.

Statistical analyses

Body condition was measured using two approaches simultaneously, both in the experiment and in the case study: i) KFI (Riney, 1955), and ii) KCOV (Serrano et al., 2008). For each of these studies and approaches, the three measures obtained from the same animal (left, right and average value from both kidneys; hereafter referred to as the 'source of the data') were included in one single model and then tested for differences in relation to a set of factors. The individual was used as a random factor in all models as a way of accounting for within-subject correlation patterns (Hurlbert, 1984). The source of the data was also included as a fixed factor. Additionally, the interaction terms between the source of the data and the predictors were included in the models to assess whether the relationships between the body condition and the predictors varied between the different sources of the data.

In the experimental study, we only included the hinds from which we were able to collect both kidneys and perirenal fat in good condition for processing in our analyses, that is, n = 12 hinds from the non-supplemented group and n = 13 hinds from the supplemented group. General linear mixed models (McCulloch et al., 2008) were used to investigate the effects of supplementary feeding (categorical variable) on KFI and FW values (dependent continuous variables) and the potential differences in their sensitivity to that factor among the sources of the data.

In the field study, the sampled animals were grouped into four biological meaningful age classes (Landete-Castillejos et al., 2004; Rodriguez-Hidalgo et al., 2010; Sáenz de Buruaga et al., 2001) for statistical purposes: calves (< 1 year old; n = 56); yearlings (1 year old; n = 54); sub-adults (2-3 years old; n = 58); adults (≥ 4 years old; n = 194). In addition, based on natural seasonal cycles and on Iberian red deer phenology (*e.g.*, Sanz and Rodríguez, 1993), two seasons were considered: autumn (September-November) and winter (December-February). As in the previous study, general linear mixed models were also used, but this time to investigate the effects of sex, age and season (including season*sex and sex*age class interactions) on KFIs and FWs as well as the potential differences in their sensitivity to those factors among the sources of the data. The response variables were log-transformed [ln(Y)] to achieve normality and reduce the effect of outliers (Zuur et al., 2007). In addition to the individual, the year was also included as a random factor to avoid temporal pseudo-replication (Hurlbert, 1984; Zuur et al., 2009).

Prior to all analyses, data exploration based on a protocol suggested by Zuur et al. (2010) was performed in order to avoid violating the assumptions of the proposed statistical procedures. This was carried out using Brodgar statistical package, version 2.6.6 (Highland Statistics Ltd., 2009). The level of significance for all tests was set at $P \leq 0.05$ (Zar, 1999). We analysed the data using SAS System for Windows, version 9.1.3 (SAS Institute Inc., 2005) and SPSS 15.0 for Windows (SPSS Inc., 2006).

2.1.3. Results

Effect of supplementary feeding on the body condition of red deer hinds

Under experimental conditions, supplementary feeding had a significant effect on all body condition indicators (Table 2.1.1). The KFIs and FWs were higher in supplemented hinds (Fig. 2.1.2). The source of these indicators did not significantly affect the relationship between body condition and supplementary feeding, as revealed by the statistically non-significant interaction between the two factors (Table 2.1.1). Furthermore, our results showed that KW (as covariate) did not significantly affect the amount of FW in the experimental hinds (Table 2.1.1).

Table 2.1.1. Effect of supplementary feeding on the body condition indicators in red deer *Cervus elaphus* hinds by using KFI and KCOV approaches (see text for details). The interaction term between the source of the body condition variables (*i.e.*, the measures of left, right and average from both kidneys) and the predictor was included in the model to assess whether the source affects the relationships between the body condition and the predictor. The individual was included as a random factor in all models and kidney weight was used as a covariate only in KCOV.

Variables	KFI	Fat weight
Source	$F_{2,46} = 4.92$ $P = 0.0116$	$F_{2,45} = 2.31$ $P = 0.1114$
Supplementary feeding	$F_{1,46} = 9.14$ $P = 0.0041$	$F_{1,45} = 17.23$ $P = 0.0001$
Source * Supplementary feeding	$F_{2,46} = 0.06$ $P = 0.9426$	$F_{2,45} = 0.01$ $P = 0.9908$
Kidney weight	–	$F_{1,45} = 2.95$ $P = 0.0926$

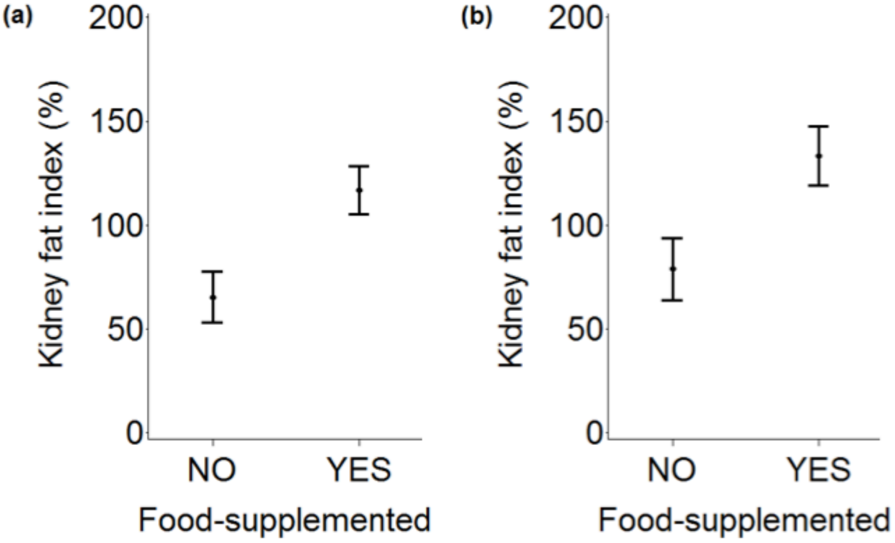

Fig. 2.1.2. Differences in the body condition (expressed as KFIs derived from the left (a) and right (b) kidneys) between non-supplemented and supplemented hinds. Bars indicate standard errors (SE).

Effects of sex, age and season on red deer body condition

Under field conditions, similar statistical results and the same ecological interpretations were generally obtained by using left, right and average body condition indicators, both in the KFI and KCOV approaches (Table 2.1.2). The source of the indicators did not statistically affect the sex and age class differences in the body condition (Table 2.1.2). However, the interaction between source and season was statistically significant (Table 2.1.2). Season and sex*age class interaction had no significant effect on the KFI and nor on FW (Table 2.1.2). Body condition indicators were statistically higher in females than in males and they increased with increasing age class (Fig. 2.1.3). The season*sex interaction evidenced sex related differences, even though they were only significant in the winter (Fig. 2.1.4). Finally, the KW affected the amount of FW in the KCOV approach under field conditions (Fig. 2.1.5; Table 2.1.2).

Table 2.1.2. Effects of season, sex, age class and season*sex and sex*age class interactions on the body condition in red deer *Cervus elaphus* by using KFI and KCOV approaches (see text for details). The interaction terms between the source of the body condition variables (*i.e.*, the measures of left, right and average from both kidneys) and the predictors were included in the models to assess whether the source affects the relationships between the body condition and the predictors. The dependent variables (KFI and perirenal fat weight in KCOV) were log-transformed [ln(Y)]. The individual and year were included as random factors and kidney weight was used as a covariate only in the KCOV model.

Variables	KFI	Fat weight
Source	$F_{2,712} = 1.75$ $P = 0.1748$	$F_{2,711} = 1.38$ $P = 0.2524$
Season	$F_{1,712} = 0.01$ $P = 0.9273$	$F_{1,711} = 0.26$ $P = 0.6103$
Sex	$F_{1,712} = 18.69$ $P < 0.0001$	$F_{1,711} = 4.73$ $P = 0.0301$
Age class	$F_{3,712} = 10.13$ $P < 0.0001$	$F_{3,711} = 26.36$ $P < 0.0001$
Season * Sex	$F_{1,712} = 38.95$ $P < 0.0001$	$F_{1,711} = 40.33$ $P < 0.0001$
Sex * Age class	$F_{3,712} = 1.94$ $P = 0.1220$	$F_{3,711} = 1.95$ $P = 0.1197$
Source * Season	$F_{2,712} = 4.00$ $P = 0.0187$	$F_{2,711} = 3.78$ $P = 0.0233$
Source * Sex	$F_{2,712} = 0.32$ $P = 0.7255$	$F_{2,711} = 0.93$ $P = 0.3932$
Source * Age class	$F_{6,712} = 0.43$ $P = 0.8620$	$F_{6,711} = 0.46$ $P = 0.8370$
Kidney weight	–	$F_{1,711} = 3.93$ $P = 0.0479$

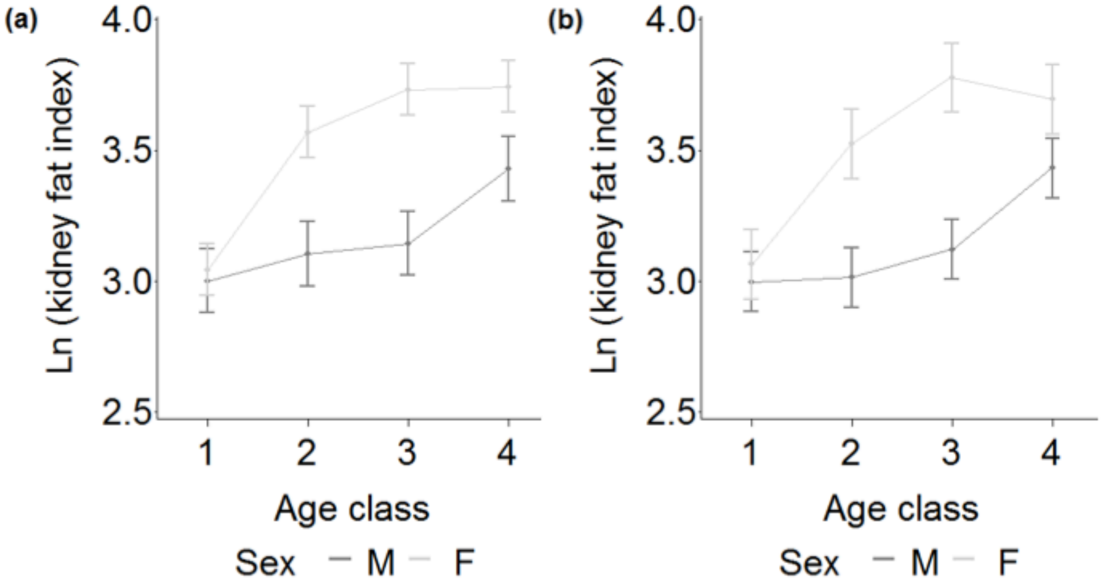

Fig. 2.1.3. Development of the body condition (expressed as KFI) across age classes (1 calves, 2 yearlings, 3 sub-adults, 4 adults) for males (M) and females (F). KFIs derived from the left (a) and right (b) kidneys were log-transformed [ln(Y)]. Bars indicate standard errors (SE).

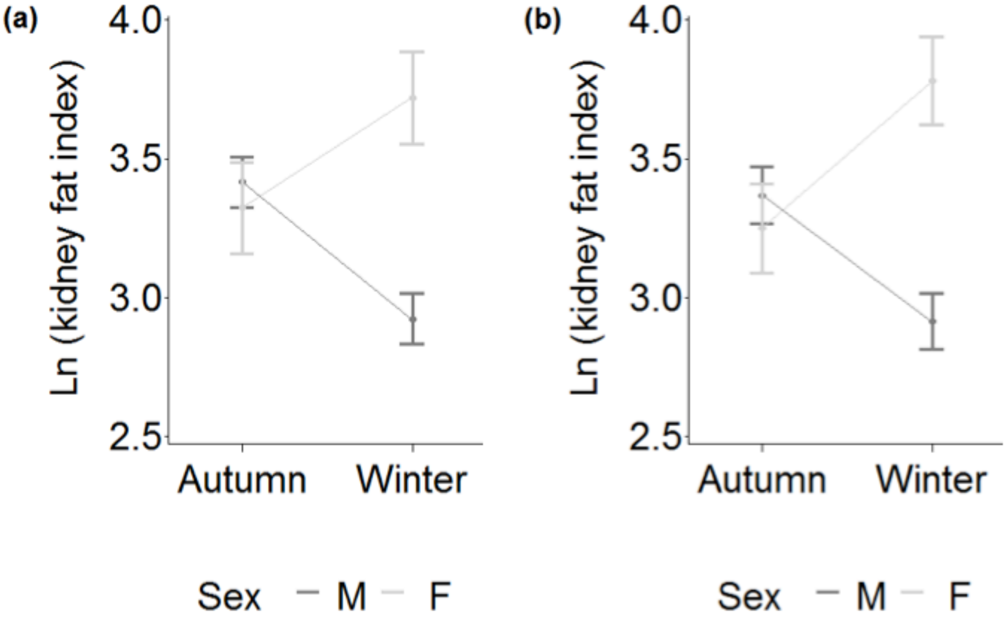

Fig. 2.1.4. Variation in the body condition (expressed as KFI) in the autumn and winter in males (M) and (F) females. KFIs derived from the left (a) and right (b) kidneys were log-transformed [ln(Y)]. Bars indicate standard errors (SE).

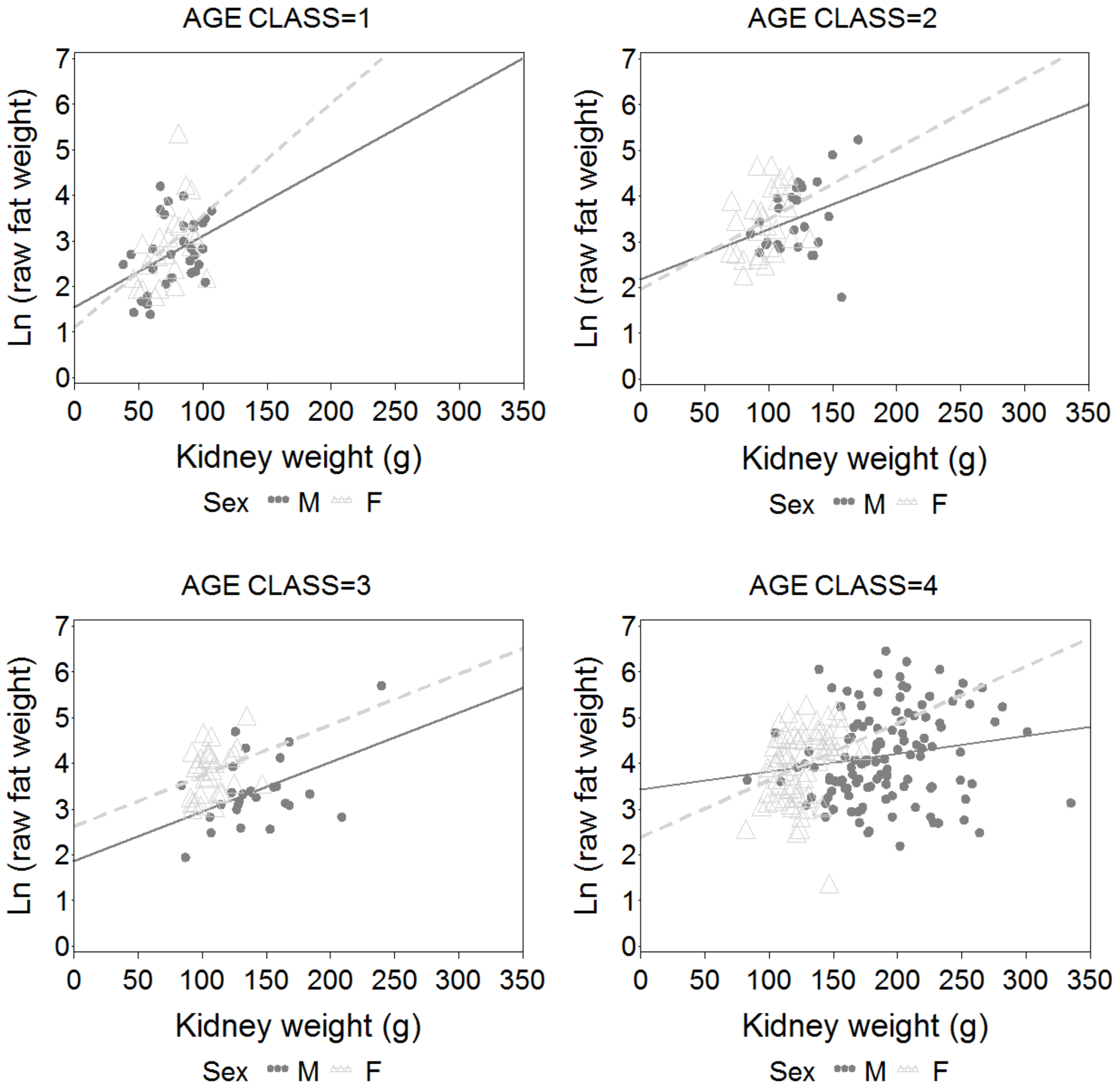

Fig. 2.1.5. Linear relationship between perirenal fat weight derived from the right kidney and respective kidney weight in males (M) and females (F) for each age class (1 calves, 2 yearlings, 3 sub-adults, 4 adults). Fat weight was log-transformed [ln(Y)].

2.1.4. Discussion

We analysed the responses of body condition indicators based on the fat reserves surrounding the kidneys to supplementary feeding, sex, age class and season by using two approaches, the KFI (Riney, 1955) and KCOV (Serrano et al., 2008). For each approach, we also evaluated the effects of the source of the data (*i.e.*, the measures derived from the left, right and the average value from both kidneys) on the relationships between predictors and the body condition variables.

Consistently for the two approaches, no differences driven by the source of the data were detected in the relationship between body condition and supplementary feeding. This suggests that all measures evaluated are equivalent when assessing the response of body condition indicators to supplementary feeding, at least under experimental conditions. The KFIs and FWs indicated that food-supplemented hinds had greater levels of fat reserves and consequently a better nutritional condition than non-supplemented hinds. Since the KFI is highly correlated with the percentage of body fat (Finger et al., 1981) and the variation in the FW occurs in parallel with body weight (Mitchell et al., 1977), these results were expected. For example, Putman and Langbein (1992) showed that supplementary feeding had a significant effect on the body mass in deer. Under experimental conditions, Kozak et al. (1994, 1995) also reported that food-supplemented wapiti *Cervus elaphus nelsoni* hinds were more able to maintain their body weight than non-supplemented animals during the wintertime. Moreover, in a comparative study in south-central Spain, Rodriguez-Hidalgo et al. (2010) found that the body mass of red deer hinds was consistently greater in a food-supplemented population than in a non-supplemented one.

Regarding the field study, the KFIs and FWs were significantly affected by sex and age class and a substantial effect of season in both sexes was also observed. On the one hand, variations in the body condition of sexually dimorphic ungulates have been documented as being linked with their contrasting life-history tactics as well as resource acquisition and energy allocation patterns (Clutton-Brock et al., 1982; McCullough, 1999). In ungulates, the absolute energy demands are usually higher for males since they have larger body sizes than females and relative physiological costs differ between sexes depending on season, for example due to specific energy requirements for reproduction (reviewed by Parker et al., 2009). On the other hand, the KFIs and the FWs in the KCOV approach were generally higher in adult animals according to previous studies (*e.g.*, Lesage et al., 2001; Matiello et al., 2009). The fat reserves in young ungulates are usually lower than in adult animals, since most of the energy is allocated to body growth instead of fat storage. This pattern may also be observed even when ungulates are under food-supplemented conditions (see Carrión et al., 2008), as in the case of our studied population. Even more relevant was that, also under field conditions, the relationship between red deer body condition and the biological traits and season (*i.e.*, predictors) was not affected, in general, by the source of the indicators. The only exception to this generalization was the predictor season, both in the KFI and KCOV approaches; a significant interaction between season and source was obtained. This result may not be very informative since the body condition did not significantly vary between seasons, nor when the source of indicators was independently considered. However, it could relate to the anatomical location of the two kidneys. In ruminants, both kidneys vary in position and according to the pressure exerted by other viscera (Dyce et al., 2010). The left kidney is often displaced and compressed by the forestomach, particularly when the rumen is full (Frandson et al., 2009). The right kidney, in turn, is not subjected to that level of pressure and probably shows a higher degree of plasticity in relation to accumulating or mobilizing fat when the body needs it. Since the energetic demands of the animals vary depending on the season, it is likely that those effects may influence the amount of fat deposits around the kidneys. Nevertheless, as previously stated, the supplementary feeding regime in the population studied is a relevant strategy to mitigate seasonal fluctuations in animals' body condition (Rodriguez-Hidalgo et al., 2010) and thus further studies carried out under fully natural conditions (*i.e.*, in unmanaged populations) may help to clarify the potential differences in the fat accumulation between the two kidneys.

Despite this result, it was possible to show that all body condition indicators responded to the biological traits assessed in the same way. Therefore, and contrary to previously established hypotheses, which suggested differences between body condition indicators when left or right kidneys were used (Anderson et al., 1972), our results show a similar pattern of response by the left, right and average indicators for the factors tested, thus similar statistical results and the same ecological interpretations can be obtained by using information from only one kidney.

When using the KW as a covariate in the KCOV approach, we obtained different results in the experimental and field studies. In the latter, the KW had a significant effect on the amount of attached FW, whereas this was not observed in the former study. These differences could be explained by the fact that a homogeneous group of hinds were considered in the experimental study, while in the other study animals belonging to different sexes and age classes were analysed. Thus, the use of the KW as a covariate successfully corrected for the size of the individuals, which was mainly relevant in the field study. According to Serrano et al. (2008), the analysis of the FW in ANCOVA (with the KW as a covariate) is a preferred method to evaluate changes in the body condition of wild ungulates whenever statistical assumptions, regarding the appropriate use of KFI in ANOVA, are not achieved. In forthcoming research, it could be interesting to continue exploring the relationship between the approaches mentioned by using real and simulated datasets where distinct situations should be tested to evaluate their performance (*e.g.*, fit and prediction properties).

In this paper, we did not evaluate potential differences in the body condition indicators between lactating and non-lactating females in the field study. Since lactation is the biological status where energetic costs are especially high for the hinds (Oftedal, 1985), it may be a factor to take into account in future investigations. For example, Matiello et al. (2009) found substantial differences in the KFI of red deer hinds between lactating and non-lactating females in the Central Italian Alps. Moreover, analysing the variation in the body condition of red deer stags by grouping them into age classes according to their potential reproductive effort (see Yoccoz et al., 2002) could also yield relevant data. Since the supplementary feeding regime in our study area may mask some of those potential differences it would be appropriate to compare the patterns of variation of body condition indicators between non-supplemented and supplemented populations in the future.

2.1.5. Implications for wildlife monitoring

Body condition is a useful indicator of the nutritional status and performance of wild ungulates, and hence optimising the sampling procedures is mandatory to reducing the time and effort required. In this study, we demonstrated that the body condition indicators derived from the left and right kidneys – and their average – were sensitive to the factors that usually shape changes in the body condition of red deer, and probably, other wild ungulates. According to our results, only one kidney and its perirenal fat may be enough, in the future, to assess body condition of red deer, which supposes a notable reduction of time both in the field (collecting the samples) and in the laboratory (processing and weighing the samples); in addition, the amount of biological residuals in the laboratories and/or in the hunting management facilities is also reduced. Assuming that differences between kidneys may still not be properly established, we recommend a standardised protocol when collecting the samples, *i.e.*, always using the same kidney and attached fat. Even though similar results were obtained from left and right kidneys, we recommend the use of the right one in red deer since it is easier to sample due to its anatomical position. Moreover, the right kidney seems to be, to some extent, more sensitive than the left kidney to changes in the nutritional status of red deer, however this issue needs a more detailed study, probably under non-supplemented conditions, to be more conclusive.

To the best of our knowledge, this was the first study evaluating the pattern of response of the body condition indicators derived from the left and the right kidneys in red deer, both in experimental and field conditions. In future research we intend to investigate those responses by using body condition indicators independent of kidney fat reserves and also simulated datasets in order to better understand the asymmetries, if present, in the amount of the animals' energy reserves on each side of their bodies. In addition, it will be important to evaluate those responses through comparative studies between supplemented and non-supplemented populations.

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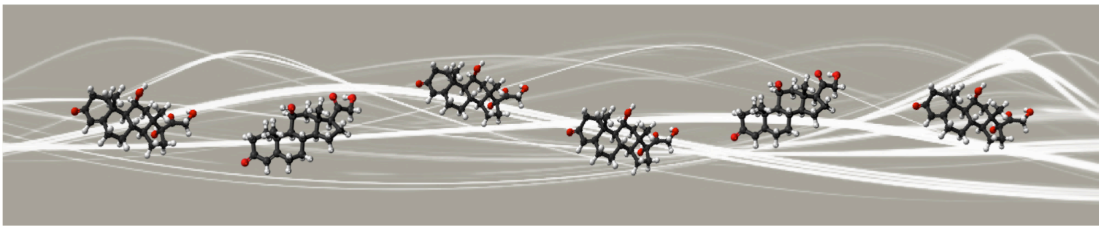

Part 2 Near infrared reflectance spectroscopy (NIRS) for predicting glucocorticoid metabolites in lyophilised and oven-dried faeces of red deer

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Summary

Interest in measuring faecal glucocorticoid metabolites (FGM) as indicators of physiological homeostasis and performance in wildlife is increasing. However, current reference techniques, specifically enzyme immunoassays (EIAs) and radioimmunoassays (RIAs), are expensive, time-consuming, reagent-based, and the samples are destroyed during their application. Conversely, near infrared reflectance spectroscopy (NIRS) is a rapid, reagent-free and non-destructive technique, which, once calibrated by standard laboratory methods, can be used at a low cost. The objectives of this study were to evaluate the feasibility of using NIRS to predict glucocorticoid metabolite concentrations in red deer (*Cervus elaphus*) faeces, as well as the effect of lyophilisation and oven drying on FGM quantification. Seventy-eight fresh faecal samples were collected directly from the rectum of hunter-harvested red deer and then divided into two equal portions; one portion of each individual sample was lyophilised and the other portion was oven-dried. After dehydration, all faecal samples were ground and then analysed by RIA (standard laboratory technique) and scanned with an NIR spectrophotometer. Modified partial least squares regression was used to generate NIRS calibration equations for both lyophilised and oven-dried samples and a cross-validation procedure was employed for their optimisation. Near infrared reflectance spectroscopy proved to be a feasible, acceptably accurate and reliable technique for predicting FGM concentrations in red deer faeces subjected either to lyophilisation or to oven drying. Calibration and cross-validation results indicated that predictive equations for lyophilised faeces were slightly more precise and robust than for the oven-dried ones (lyophilised: $R^2 = 0.90$, $r^2_{cv} = 0.81$, RPD = 2.72; oven-dried: $R^2 = 0.88$, $r^2_{cv} = 0.79$, RPD = 2.26; CV: cross-validation, RPD: ratio of performance to deviation). Nevertheless, oven-dried faeces may be used as an alternative to lyophilised ones to quantify FGM levels accurately, provided that an appropriate combination of dehydration time and temperature is used during the desiccation process. High degrees of association and statistically significant positive correlations ($p < 0.001$) were found between the lyophilised and oven-dried samples regarding their FGM content, both for RIA assays and NIRS analyses. This study provides a new approach for assessing stress levels in free-ranging populations and has practical implications concerning wildlife monitoring as it makes it possible to improve the efficiency and reduce the cost and time constraints of current analytical techniques.

Keywords: *Cervus elaphus*, faecal indicators, glucocorticoid metabolites, NIRS, stress

2.2.1. Introduction

Measuring stress in wildlife is increasingly relevant for conservation and management programmes, as it allows the assessment of the physiological status and performance of both individuals and populations (Busch and Hayward, 2009; Tarlow and Blumstein, 2007; Wikelski and Cooke, 2006). Glucocorticoids (*i.e.*, cortisol and corticosterone) and their metabolites have been used as biomarkers to monitor the physiological stress response in domestic animals (Morrow et al., 2002; Möstl and Palme, 2002; Palme et al., 2000) and wildlife (Azorit et al., 2012; Dalmau et al., 2007; Denhard et al., 2001; Huber et al., 2003; Zwijacz-Kozica et al., 2013). Glucocorticoids are steroid hormones secreted by the adrenal cortex and play a key role in helping organisms to overcome stressful situations, but chronic high glucocorticoid levels may produce deleterious effects on an individual's health and overall fitness (Pride, 2005; Reeder and Kramer, 2005; Sapolsky et al., 2000).

Over the past years, the measurement of glucocorticoid concentrations in blood, saliva, urine, faeces, and more recently in hair and feathers, has been used to assess stress in wildlife (reviewed by Sheriff et al., 2011). Faecal hormone monitoring has recently gained particular notoriety in wildlife research and conservation biology mainly due to the non-invasiveness and ease of sampling (Keay et al., 2006; Millspaugh and Washburn, 2004; von der Ohe and Servheen, 2002). Faecal glucocorticoids are previously metabolised by the liver and later by gut bacteria, and therefore the relative composition of the metabolites formed differ considerably among species (Möstl and Palme, 2002; Palme et al., 2005; Touma and Palme, 2005), and is also affected by individual factors such as gut microbiota, diet, sex and metabolic rate (Goymann, 2012). Enzyme immunoassays (EIAs) and radioimmunoassays (RIAs) have been widely used as reference methods to quantify glucocorticoid metabolites in faecal samples (Möstl and Palme, 2002; Sheriff et al., 2011). However, these techniques are expensive, require chemical reagents or radioactive substances (in the case of RIAs), and the samples are destroyed by analysis. Furthermore, they are time consuming since glucocorticoid extraction is needed before biochemical assays (Pappano et al., 2010; Sheriff et al., 2011).

During the last decades, near infrared reflectance spectroscopy (NIRS) has shown a huge potential for assessing the physicochemical properties of many substances and materials. It has the advantage of being a non-destructive and reagent-free technique that provides a rapid analysis of complex samples containing a wide range of components (Williams, 2001). In addition, the samples require little or no preparation before analysis (Roggo et al., 2007; Siesler et al., 2002). Near infrared reflectance spectroscopy has been widely and successfully applied in various research fields and industries, especially in those linked to agriculture and evaluation of food quality (Cen and He, 2007; Ozaki et al., 2007; Roberts et al., 2004; Williams and Norris, 2001), but also in soil science research (Ludwig et al., 2002; Vendrame et al., 2012) and physiological studies (Banaji et al., 2008; Obrig and Villringer, 2003). The analysis of faecal material by NIRS has also been extensively used to assess numerous aspects of nutrition, physiology and ecology, particularly of domestic and free-ranging herbivores (Dixon and Coates, 2009; Dryden, 2003; Foley et al., 1998; Gálvez-Cerón et al., 2013; Lyons and Stuth, 1992). However, to our best knowledge, no studies have been reported using NIRS to predict the concentrations of stress hormone metabolites in faeces. In fact, the use of NIRS as an analytical method in hormonal studies is still scarce (Fountain et al., 2003; Kinoshita et al., 2012; Pérez et al., 2004; Xia et al., 2007). Near infrared reflectance spectroscopy is a predictive (indirect) method, which requires the development of calibration models against appropriate standard laboratory methods. Hence, the performance of NIRS as a procedure for quantitative analysis will depend on the accuracy and precision of those reference methods (Cen and He, 2007).

In this paper, our intent was to explore cost-effective and timesaving methods for assessing stress in wild populations, in particular, that of free-ranging herbivores. The objectives of this study were two-fold. Firstly, we evaluated the feasibility of using NIRS to predict the concentration of faecal glucocorticoid metabolites (FGM) in red deer (*Cervus elaphus*) faeces. The red deer has a widespread distribution in the northern hemisphere (Wemmer, 1998) and is one of the most important game species in many European countries, where their populations are subjected to different management and exploitation regimes (Apollonio et al., 2010). In some cases, those populations are overabundant and intensively managed for hunting (Gortázar et al., 2006). This species can also suffer from various diseases, many of which are shared with livestock and some are zoonoses of importance for public health (Ferroglio et al., 2011; Gortázar et al., 2007). Considering the variety of potential stress-inducing factors to which red deer are exposed, monitoring stress in their wild populations is of general interest. Secondly, we investigated the effect of two drying methods (lyophilisation and oven drying) on the quantification of FGM and developed NIRS calibration models for predicting their concentrations for each drying treatment. Previous studies have shown that different drying procedures affect FGM concentrations and, until now, lyophilisation has been the preferred method used for drying faecal samples in stress research, as it allows for effective preservation/recovery of FGM (Terio et al., 2002). However, apart from the fact that lyophilisers are not always available in laboratories, this freeze-drying process can take up to three or four days to complete (Goymann et al., 1999; Millspaugh et al., 2001). On the other hand, oven-dried samples can be ready for analysis in half the time, but their use is generally not recommended since the oven drying process may cause FGM degradation (Terio et al., 2002). Therefore, we intended to verify how effective and reliable the oven drying method is in preserving FGM in relation to lyophilisation.

2.2.2. Materials and methods

Sample collection and storage

Fresh faecal samples (*n* = 78) were collected directly from the rectum of hunter-harvested red deer at eight different locations in the Iberian Peninsula during three consecutive regular game seasons (September to February 2010-11 to 2012-13). Young and adult male and female deer were sampled to take into account any potential variations in FGM concentrations due to age and sex (Goymann, 2012; Millspaugh and Washburn, 2004). Upon collection, the faecal samples were placed in individual appropriately identified plastic bags, and then transported in a cooler box with freeze gel packs to the laboratory, to prevent any degradation of the hormone metabolites (Palme, 2005; Sheriff et al., 2011). Once in the laboratory, the samples were stored in a freezer at –20 °C, without any chemical treatment (Millspaugh and Washburn, 2004), until processing.

Drying and preparation of faecal samples

Individual faecal samples were separated into two equal portions: one portion was lyophilised at –20 °C for 96 h, whereas the other portion was oven-dried at 60 °C for 48 h. After dehydration, all samples were ground in a cyclone-type mill to pass a 1 mm screen and stored in zip-lock bags in a cool, dry place, before the NIRS analysis.

FGM quantification by a reference method

The concentrations of FGM were determined in the lyophilised and oven-dried samples with a ^{125}I-corticosterone radioimmunoassay (RIA) kit (#07-120103, MP Biomedicals, LLC, Orangeburg, NY) that has already been biologically and analytically validated for elk (*Cervus elaphus canadensis*) (Wasser et al., 2000; see also Millspaugh et al., 2001). Prior to the RIA assays, FGM were extracted by placing 0.5 ± 0.05 g of dry faeces in a test tube with 4 mL of 100% methanol plus 1 mL of distilled water and shaking in a vortex for 1-2 min, and subsequently in an orbital shaker at 230 rpm for 45 min. Samples were then centrifuged at 3000 rpm for 20 min and the supernatant saved and stored at –20 °C until FGM determination. We followed the manufacturer’s protocol to quantify FGM concentrations. Faecal extracts were diluted 1:10 in the kit buffer to avoid methanol interference. The concentrations of FGM, which represent pooled fractions of blood-circulating glucocorticoids that have been secreted and metabolised over a period of approximately 24 h (Bradshaw, 2003; Huber et al., 2003; Wasser et al., 2000), were expressed in ng/g of dry matter (DM) as in previous studies (*e.g.*, Pereira et al., 2006; Zwijacz-Kozica et al., 2013). The manufacturer indicated the following cross-reactivities for the antiserum used in this study: 100% for corticosterone, 0.34% for desoxycorticosterone, 0.10% for testosterone, 0.05% for cortisol, 0.03% for aldosterone, 0.02% progesterone, 0.01% androstenedione, 0.01% 5α-dihydrotestosterone and < 0.01% for other steroids.

NIRS analysis

Dried and ground faecal samples were packed into 35 mm diameter circular cups, with quartz glass windows, and scanned using an NIRSystems 5000 (FOSS, Hillerød, Denmark) over a wavelength range of 1108-2492 nm. The data were recorded at 2 nm intervals as *log 1/R* (where R = reflectance), resulting in 692 points per sample. Each sample was scanned twice, by manually rotating the sample cup approximately 180° relative to the previous scan. Hence, two spectra were collected per sample. The same operator performed the entire scanning procedure.

The spectral data processing and statistical analyses were performed using WinISI III (v.1.6) software. Prior to development of calibration models, the original spectra were subjected to different pre-treatment methods in order to improve signal-to-noise ratio and, therefore, maximise the signal intensity for the analytes of interest (Heise and Winzen, 2002). Spectral correction algorithms – standard normal variate (SNV), detrend (DT) and multiplicative scatter correction (MSC) – were applied to reduce the effects of light scattering due to particle size. Furthermore, different mathematical treatments using the first, second and third derivatives, with different subtraction gaps and smoothing intervals were also tested. A total of sixteen spectral models for each predicted parameter were developed, resulting from the evaluation of four scatter correction techniques (SNV; DT; SNV+DT; MSC) and four mathematical treatments (1,4,4,1; 2,4,4,1; 3,4,4,1; 2,10,10,1 – derivative number, subtraction gap, smooth, second smooth).

Modified partial least squares (MPLS) regression was used to develop the calibration models (Martens and Martens, 2001) and '*leave-n-out*' cross-validation (Shao, 1993) was then performed for their optimisation. Cross-validation was also used to identify those samples whether chemical (t) or spectral (H) outliers (about 8% of the samples). The t outliers are samples that have a relationship between their reference values and spectra that is different from the relationship of the other samples in the set and with large residuals (t values > 2.2). Samples with a large t statistic often cast doubt on the chemical reference value. An H outlier identifies a sample that is spectroscopically different from other samples in the population and has a standardized H value (modification of the Mahalanobis distance) greater than 3.

The quality and predictive ability of the calibration equations were evaluated by using the following statistical parameters (for details see Shenk and Westerhaus, 1996; Williams and Sobering, 1996): coefficient of determination of the calibration (R^2), standard error of the calibration (SEC), coefficient of determination of the cross-validation (r^2_{cv}), standard error of the cross-validation (SECV), the range error ratio (RER, which was calculated by dividing the range in the reference data by the SECV), and the ratio of performance deviation (RPD, which was calculated by dividing the standard deviation of the reference values by the SECV).

Statistical analysis

Linear regression was used to investigate the relationship between the concentrations of FGM in the lyophilised (standard drying method) and oven-dried samples (Montgomery et al., 2012). The level of agreement between the drying methods on the quantification of FGM was assessed using *t*-tests for the hypothesis for the intercept (α) and slope (β): H_0: $\alpha = 0$ and $\beta = 1$ (Passing and Bablok, 1983), that is, we tested whether the slope and intercept of the fitted regression line significantly differed from the line of identity (y = x). The degree of association between the two drying procedures was assessed using Pearson's correlation test (Sokal and Rohlf, 2009). Moreover, we analysed whether the NIRS prediction of FGM was influenced by the drying treatment by testing the interaction between the drying procedure and FGM predicted by NIRS. The level of significance of all tests was set at 0.05 (Zar, 1999). All analyses were performed using R statistical software, version 3.0.3 (R Core Team, 2014).

2.2.3. Results

NIRS calibration and validation

The calibration sets used as reference data for lyophilised and oven-dried samples covered a wide range of FGM concentrations (Table 2.2.1), thus ensuring its usefulness to calibrate the NIRS technique.

The spectral pre-treatment methods that gave the best results were the second derivative combined with MSC for lyophilised samples and with SNV+DT for oven-dried samples (Table 2.2.1). The estimated parameters for the best calibration equations and cross-validation procedures indicated that good calibration models were obtained for predicting FGM levels both in lyophilised and oven-dried faeces (Fig. 2.2.1; Table 2.2.1).

Table 2.2.1. Statistical and data processing results relating near infrared reflectance spectroscopy (NIRS) predicted values for faecal glucocorticoid metabolites (FGM, expressed as ng/g of dry matter) to laboratory values obtained by radioimmunoassay in lyophilised and oven-dried faeces of red deer (*Cervus elaphus*).

	Descriptive statistics [a]			**Spectral pre-treatments**		**NIRS calibration and cross-validation statistics**					
	Min - Max	**Average**	**Standard deviation**	**Math treatment** [b]	**Scatter correction** [c]	R^2	**SEC**	r^2_{cv}	**SECV**	**RER**	**RPD**
FGM (lyophilised)	24.33 - 482.99	141.89	80.24	2,4,4,1	MSC	0.90	21.89	0.81	29.50	15.55	2.72
FGM (oven-dried)	24.90 - 408.42	155.33	91.44	2,4,4,1	SNV+DT	0.88	32.23	0.79	40.42	9.49	2.26

R^2 = coefficient of determination of calibration; SEC = standard error of calibration; r^2_{cv} = coefficient of determination of cross-validation; SECV = standard error of cross-validation; RER = range error ratio (max–min/SECV); RPD = ratio of performance deviation (SD/SECV).

[a] Descriptive statistics of FGM concentrations determined by radioimmunoassay (standard laboratory technique) used for NIRS calibration.

[b] Mathematical treatment: derivative order, gap, first smoothing, second smoothing.

[c] Spectra correction algorithms: SNV = standard normal variate, DT = detrend, MSC = multiplicative scatter correction.

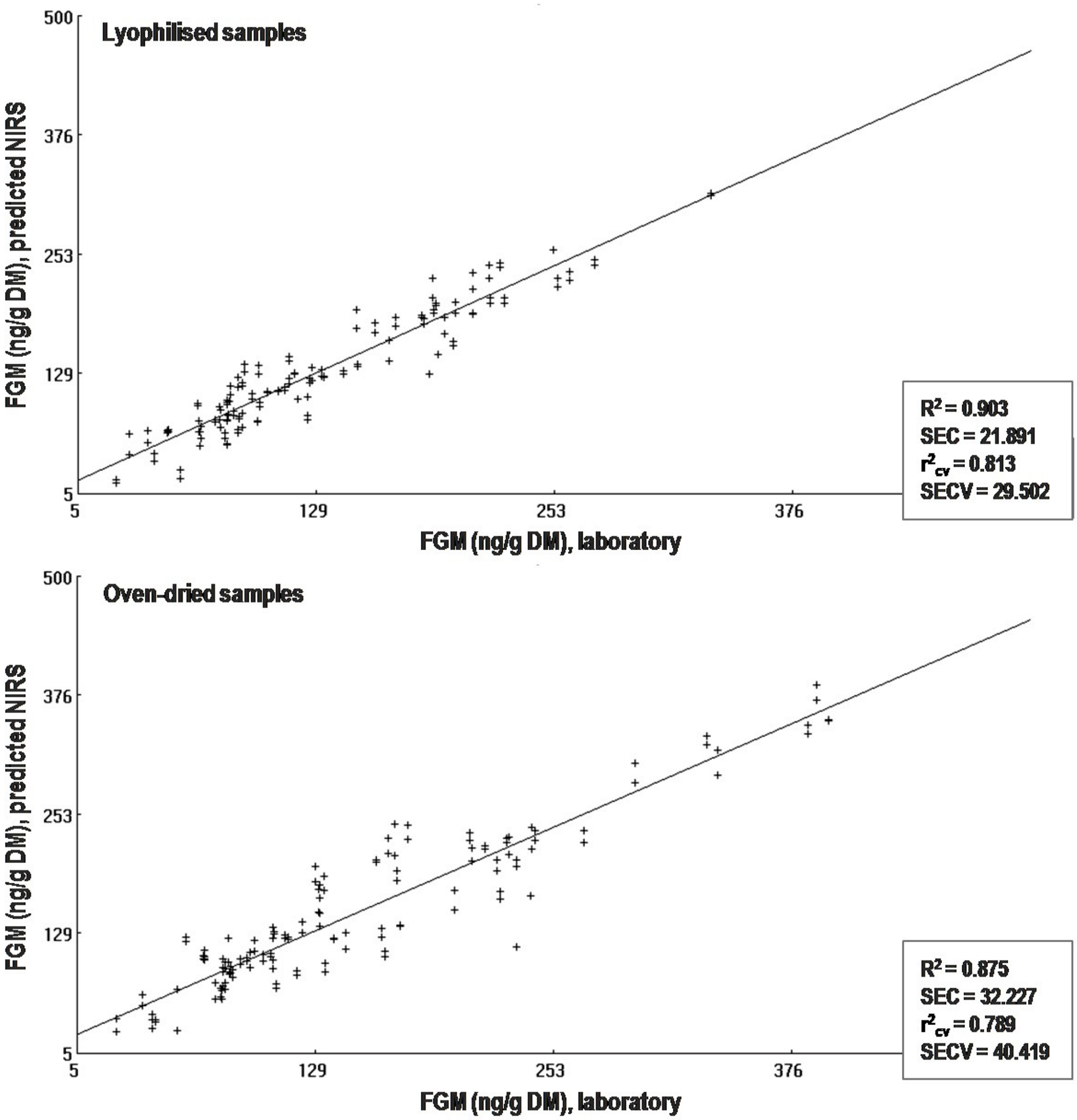

Fig. 2.2.1. Linear relationship between the concentrations of faecal glucocorticoid metabolites (FGM, expressed as ng/g of dry matter) determined by radioimmunoassay (standard laboratory method) and near infrared reflectance spectroscopy (NIRS) predicted values, for lyophilised and oven-dried samples. R^2 = coefficient of determination of calibration; SEC = standard error of calibration; r^2_{cv} = coefficient of determination of cross-validation; SECV = standard error of cross-validation.

Effect of drying treatments on FGM levels

A statistically significant positive correlation was observed between RIA-determined FGM levels for lyophilised and oven-dried samples (*r* = 0.910, n = 78, $p < 0.001$). The slope of the regression did not significantly differ from 1 (β: *t* = 0.696, df = 77, p = 0.489) and the intercept did not significantly differ from 0 (α: *t* = 0.921, df = 77, p = 0.360), thus indicating a close relationship between the results obtained from the two sets of samples. This relationship was better at lower than at higher FGM concentrations, when oven-dried faeces showed overall higher values than the lyophilised ones (Fig. 2.2.2a). This might be due to the reduced number of observations (data points) at higher FGM concentrations. Similarly, a high level of agreement (β: *t* = 0.125, df = 77, p = 0.901 and α: *t* = 1.228, df = 77, p = 0.223) and a statistically significant positive correlation (*r* = 0.751, n = 78, $p < 0.001$) was observed between the lyophilised and oven-dried faecal samples for NIRS-predicted values (Fig. 2.2.2b). Finally, the relationship between RIA-determined FGM and NIRS-predicted FGM was not influenced by the drying treatment as indicated by the non-significant interaction (*t* = -1.232; df = 152; p = 0.220).

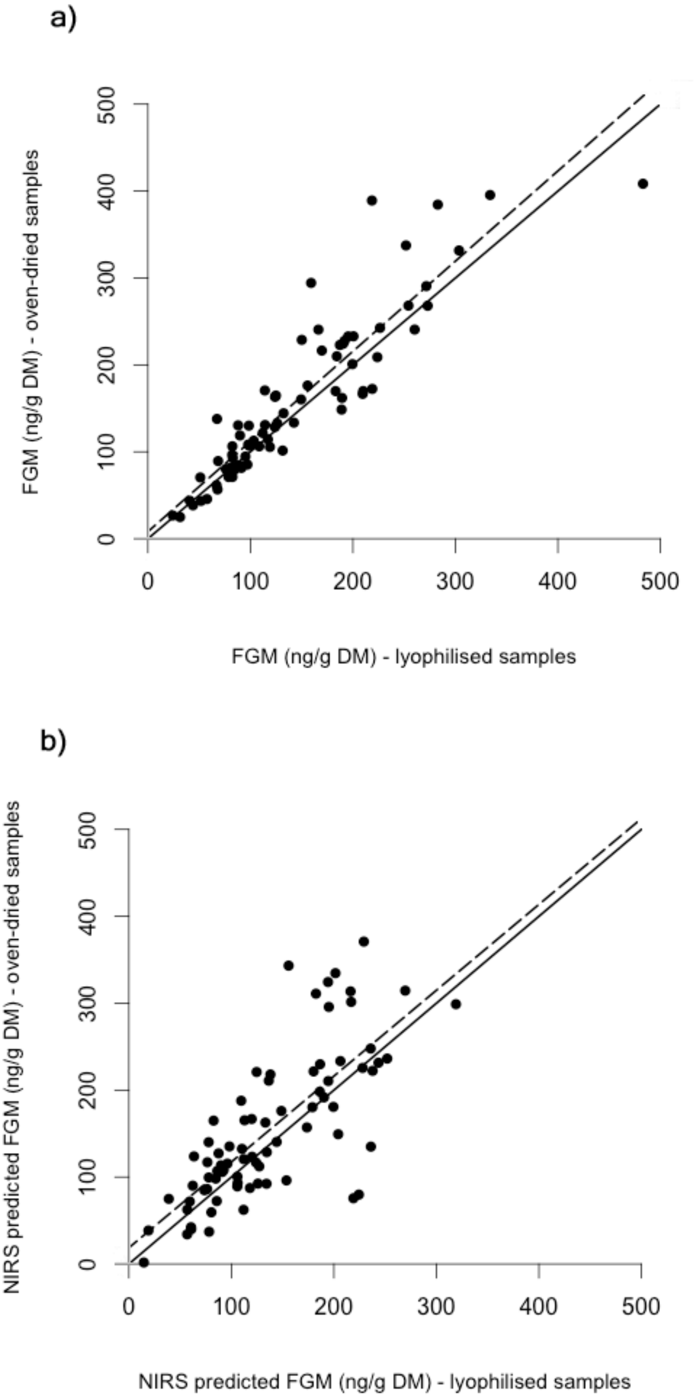

Fig. 2.2.2. Relationship between lyophilised and oven-dried samples in terms of faecal glucocorticoid metabolite content (FGM, expressed as ng/g of dry matter) for (a) radioimmunoassay and (b) near infrared reflectance spectroscopy (NIRS) analyses. Solid black line: y = x. Dashed black line: fitted regression line.

2.2.4. Discussion

NIRS calibration and validation

This study shows the feasibility and reliability of using NIRS to predict FGM concentrations in faeces subjected either to lyophilisation or to oven drying, in particular in red deer faeces, for the first time. Although good calibration models were obtained for both drying methods, the calibration equations for the lyophilised faeces were slightly more precise and robust than the equations for the oven-dried ones. In both cases, the R^2 and r^2_{cv} values obtained showed a strong relationship between the reference data and the NIRS predicted values, thus indicating that NIRS can predict FGM concentrations in faeces with good precision (Shenk and Westerhaus, 1996). Prediction equations for FGM content were satisfactory, probably due to the identification of absorption bands related to OH and CH bonds contained in the samples.

Williams and Sobering (1996) reported that RPD values should ideally be at least 3 and the RER at least 10. In this study, the RER value obtained for lyophilised faeces was higher than 10, whereas the estimated RPD values for both drying treatments were lower than 3 (Table 2.2.1). However, Saeys et al. (2005) suggested that RPD values ranging from 2.0 to 2.5 might be used to make acceptable quantitative predictions for some compounds, while good predictions may be achieved whenever the RPD values are greater than 2.5. Our results are consistent with these criteria, thus showing that NIRS can be a valuable tool for predicting FGM levels in red deer faeces, and probably those of other ruminants, with an acceptable accuracy. Moreover, taking into account that the predictive performance of NIRS may decrease with the increasing complexity of the compound analysed (Andrés et al., 2005; Scholtz et al., 2009), our results are very promising since NIRS was able to detect and predict a diverse array of stress hormone metabolites, whose formation and composition vary depending on the biological and physiological traits of species and individuals (Goymann, 2012; Palme et al., 2005). Probably, more robust calibration equations may still be achieved in further research by increasing the number of samples in the calibration set. Furthermore, red deer is a highly sexually dimorphic species (Clutton-Brock et al., 1982) and differences both in stress response and excreted glucocorticoid metabolites have been reported between males and females (Goymann, 2012; López-Olvera et al., 2007). Differences in the faecal chemistry among sexes can greatly affect NIRS spectra profiles, as seen in domestic goats (Walker et al., 2007) and also in red deer and fallow deer (*Dama dama*), where sex could be identified according to spectral patterns (Tolleson et al., 2005). Therefore, separate calibration models for each sex should also be tested in order to try to achieve more robust calibrations.

Effect of drying treatments on FGM levels

The overall high agreement between FGM concentrations in lyophilised and oven-dried samples suggests that oven drying does not cause FGM degradation, and may be, therefore, as valid and effective as the lyophilisation process. These results differ from those obtained by Terio et al. (2002), who found significantly lower FGM concentrations in oven-dried cheetah (*Acinonyx jubatus*) faeces, as compared to lyophilised faecal samples. However, Terio et al. (2002) oven-dried the faecal samples at 71 °C for 72 h (3 days), while in the present study the samples were oven-dried at 60 °C for 48 h (2 days). These differences in the drying protocol may have led to FGM degradation or changes in the immunodominant metabolites, as also discussed by those authors. In brief, our results suggest that oven-dried samples may be used as an alternative to lyophilised faeces to accurately quantify FGM levels, provided that an appropriate combination of dehydration time and temperature is used during the desiccation process.

2.2.5. Conclusions and implications for wildlife monitoring

The development of reliable, fast and inexpensive procedures to assess stress in wild populations is essential to reducing the cost and time constraints of the analyses, as well as to minimising the effort required for wildlife surveys. In this study it has been demonstrated that NIRS is a valid tool for predicting the concentration of FGM in red deer faeces subjected to different drying treatments. This technique revealed a great potential for quantitative analysis in hormonal studies using faeces and offers various advantages over traditional laboratory methods that may help to improve the cost-effectiveness of such analytical procedures. Besides being a non-destructive and reagent-free technique, one of its major advantages is that, once calibrations are fine-tuned, it allows a rapid prediction of multiple constituents from a single sample at a low cost. Although good calibration equations were obtained in our study, further research, preferably using an independent validation scheme, as well as experimental determination of FGM allowing comparison between stressed and non-stressed red deer, would increase the robustness of the equations and, therefore, reinforce the reliability and usefulness of NIRS to predict FGM in this species. It is also important to point out that NIRS must not be seen as a replacement technique for standard laboratory methods; instead, it should be used together with those reference methods in order to reduce the cost and improve the efficiency of ecological studies (Foley et al., 1998).

This study has also shown that oven-dried faeces might be used to quantify FGM levels with a good degree of accuracy in relation to lyophilised faeces. For that method to be reliable, certain time and temperature requirements must be met in order to avoid FGM degradation. This will allow a considerable reduction in the time required for the dehydration of faecal samples. Moreover, since lyophilisers are expensive and are not always available in the laboratories (or in other facilities), it will be possible to use an alternative method, that also ensures the preservation of FGM, to dry faecal material. Although no significant differences were found between the concentrations of FGM in oven-dried and lyophilised faeces, the NIRS predictions were slightly more accurate for the lyophilised samples.

This study represents an important step in the development of new approaches for evaluating stress levels in the faeces of wild animals, in particular those of free-ranging herbivores.

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Chapter 3

Nutritional Ecology of Red Deer in Mediterranean Ecosystems

Determining changes in the nutritional condition of red deer in Mediterranean ecosystems: effects of environmental, management and demographic factors

Summary

Monitoring changes in the nutritional status of wild populations is crucial to understanding how species respond to natural or human-mediated variations in food availability and quality. It is a fundamental step for decision-making in wildlife management. Moreover, such monitoring can only be made effective by establishing indicators which reliably reflect those changes. Many studies on nutritional ecology still tend to rely on the use of single indicators. Nonetheless, nutrition has multiple interdependent facets and researchers should combine different indicators reflecting different nutritional ‘currencies’ to better understand how organisms interact with their environment through feeding. In this paper, data from a large-scale cross-sectional survey conducted between 2010 and 2013 was used to examine the influence of environmental conditions and management practices on the variation of faecal nitrogen content (FN, index of dietary quality) and kidney fat index (KFI, indicator of an animal’s energy status) in red deer (*Cervus elaphus*) in the Mediterranean ecosystems of Iberia. Variations in the nutritional status in relation to an individual’s sex, age, dietary aspects and faecal counts of lungworm larvae were also analysed. Our results showed that FN levels were statistically and positively correlated with vegetation primary productivity. In contrast, dietary fibre fractions and the average altitude of the study areas were negatively associated with FN concentrations. The highest ranked models obtained for KFI indicated that the nutritional condition of red deer was related to sex, dietary quality and faecal parasite counts, as well as to changes in vegetation productivity and latitude. The effect of high-quality diets on deer kidney fat deposits was gradually lower as the population density increased. This result was more marked in non-food-supplemented populations. Both FN and KFI were statistically higher during the winter in relation to the autumn. This study shows that FN can be used to monitor and predict changes in the dietary quality, and the KFI in the nutritional status, in red deer in Mediterranean environments, reliably. Since both indicators showed different sensitivity to biological, ecological and management determinants, this research also highlights their value and complementary use when studying populations of large wild herbivores.

Keywords: *Cervus elaphus*, faecal nitrogen, Iberian Peninsula, kidney fat index, nutritional ecology

3.1. Introduction

Nutritional condition (*sensu* Harder and Kirkpatrick, 1994) is a major determinant of an animal's performance, influencing diverse aspects of its life history such as growth, reproduction and survival (Barboza et al., 2009; Randall et al., 2002), with consequent implications for population dynamics (Cook et al., 2004; Owen-Smith and Mills, 2006). For most large wild herbivores, nutritional condition is the result of the physiological and behavioural responses of individuals to their biophysical and social environments (Ceacero et al., 2012; Long et al., 2014; Parker et al., 2009). From a physiological perspective, it can be viewed as an outcome of dynamic processes that involve allocation and mobilisation of somatic reserves (*i.e.*, lipids and proteins), which, in turn, are regulated by a combination of intrinsic traits, environmental factors and ecological interactions (Barboza et al., 2009; Monteith et al., 2013; Parker et al., 2009).

Fat and proteins are essential nutrients for animals and their deficit can disrupt numerous physiological processes. They are important sources of metabolic energy (especially fats) and are also basic structural components of tissues (*e.g.*, muscle proteins) (Randall et al., 2002). In dimorphic ungulates, nutritional or energy requirements may vary according to differences in body size and digestive efficiency (Barboza and Bowyer, 2000; Clauss and Hummel, 2005), activity time (Hamel and Côté, 2008; Long et al., 2014), as well as with sex, age, and reproductive status (Forsyth et al., 2005; Hamel and Côté, 2008; Santos et al., 2013). Furthermore, pathogens can negatively affect both nutrient metabolism and absorption (Coop and Kyriazakis, 1999; Gálvez-Céron et al., 2015), therefore increasing the host's nutritional demands and consequently its susceptibility to environmental constraints (Ezenwa, 2004).

Natural populations, inhabiting seasonally changing environments, typically face annual cycles of forage availability and quality, which are strongly influenced by weather conditions (Marshal et al., 2005; Moen et al., 2006). Such fluctuations determine changes in an individual's nutritional condition, with plant-growing seasons corresponding to high-quality forage periods, in which animals gain body mass and restore their energy reserves, while in periods of plant senescence or dormancy they usually have to cope with nutritional restrictions and have to mobilise their fat and protein stores for body maintenance (Parker et al., 1999; WallisDeVries, 1998). The periods of food shortage for wild herbivores differ throughout the regions of the globe; in many temperate zones the most limiting season is the winter, whereas in tropical and subtropical areas the most critical period corresponds to the dry season (Moen et al., 2006; Parker et al., 2009). The way animals cope with seasonal nutritional constraints is therefore context-dependent, thus highlighting the importance of considering environmental conditions on different geographical scales to understand the nutritional ecology of large wild herbivores (*e.g.*, Martínez-Jauregui et al., 2009). The influence of environmental variation on both the diet and nutritional condition of wild ungulates is broadly documented, in particular for populations living in northern latitudes of Eurasia and North America (*e.g.*, Christianson and Creel, 2007; Cook et al., 2013; Pettorelli et al., 2005a). However, fewer efforts have been made to elucidate how seasonal environmental changes affect the feeding ecology and nutritional condition of wild deer in the Mediterranean ecosystems (*e.g.*, Miranda et al., 2012; Rodriguez-Hidalgo et al., 2010), where the summer drought is the most critical factor influencing the availability and nutritive value of forage (Bugalho and Milne, 2003).

Demographic factors have also been shown to be important drivers of nutritional condition among ungulates. Population densities above the ecological carrying capacity (*sensu* Caughley, 1979) often result in competition for food resources between individuals (Stewart et al., 2005), with additional negative impacts on plant communities and the nutritive value of vegetation (Blanchard et al., 2003; Perea et al., 2014). Moreover, the interactive effects between high densities and unfavourable environmental conditions affecting plant productivity may also have profound short- and long-term consequences on the animals' condition and, ultimately, on the dynamics of wild populations (Forchhammer et al., 2001; Post and Stenseth, 1999; Rodriguez-Hidalgo et al., 2010; Simard et al., 2010). In recent decades, ecological and socio-economic changes have contributed to a significant increase of deer populations in many temperate regions of the Northern Hemisphere (Apollonio et al., 2010; Gordon et al., 2004), including in the Mediterranean areas of southwestern Europe (Acevedo et al., 2011; González et al., 2013). Despite this, the impact of population densities on the nutritional status and dietary quality of wild deer remains essentially unknown for this geographical region, where both plant phenology and productivity of habitats differ from those observed at northern temperate latitudes (*e.g.*, Gillman et al., 2015; Gordo and Sanz, 2010).

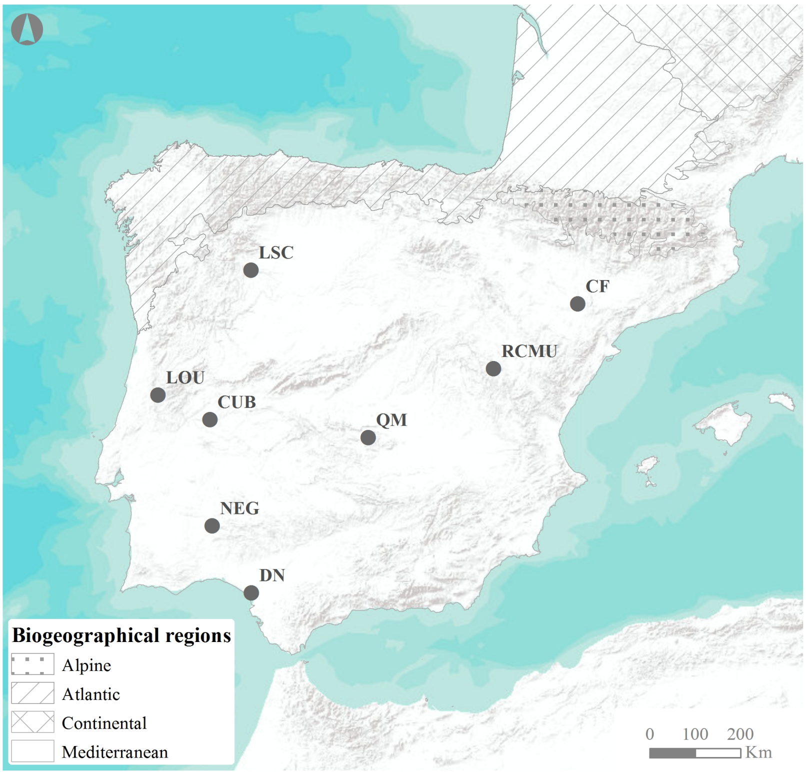
Management practices can also affect the nutritional condition of large herbivores in numerous ways, either through interventions in the structure and composition of habitats (Simard et al., 2014; WallisDeVries, 1998), or by directly providing supplemental food, a practice traditionally used in areas managed for hunting (Milner et al., 2014; Putman and Staines, 2004). In the Iberian Peninsula, where big game hunting, particularly of red deer *Cervus elaphus*, represents an important economic activity (Martínez-Jauregui et al., 2011), supplementary feeding practices are usually related to the maintenance of artificially high population densities and/or production of high-quality trophies. Although management practices are recognised as important factors influencing the nutritional condition of ungulate species, their effects have rarely been studied in detail in wild populations, especially in Mediterranean environments (but see Rodriguez-Hidalgo et al., 2010; Santos et al., 2013).

In this paper, a large-scale cross-sectional approach was used in order to assess the influence of environmental factors, management practices and population densities on the dietary quality and nutritional status of red deer in the Mediterranean habitats of the Iberian Peninsula, during the seasons following the period of greatest food restriction. In this region, as well as in many other parts of Europe, the red deer is one of the most widespread wild ungulates and is extremely important both ecologically, as a key species for the functioning of ecosystems (Smit and Putman, 2011), and economically, as a major game species (Martínez-Jauregui et al., 2011; Milner et al., 2006). Due to its ecological and economic relevance, knowledge of the factors that may affect nutritional condition of red deer is therefore fundamental to developing appropriate management practices. By using two nutritional indices – faecal nitrogen (FN) as a proxy for dietary quality (Leslie et al., 2008) and kidney fat index (KFI) as an indicator of the amount of energy reserves in an animal's body (Santos et al., 2013) – the following hypotheses were tested in this study: (H1) periods of increased vegetation productivity, which are greatly influenced by accumulated rainfall during the autumn and winter in Mediterranean areas (Rodriguez-Hidalgo et al., 2010), are expected to affect positively both FN and KFI (Blanchard et al., 2003; Hamel et al., 2009; Kucera, 1997); (H2) significant effects of habitat composition and supplementary feeding practices are also predictable, with high-quality habitats and increased forage supply having positive effects on deer nutritional condition (Santos et al., 2013); (H3) high population densities are expected to have a negative effect on deer nutritional condition (Blanchard et al., 2003; Stewart et al., 2005). Finally, the effects of sex, age and parasite load on the measured nutritional indices were also analysed.

3.2. Materials and methods

Study areas and red deer populations

The present study was conducted in eight areas which are representative of a variety of Mediterranean environments found across the Iberian Peninsula (Fig. 3.1): *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). The sampling sites are predominantly characterised by a Mediterranean climate, but show different temperature and precipitation regimes throughout the year, which are determined by their geographic position and different climatic influences (Fig. 3.1). The types of vegetation and land-use patterns found at each location result from the combination of environmental determinants and land management practices carried out by landowners (public or private entities, or both; Table 3.1). Red deer densities vary among study areas (Table 3.1) and reflect the type of management applied. In most of the populations studied, management objectives are oriented to deer hunting. However, in some areas, those objectives are related to damage control and population control. The provision of supplementary food for red deer is a common management practice only in some study sites, especially in private hunting grounds (Table 3.1).



Legend
Latitude/Longitude[a]
Elevation[b]
Average annual temperature[c]
Sum of average monthly precipitation[c]
Köppen-Geiger Climate Classification[d]

LSC
41° 56'N / 6° 33'W
536 - 1235 m.a.s.l.
10.3 °C
657 mm
Csb

LOU
40° 03'N / 8° 15'W
105 - 1205 m.a.s.l.
13.2 °C
1149 mm
Csb

CUB
39° 40'N / 7° 13'W
90 - 364 m.a.s.l.
16.0 °C
710 mm
Csa

NEG
38° 03'N / 7° 11'W
192 - 442 m.a.s.l.
15.9 °C
547 mm
Csa

DN
36° 59'N /6° 24'W
0 - 46 m.a.s.l.
17.1 °C
534 mm
Csa

QM
39° 24'N / 4° 06'W
722 - 1257 m.a.s.l.
12.5 °C
470 mm
Csa

RCMU
40° 26'N / 1° 37'W
1100 - 1935 m.a.s.l.
7.6 °C
615 mm
Cfb

CF
41° 24'N / 0° 02'E
116 - 390 m.a.s.l.
14.0 °C
414 mm
BSk

Fig. 3.1. Location of the study areas in the Iberian Peninsula, within the Mediterranean biogeographic region (from European Environment Agency, 2011a). Specific 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 25x25 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 (°C) 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).

Table 3.1. Main characteristics of study areas regarding management of red deer (*Cervus elaphus*) populations and land cover (% of occupancy of habitat types). Sample size (*n*) and results of descriptive statistics (average ± standard errors, SE) for the variables related to supplementary feeding practices, population densities, dietary components, nutritional condition, and deer parasite load obtained for each study site are also shown. Statistics refer to data collected during a three-year sampling period (game seasons from 2010/2011 to 2012/2013). Study areas correspond with those in Fig. 3.1.

		Study areas							
	Descriptor	**LSC**	**LOU**	**CUB**	**NEG**	**DN**	**QM**	**RCMU**	**CF**
Game management	Area (ha)	48,740	32,517	1,561	1,722	50,720	6,864	49,778	31,327
	Type of management	Public	Public/Private	Private	Private	Public	Public	Public	Private
	Type of estate	Open	Open	Open	Semi-fenced	Semi-fenced	Fenced	Open	Open
	Supplementary feeding	Yes	Yes	Yes	Yes	No	No	No	No
	Feeding (Kg/deer/year ± SE)	12.3 ± 0.93	4.6 ± 0.46	57.3 ± 6.50	63.0 ± 4.59	0.0	0.0	0.0	0.0
	Feeding (Kg/Km^2/year ± SE)	37.6 ± 3.40	28.8 ± 2.03	2071.3 ± 170.83	975.6 ± 0.00	0.0	0.0	0.0	0.0
	Population density (deer/Km^2 ± SE)	3.1 ± 0.08	6.3 ± 0.33	36.5 ± 2.39	15.7 ± 1.20*	10.1 ± 0.85	27.9 ± 1.85	7.3 ± 0.24	6.4 ± 0.89
Land use and habitat types	Coniferous forests (%)	23.0	16.7	0.0	6.3	9.7	35.4	54.1	23.7
	Broadleaved forests (%)	5.6	13.9	71.3	44.5	0.4	26.6	3.1	0.0
	Mixed forests (%)	3.3	19.3	0.0	0.0	1.1	9.3	1.4	0.0
	Scrublands (%)	56.8	39.6	0.0**	0.0**	23.3	13.7	25.3	30.7
	Natural grasslands (%)	1.1	0.0	12.4	0.0	1.5	0.0	4.2	0.0
	Agricultural lands (%)	9.4	9.0	10.4	28.8	4.6	9.2	11.5	45.4
	Food plots for big game (%)	< 1.0	< 1.0	3.5	20.3	0.0	5.8	0.0	0.0
	Wetlands (%)	0.0	0.0	0.0	0.0	39.2	0.0	0.0	0.0
Diet components and deer condition	Sample size (*n*)	17	38	28	45	18	39	42	45
	KFI (% ± SE)	35.6 ± 4.06	26.5 ± 3.28	30.7 ± 3.35	54.6 ± 3.92	17.2 ± 2.21	23.2 ± 2.15	34.7 ± 4.05	20.1 ± 1.51
	FN (% ± SE)	2.0 ± 0.04	2.3 ± 0.07	2.6 ± 0.07	2.7 ± 0.05	2.4 ± 0.08	2.6 ± 0.04	2.1 ± 0.07	2.6 ± 0.04
	Hemicellulose (% ± SE)	14.1 ± 0.67	12.4 ± 0.30	14.8 ± 0.29	14.3 ± 0.26	15.6 ± 0.72	14.1 ± 0.24	15.7 ± 0.27	12.7 ± 0.19
	Cellulose (% ± SE)	21.3 ± 1.33	18.9 ± 0.60	19.1 ± 0.85	21.1 ± 0.64	21.5 ± 1.08	16.8 ± 0.57	20.0 ± 0.43	16.7 ± 0.36
	Lignin (% ± SE)	13.1 ± 1.43	20.9 ± 1.05	10.4 ± 0.42	11.3 ± 0.40	17.0 ± 1.02	15.9 ± 0.48	16.0 ± 0.44	15.5 ± 0.41
	Elaphostrongylus cervi (lpg ± SE)	1079.2 ± 279.77	195.4 ± 49.43	47.6 ± 13.12	73.5 ± 16.07	97.4 ± 41.45	43.4 ± 11.17	108.4 ± 27.03	0.0

* Deer population density for the NEG hunting ground was derived from Lopes (2008).

** Scrublands dominated by *Cistus* spp. occur mainly associated with broadleaved forests of *Quercus* spp.

Sampling and data collection

The fieldwork was conducted over three consecutive hunting seasons (September–February, from 2010/2011 to 2012/2013). During this period, samples were collected from 272 red deer (147 males and 125 females), which were shot by hunters or game rangers in the context of commercial hunting or population control. Fresh faecal pellets were taken from the rectal area of each animal sampled during field necropsies, placed in individual, appropriately identified plastic zip bags, and then transported in a cooler box to the laboratory for further analysis. Once in the laboratory, the faecal samples were divided into portions: one portion was frozen at –20 ºC until the determination of the dietary quality, whereas the other portion was maintained in a fridge at 4–5 ºC for a maximum of 48 h before parasitological analysis. The right kidney (along with its perirenal fat) was also collected from each deer and transported under similar conditions for later processing and evaluation of its nutritional condition (see below for detailed laboratory procedures). An average of 34 deer (Min-Max: 17-45) was sampled per study site (Table 3.1). Whenever the number of animals culled per hunting event was large enough, a subset of individuals of different sex and age was randomly selected for sampling from each population surveyed. Deer age was determined by examining the sequence of eruption and replacement of mandibular teeth in animals up to 2 years old (Sáenz de Buruaga et al., 2001) or by counting annual cementum layers in longitudinal sections of permanent first incisors in individuals older than 2 (Hamlin et al., 2000). Four age classes were subsequently defined according to biological criteria, as in other studies performed previously with red deer (*e.g.*, Ruiz-Fons et al., 2013; Santos et al., 2013): calves (< 1 year old, n = 22); yearlings (1 year old, n = 49); sub-adults (2–3 years old, n = 38); adults (≥ 4 years old, n = 163).

Nutritional indicators

Deer nutritional condition was estimated by calculating the kidney fat index (KFI), which consists of expressing the weight of perirenal fat in relation to the fat-free kidney weight (Riney, 1955). In deer species, the amount of fat surrounding the kidneys is considered a good predictor of the total body fat, *i.e.*, the amount of energy stores in an animal's body (Finger et al., 1981), especially for capital breeders such as the red deer (Mysterud et al., 2008; see also Serrano et al., 2008). Moreover, fluctuations in body weight are closely associated with those in kidney fat deposits (Mitchell et al., 1976). The KFI has proven to be a sensitive indicator of changes in food availability (Santos et al., 2013) and therefore can also provide clues concerning the quality of habitats for large herbivores. Since KFI values derived from one kidney are similar to those obtained by pooling the data from both kidneys (Santos et al., 2013), the KFI was calculated based on the data collected from the right kidney only, as follows: KFI = (FW / KW) x 100, where FW is the weight of the perirenal fat and KW is the weight of the kidney free of fat (Riney, 1955). Both FW and KW were weighed on an electronic precision balance to the nearest 0.1 g.

The nitrogen content in the deer faeces (FN) was used as an index of dietary quality among the populations studied (Leslie et al., 2008); some authors use faecal crude protein (FCP = FN x 6.25) as an equivalent measure of forage quality in studies with wild herbivores (*e.g.*, Côté and Festa-Bianchet, 2001; Hamel et al., 2009). In ruminants, the FN depends on the nutritional value in terms of the nitrogen contained in the plants consumed, but can also be greatly affected by other dietary components (*e.g.*, fibres and tannins), which alter the animals' digestive efficiency, thus influencing the amount of FN excreted (Barboza et al., 2009). To take this into account, the insoluble dietary fibre fractions (*i.e.*, structural components of plant cell walls) – neutral detergent fibre (NDF), acid detergent fibre (ADF) and acid detergent lignin (ADL) – were also determined in the faecal samples and used as predictors of the nutritive value of the diets and digestibility of the organic matter (Barboza et al., 2009; Gálvez-Céron et al., 2015). In the laboratory, the faecal pellets (approximately 15–20 g per individual sample) were oven-dried at 60 °C for 48 h and then ground in a cyclone-type mill to pass through a 1 mm screen before chemical analysis. The FN, NDF, ADF, and ADL contents in the faecal samples were estimated by using near infrared reflectance spectroscopy (NIRS). This technique has been widely used to predict a number of dietary attributes of domestic and wild ungulate species from faecal material, including nitrogen and fibre contents (reviewed by Dixon and Coates, 2009). For quantitative analysis, NIRS instruments first have to be calibrated using reference data obtained from the application of standard laboratory techniques and the derived equations validated (Siesler et al., 2002). In this study, a subset of $n = 96$ faecal samples (nearly 35% of the total sample size), representative of the same deer populations studied and sampling years (2010–2013), was used for calibration purposes (see Appendix 1 for detailed procedures). By using samples with the same origin to perform NIRS calibrations, more robust equations are obtained and, consequently, a better precision and accuracy of dietary component estimates is ensured (Siesler et al., 2002). Samples from males and females, as well as young and adult animals, were also included in the calibration set in order to try to cover the widest range of variation in the components analysed, due to any potential differences related to sex and age factors. The determination of the FN content in the samples used for NIRS calibration was made in 0.2 ± 0.05 g of dry faeces by the Dumas combustion method in a LECO analyser (LECO Corporation, St. Joseph, MI, USA). The NDF, ADF, and ADL contents were determined sequentially in 0.5 ± 0.05 g of dry faeces following the detergent method (Van Soest et al., 1991), using an ANKOM 220 Fibre Analyser (Ankom Technology Corporation, Fairport, NY, USA) with the application of the filter bag technique. All analyses were carried out in duplicate and the diet components were expressed as a percentage (%) of dry matter (DM). The three main plant cell wall components (*i.e.*, hemicellulose, cellulose, and lignin) were further calculated for all samples from NDF, ADF, and ADL fractions as follows: Hemicellulose (%) = NDF – ADF; Cellulose (%) = ADF – ADL; Lignin (%) = ADL.

Parasite counts

Because infections by parasitic helminths can have a considerable impact both on an animal's condition (Vicente et al., 2007a; 2007b), as well as in the amount of FN excreted (Gálvez-Céron et al., 2015), the abundance of first-stage larvae (L1) of *Elaphostrongylus cervi* Cameron 1931 (Nematoda: Protrostrongylidae) was quantified in the deer faeces. This nematode is a highly specific parasite of red deer, but infected animals rarely show clinical signs of disease. Adult worms are found in the central nervous system and skeletal musculature of their hosts. The parasite's eggs are released into the blood vessels and transported to the lungs, where the L1 hatch. Then, the larvae migrate into the alveolar spaces, ascend the bronchial tree, are swallowed and excreted later in the faeces. Deer become re-infected by the accidental ingestion of terrestrial gastropods (intermediate hosts) that are infected with the third-stage larvae (L3) (Handeland, 2000; Lankester, 2001). *Elaphostrongylus cervi* is widely distributed in the Iberian populations of red deer (Vicente and Gortázar, 2001; Vicente et al., 2006) and the seasonal patterns of L1 excretion have already been described in the Mediterranean habitats of south-central Spain (Vicente et al., 2005). *E. cervi* L1 were extracted from 8–10 g of faeces using a beaker-modified Baermann technique (Forrester and Lankester, 1997). The larvae were then quantified at the microscope in 1 mL of faecal solution in a Favatti counting chamber and expressed as the number of L1 per gram (lpg) of faeces, by multiplying the total number of larvae by the dilution factor of the faecal solution and then dividing by the initial weight of the sample. The identification of *E. cervi* L1 was based on their morphological characteristics and linear dimensions (Vicente and Gortázar, 2001).

Seasonality, weather, climate and vegetation productivity

Weather conditions are important in determining the cycles of vegetation growth and senescence, and therefore influence the availability and quality of food resources for wild herbivores (Moen et al., 2006). In environments with a marked seasonality, as in the case of Mediterranean ecosystems, accumulated rainfall during the autumn and winter plays a key role in plant regeneration following the summer drought (*e.g.*, Bugalho and Milne, 2003; Martínez-Jauregui et al., 2009; Rodríguez-Hidalgo et al., 2010). Hence, variables of rainfall occurrence are expected to have positive effects on deer nutritional indices. On the other hand, low ambient temperatures usually increase basal metabolic rate to maintain body thermoregulation (Randall et al., 2002), making it difficult for animals to accumulate large amounts of energy reserves, especially fat. Records of total daily precipitation (mm) and daily ambient temperatures (ºC) were obtained from the weather stations closest to each study area for the entire sampling period (years 2010–2013). The Spanish and Portuguese National Meteorology Institutes provided the data. Since the proximate factors that may influence the nutritional condition of red deer were of main interest, variables on a short-time scale were derived from the raw weather databases. Average monthly temperatures and the accumulated rainfall during the last t – 30, t – 60, and t – 90 days in relation to the day (t) on which animals were culled were calculated. The normalised difference vegetation index (NDVI), which has been found to reflect variation in rainfall occurrence (Anyamba and Tucker, 2005; Immerzeel et al., 2009; Wang et al., 2003), was also used in our analyses as an indicator of vegetation productivity (Pettorelli et al., 2005b). The NDVI values for each study area were obtained from satellite images with a spatial resolution of 250 m, provided by MODIS (Moderate Resolution Imaging Spectroradiometer; http://modis.gsfc.nasa.gov) for the nearest periods (approximately 2 weeks) preceding the date on which the deer were shot. The images consisted of composites of satellite measurements collected over a 16-day cycle, after the application of the maximum value composite (MVC) algorithm; that is, the pixel with the highest NDVI value was selected to represent the composite for that period. In the Iberian Peninsula, there are important regional differences in both plant phenology and productivity, which are associated with geographical and altitudinal gradients (Alcaraz et al., 2006). Hence, the latitude and longitude coordinates (in decimal degrees) of geometric centroids were obtained for each study area and used as a measure of the geographic position of the populations studied, as well as a proxy for climatic conditions on a larger spatial scale. Moreover, because interactions between climate and topography may determine asynchronies in plant phenology (Pettorelli et al., 2005a), the average altitude (measured as meters above sea level, m.a.s.l.) of each study site was also determined. Food intake and diet selection may also vary seasonally among sexes in red deer, due to environmental factors and reproductive events (Clutton-Brock et al., 1989; Miranda et al., 2012), and are therefore likely to influence their nutritional condition. For analytical purposes, the sampling months were grouped into two categories according to natural seasonal cycles and the reproductive biology of red deer in Iberia: autumn (September, October, November) and winter (December, January, February).

Game management practices and habitat composition

Information on the management of deer populations at the level of the study site was obtained using standardised questionnaires and/or personal interviews with game managers. Supplementary feeding of red deer is frequently used as a management practice in many hunting grounds, particularly in those of south-central Iberia, where deer populations are more intensively managed for hunting. Moreover, in some of those areas, such practices are often associated with the maintenance of high population densities, many of them above the ecological carrying capacity (Gortázar et al., 2006; Vicente et al., 2007a). Contrary to what happens in northern European countries and in North America, where deer are fed mainly during the winter (Putman and Staines, 2004), in the Mediterranean environments of the Iberian Peninsula supplementary food is usually provided during the summer months and early autumn, which correspond to the period of natural food shortage for wild herbivores (Bugalho and Milne, 2003). However, depending on the management system, food supplementation may also be maintained throughout the hunting seasons (from September to February) or all year round (*e.g.*, Rodriguez-Hidalgo et al., 2010). Since food supply may affect the nutritional condition of red deer (Osborn and Jenks, 1998; Santos et al., 2013), our study areas were categorised as having, or not, a supplementary feeding regime (Table 3.1). In those areas where deer were artificially fed, food was distributed directly on the ground, usually at permanent feeding spots, and the food type consisted mainly of grain (especially maize *Zea mays*) and alfalfa *Medicago sativa*; in some areas, fruit was also provided periodically. The reasons for game managers feeding deer were related to the compensation of seasonal constraints in food availability, the enhancement of individual traits (*e.g.*, high-quality trophies in males) and overall body condition, and to attract animals to specific areas in order to be hunted. To evaluate the effects of the amount of food supplied on the nutritional status of the deer, two measures were derived from the data provided by managers and from estimates of population size (see below): i) the amount of food supplied per deer per year (feeding/deer/year), and ii) the amount of supplementary food per surface area per year (feeding/Km^2/year) (Table 3.1).

Land use and habitat composition may also influence the nutritional condition of deer by offering animals different feeding opportunities and types of diet with different nutritive value (*e.g.*, Osborn and Jenks, 1998; Simard et al., 2014). For each study area, the percentage of the main vegetation communities was calculated using a Corine Land Cover map for 2006 (100 m spatial resolution; European Environment Agency, 2011b). Seven broad categories of vegetation that seemed likely to affect nutritional indicators were defined: coniferous forests, broadleaved forests, mixed forests, scrublands, natural grasslands, agricultural lands, and wetlands (Table 3.1). Since the creation of food plots for deer was a management option in some areas, an eighth category (defined as ‘food plots for big game’; Table 3.1) was subsequently derived from the surface area occupied by agricultural land using data provided by the game managers. Food plots were cultivated mainly with leguminous plants and cereals.

Estimation of red deer population densities

Vehicle-based spotlight counts combined with distance sampling techniques were conducted annually in each study area in order to estimate red deer densities (Acevedo et al., 2008). Surveys were carried out following a line transect sampling design in all study locations (Buckland et al., 2001). Transects were evenly distributed within each survey area, and covered different types of habitat and topography. The same transect routes were used throughout the whole study. Red deer counts were done during the species' breeding season, which in Iberia occurs mainly in September and October, although it may extend to November (*e.g.*, Sanz and Rodríguez, 1993); this period also coincides with the time of the year at which individuals of both sexes are most easily observed (Marco and Gortázar, 2002). While conducting field surveys, the area on both sides of the transect routes was scanned using a 100-W halogen spotlight in search of deer. Once deer were detected, the perpendicular distances of deer 'clusters' (defined as single animals or groups of animals) in relation to the transect lines were measured, and the 'cluster' sizes recorded as well (Acevedo et al., 2008; Buckland et al., 2001). Red deer density estimates at study site level were obtained by analysing field records in the software *Distance 6.0 release 2* (Thomas et al., 2010). Data from different populations were analysed separately. Global detection functions were generated for each study area by pooling the data from annual surveys and the analyses were post-stratified by year, in order to derive annual estimates of density (expressed as deer/Km^2; Table 3.1). To determine the most parsimonious models for the detection functions of each area, different models were tested and compared serially. Once this was completed, the models with the lowest Akaike's Information Criterion (AIC) value were selected (Akaike, 1974). Detection function model fitting was also evaluated by using chi-squared (χ^2) goodness-of-fit tests and by visually checking the histograms during the modelling process (Buckland et al., 2001). Deer count surveys were not possible to conduct in the NEG hunting ground because of the terrain conditions and the small number of passable paths. As a result, density estimates obtained for a neighbouring area with similar characteristics, where a line transect sampling scheme was also applied (Lopes, 2008), were used as an approximation of the population densities in the NEG study area.

Statistical analyses

Two nutritional indicators – faecal nitrogen (FN) and kidney fat index (KFI) – were modelled against a set of biological, environmental, management, and demographic determinants (see Appendix 1 for a summary of the explanatory variables used in our analyses; detailed description is given in the previous sections).

Prior to the development of the statistical models, a preliminary exploratory analysis was conducted in order to investigate the characteristics of the data and the relationship between the variables (Zuur et al., 2007; 2010). The data exploration mainly consisted in checking the distribution of variables, detection of outliers, and collinearity diagnostics among the explanatory variables, as well as the identification of potential interactions between them. A combination of descriptive statistics, graphical tools, and correlation analyses was used to examine data patterns. Correlation between predictor variables (*i.e.*, multicollinearity) is an important issue to consider when applying regression-based techniques, as it may result in inflated estimates of the standard error of coefficients, often leading to statistical problems and erroneous conclusions. Multicollinearity between candidate predictors was assessed by means of pairwise correlations and by checking variance inflation factors (VIF). As some of the predictors tested had multiple degrees of freedom (df), adjusted generalised VIF values ($GVIF_{adj} = GVIF^{1/2*df}$, Fox and Weisberg, 2011) were used preferentially to rule out multicollinearity among explanatory variables. A $GVIF_{adj}$ value of 2.5 was used as a cut-off criterion for identifying the presence of highly correlated predictors. Accordingly, the GVIF values were calculated for the set of variables and the predictor with the largest GVIF was excluded. This process was repeated sequentially until all the predictors reached a $GVIF_{adj}$ smaller than 2.5 (Zuur et al., 2009; 2010). Among all the continuous explanatory variables, only parasite abundance data (*E. cervi* L1 counts) showed an extremely skewed distribution (to the right) with a large amount of extreme observations. To reduce the effects of those outliers and better deal with non-linearity problems, the data of *E. cervi* abundance was subjected to a natural logarithmic transformation [ln (x+1)] before the application of the statistical models (Zuur et al., 2007).

Mixed effects models (McCulloch et al., 2008; Zuur et al., 2009) were used to analyse the relationships between the nutritional indicators of red deer and the explanatory variables selected. This analytical approach was chosen on the basis of the nature of the data and type of inferences under consideration. Here, our study sites were assumed to be a random sample of a larger number of wild red deer populations found across the Mediterranean ecosystems of the Iberian Peninsula. Therefore, the 'study area' was included as a random factor in the all models developed. Moreover, as our sampling was conducted over three consecutive years, the 'hunting season' (as a categorical variable) was also incorporated in the models. However, due to the reduced number of levels available (only 3), it was treated as a fixed effect term, instead of a random effect (Zuur et al., 2013).

All analyses were performed using the R statistical package, version 3.1.2 (R Core Team, 2014). The variation in FN content (normally distributed continuous variable; Fig. A.1.2, Appendix 1) was modelled using linear mixed models (LMM). On the other hand, the KFI (continuous response variable containing only positive values and with a distribution moderately skewed to the right; Fig. A.1.2, Appendix 1) was modelled using generalised linear mixed models (GLMM) with a gamma distribution and a log link function (Zuur et al., 2009; 2013). For each modelling approach and in addition to the main effects of predictor variables, a series of two-way interaction terms that could potentially affect the response variables were created and incorporated into the regression models for testing. These included all possible interactions with biological and/or ecological significance based on our predictions. Because of the large number of potential explanatory variables, a stepwise variable selection algorithm based on AIC (Akaike, 1974) was run to help in identifying the most important predictors and find the most parsimonious models (Weisberg, 2005). Those models with the lowest AIC score were considered the most parsimonious, *i.e.*, as having the most favourable trade-off between the number of parameters and model fit. Models separated by less than two AIC units (ΔAIC < 2) were considered as having equivalent support (Burnham and Anderson, 2002). All models (both LMM and GLMM) were run using the 'lme4' package, version 1.1-7 (Bates et al., 2014). The 'lmer' function with restricted maximum likelihood (REML) was used to fit LMM, while the 'glmer' function with the maximum likelihood (ML) was applied in the case of GLMM. For each model fitted, residual plots were checked to ensure that model assumptions were not violated (Zuur et al., 2010). Parameter estimates ± standard errors (SE) and *t*-tests for the significance of each parameter coefficient, when all other predictors are present in the model, are only given for the LMM and GLMM with the lowest AIC (see Results). Significance tests for fixed effects were also performed to evaluate the importance of predictors in the final models. The 'anova' function in the 'lmerTest' package, version 2.0-20 (Kuznetsova et al., 2014), was used to calculate approximate denominator degrees of freedom by Satterthwaite's method, as well as *F*-statistics along with *P*-values for all LMM fitted. In relation to GLMM, likelihood ratio tests (LRT) were used to derive *P*-values and assess the significance of fixed effects; here, the 'mixed' function included in the 'afex' package, version 0.13-145 (Singmann et al., 2015), was used. Statistical significance was set at $P \leq 0.05$ for all tests. Coefficients of determination (R^2) were also calculated according to Nakagawa and Schielzeth (2013), to assess the amount of variation explained by each LMM.

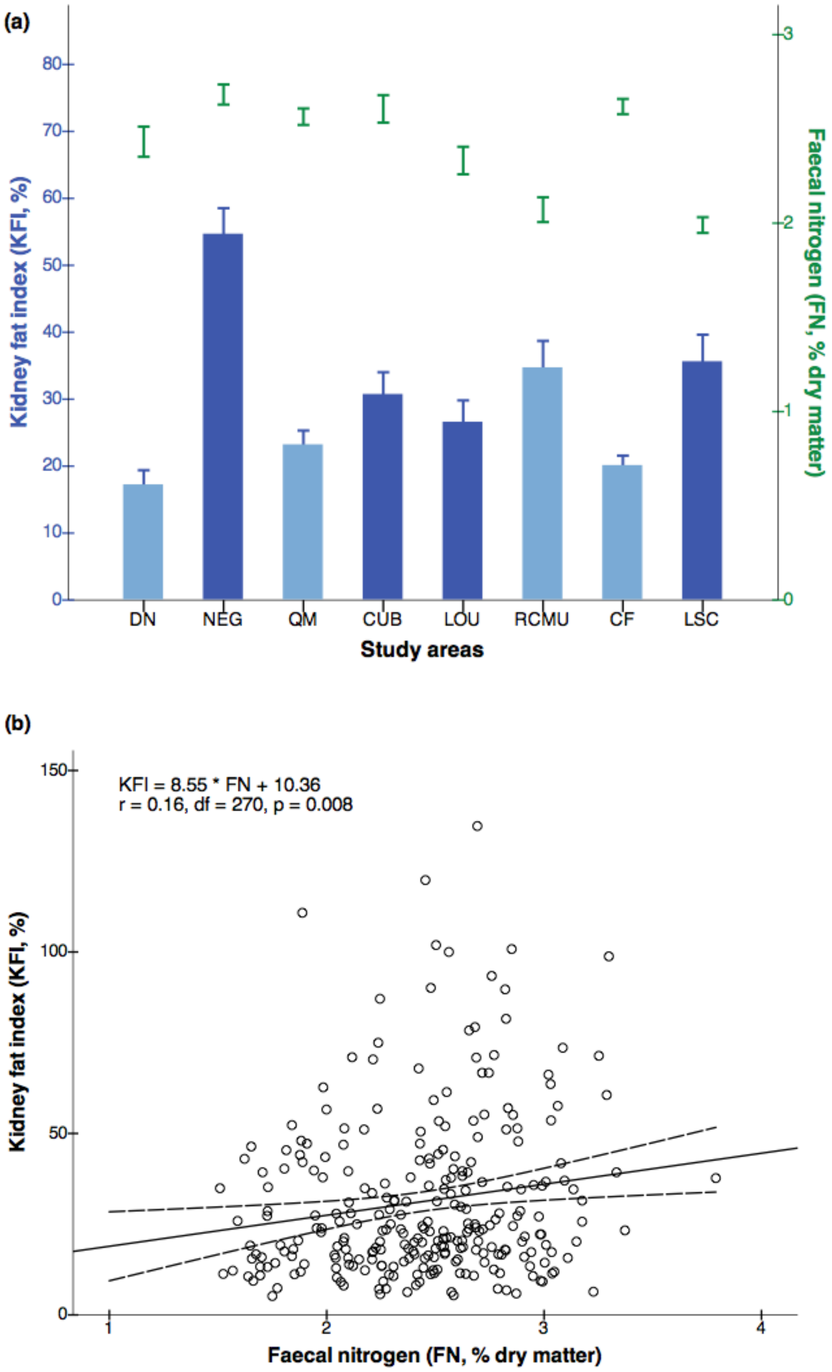


Fig. 3.2. (a) Average values (± SE) of the kidney fat index (KFI, %; in blue) and faecal nitrogen (FN, % of dry matter; in green) of red deer *Cervus elaphus* in eight study areas located in the Mediterranean Iberian Peninsula during three consecutive hunting seasons (from September to February, 2010/2011 – 2012/2013). Deer populations are ordered by latitude, from south to north. Light and dark blue colours in the KFI bars differentiate non-supplemented from food-supplemented populations, respectively. (b) Linear relationship between FN and KFI for the entire set of red deer populations studied. Solid black line: fitted regression line ($y = 8.55\ x + 10.36$). Dashed black line: estimated confidence intervals for the mean.

3.3. Results

Throughout the whole study period, the amount of nitrogen measured in the faeces (FN) of red deer varied between 1.51 and 3.79% DM (average ± SE: 2.44 ± 0.03%), while the kidney fat index (KFI) values ranged from 5.2 to 134.7% (average ± SE: 31.3 ± 1.4%). The two nutritional indices were positively and significantly correlated ($r = 0.16$, df = 270, $p < 0.01$) and their average values varied among the study areas (Fig. 3.2; Table 3.1).

From the multicollinearity diagnostics using Pearson's pairwise correlation tests, important levels of association were identified between the following groups of continuous explanatory variables (see correlation matrices in Appendix 1): residual dietary fibre components (especially lignin) and some habitat types (Fig. A.1.3); index of vegetation productivity and accumulated rainfall (Fig. A.1.4); monthly ambient temperatures, geographical position and average altitude of study areas (Fig. A.1.5); red deer density and amount of food supplied by game managers (Fig. A.1.6). Based on VIF analysis, ten predictors were excluded sequentially before modelling: HConFor; HBlFor; T.mean; Feed.Kg.Km^2; LONG; HScrub; SUPPL.01; T.max; Rain.60; Rain.90 (see Table A.1.2 for variable description). A second inspection of VIF values after model selection did not reveal major collinearity problems among the predictors retained in the final models. As expected, inflated values were only observed when interactions were entered.

Factors affecting dietary quality of red deer

The most parsimonious LMM describing the variation in the FN content in red deer included the effects of residual dietary fibre, seasonality, vegetation productivity and topography (Table 3.2). The amount of variation explained exclusively by the fixed effects in the LMM was 47.9%, while the combined effects of fixed and random factors explained 68.4% of the total variation in the

FN levels. All faecal fibre components were negatively and significantly associated with the amount of FN excreted (Hemicellulose: $F_{1,\ 260.511} = 5.124$, $P = 0.024$; Cellulose: $F_{1,\ 261.993} = 51.307$, $P<0.0001$; Lignin: $F_{1,\ 250.818} = 25.783$, $P<0.0001$; Fig. 3.3, Table 3.2). The FN levels were significantly higher in the winter in relation to the autumn ($F_{1,\ 256.838} = 18.853$, $P<0.0001$), and a more pronounced negative relationship between FN and the lignin fraction was also observed during the winter period, as revealed by the significant interaction between the season and faecal lignin content ($F_{1,\ 260.007} = 22.816$, $P<0.0001$; Fig. 3.3, Table 3.2). Increased primary productivity, as measured by the NDVI, was positively correlated with FN ($F_{1,\ 18.26\ 9} = 11.442$, $P = 0.003$; Table 3.2), and a negative, marginally significant relationship between FN and the average altitude of the study areas was also found ($F_{1,\ 3.676} = 7.700$, $P = 0.055$; Fig. 3.4; Table 3.2).

Table 3.2. Parameter estimates$^{(\Psi)}$ for the best linear mixed-effect model (LMM) explaining the variation in the faecal nitrogen content (FN, expressed as a % of dry matter and used as an indicator of protein quality of the diet) in red deer *Cervus elaphus*, during three consecutive hunting seasons, in the Mediterranean environments of the Iberian Peninsula. 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. Marginal significance is indicated by a superscript '#'.

Dependent variable: Faecal nitrogen (FN, % DM)

Model terms	**Estimate**	**Std. Error**	***t* value**	***p* value**
Intercept	3.004e+00	3.490e-01	8.609	**<0.0001**
Hunting season (2011-2012)	– 7.171e-02	4.140e-02	– 1.732	0.084$^{\#}$
Hunting season (2012-2013)	– 3.901e-02	9.409e-02	– 0.415	0.679
	Positive coefficients			
Season (Winter)	5.580e-01	1.285e-01	4.342	**<0.0001**
NDVI	1.631e+00	4.822e-01	3.383	**0.003**
	Negative coefficients			
Average altitude	– 5.161e-04	1.860e-04	– 2.775	0.055$^{\#}$
Hemicellulose	– 2.265e-02	1.001e-02	– 2.264	**0.024**
Cellulose	– 3.588e-02	5.009e-03	– 7.163	**<0.0001**
Lignin	– 6.391e-03	6.470e-03	– 0.988	0.324
Season (Winter) * Lignin	– 3.855e-02	8.071e-03	– 4.777	**<0.0001**

$^{(\Psi)}$ Parameter estimates for the levels of fixed factors were computed by considering a reference value of 0 for: level '2010-2011' for hunting season; and level 'Autumn' for season.

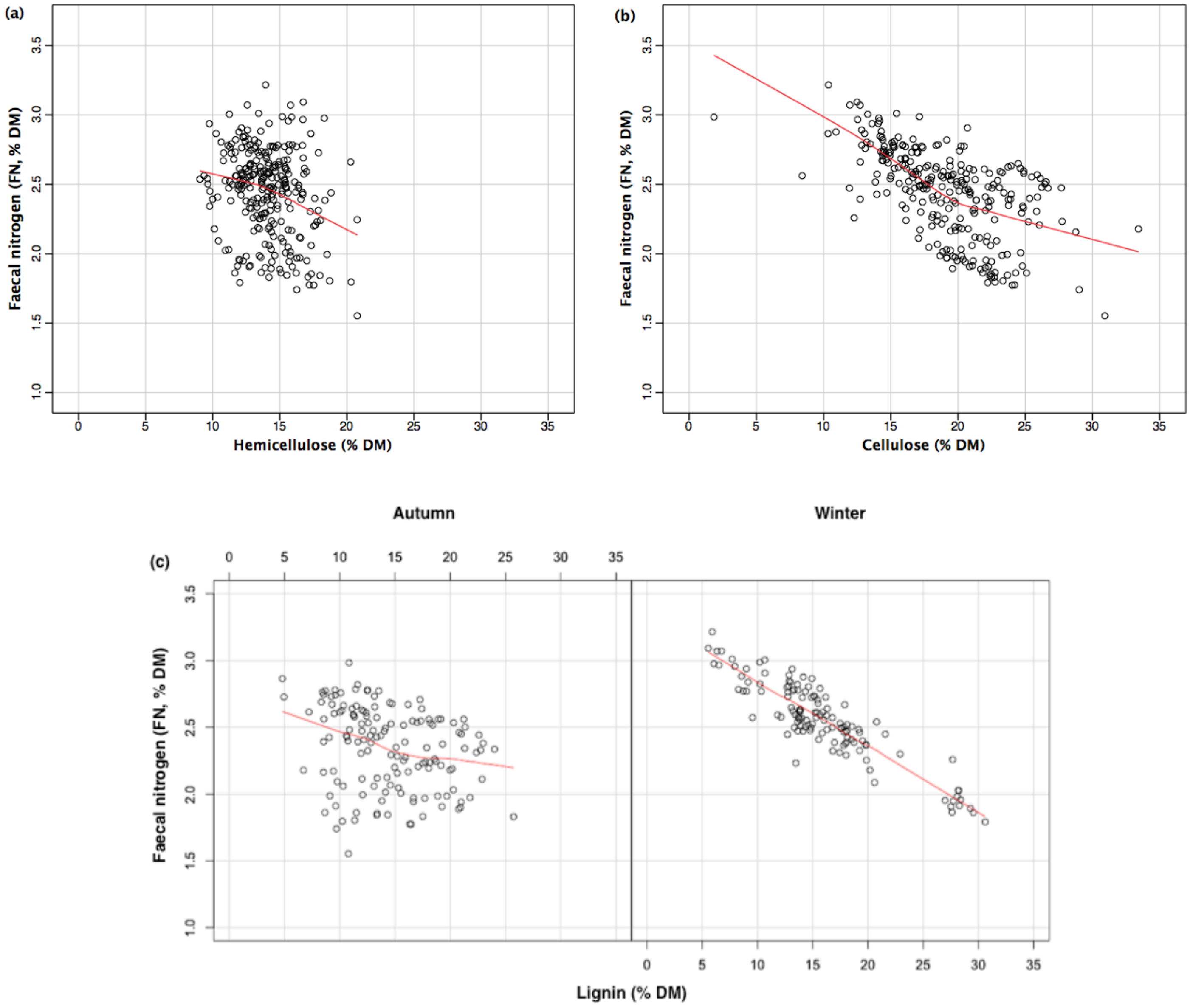


Fig. 3.3. Scatterplots showing the relationship between fibre components (a. hemicellulose; b. cellulose; c. lignin) and faecal nitrogen (all expressed as a % of dry matter, DM) in faeces of red deer *Cervus elaphus*. A conditional plot (panel c, below) is used to show the relation between the lignin fraction and faecal nitrogen in the autumn and winter (left and right panels, respectively). Loess smoothing curves (red lines) were fit to data points in all plots.

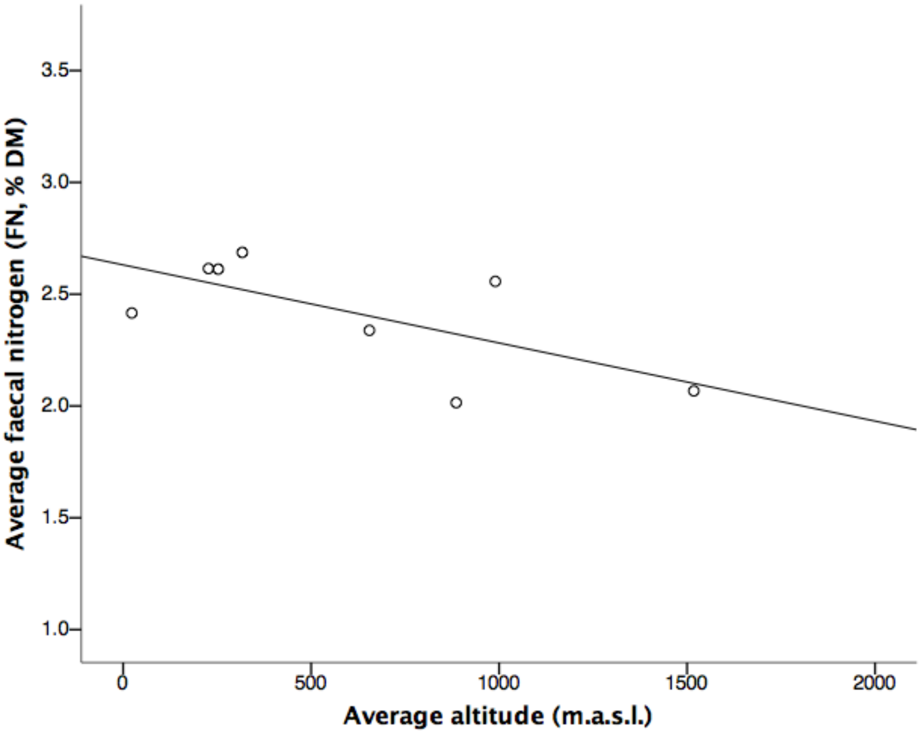


Fig. 3.4. Linear relationship between the average altitude of study areas (in meters above sea level, m.a.s.l.) and average faecal nitrogen levels (expressed as a % of dry matter, DM) in red deer.

A similar model, in terms of AIC support (*i.e.*, ΔAIC < 2; Appendix 1), included the interaction between NDVI and season. However, its effect on FN was not statistically significant ($F_{1,\ 29.591}$ = 0.034, P = 0.855). The variables related to biological traits, parasite counts, population density and management practices, as well as their interactions, were not important predictors of FN levels in red deer. None of these variables had a significant influence on FN levels, nor contributed to improving the fit of the models (see Table A.1.4 in the Appendix 1 for the results of stepwise model selection, and measures of fit and parsimony for each model).

Factors affecting fat reserves in red deer

Four competitive (equivalent) GLMM, in terms of AIC, were obtained to explain the variation in the KFI in red deer (Appendix 1). All models included the effects of individual traits, such as sex, dietary attributes, and parasite counts, as well as the effects of seasonality, vegetation productivity, latitude, population density, and supplementary feeding. Small differences between those models were mainly due to the number and type of interactions present.

Considering the GLMM with the lowest AIC score (model 1; Table A.1.5), it was found that females had significantly higher average KFI values than males (χ^2 (1) = 40.36, $P<0.0001$; Table 3.3). Parasite abundance was negatively associated with the KFI, despite the absence of significant effects (χ^2 (1) = 1.11, $P = 0.29$; Table 3.3). Also at the individual level, the lignin content of the diet had a significant negative relationship with the KFI (χ^2 (1) = 31.76, $P<0.0001$; Table 3.3), while faecal nitrogen associated positively with the amount of fat reserves in red deer (χ^2 (1) = 6.53, $P = 0.01$; Table 3.3). Furthermore, the additive effects of FN and deer population density interacted significantly to explain KFI (χ^2 (1) = 5.61, $P =$ 0.02), *i.e.*, it was observed that the effect of FN on the KFI was progressively lower with increased deer density (Fig. 3.5; Table 3.3). Fat stores were also positively associated with the amount of supplementary food provided (χ^2 (1) = 4.15, $P = 0.04$), as well as with the NDVI (χ^2 (1) = 10.45, $P = 0.001$; Fig. 3.6; Table 3.3). In addition, the KFI was significantly affected by the season (χ^2 (1) = 7.89, $P =$ 0.005), with higher average KFI values in the winter, relative to the autumn (Table 3.3), and its effect interacted significantly with latitude (χ^2 (1) = 10.24, $P = 0.001$). In this case, the KFI increased slightly with increasing latitude in the autumn, while a stronger inverse relationship was observed in the winter (Fig. 3.7). On the other hand, the interactive effect between the season and the NDVI had no significant influence on KFI (χ^2 (1) = 2.47, $P = 0.12$; Table 3.3).

Table 3.3. Parameter estimates(Ψ) for the best generalised linear mixed model (GLMM) explaining the variation in the kidney fat index (KFI, expressed as % and used as an indicator of the nutritional condition) in red deer *Cervus elaphus*, during three consecutive hunting seasons, in different Mediterranean environments of the Iberian Peninsula. 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. Marginal significance is indicated by a superscript '#'.

Dependent variable: Kidney fat index (KFI, %)

Model terms	**Estimate**	**Std. Error**	***t* value**	***p* value**
Intercept	– 1.517	1.582	– 0.959	0.338
Hunting season (2011-2012)	– 0.114	0.067	– 1.711	$0.087^{\#}$
Hunting season (2012-2013)	– 0.017	0.113	– 0.153	0.878
	Positive coefficients			
Sex (Females)	0.395	0.060	6.615	**<0.0001**
Season (Winter)	9.185	2.957	3.106	**0.002**
NDVI	0.452	0.456	0.992	0.321
Latitude	0.116	0.037	3.102	**0.002**
Food supply, Kg/deer/year	0.005	0.003	2.164	**0.030**
Deer density	0.029	0.021	1.400	0.161
Faecal nitrogen	0.373	0.140	2.665	**0.008**
Season (Winter) * NDVI	1.372	0.823	1.666	$0.096^{\#}$
	Negative coefficients			
Lignin	– 0.053	0.009	– 5.931	**<0.0001**
Log$_e$ (E.cervi.lpg + 1)	– 0.019	0.018	– 1.059	0.290
Deer density * Faecal nitrogen	– 0.020	0.008	– 2.436	**0.015**
Season (Winter) * Latitude	– 0.248	0.068	– 3.661	**0.0003**

(Ψ) 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; and level 'Autumn' for season.

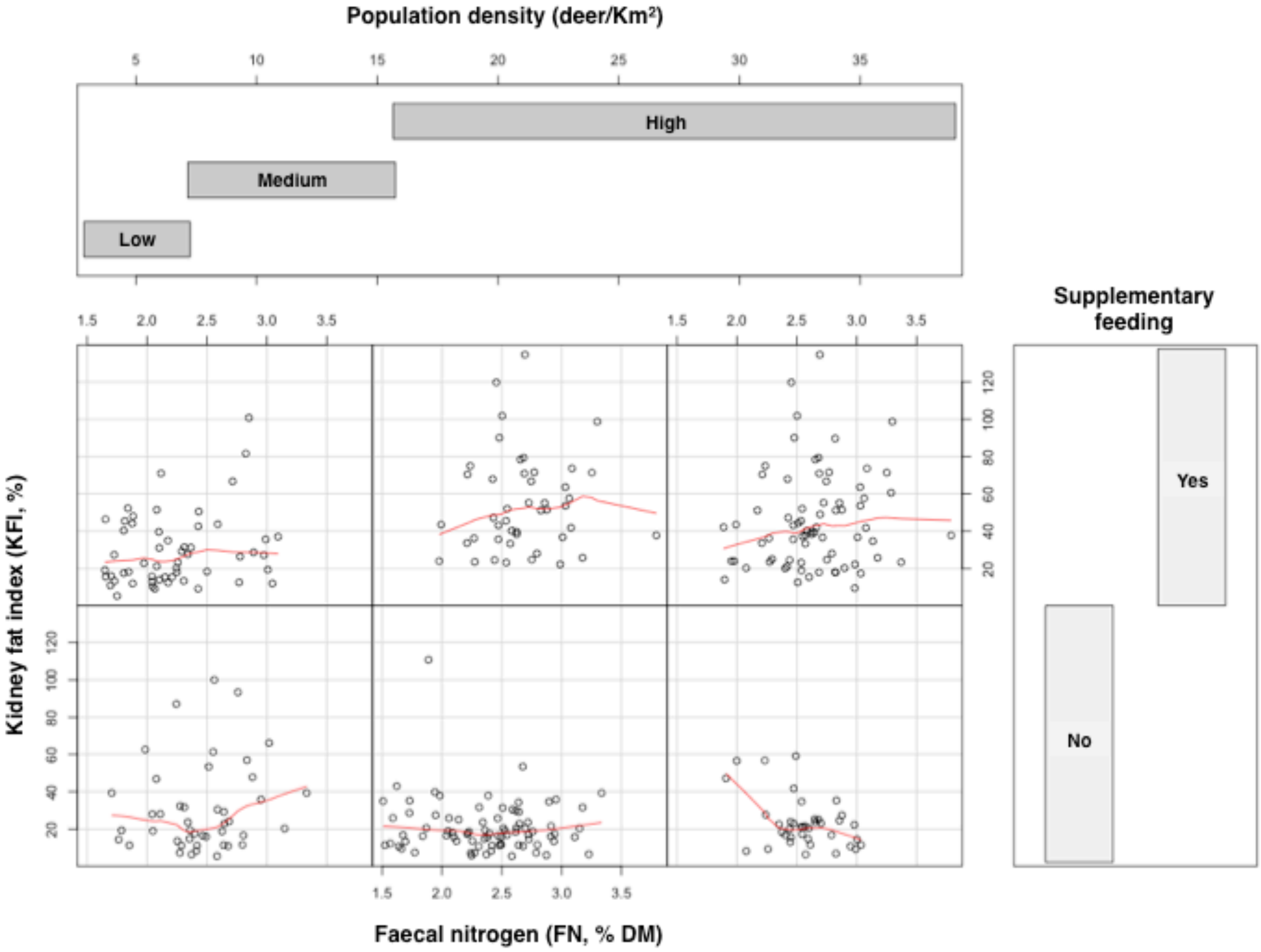


Fig. 3.5. Conditional plot showing the relationship between faecal nitrogen (expressed as % of dry matter, DM) and kidney fat index (KFI, %) for different red deer density ranges (divided into low, medium, and high, and expressed as deer per Km^2), and different supplementary feeding regimes (this is represented in terms of the presence or absence of supplementary feeding practices for clarity). Loess smoothing curves (red lines) were fit to data points in all plots. The lower panels show the relationship between FN and KFI for different deer densities in non-supplemented populations, while the upper panels illustrate the same relationship in food-supplemented populations. Deer population densities increase from left to right (the two leftmost panels show FN versus KFI at low densities, the middle panels for medium densities, and the two rightmost panels for high densities).

The other three equivalent GLMM (models 2, 3 and 4; Table A.1.5) did not include the season*NDVI interaction term. Instead, it was replaced by an interaction between deer density and the amount of food supplied in model 2, and by an interaction between parasite load and supplementary feeding in model 4. However, in both situations, the effects of such interaction terms also had no significant influence on the KFI (model 2: $\chi^2(1) = 2.32$, $P = 0.13$; model 4: $\chi^2(1) = 1.16$, $P = 0.28$). Especially noteworthy, was that the effect of the interaction mentioned in model 2 was close to marginal significance and the regression coefficient was positive and statistically significant ($t = 2.106$; $P = 0.035$). Model 3 just maintained the significant interactions of model 1 (described in detail above).

Finally, no significant changes in the response variable were detected when testing for the main effects of age class, as well as for the interactions between sex and age class, or sex and season. Measures of fit and parsimony of models that resulted from stepwise model selection based on AIC are shown in Table A.1.5 (Appendix 1).

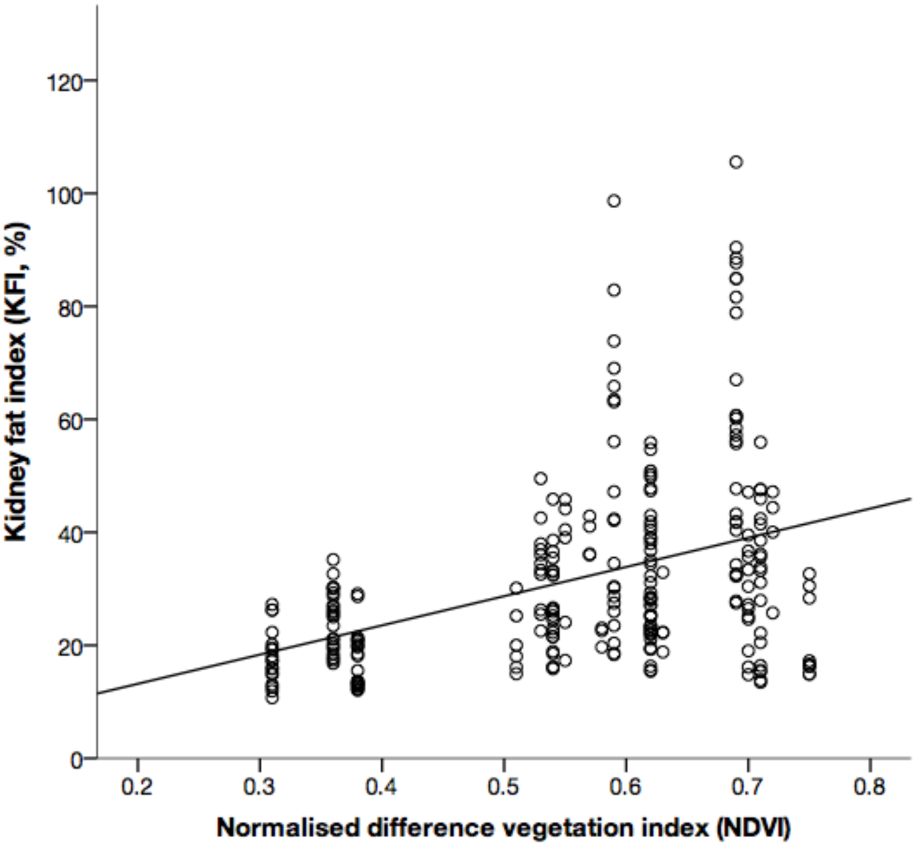


Fig. 3.6. Relationship between the normalised difference vegetation index (NDVI) and kidney fat index (KFI, %) in red deer.

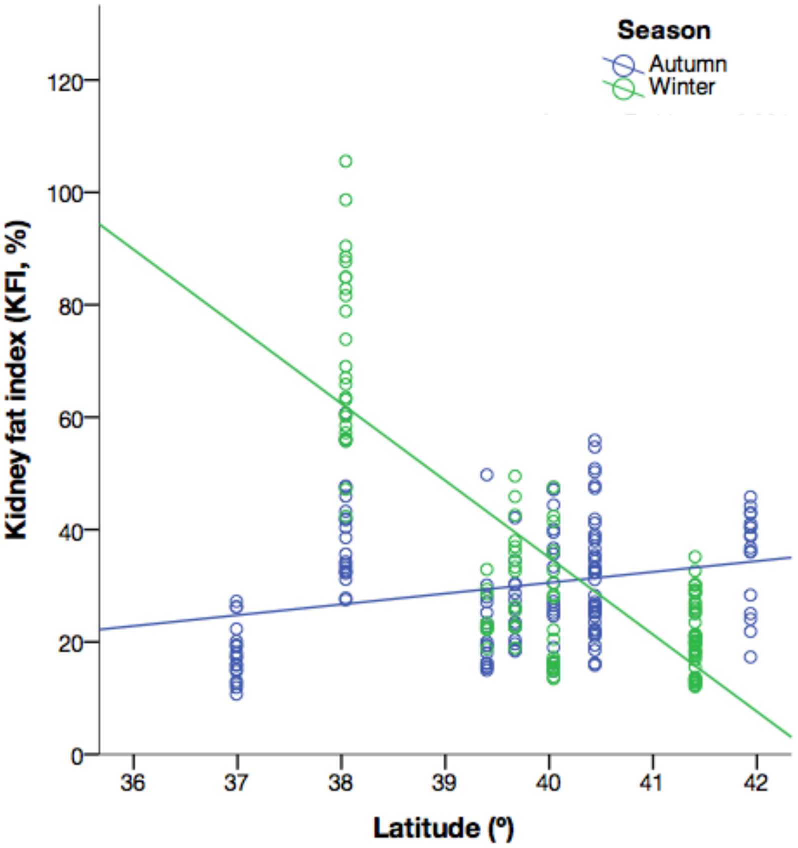


Fig. 3.7. Relationship between latitude (in decimal degrees) and kidney fat index (KFI,%) in red deer in the autumn and winter.

3.4. Discussion

The influence of proximate factors related to seasonal changes in food availability and quality, game management practices and population densities on the dietary quality and nutritional status of red deer in areas characterised by a Mediterranean-type climate was assessed. Moreover, differences in the nutritional condition, according to the deer's biological traits and parasitic infections, were evaluated.

Before going deeply into the interpretation of the results obtained, some general comments regarding the meaning of the nutritional indices used must be made. The concentrations of FN (as well as those of faecal fibre fractions) reflect short-term dietary aspects related to the nutritional value of the plants consumed and the digestibility of materials. The KFI, on the other hand, reflects more long-term dietary factors and cumulative energy balance over time. The two indices showed different relationships with the predictors tested, especially with those associated with individual characteristics, management and demographic factors, which emphasises the value of their complementary use in studies of nutritional ecology. All faecal fibre components analysed (cell wall polysaccharides and lignin) were negatively related to FN levels, and a negative relationship was also found between the lignin fraction and KFI. In ruminants, forage digestibility is inversely related to dietary fibre content (Van Soest, 1994). Diets containing high fibre content are less digestible because increased levels of these components tend to suppress microbial function in the rumen, thus reducing digestive efficiency. In such situations, animals may consume more food in order to compensate for the decreasing digestibility of their diets. This strategy usually results in more faeces produced and in lower FN concentrations ('dilution effect'; Barboza et al., 2009). According to the general principles of forage digestibility and intake, it is also expected that animals relying on diets characterised by high levels of lignin (the most indigestible fraction in plant material), over a relatively large time interval, would have a poorer body condition. Conversely, diets with low fibre content and protein-rich are more digestible, thus allowing animals to extract the maximum nutritional value from the food ingested. High-quality diets are usually associated with increased FN levels and improved body condition. In this study, FN and KFI were associated positively, which was what one would expect considering that the accumulation of fat reserves by animals also depends on the concomitant intake of adequate levels of forage protein (Barboza et al., 2009; Randall et al., 2002). A similar relationship between FN and KFI has already been described in a population of mule deer (*Odocoileus hemionus*). However, in this particular case, the KFI of individuals that were shot in March was related to the FN levels measured in faeces collected in the preceding month of December (Kucera, 1997). Furthermore, the concentrations of FN (or faecal crude protein) have been reported to correlate positively with several other measures of condition and performance in populations of large wild herbivores (Blanchard et al., 2003; Côté and Festa-Bianchet, 2001; Ryan et al., 2012; van der Waal et al., 2003).

Periods of high-quality forage (protein and energy-rich) coincide with a build up of structural protein and restoration of fat reserves in wild ungulates, thus allowing them to gain body mass (Parker et al., 2009; WallisDeVries, 1998). Our results indicated that both FN and KFI varied seasonally, showing higher values in the winter in relation to autumn, and were positively correlated with the NDVI (an index of the greenness of vegetation which is directly associated with photosynthetic activity) under Mediterranean conditions. These findings support our first hypothesis, which stated that periods of increased primary productivity would influence positively both nutritional indices in red deer. In addition, it was predicted that vegetation productivity would be related to accumulated rainfall. The relationship between the NDVI and precipitation occurrence is well documented in temperate and subtropical areas (*e.g.*, Anyamba and Tucker, 2005; Immerzeel et al., 2009; Wang et al., 2003); for our data, the two variables showed a positive curvilinear relationship. Moreover, an increasing number of studies have demonstrated the positive effects of time-lagged NDVI or rainfall on the nutritional indicators of wild herbivores inhabiting different seasonal environments (Blanchard et al., 2003; Couturier et al., 2009; Hamel et al., 2009; Marshal et al., 2005, 2008; Pettorelli et al., 2005a; Ryan et al., 2012). For most of our study areas, maximum NDVI values are usually observed between mid-autumn and late winter, and are strongly dependent on the rate of rainfall during that period (Alcaraz et al., 2006). In the Mediterranean ecosystems of the Iberian Peninsula, the autumn and winter rainfall is key for triggering vegetation regeneration after the summer drought, particularly in promoting the development of the herbaceous layer (mainly dominated by forbs and grasses, with a high protein content and a relatively low degree of lignification), and activating the vegetative growth of shrubs (with a relatively high lignin content and intermediate levels of protein) (Figueroa and Davy, 1991; Peñuelas et al., 2004; WallisDeVries, 1998). Also in Mediterranean areas dominated by *Quercus* woodlands, wild ungulates usually have access to large amounts of acorns (with a fat-rich content), which may represent a significant proportion of their diets between mid-autumn and early winter (San Miguel et al., 2000). Therefore, after the nutritional constraints of the summer, in which the vegetation available is of low nutritive quality (Bugalho and Milne, 2003), red deer can benefit from the improved feeding conditions along the autumn-winter continuum in order to enhance their body condition. Previous studies conducted during the same seasonal period in south-central Iberia have already demonstrated a progressive increase in the protein content of the diet in red deer (Miranda et al., 2012) and an overall improvement of their nutritional condition (Rodriguez-Hidalgo et al., 2010; Santos et al., 2013). This pattern contrasts with condition cycles documented in ungulate populations living in northern regions of the Holarctic (*e.g.*, Clutton-Brock et al., 1989; Garroway and Broders, 2005), but also with those reported for populations inhabiting high mountain ranges of the Mediterranean Iberian Peninsula (Serrano et al., 2011) or other Iberian regions characterised by an Atlantic- or Alpine-type climate regime (Galvez-Cerón et al., 2013; Garín et al., 2001). In these environments, dietary quality and body condition are usually lower during late autumn and winter because of reduced access to high-quality forage and adverse weather conditions.

The average altitude of our study areas correlated negatively with FN levels, probably due to asynchronies in vegetation productivity mediated by differences in temperature regimes. Plant phenology patterns can vary substantially with altitude, and even within short spatial distances (Alcaraz et al., 2006; Pettorelli et al., 2005a). At higher altitudes, low temperatures are a limiting factor for vegetation growth during the coldest months of the year. As a consequence, the timing of the peak of vegetation productivity (*i.e.*, when the protein content of plants is higher) occurs later in those areas, typically in the spring months, when temperatures are more favourable (Alcaraz et al., 2006). Likewise, the lower temperatures and reduced photoperiod verified at northern latitudes during the winter, apart from affecting plant growth and availability of food resources for herbivores (WallisDeVries, 1998), also increase the deer's energy demands for body thermoregulation (Randall et al., 2002). Therefore, animals confronted with such conditions are expected to activate catabolic pathways more frequently, in order to maintain their normal metabolic functions, which prevents the accumulation of high amounts of energy reserves. These observations support the pronounced decrease in the reserves of fat in red deer verified during the winter with the increasing latitude of our study region.

Females exhibited better nutritional condition than males, and this difference tended to be more marked at older ages. However, no differences were found among sexes and age groups with respect to the dietary fractions detected in faecal samples, which allows us to assume that young and adult male and female deer fed basically on the same type of food resources during our sampling period. The fact that females had a higher body condition in relation to the males has been described consistently in Iberian populations of red deer, in particular during the autumn-winter period (Santos et al., 2013; Vicente et al., 2007a, 2007b). Two main factors may explain this result. First, males have larger body sizes and therefore greater energy demands in absolute terms (Clutton-Brock et al., 1989; Parker et al., 2009; Randall et al. 2002). Second, hinds begin to recover their condition earlier in the autumn, during late lactation/early gestation, and when forage conditions become more favourable (Rodriguez-Hidalgo et al., 2010), while stags are investing much of their energy on reproductive activities during the rut. Only after this period (normally in late autumn), do males start to regain condition. Concerning dietary aspects, our results agree, in general, with those of Miranda et al. (2012), who found no differences in dietary components between sexes and age classes from October to March; the only exception was in September, when adult males relied essentially on a poor-quality diet based on senescent grasses and forbs, while hinds and juveniles of both sexes fed preferentially on shrubs, which are more nutritive than the herbaceous layer in late summer and early autumn (Bugalho and Milne, 2003). In our study, these differences may have been masked by the different grouping of sampling months. The results obtained therefore suggest an overall dietary overlap between sexes and age classes in the autumn and winter. The apparent absence of sexual trophic segregation could probably be due to the improved feeding conditions during that period in Mediterranean environments and the concomitant need of both sexes to regain condition (Miranda et al., 2012). According to some hypotheses, dietary sexual segregation in dimorphic cervids is more likely to occur when high-quality food resources are scarce (Barboza and Bowyer, 2000) and, as already mentioned, this period coincides with the summer in our study region (Bugalho et al., 2001; Bugalho and Milne, 2003). Our findings also indicate a change in the diet of red deer between the two seasons analysed. In the winter, faecal fractions revealed higher concentrations of lignin and nitrogen, possibly related to an increased consumption of shrubs (Miranda et al., 2012; San Miguel et al., 2000), which usually become more nutritive with the approach of springtime.

Deer in food-supplemented populations had higher levels of energy reserves than those in non-supplemented populations, and their nutritional status increased as a function of the amount of food provided by game managers. These results are consistent with our second hypothesis and are in agreement with previous research conducted under experimental and natural field conditions (*e.g.*, Santos et al., 2013; Vicente et al., 2007a). Intuitively, such situation would be expected, since animals that are less subjected to food restrictions are more likely to maintain a better nutritional condition over time. Similar conclusions were derived from studies in which body mass was used as a measure of condition. In Great Britain, Putman and Langbein (1992) demonstrated that the provision of supplementary food was positively correlated with the body mass of captive deer. Similarly, in a 12-year comparative study performed under natural conditions in south-central Iberia, it was found that the body mass of female red deer was consistently greater in a food-supplemented population than in a non-supplemented one (Rodríguez-Hidalgo et al., 2010). On the other hand, no significant effect of supplementary feeding practices on dietary quality was observed in our study. It is suspected that this result arises from the fact that the high-quality forage naturally available in the Mediterranean habitats during our sampling period (autumn-winter) tended to attenuate the differences between food-supplemented and non-supplemented populations. Different results were reported in two other studies with wild herbivores that were conducted in different environments (Osborn and Jenks, 1998; van der Waal et al., 2003). In both cases, the authors reported significant increases in FN concentrations in populations where animals had access to supplementary forage. However, such effects were detected during periods of natural food shortage, which contrasts with our observations.

Parasite load was negatively associated (although not significantly) with the nutritional status of red deer, which is in line with previous works performed with this species (Irvine et al., 2006; Vicente et al., 2007a). The relationship between these variables can be bidirectional. Not only may parasites cause loss of condition (*e.g.*, Gunn and Irvine, 2003; Irvine et al., 2006), but also poor nutritional condition may favour increased parasitism, due to the host's increased susceptibility to infections (*e.g.*, Irvine et al., 2006). In general, animals exhibiting superior body condition are able to deal better with parasite infections because they tend to invest more in immune defence (Møller et al., 1998; Vicente et al., 2007b).

With respect to our last hypothesis, high population densities alone were not statistically associated with decreases in the nutritional status in red deer. However, there was an evident negative density-dependent response of deer body reserves when considering the combined effects of population density and dietary quality, especially in populations without a supplementary feeding regime. These findings are consistent with the ecological principles on the relationship between herbivore density and availability of high-quality food items (Crawley, 1983; Danell et al., 2006). The adverse effects of density dependence on an animal's nutritional status are usually exacerbated in natural populations because high-quality forage tends to become scarce faster due to increased intraspecific competition (Blanchard et al., 2003; Stewart et al., 2005; van der Waal et al., 2003), consequently leading to increased nutritional stress (Santos et al., submitted). In contrast, these negative effects are less likely to be observed in populations where supplementary food is provided artificially (Rodriguez-Hidalgo et al., 2010; Vicente et al., 2007a). Nevertheless, despite the apparent effectiveness of such practices in the maintenance or improvement of individuals' condition and overall performance, they are frequently linked to the existence of overabundant populations, which, in turn, can have severe undesirable effects on the ecosystems, apart from the increased risks of disease transmission and several other demographic and physiological effects (Gortázar et al., 2006; Milner et al., 2014; Perea et al., 2014; Putman and Staines, 2004).

3.5. Conclusions and implications for population management

This research represents an important contribution for understanding the complex links between nutrition and condition in wild herbivores. The nutritional indicators used in the present study proved to be sensitive to seasonal changes in vegetation productivity, thus demonstrating its potential for evaluating the responses of red deer to changes in the quality of habitats in Mediterranean ecosystems, at least during the autumn and winter. Under our study conditions, FN levels correlated positively with periods of high-quality forage and predicted changes in the body condition of red deer. The use of FN as an index of dietary quality in herbivores has generated some controversy, mainly due to the influence of plant secondary metabolites (PSM), especially tannins, in the amount of FN excreted. It has been suggested that these compounds tend to increase FN concentrations and, therefore, a direct relationship between FN and dietary protein should not be assumed (see Leslie et al., 2008 for a review). The effects of tannins were not evaluated in this study. However, it has been argued that the effects of PSM may be attenuated in herbivores that feed on mixed diets, such as the case of red deer (Leslie et al., 2008; Verheyden-Tixier et al., 2008). Furthermore, Leslie et al. (2008) defended that FN may be a suitable proxy for dietary quality in wild herbivores if assessments are performed within the same species and between comparable study units, which was supported by our findings.

The body condition of red deer was closely associated with game management practices. Deer in populations with a supplementary feeding regime showed a better nutritional status and were less affected by the effects of density dependence. Despite this, it is important to stress that the implementation of such practices has associated risks, with numerous implications at the population and ecosystem levels. Therefore, it is recommended that game managers consider these real problems and weigh the pros and cons of maintaining such management options. It is also considered that future research should focus on more complex issues, in particular on estimates of the sustainable carrying capacity of habitats according to the specific context. Although hard to achieve, it would provide a firmer basis for management decisions. Certainly, this will require integrative approaches linking data on: the nutritional requirements of species, indicators of physiological change, quantitative measures of resource availability and nutritive value of plants, as well as demographic information.

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Chapter 4

Stress Physiology of Red Deer in Mediterranean Environments

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

Summary

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. 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. 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. Our results showed 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. 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 elaphus*, chronic stress, environmental variation, faecal glucocorticoid metabolites, hunting management, Mediterranean habitats

4.1. Introduction

During their evolutionary history, organisms have developed different adaptive physiological and behavioural mechanisms to cope with adverse conditions or stressors (Bijlsma and Loeschcke, 2005; Boonstra, 2005). However, persistent exposure to harmful stimuli can seriously affect their physiology and overall condition (Romero and 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 et al., 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, a key element of the neuroendocrine system that regulates the body's reactions to internal and environmental challenges (Ulrich-Lai and Herman, 2009). Stress can be divided into acute or chronic, depending on the duration of the exposure to the stressors. While an acute stress response is limited in time and operates as an adaptive survival mechanism that allows organisms to restore physiological homeostasis, chronic stress elicits prolonged hypersecretion of glucocorticoids and can have detrimental effects on the animals' health, growth, reproductive performance (Boonstra, 2005; Reeder and Kramer, 2005; Wingfield and 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 and Washburn, 2004; Wikelski and 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; von der Ohe and Servheen, 2002). Moreover, because faecal glucocorticoid metabolite (FGM) levels represent pooled fractions of the amount of hormones that have been secreted and metabolised over a broad period of time (Palme et al., 2005; Wasser et al., 2000), they can provide a long-term and integrated profile of the animals' physiology (Sheriff et al., 2010). Owing to its great potential, FGM analysis has been increasingly applied to monitoring stress responses in a wide variety of wild animals (Chinnadurai et al., 2009; Wasser et al., 2000; Young et al., 2004).

Several factors may act as important sources of stress for mammals. Human activities such as hunting (Bateson and Bradshaw, 1997; Burke et al., 2008), tourism (Rehnus et al., 2014; Zwijacz-Kozica et al., 2013), 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 (Navarro-Castilla et al., 2014; Rimbach et al., 2013). Apart from human-induced stressful situations, social factors (Creel et al., 2013), an elevated risk of predation (Sheriff et al., 2009) as well as seasonal variations in the ambient temperature or in the availability of food resources (Beehner and McCann, 2008; Dalmau et al., 2007) 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).

Wild ungulates have a wide global distribution and are important both ecologically and economically (Gordon et al., 2004). In Europe, ungulate populations have expanded significantly over the last decades as a result of direct human intervention or by natural processes, which has led to increased concerns regarding their management (Apollonio et al., 2010). Such expansion poses new challenges for ungulates, because they have to cope with different forms of anthropogenic pressure, such as changes in the landscape, hunting, and various other human-induced disturbances (Acevedo et al., 2011; Stankowich, 2008). Moreover, in a scenario of climate change, ungulates may have to adapt to new environmental conditions (Mysterud and Sæther, 2011). The red deer *Cervus elaphus* is currently abundant and widespread in the Iberian Peninsula, where its populations are subjected to different management systems and exploitation regimes. Due to its socioeconomic value as a game species, red deer is principally managed for hunting purposes. However, in some situations, their management is linked to damage control and population regulation (Perea et al., 2014). Game management strategies vary throughout the regions and management units and largely depend on the goals and objectives of the administrative authorities and gamekeepers (Carranza, 2010; Vingada et al., 2010). In certain areas, game management practices promote overabundant populations, which, in turn, favour the aggregation of animals, probably increasing social stress, but also the probability of disease transmission among individuals or different species (Gortázar et al., 2006; Vicente et al., 2007a). On the other hand, culling regimes and past demographic history may affect populations' structure and fitness (Queirós et al., 2014; Torres-Porras et al., 2014), and the hunting methods practised can have severe consequences on red deer physiology (Bateson and Bradshaw, 1997; see also Koster and Noss, 2014).

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 et al., 1997; Huber et al., 2003a; Ingram et al. 1999), and even less was performed in the wild (*e.g.*, Corlatti et al., 2011; Sauwerwein et al., 2004). 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.

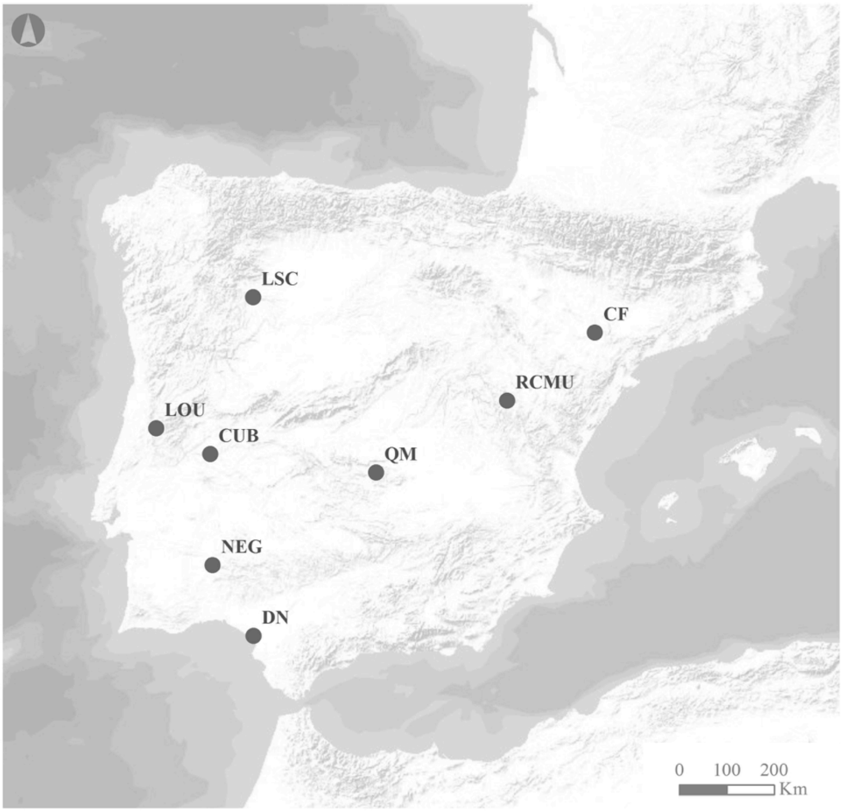
4.2. Materials and methods

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. 4.1): *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. 4.1). 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 4.1.

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. The collection, transportation and storage of samples were done according to Santos et al. (2014). The number of samples collected in each study area varied between n = 16 in LSC and n = 47 in NEG and CF (Table 4.1). 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 et al., 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).



Legend
Latitude/Longitude [a]
Elevation [b]
Average annual temperature [c]
Sum of average monthly precipitation [c]
Köppen-Geiger Climate Classification [d]

LSC 41° 56'N / 6° 33'W 536 - 1235 m.a.s.l. 10.3 °C 657 mm Csb	**LOU** 40° 03'N / 8° 15'W 105 - 1205 m.a.s.l. 13.2 °C 1149 mm Csb
CUB 39° 40'N / 7° 13'W 90 - 364 m.a.s.l. 16.0 °C 710 mm Csa	**NEG** 38° 03'N / 7° 11'W 192 - 442 m.a.s.l. 15.9 °C 547 mm Csa
DN 36° 59'N /6° 24'W 0 - 46 m.a.s.l. 17.1 °C 534 mm Csa	**QM** 39° 24'N / 4° 06'W 722 - 1257 m.a.s.l. 12.5 °C 470 mm Csa
RCMU 40° 26'N / 1° 37'W 1100 - 1935 m.a.s.l. 7.6 °C 615 mm Cfb	**CF** 41° 24'N / 0° 02'E 116 - 390 m.a.s.l. 14.0 °C 414 mm BSk

Fig. 4.1. Location of the study areas in the Iberian Peninsula. Climatic and topographical features of each sampling site are also shown.

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

[b] Elevation ranges (expressed as meters above sea level, m.a.s.l.) were derived either from the 25x25 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).

Table 4.1. 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. 4.1 and sample size (*n*) is also indicated. For abbreviations, see materials and methods section.

Hunting ground (*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.

FGM concentrations

Faecal pellets (approx. 15-20 g per sample) were dried in a conventional oven at 60 °C 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. As a predictive (indirect) technique, NIRS has to be first calibrated against standard laboratory methods for quantitative analysis (Foley et al., 1998; Siesler et al., 2002). To meet this requirement, we used a subset of 78 samples (approx. 27% of the total sample size), representative of the same populations and sampling years (2010-2013) studied, to calibrate and validate NIRS technology for predicting stress hormone metabolite concentrations in red deer faeces (for details see Santos et al., 2014). By using faecal samples with similar characteristics for calibration purposes, a better precision and accuracy of FGM estimates by NIRS is ensured (Siesler et al., 2002). The same procedures outlined in Santos et al. (2014) to scan the faecal samples and collect NIR spectra were followed. 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 4.2).

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 (Dantzer et al., 2014; Wasser et al., 2000), 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 4.2), and represent spatial gradients of climatic conditions at a macro-scale level (Eikenaar et al., 2012). Finally, based on life-history events and on seasonal patterns in glucocorticoid secretion previously reported for red deer (Corlatti et al., 2011; Huber et al. 2003a), the sampling months were grouped in two categories: autumn (September to November) and winter (December to February) (Table 4.2).

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 and Bradshaw, 1997; Burke et al., 2008). In the Iberian Peninsula, red deer are typically hunted either by stalking, or through drive hunts or ‘battues’. Stalking involves a hunter with or without a game guide and is targeted at a specific animal, whereas drive hunts and ‘battues’ are collective hunting events where a number of red deer are hunted with the aid of beaters with or without hounds (Vingada et al., 2010). 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 4.1 and 4.2). 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 4.1 and 4.2).

Supplementary feeding is a commonly used practice in areas managed for hunting. The reasons for feeding deer can vary from compensating seasonal nutritional constraints (*i.e.*, helping animals to maintain or enhance their body condition and overall performance in periods of food shortage, and also to ensure high quality trophies), and/or to attract animals in order to be hunted (Carranza, 2010; Brown and Cooper, 2006; Putman and Staines, 2004). 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 (*e.g.*, 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 hunting seasons (Tables 4.1 and 4.2).

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 4.2). The 'Wild Ungulates Land Avoidance Index' (WULAI) 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), as previously described by Cassinello et al. (2006). The WULAI values calculated correspond to the average scores for each study area (Table 4.1).

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 (Forristal et al., 2012; Li et al., 2007). 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; Buckland et al., 2001). 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. The most parsimonious models were selected based on the lowest Akaike's Information Criterion (AIC) value (Akaike, 1974). Chi-squared (χ^2) goodness-of-fit tests and visual inspection of histograms were also used to evaluate model fitting (Buckland et al., 2001). 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).

Table 4.2. 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 (°C) at time *t* – 1 day
TEMPAvg_m1 (*)	Average temperature (°C) of the last 30 days
TEMPAvg_m2 (*)	Average temperature (°C) of the last 60 days
TEMPAvg_m3 (*)	Average temperature (°C) of the last 90 days
TEMPMax_24h (*)	Maximum temperature (°C) at time *t* – 1 day
TEMPMax_m1 (*)	Average maximum temperature (°C) of the last 30 days
TEMPMax_m2 (*)	Average maximum temperature (°C) of the last 60 days
TEMPMax_m3 (*)	Average maximum temperature (°C) of the last 90 days
TEMPMin_24h (*)	Minimum temperature (°C) at time *t* – 1 day
TEMPMin_m1 (*)	Average minimum temperature (°C) of the last 30 days
TEMPMin_m2 (*)	Average minimum temperature (°C) of the last 60 days
TEMPMin_m3 (*)	Average minimum temperature (°C) 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

Statistical analyses

A preliminary exploration of the data was performed to obtain a better understanding of their characteristics, and to avoid violating assumptions of statistical procedures (Zuur et al., 2010). The approach mainly included an examination of variable distribution, outlier detection, and collinearity diagnostics between the candidate predictors. 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 and 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 et al., 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 algorithm (Akaike, 1974) was run to find the most parsimonious model. In this procedure, variables are added step-by-step until no improvements are found in the AIC value. In general, a model is selected over another model when the former reduces the AIC by 2 units (Burnham and Anderson, 2002). 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 et al., 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 (Goymann, 2012; López-Olvera et al., 2007), as well as between young and adult individuals (Goymann, 2012; Millspaugh and Washburn, 2004). 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 et al., 2007b), season*temperature at time $t - 1$ day (*e.g.*, Corlatti et al., 2011; Huber et al., 2003a), and supplementary feeding*population density (*e.g.*, Vicente et al., 2007a). The data exploration and statistical analyses were conducted with R statistical software, version 3.0.3 (R Core Team, 2014). The LMMs 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 2.0-6 (Kuznetsova et al., 2014) for R. Statistical significance was set at $P \leq 0.05$.

As a final step, variation-partitioning techniques (Borcard et al., 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 three main components (*i.e.*, sets of related predictors): individual, environmental, and human (see Tables 4.3 and 4.4). The variables 'study area' and 'hunting season' were also incorporated into a distinct component, the so-called 'spatio-temporal' component. Thus, four components were defined and partitioned 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: 'MainComp') and the amount of variation of the final model (SpaTemp+MainComp), 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. A.2.1 in Appendix 2 for details on procedures; see also Acevedo et al., 2010; Legendre and Legendre, 2012).

The proportion of variation explained (R^2) by the abovementioned models was calculated according to Nakagawa and 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.

4.3. Results

An average of 36.13 ± 4.40 (SE, see Table 4.1) 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 from 0.59 to 391.04 ng/g DM. 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. The average FGM concentrations varied among study areas (Fig. 4.2).

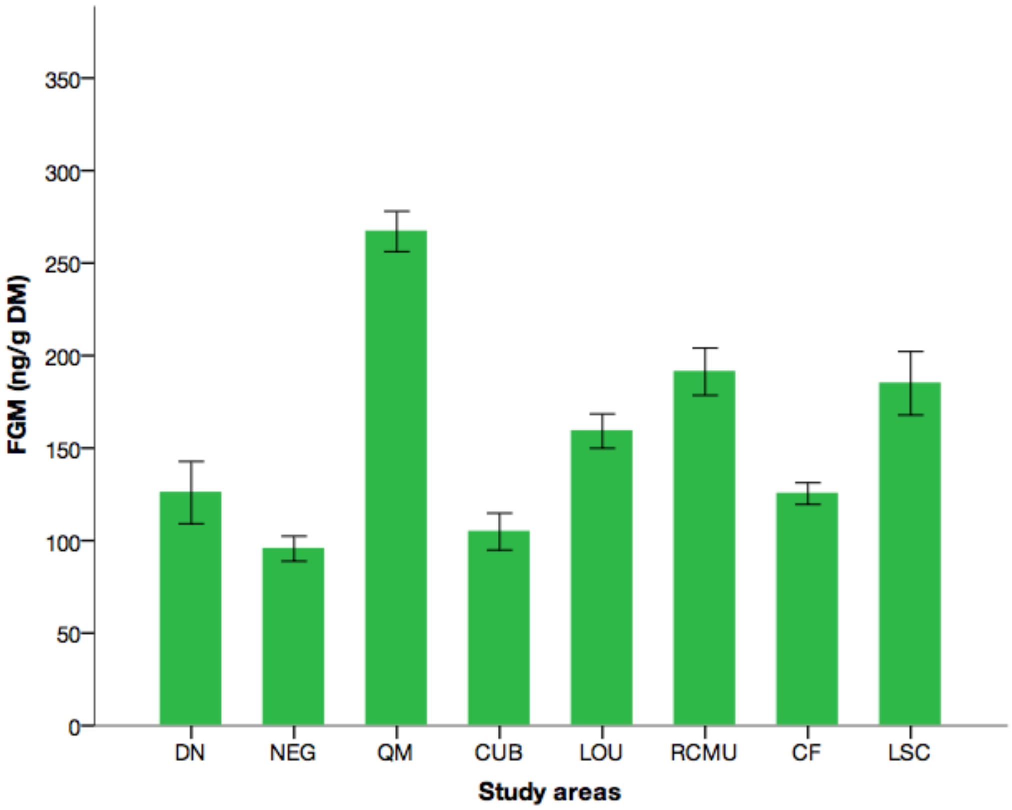


Fig. 4.2. Average concentrations of faecal glucocorticoid metabolites (FGM, expressed as ng/g of dry matter, DM) measured in the faeces of red deer *Cervus elaphus* during three consecutive game seasons in eight study areas located in the Iberian Peninsula. Deer populations are ordered by latitude, from south to north. Bars indicate standard errors (SE).

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 4.2). The final model included 10 predictors and three interaction terms (see Table A.2.1 in Appendix 2 for results of the stepwise model selection analysis). Results of linear mixed models are summarised in Tables 4.3 and 4.4.

Table 4.3. Results of tests of fixed effects and their interactions in a linear mixed model explaining the 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
Individual	Sex	1, 267	0.018	0.893
	Age class	3, 267	5.410	**0.001**
	Sex * Age class	3, 267	0.494	0.686
Environmental	Season	1, 267	0.068	0.794
	Average temperature on t – 1 day	1, 267	8.819	**0.003**
	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**
Human	WULAI	1, 267	32.013	**<0.0001**
	Supplementary feeding	1, 267	2.719	0.100
	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**

Regarding individual-level factors, no significant differences were found in FGM concentrations between males and females ($F_{1,267}$ = 0.018, P = 0.893), but such concentrations differed significantly among age classes ($F_{3,267}$ = 5.410, P = 0.001), with younger animals showing higher levels of hormone metabolites when compared to adult animals (Table 4.4). The interaction sex*age class had no significant effect on FGM concentrations ($F_{3,267}$ = 0.494, P = 0.686).

Table 4.4. 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
Individual	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
	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
Environmental	Season (Winter)	-8.51	32.51	-0.262	0.794
	Average temperature on *t* – 1 day	2.09	2.13	0.984	0.326
	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**
Human	WULAI	-25.02	4.42	-5.658	**<0.0001**
	Supplementary feeding (Yes)	-39.47	23.94	-1.649	0.100
	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.

In relation to environment-related factors, no significant differences were observed in FGM levels between the autumn and winter period ($F_{1,267}$ = 0.068, *P* = 0.794), 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 ($F_{1,267}$ = 4.048, *P* = 0.045; Fig. 4.3). The precipitation accumulated one month before sampling was negatively associated with FGM concentrations ($F_{1,267}$ = 5.514, *P* = 0.020; Table 4.4), whereas the latitude of the study areas had a positive significant effect on the hormonal levels in red deer ($F_{1,267}$ = 35.944, *P* < 0.0001; Table 4.4).

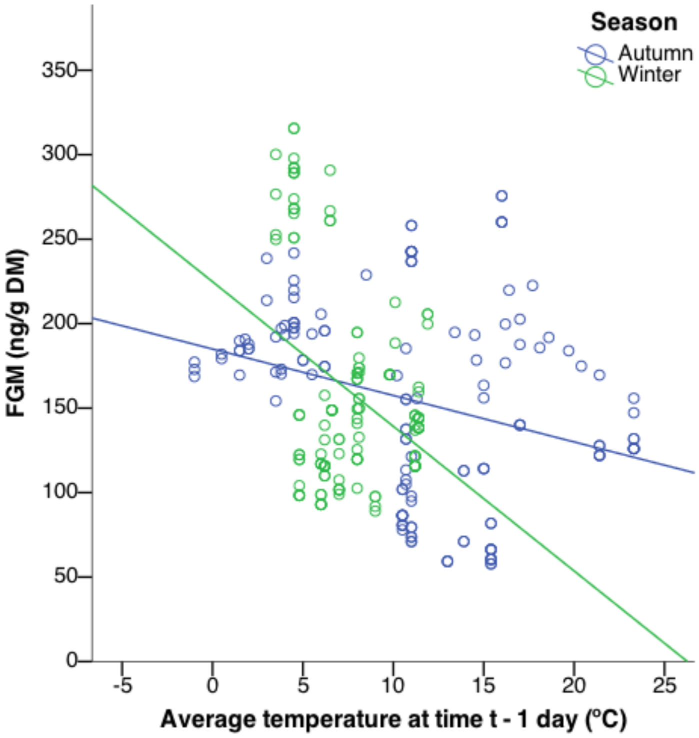


Fig. 4.3. Relationship between the average daily temperature (°C) and the concentrations of faecal glucocorticoid metabolites (FGM, expressed as ng/g of dry matter, DM) in red deer *Cervus elaphus* in the autumn and winter. The average daily temperature refers to the average temperature recorded one day before the date on which the animals were culled.

Significant relationships between stress hormone concentrations and game management practices, as well as other anthropogenic factors, were also found. Supplementary feeding and population density alone had no significant effects on FGM levels ($F_{1,267} = 2.719$, $P = 0.100$; $F_{1,267} = 0.061$, $P = 0.805$). However, the interaction between these variables significantly affected FGM concentrations in red deer ($F_{1,267} = 10.356$, $P = 0.001$). 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. 4.4). The presence or absence of big hunting events also significantly affected FGM concentrations ($F_{1,267} = 4.158$, $P = 0.042$), with such concentrations being higher in areas where hunting methods involve the use of hounds and beaters (Table 4.4). In addition, a statistically negative relationship was found between hormone levels and WULAI ($F_{1,267} = 32.013$, $P < 0.0001$; Table 4.4).

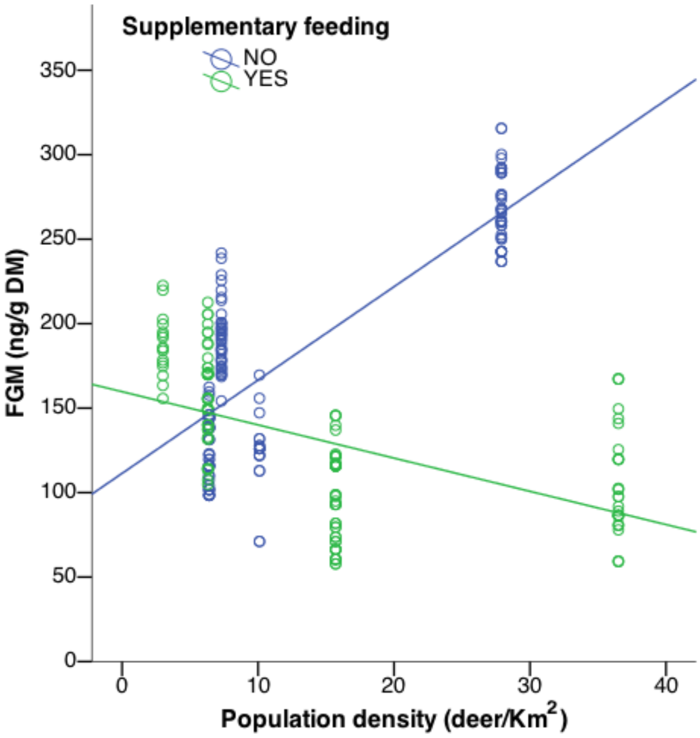


Fig. 4.4. Relationship between population density (calculated as deer/Km^2) and the concentrations of faecal glucocorticoid metabolites (FGM, expressed as ng/g of dry matter, DM) in food-supplemented and non-food-supplemented populations of red deer *Cervus elaphus*.

The final model explained 51.2% of the total variation in FGM concentrations in red deer (Fig. 4.5a). 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. 4.5a). 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. 4.5b). The overlaid effect of the three components accounted for approximately 50% of the variation, while all the effects shared between two components were negatively associated (Fig. 4.5b).

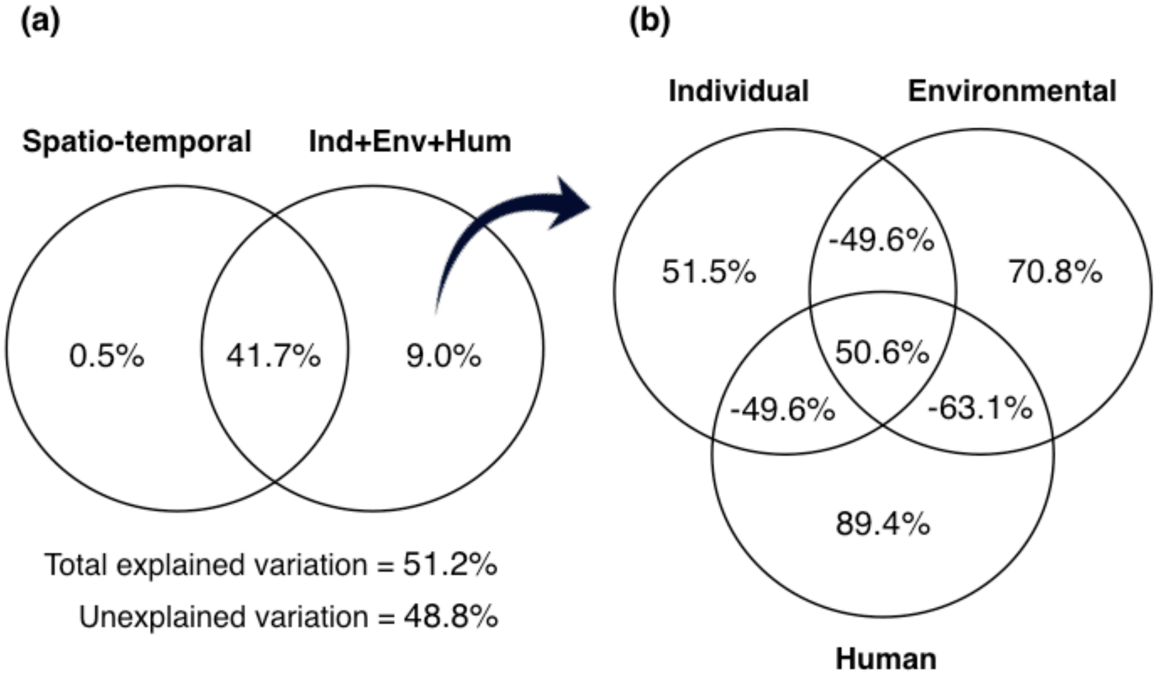


Fig. 4.5. Venn diagrams showing (a) the percentage of variation explained by the spatio-temporal factors versus the remaining set of predictors included in the final model, as well as their overlaid effects; and (b) the percentage of variation explained by the individual (Ind), environmental (Env) and human (Hum) components, and their intersections, on the variation of faecal glucocorticoid metabolite concentrations in red deer *Cervus elaphus*. See Fig. A.2.1 in Appendix 2 for details of the procedures used to separate out the explained variation into their pure and shared effects.

4.4. 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 (Azorit et al., 2012; Corlatti et al., 2011; Forristal et al., 2012; Huber et al., 2003a), 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 David's deer *Elaphurus davidianus* (Li et al., 2007), and Pampas deer *Ozotoceros bezoarticus* (Pereira et al., 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 (Crespi et al., 2013; Sands and Creel, 2004; Wada et al., 2006). 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 and months of the year. 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 et al., 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 and 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). For example, in an experimental study with red deer, Azorit et al. (2002) found different individual responses to the same stressor. Furthermore, 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 (Bonier et al., 2009; Reeder and Kramer, 2005). 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 (Corlatti et al., 2011; Huber et al., 2003a; 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 (Chinnadurai et al., 2009; Pereira et al., 2006). 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 and 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 and 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 (Grignolio et al., 2011; Stankowich, 2008). Under such circumstances, they increase vigilance and can alter their distribution patterns or habitat use, as well as their feeding behaviour (Benhaiem et al., 2008; Grignolio et al., 2011; Jayakody et al., 2008). They also show differentiated behaviour throughout the year and time of the day according to the risks of being predated or hunted (Sönnichsen et al., 2013). Moreover, there is evidence that ungulates become less tolerant to hunting activities with the progression of the game seasons (Millspaugh et al., 2000), thus highlighting the importance of previous cumulative experiences on their behaviour. Other studies have provided evidence on the short- and long-term physiological effects of hunting activities on wild herbivores. For example, Bateson and 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 and 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. Studies integrating field endocrinology and spatial ecology have an enormous potential for improving our understanding of how landscape structure (*e.g.*, fragmentation levels) affect species' physiology (Ellis et al., 2012).

4.5. 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. For example, sex determination from unknown samples collected in the field often requires genetic analysis, which has prohibitive costs when applied to a large amount of samples. 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 and 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.

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Chapter 5

Summary and Conclusions

This last chapter is intended to summarise the main topics and results and highlight the key findings, in order to provide an overview of this thesis. The implications of the results achieved for red deer monitoring and management are discussed in more detail at the end of the respective chapters and, therefore, such implications are only summarised here.

***

Two general hypotheses were formulated at the beginning of this thesis (Chapter 1, Section 1.2). The first hypothesis stated that the physiological status and physical condition of red deer could be evaluated in a simpler way, using more cost-effective and timesaving methods. Both studies conducted in Chapter 2 (here referred to as #1 and #2) confirmed our working hypothesis.

Study #1

Optimising the sampling effort to evaluate body condition in ungulates:
a case study on red deer

Context and purpose. The central question in this study was whether one kidney and its perirenal fat could provide the same information as the average of the two kidneys (classical approach), when assessing the nutritional condition in wild ungulates.

Methodologies. The potential differences between indicators of nutritional status derived from the left and right kidneys, as well as from both kidneys, were investigated, using two types of indicators: KFI (kidney fat index) and KCOV (fat weight as the dependent variable and the kidney weight as a covariate). The responses of those indicators to biological and ecological factors, that usually shape changes in the nutritional condition in wild ungulates, were tested and described.

Main results. By using red deer as a model species, both under experimental and field conditions, no differences were observed when testing the indicators derived from the left and right kidneys and their average, both in the KFI and KCOV approaches.

Conclusions and implications. Only one kidney and its associated fat is enough to assess the nutritional condition in red deer and probably in other wild ungulates, which represents a considerable reduction of time and effort, both in the field and in the laboratory.

Study #2

Near infrared reflectance spectroscopy (NIRS) for predicting glucocorticoid metabolites in lyophilised and oven-dried faeces of red deer

Context and purpose. Cost-effective and time-efficient ways for assessing physiological stress levels in wildlife, particularly in red deer, were explored as an alternative to the conventional immunochemical techniques (radioimmunoassays or enzyme immunoassays).

Methodologies. The feasibility of using NIRS technology to predict the concentration of faecal glucocorticoid metabolites (FGM), which have been increasingly used as physiological indicators of the stress response in many vertebrates, was evaluated using faeces of red deer for the first time. In addition, the effect of oven drying, on the quantification of FGM, in comparison to lyophilisation (standard drying method) was examined; NIRS calibration equations were developed for predicting FGM concentrations for each drying treatment.

Main results. Good calibration equations were obtained for predicting FGM levels both in lyophilised and oven-dried faeces. The concentrations of FGM measured in lyophilised and oven dried-faeces were similar.

Conclusions and implications. NIRS may be a feasible, acceptably accurate and reliable technique to predict FGM concentrations in faecal material, and oven-dried faeces may be used as an alternative to lyophilised faeces to quantify FGM levels accurately. The results obtained from this study have clear practical implications concerning wildlife monitoring, as they make it possible to improve the efficiency and reduce the cost and time constraints of current analytical techniques.

Both studies #1 and #2 deal with aspects of high interest for wildlife ecologists and game managers, as they integrate monitoring and assessment of indicators of ecological change with management goals.

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After fine-tuning procedures to assess the physical condition in red deer, two large-scale cross-sectional studies were conducted to evaluate the effects of management practices, environmental factors and individual traits on the nutritional condition and stress physiology of the species in the Mediterranean environments of the Iberian Peninsula (here referred to as studies #3 and #4, respectively). The second general hypothesis of this thesis, which affirmed that the evaluation of physiological and nutritional status could provide important insights concerning red deer ecology and may serve as a basis for decision-making, was supported with the completion of the observational studies of Chapters 3 and 4.

Study #3

Determining changes in the nutritional condition of red deer in Mediterranean ecosystems: effects of environmental, management, and demographic factors

Context and purpose. This study was aimed at investigating the effects of environmental conditions and game management practices on the quality of the diet and nutritional status of red deer in Mediterranean areas of Iberia. Variations in the nutritional condition in relation to the sex, age, parasite load and dietary attributes of individuals were also examined and described.

Methodologies. The amount of nitrogen excreted in faeces (faecal nitrogen, FN) was used as an indicator of the diet quality, in terms of the protein content, while the kidney fat index (KFI) was used as an indicator of the nutritional condition of the deer. The FN levels were predicted by NIRS, after the appropriate calibration of the technique (see details in Chapter 3), and the KFI was calculated using the right kidney only with its associated fat, according to the recommendations made in Chapter 2 (Study #1).

Main results. Both FN and KFI levels showed seasonal variations, with higher values in the winter in relation to the autumn, and were positively correlated with the productivity and quality of the vegetation. In contrast, both nutritional indicators were negatively associated with the fibre content of the diets. Overall, the amount of kidney fat increased with increasing dietary quality in red deer. However, the effect of high quality diets on deer nutritional status was progressively lower with increasing population density and this was more evident in populations that depended exclusively on natural food resources. The concentrations of FN were also negatively related with the average altitude of the study areas, whereas the KFI decreased with increasing latitude only in the winter. Sex-related differences were also observed in the KFI levels, with females having superior amounts of fat reserves than males.

Conclusions and implications. It was demonstrated that FN and KFI can be used as reliable indicators to monitor changes in the dietary quality and nutritional status, respectively, in red deer in Mediterranean areas, since they were sensitive to changes in environmental conditions and diverse management actions. As both indices responded differently to ecological and biological factors, in particular to those related to individual characteristics, management and demographic variables, this study also emphasised the value of using them in a complementary way to better understand the nutritional ecology of red deer, in order to adopt more efficient and suitable management measures.

Study #4

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

Context and purpose. The main aim of this study was to examine the impact of a set of anthropogenic and environmental factors on the physiology of chronic stress in free-ranging red deer in a variety of Mediterranean environments, where the species is subjected to differing management systems and weather regimes.

Methodologies. Faecal glucocorticoid metabolite (FGM) concentrations were predicted in the faeces of red deer by means of NIRS technology (validated in Chapter 2, Study #2) and were used as indicators of stress. An integrated approach was used to estimate the relative contribution of individuals' biological attributes, as well as natural environmental and human factors, in modulating the stress levels in the various populations studied.

Main results. Factors related to hunting management practices accounted for most of the variation in the stress levels in red deer, followed by the environmental factors and individuals' biological traits. Two results were of particular interest from the point of view of management because of their significance as potential sources of chronic stress for the species: firstly, it was observed that stress levels were significantly higher, in areas where hunting methods involved large drive hunts and the use of hounds, than in areas where deer were hunted selectively by stalking; secondly, it was found that FGM concentrations rose with increasing deer population density in areas where supplementary feeding was not provided, whereas almost no effect was observed in food-supplemented populations. This suggests that the effects of an overabundance of deer on chronic stress were mitigated by the provision of supplementary food. Conversely, deer in dense populations and without supplementary feeding were affected by the negative effects of density-dependence more markedly and exhibited signs of stress that were probably caused by nutritional constraints. This fact had already been implicit in Chapter 3, from a purely nutritional perspective.

Conclusions and implications. This study is an important contribution to a better understanding of the mechanisms that influence the stress physiology of large herbivores in the wild, particularly of red deer, as it provides an alternative perspective on how multiple factors can operate, independently or synergistically, to modulate their stress levels. This research also has some implications regarding animal welfare and may serve as a basis to support game management decisions.

Based on the main results of studies #3 and #4, it is suggested that wildlife managers and gamekeepers should ideally promote hunting practices that cause lower levels of disturbance for wildlife, and their efforts should also be directed to maintaining a balance between animal densities and the environment.

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Final remarks

From the experimental and cross-sectional studies conducted within the framework of this thesis, the following general conclusions were derived:

1. Data collected from hunted animals are a valuable source of information that makes it possible to evaluate their physical condition and make inferences about the status of the remaining individuals in a population and are, therefore, extremely useful for management purposes;

2. Monitoring the physical condition of wild ungulates and particularly of red deer can be performed using simpler procedures than those traditionally used. This will optimise the time and effort required to take samples and conduct the laboratory analyses. As the amount of fat surrounding the kidneys is sensitive to changes in food availability, it can therefore be used as an indicator to assess and monitor the nutritional status in red deer. The indices of nutritional condition derived from one or two kidneys provide equivalent results, thus allowing the same conclusions to be reached. Therefore, the data gathered from a single kidney is enough to monitor changes in the nutritional status of red deer and probably of other wild deer species;

3. Near infrared spectroscopy (NIRS) can be used reliably to make quantitative predictions of concentrations of stress hormone metabolites, faecal nitrogen and dietary fibre fractions in red deer faeces. As such concentrations can be estimated from a single faecal sample, NIRS technology represents a cost-effective approach to assessing indicators of ecological relevance;

4. Interdisciplinary studies integrating nutrition, physiology and ecology considerably enhance our understanding of species-environment relationships;

5. The red deer populations living in the Mediterranean Iberia are subjected to different management regimes and environmental conditions and this has consequences on the animals’ physiological status and physical condition. Variables reflecting the type of game management are important drivers of both nutritional and physiological status in red deer in Mediterranean areas of the Iberian Peninsula and, consequently, should be incorporated in studies which intend to model variations in such indicators;

6. High population densities had adverse effects on the nutritional condition and stress levels in red deer in natural populations without supplementary feeding;

7. Populations subjected to hunting methods involving massive hunting events and the use of hounds presented higher levels of chronic stress than populations where hunting was performed using less disturbing methods. This lead us to presume that the cumulative effects of hunting may persist in the populations for some time after the hunting events;

8. The use of indicators that provide information about the internal status and overall physical condition of individual wild animals and their populations should be encouraged in order to support wildlife management decisions.

***

The research presented in this thesis represents a contribution towards a better understanding of the ecology of red deer in Mediterranean ecosystems, from a nutritional and physiological perspective. It is expected that the development of new tools and the knowledge about the factors that affect the physical condition of deer may help wildlife managers to implement more effective monitoring programmes and sustainable management practices.

Supplementary material

Appendix 1

Supplementary material for **Chapter 3**

Prediction of diet components from the faeces of red deer using near infrared reflectance spectroscopy (NIRS): Equipment, procedures and main results

A.1.1. Aims

The aim of this section is to detail the equipment and procedures used to estimate the diet components in the faeces of red deer, using near infrared reflectance spectroscopy (NIRS). The dietary fractions analysed in the faecal material were the following: faecal nitrogen (FN); neutral detergent fibre (NDF); acid detergent fibre (ADF); and acid detergent lignin (ADL).

A.1.2. Materials and methods

NIRS analysis: scanning, calibration and validation procedures

Dried and ground faecal samples (n = 96) were packed into 35 mm diameter circular cups, with quartz glass windows, and scanned using an NIRSystems 5000 (FOSS, Hillerød, Denmark) over a wavelength range of 1108-2492 nm. The data were recorded at 2 nm intervals as *log 1/R* (where R=reflectance), resulting in 692 points per sample. Each sample was scanned twice, by manually rotating the sample cup approximately 180° relative to the previous scan. Hence, two NIR spectra were collected per sample and averaged for subsequent chemometric analysis. The same operator performed the entire scanning procedure.

The spectral data processing and statistical analyses were performed using WinISI III (v.1.6) software. Prior to developing the calibration models, the NIR spectra were subjected to different pre-treatment methods, in order to improve signal-to-noise ratio and, therefore, maximise the signal intensity for the analytes of interest (Heise and Winzen, 2002). Spectral correction algorithms – standard normal variate (SNV), detrend (DT) and multiplicative scatter correction (MSC) – were applied to reduce the effects of light scattering due to particle size. Furthermore, different mathematical treatments using the first, second and third derivatives, with different subtraction gaps and smoothing intervals were also tested. Sixteen spectral models for each dietary attribute to be predicted were developed, in total, resulting from the evaluation of four scatter correction techniques (SNV; DT; SNV+DT; MSC) and four mathematical treatments (1,4,4,1; 2,4,4,1; 3,4,4,1; 2,10,10,1 – derivative number, subtraction gap, first smooth, second smooth).

Modified partial least squares (MPLS) regression was used to develop the calibration models (Martens and Martens, 2001) and '*leave-n-out*' cross-validation (Shao, 1993) was then performed for their optimisation. Cross-validation was also used to identify those samples, whether chemical (*t*) or spectral (*H*) outliers. The *t* outliers are samples that have a relationship between their reference values and spectra that is different from the relationship of the other samples in the set and with large residuals ($t > 2.2$). Samples with a large *t* statistic often cast doubt on the chemical reference value. An *H* outlier identifies a sample that is spectroscopically different from the other samples in the population and has a standardized *H* value (modification of the Mahalanobis distance) greater than 3.

The quality and predictive ability of the calibration equations were evaluated by using the following statistical parameters (for details see Shenk and Westerhaus, 1996; Williams and Sobering, 1996): coefficient of determination of the calibration (R^2), standard error of the calibration (SEC), coefficient of determination of the cross-validation (r^2_{cv}), standard error of the cross-validation (SECV), the range error ratio (RER, which was calculated by dividing the range in the reference data by the SECV), and the ratio of performance deviation (RPD, which was calculated by dividing the standard deviation of the reference values by the SECV).

A.1.3. Results and brief discussion

The spectral pre-treatment techniques that gave the best results were the first derivative combined with MSC for FN, the second derivative with SNV for NDF and ADF fractions, and the second derivative combined with MSC for ADF fraction (Table A.1.1). For all faecal constituents, the R^2 and r^2_{cv} values showed a strong relationship between the data obtained using standard laboratory techniques (reference data) and the values predicted by NIRS (Fig. A.1.1; Table A.1.1). Overall, the coefficients of determination for both calibration and validation were above 0.9, thus indicating that NIRS can generate excellent quantitative predictions for all the components analysed (Shenk and Westerhaus, 1996). Moreover, the calibration equations developed in this study may be considered very robust according to the criteria defined by Williams and Sobering (1996), who indicated that RER values should, ideally, be at least 10 and the RPD at least 3 (Table A.1.1). A compilation of the calibration and validation statistics for dietary attributes predicted by NIRS in faeces of wild and domestic herbivores can be consulted in a review article by Dixon and Coates (2009).

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Table A.1.1. Statistical and data processing results relating near infrared reflectance spectroscopy (NIRS) predicted values for faecal nitrogen (FN), neutral detergent fibre (NDF), acid detergent fibre (ADF) and acid detergent lignin (ADL) to values obtained by standard laboratory techniques in faecal samples of red deer (*Cervus elaphus*). All values are expressed as a % of dry matter (DM).

	Descriptive statistics [a]				**Spectral pre-treatments**		**NIRS calibration and cross-validation statistics**					
	N	**Min - Max**	**Average**	**Standard deviation**	**Maths treatment [b]**	**Scatter correction [c]**	R^2	**SEC**	r^2_{cv}	**SECV**	**RER**	**RPD**
FN (% DM)	96	1.52 - 3.39	2.42	0.49	1,4,4,1	MSC	0.97	0.09	0.95	0.11	17.00	4.47
NDF (% DM)	96	25.80 - 64.71	49.54	8.08	2,4,4,1	SNV	0.96	1.62	0.89	2.66	14.64	3.04
ADF (% DM)	96	16.43 - 53.24	35.23	7.50	2,4,4,1	SNV	0.97	1.40	0.92	2.21	16.66	3.39
ADL (% DM)	96	6.12 - 31.59	15.66	5.93	2,4,4,1	MSC	0.98	0.98	0.95	1.38	18.52	4.31

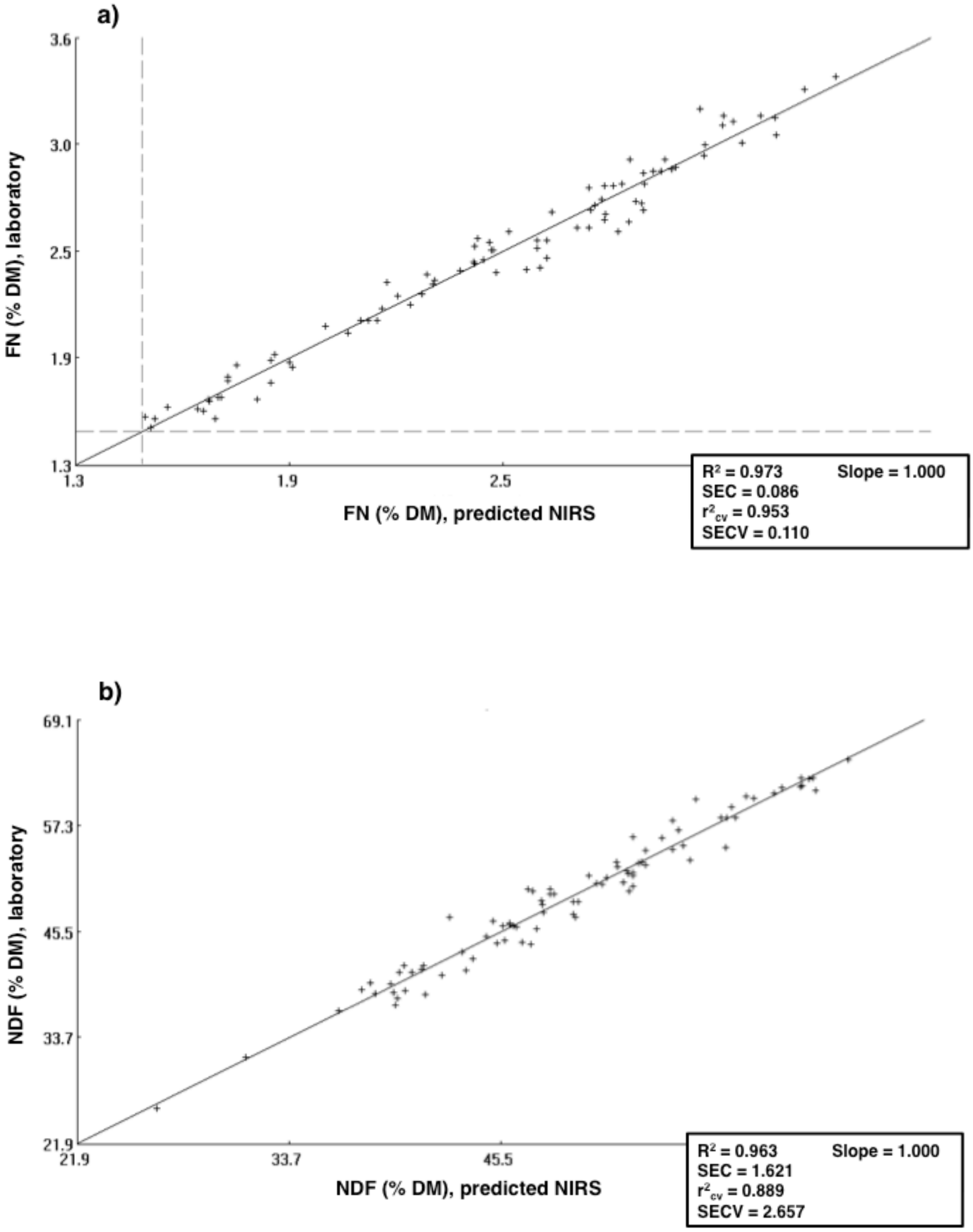
R^2 = coefficient of determination of calibration; SEC = standard error of calibration; r^2_{cv} = coefficient of determination of cross-validation; SECV = standard error of cross-validation; RER = range error ratio (Max – Min/SECV); RPD = ratio of performance deviation (SD/SECV).

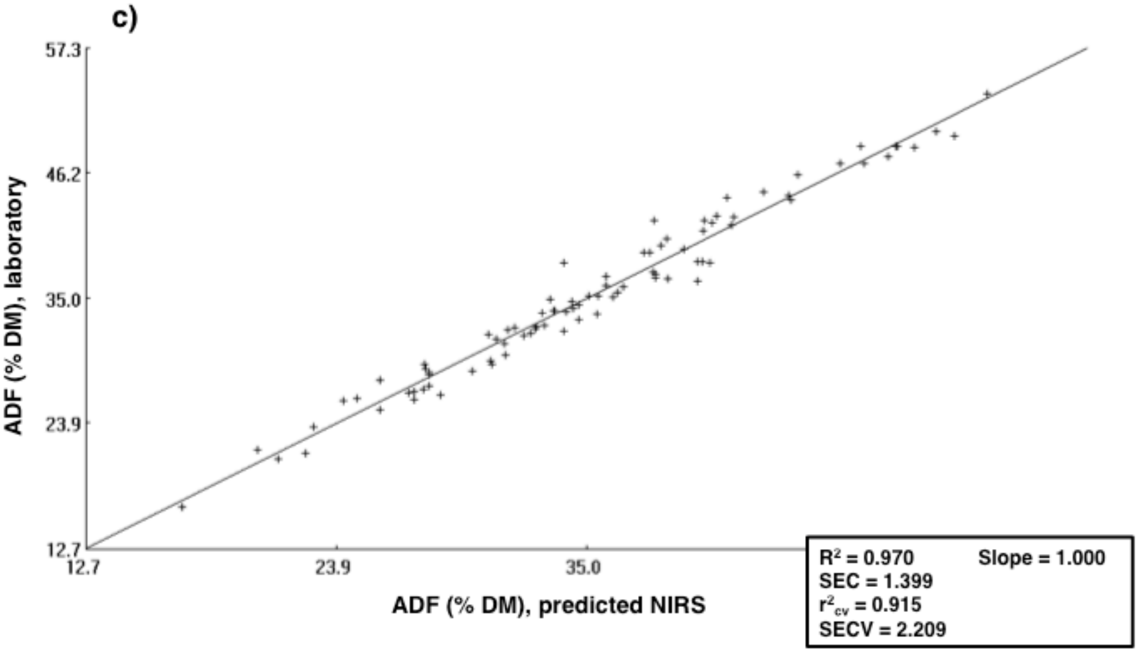
[a] Descriptive statistics of FN, NDF, ADF, and ADL content determined by standard laboratory techniques and used for NIRS calibration. The Dumas combustion method was used to determine FN content, whereas NDF, ADF, and ADL fractions were determined sequentially according to the Ankom protocol (ANKOM Technology, Fairport, NY, USA).

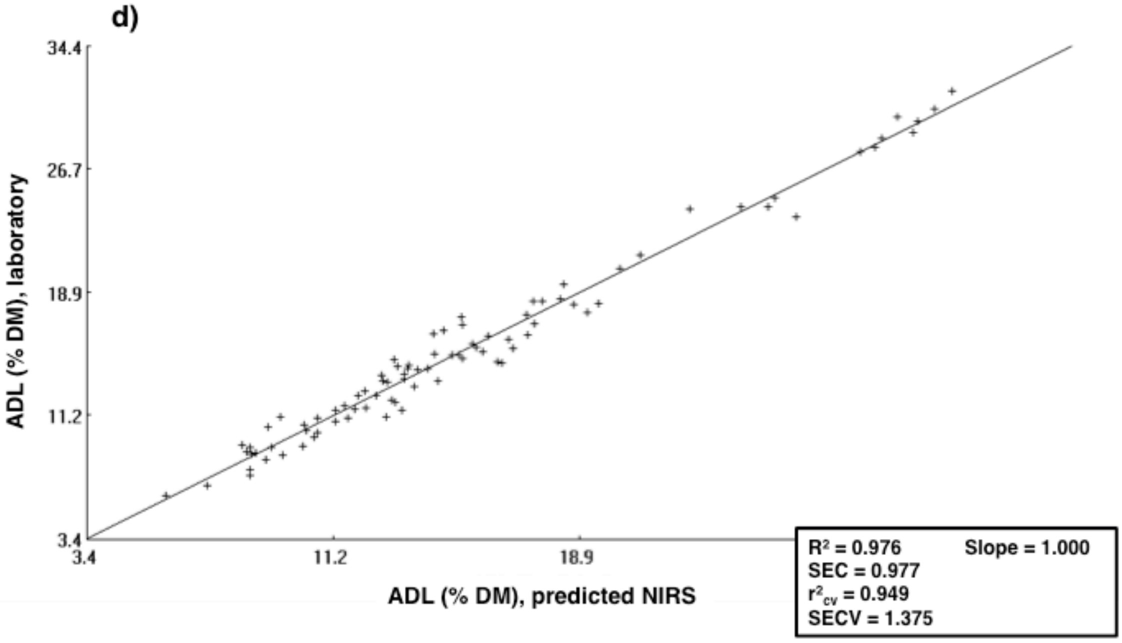
[b] Mathematical treatment: derivative order, gap, first smoothing, second smoothing.

[c] Spectra correction algorithms: SNV = standard normal variate, MSC = multiplicative scatter correction.

Fig. A.1.1. Linear relationship between values predicted by near infrared reflectance spectroscopy (NIRS) and values obtained from standard laboratory techniques for: (a) faecal nitrogen (FN); (b) neutral detergent fibre (NDF); (c) acid detergent fibre (ADF); and (d) acid detergent lignin (ADL). Values are expressed as a % in terms of dry matter (DM). R^2 = coefficient of determination of calibration; SEC = standard error of calibration; r^2_{cv} = coefficient of determination of cross-validation; SECV = standard error of cross-validation.




c)
57.3
46.2
35.0
23.9
12.7
12.7
23.9
35.0
ADF (% DM), laboratory
ADF (% DM), predicted NIRS
$R^2 = 0.970$ Slope = 1.000
SEC = 1.399
$r^2_{cv} = 0.915$
SECV = 2.209


d)
34.4
26.7
18.9
11.2
3.4
3.4
11.2
18.9
ADL (% DM), laboratory
ADL (% DM), predicted NIRS
$R^2 = 0.976$ Slope = 1.000
SEC = 0.977
$r^2_{cv} = 0.949$
SECV = 1.375

Appendix 1 (cont.)

Effects of biological, environmental, management, and demographic factors on the nutritional condition of red deer in the Iberian Peninsula: Variables, relationships and models

Table A.1.2. Summary of the variables used to model the dietary quality (measured as the amount of nitrogen excreted in faeces, FN) and nutritional condition (measured using the kidney fat index, KFI) of wild red deer *Cervus elaphus* in the Mediterranean ecosystems of the Iberian Peninsula.

Variables/Codes	Type of variable	Variable description and units
Individual traits		
Sex	Categorical	2 levels: 1 = Males; 2 = Females
Age.class	Categorical	4 levels: 1 = Calves; 2 = Yearlings; 3 = Sub-adults; 4 = Adults
Parasite load		
E.cervi.lpg (1)	Continuous	Abundance of excreted first-stage larvae (L1) of *Elaphostrongylus cervi* (L1 per gram of faeces, lpg)
Dietary fibre		
Hemicell (2)	Continuous	Hemicellulose content (% of dry matter, DM)
Cell (2)	Continuous	Cellulose content (%, DM)
Lign (2)	Continuous	Lignin content (%, DM)
Seasonality		
SEASON	Categorical	2 levels: Autumn = (Sep., Oct., Nov.); Winter = (Dec., Jan., Feb.)
Weather and climate		
Rain.30 (3)	Continuous	Accumulated rainfall (mm) in the last 30 days
Rain.60 (3)	Continuous	Accumulated rainfall (mm) in the last 60 days
Rain.90 (3)	Continuous	Accumulated rainfall (mm) in the last 90 days
T.min (3)	Continuous	Average monthly minimum temperature (ºC)
T.mean (3)	Continuous	Average monthly mean temperature (ºC)
T.max (3)	Continuous	Average monthly maximum temperature (ºC)
Vegetation productivity		
NDVI (4)	Continuous	Normalised Difference Vegetation Index
Geographic		
LAT	Continuous	Latitude (decimal degrees)
LONG	Continuous	Longitude (decimal degrees)
Topographic		
ALT.m	Continuous	Average altitude (meters above sea level, m.a.s.l.)
Habitat composition		
HConFor (5)	Continuous	Coniferous forests (%)
HBlFor (5)	Continuous	Broadleaved forests (%)
HMxFor (5)	Continuous	Mixed forests (%)
HScrub (5)	Continuous	Scrublands (%)
HNatGrass (5)	Continuous	Natural grasslands (%)
HAgr (5)	Continuous	Agricultural lands (%)
HWet (5)	Continuous	Wetlands (%)
HPlotGame (6)	Continuous	Food plots for big game (%)
Game management		
SUPPL.01	Categorical	Supplementary feeding, 2 levels: 0 = absence; 1 = presence
Feed.Kg.Deer	Continuous	Amount of food supplied per deer per year (Kg/deer/year)
Feed.Kg.Km2	Continuous	Amount of food supplied per surface area per year (Kg/Km^2/year)
Demographic		
D.deer	Continuous	Population density (red deer/Km^2)

(1) Log transformed: ln (x + 1); (2) Fractions derived from neutral detergent fibre (NDF), acid detergent fibre (ADF), and acid detergent lignin (ADL); (3) Data provided by the Spanish and Portuguese National Meteorology Institutes for the weather stations closest to each study area. Accumulated rainfall variables were calculated in relation to the date on which the deer were culled; (4) NDVI was obtained from satellite images from MODIS (Moderate Resolution Imaging Spectroradiometer), available at: http://modis.gsfc.nasa.gov; (5) Information derived from Corine Land Cover maps for 2006 (European Environment Agency, 2011); (6) Values were calculated from the percentage of agricultural land in each study area, based on the information provided by game managers.

Table A.1.3. Simple correlation coefficients (Pearson's *r*) for the relationship between the nutritional indicators – faecal nitrogen (FN, % of dry matter, DM) and kidney fat index (KFI, %) – with all the candidate continuous explanatory variables.

Variables	**Nutritional indicators**			
	Faecal nitrogen (FN, % DM)		**Kidney fat Index (KFI, %)**	
	r	*p*	*r*	*p*
Parasite load				
Elaphostrongylus cervi (L1) per gram of faeces (lpg) (1)	– 0.251	***	+ 0.100	NS
Dietary fibre				
Hemicellulose content (%, DM)	– 0.181	**	+ 0.093	NS
Cellulose content (%, DM)	– 0.439	***	+ 0.078	NS
Lignin content (%, DM)	– 0.378	***	– 0.342	***
Weather and climate				
Accumulated rainfall (mm) in the last 30 days	– 0.206	***	+ 0.143	*
Accumulated rainfall (mm) in the last 60 days	– 0.118	#	+ 0.192	**
Accumulated rainfall (mm) in the last 90 days	– 0.017	NS	+ 0.133	*
Average monthly minimum temperature (ºC)	– 0.060	NS	– 0.109	#
Average monthly mean temperature (ºC)	– 0.020	NS	– 0.048	NS
Average monthly maximum temperature (ºC)	+ 0.002	NS	– 0.008	NS
Vegetation productivity				
Normalised Difference Vegetation Index (NDVI)	– 0.081	NS	+ 0.297	***
Geographic				
Latitude (decimal degrees)	– 0.230	***	– 0.194	**
Longitude (decimal degrees)	– 0.047	NS	– 0.205	***
Topographic				
Average altitude (meters above sea level, m.a.s.l.)	– 0.410	***	+ 0.017	NS
Habitat composition				
Coniferous forests (%)	– 0.348	***	– 0.120	*
Broadleaved forests (%)	+ 0.295	***	+ 0.253	***
Mixed forests (%)	– 0.112	#	– 0.152	*
Scrublands (%)	– 0.348	***	– 0.247	***
Natural grasslands (%)	– 0.042	NS	+ 0.004	NS
Agricultural lands (%)	+ 0.290	***	+ 0.045	NS
Wetlands (%)	– 0.008	NS	– 0.167	**
Food plots for big game (%)	+ 0.297	***	+ 0.433	***
Game management				
Amount of food supplied per deer per year (Kg/deer/year)	+ 0.283	***	+ 0.416	***
Amount of food supplied per surface area per year (Kg/Km^2/year)	+ 0.266	***	+ 0.256	***
Demographic				
Population density (red deer/Km^2)	+ 0.283	***	+ 0.018	NS

Significance levels: NS = not significant; # = 0.1 < p < 0.05; * p < 0.05; ** p < 0.01; *** p < 0.001;

(1) Log transformed: ln (x + 1)

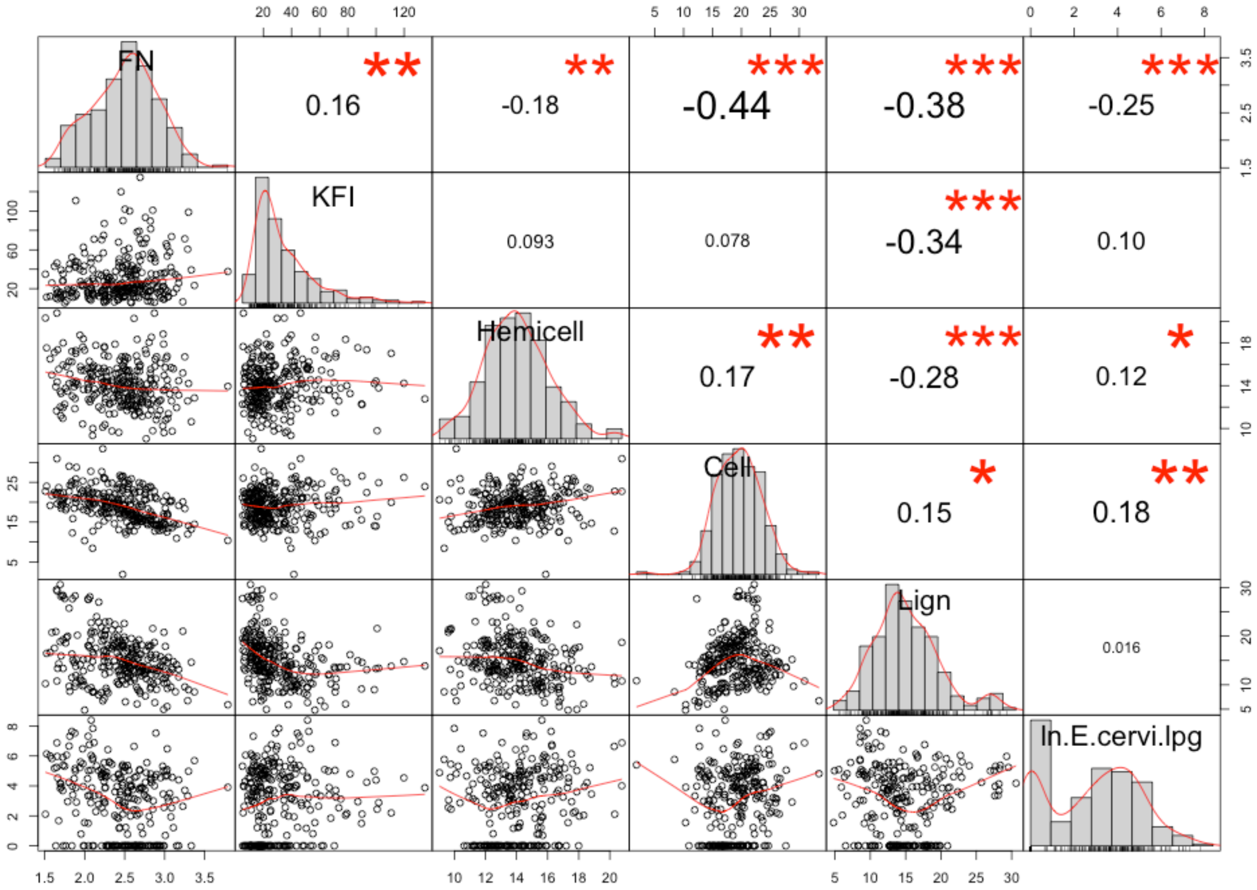


Fig. A.1.2. Correlation matrix for the two response variables – faecal nitrogen (FN, % of dry matter, DM) and kidney fat index (KFI, %) – and continuous explanatory variables related to dietary fibre components and the parasitological status of red deer. The main diagonal shows histograms of the data distribution for each variable. The lower diagonal part shows bivariate scatterplots with a fitted line (red lines). The upper diagonal part shows the absolute Pearson's correlation coefficients and associated *p*-values as stars (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). The font size of the correlation is proportional to its strength. Explanatory variable codes: Hemicell = hemicellulose (% DM); Cell = cellulose (% DM); Lign = lignin (% DM); ln.E.cervi.lpg = log transformed *Elaphostrongylus cervi* counts (larvae per gram of faeces, lpg).

Fig. A.1.3. Correlation matrix for explanatory variables associated with residual dietary fibre components (Hemicell, Cell, and Lign, all expressed as a % of dry matter, DM) and habitat composition of study areas (in a % of occupancy in relation to surface area). The lower diagonal part shows bivariate scatterplots with a fitted line (red lines). The upper diagonal part shows the absolute Pearson's correlation coefficients and associated *p*-values as stars (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). The font size of the correlation is proportional to its strength. Variable codes: Hemicell = hemicellulose; Cell = cellulose; Lign = lignin; HConFor = coniferous forests; HBlFor = broadleaved forests; HMxFor = mixed forests; HScrub = scrublands; HNatGrass = natural grasslands; HAgr = agricultural lands; HPlotGame = food plots for big game. The habitat variable "HWet = Wetlands" is not shown, because it was present in only one of the study areas.

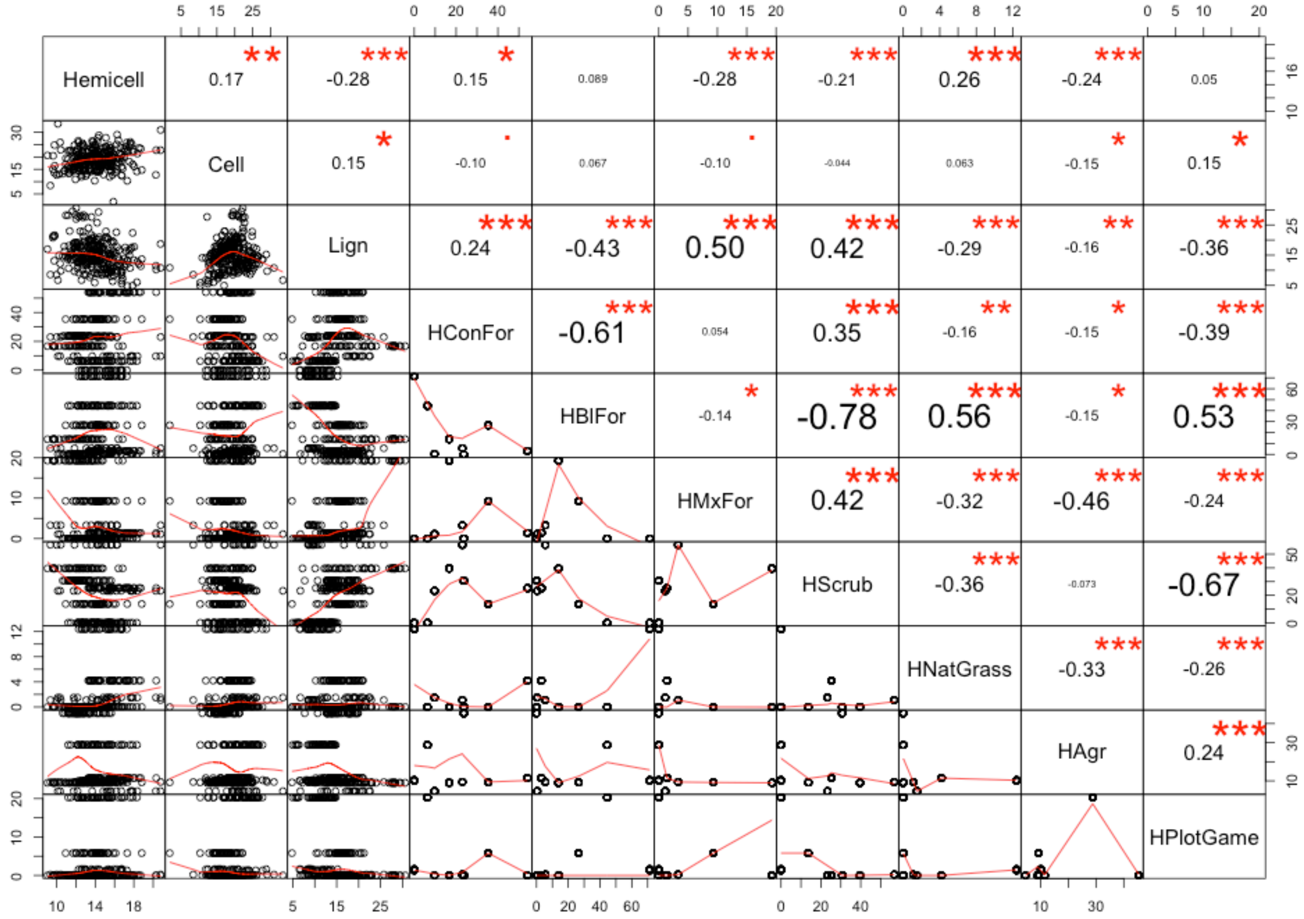


Fig. A.1.4. Correlation matrix for explanatory variables associated with vegetation productivity (measured by normalised vegetation difference index, NDVI) and accumulated rainfall (mm) in the last 30, 60 and 90 days in relation to the date on which deer were culled. The NDVI was calculated from satellite images obtained for the nearest periods (approximately 2 weeks) preceding the date on which the deer were shot. The lower diagonal part shows bivariate scatterplots with a fitted line (red lines). The upper diagonal part shows the absolute Pearson's correlation coefficients and associated *p*-values as stars (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). The font size of the correlation is proportional to its strength.

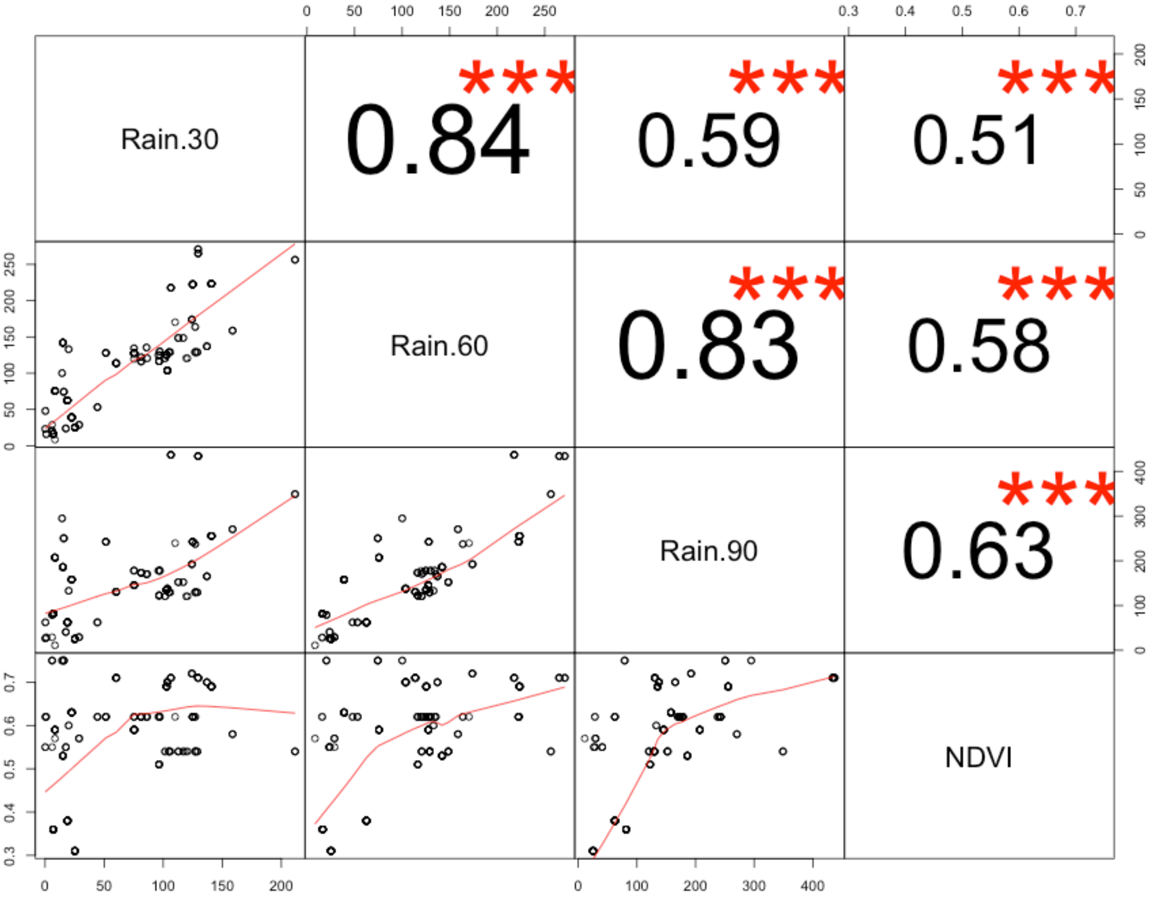


Fig. A.1.5. Correlation matrix for explanatory variables associated with temperature regimes, geographical position and topographical features of study areas. The lower diagonal part shows bivariate scatterplots with a fitted line (red lines). The upper diagonal part shows the absolute Pearson's correlation coefficients and associated *p*-values as stars (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). The font size of the correlation is proportional to its strength. Variable codes: T.min = average monthly minimum temperature (ºC); T.mean = average monthly mean temperature (ºC); T.max = average monthly maximum temperature (ºC); LAT = latitude (decimal degrees); LONG = longitude (decimal degrees); ALT.m = average altitude (meters above sea level, m.a.s.l.). Monthly temperatures were calculated for the months on which the deer were culled.

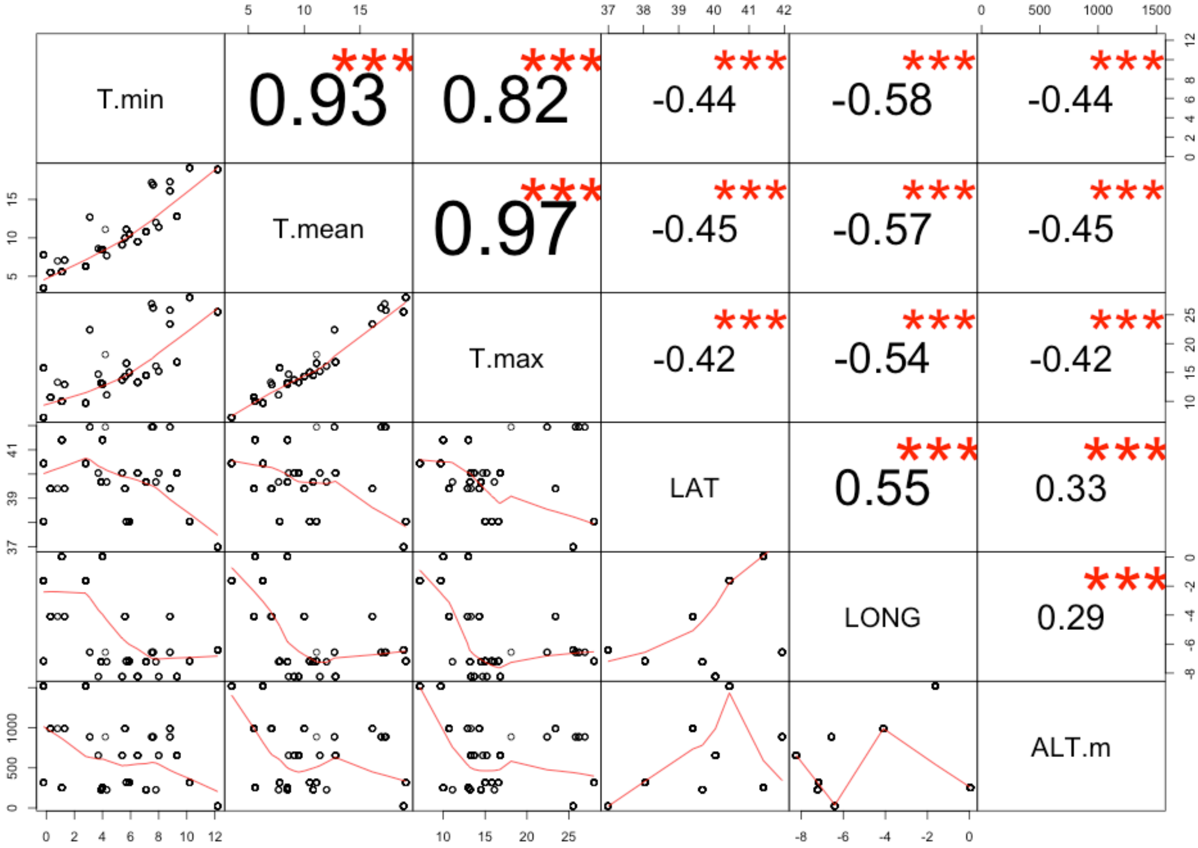


Fig. A.1.6. Correlation matrix for explanatory variables related to the relative abundance of red deer and supplementary feeding practices. The lower diagonal part shows bivariate scatterplots with a fitted line (red lines). The upper diagonal part shows the absolute Pearson's correlation coefficients and associated *p*-values as stars (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). The font size of the correlation is proportional to its strength. Variable codes: D.deer = deer density (expressed as red deer per square kilometre, Km^2); Feed.Kg.Deer = amount of food supplied per deer per year (expressed as Kg/deer/year); Feed.Kg.Km2 = amount of food supplied per surface area per year (in Kg/Km^2/year).

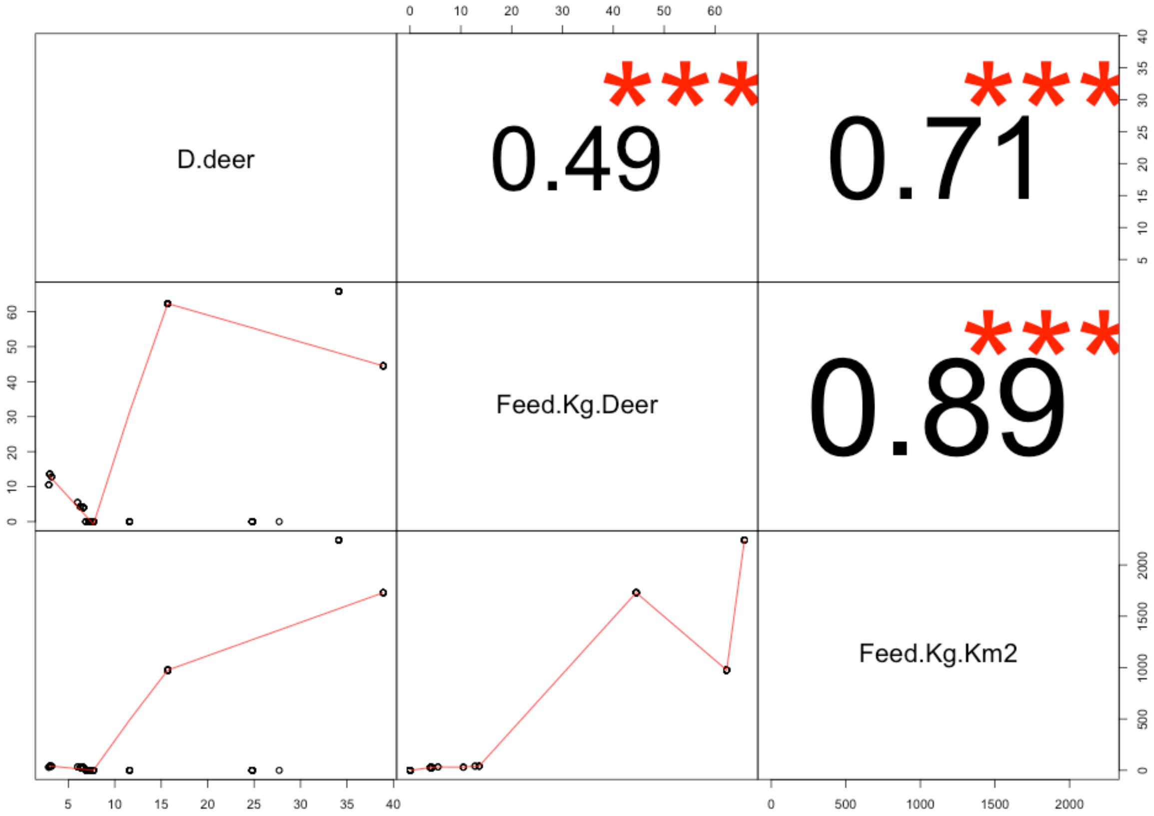


Table A.1.4. List of the best linear mixed models (LMM) obtained to describe the variation in faecal nitrogen content (FN, % of dry matter) in red deer *Cervus elaphus* in the Mediterranean environments of the Iberian Peninsula. A stepwise algorithm based on Akaike's Information Criteria (AIC) was run to help in selecting the most important predictors and find the most parsimonious models. Models are ordered according to AIC. Model parsimony increases with decreasing AIC. All LMM were fit using restricted maximum log-likelihood (REML) estimation: REML = Log($\mathcal{L}$)*(–2), where Log ($\mathcal{L}$) is the log-likelihood, *K* is the number of parameters, and ΔAIC is the difference in the AIC value of a given model in relation to the best model (on the top). Models that differ by less than 2 AIC units (ΔAIC < 2) were considered to have equivalent support (Burnham and Anderson, 2002). Both marginal and conditional coefficients of determination (R^2) were calculated for each model according to Nakagawa and Schielzeth (2013). The 'study area' was included in all models as a random factor and is indicated as [RF]. Variable abbreviations are shown in Table A.1.2.

Models: Faecal Nitrogen, FN [Linear Mixed Models]	**Interaction terms**	**Log ($\mathcal{L}$)**	**REML**	***K***	**AIC**	**ΔAIC**	***Marginal R^2***	***Conditional R^2***
FN = Hunt_season + Season + Hemicell + Cell + Lign + ALT.m + NDVI + Season*Lign + [RF]	1	– 80.0	160.0	12	184.0	0.0	0.48	0.68
FN = Hunt_season + Season + Hemicell + Cell + Lign + ALT.m + NDVI + Season*Lign + Season*NDVI + [RF]	2	– 79.5	159.0	13	185.0	1.0	0.48	0.69
FN = Hunt_season + Season + Hemicell + Cell + Lign + ALT.m + NDVI + Rain.30 + Season*Lign + [RF]	1	– 80.5	161.0	13	187.0	3.0	0.46	0.69
FN = Hunt_season + Season + Hemicell + Cell + Lign + ALT.m + NDVI + LAT + Season*Lign + [RF]	1	– 81.6	163.1	13	189.1	5.1	0.48	0.70
FN = Hunt_season + Season + Hemicell + Cell + Lign + ALT.m + NDVI + Season*Lign + ALT.m*NDVI + [RF]	2	– 81.6	163.1	13	189.1	5.1	0.44	0.60
FN = Hunt_season + Season + Hemicell + Cell + Lign + ALT.m + NDVI + Sex + Season*Lign + [RF]	1	– 82.4	164.8	13	190.8	6.8	0.48	0.68

Continuation of **Table A.1.4** (previous page)	**Interaction terms**	**Log ($\mathcal{L}$)**	**REML**	***K***	**AIC**	**ΔAIC**	***Marginal R^2***	***Conditional R^2***
FN = Hunt_season + Season + Hemicell + Cell + Lign + ALT.m + NDVI + D.deer + Season*Lign + [RF]	1	– 83.6	167.2	13	193.2	9.2	0.44	0.73
FN = Hunt_season + Season + Hemicell + Cell + Lign + ALT.m + NDVI + Rain.30 + Season*Lign + NDVI*Rain.30 + [RF]	2	– 82.6	165.3	14	193.3	9.3	0.47	0.73
FN = Hunt_season + Season + Hemicell + Cell + Lign + ALT.m + NDVI + LAT + Season*Lign + Season*LAT + [RF]	2	– 82.8	165.6	14	193.6	9.6	0.50	0.60
FN = Hunt_season + Season + Hemicell + Cell + Lign + ALT.m + NDVI + Feed.Kg.Deer + Season*Lign + [RF]	1	– 84.6	169.3	13	195.3	11.3	0.47	0.67
FN = Hunt_season + Season + Hemicell + Cell + Lign + ALT.m + NDVI + [RF]	0	– 86.8	173.6	11	195.6	11.6	0.44	0.58
FN = Hunt_season + Season + Hemicell + Cell + Lign + ALT.m + NDVI + Sex + Season*Lign + Season*Sex + [RF]	2	– 83.9	167.8	14	195.8	11.8	0.48	0.68
FN = Hunt_season + Season + Hemicell + Cell + Lign + ALT.m + NDVI + $\log_e$(E.cervi.lpg) + Season*Lign + [RF]	1	– 85.5	171.0	13	197.0	13.0	0.48	0.64
FN = Hunt_season + Season + Hemicell + Cell + Lign + ALT.m + NDVI + Age.class + Season*Lign + [RF]	1	– 84.6	169.2	15	199.2	15.2	0.48	0.69
FN = Hunt_season + Season + Hemicell + Cell + Lign + ALT.m + NDVI + Season*Lign + Season*ALT.m + [RF]	2	– 86.9	173.8	13	199.8	15.8	0.48	0.65
FN = Hunt_season + Season + Hemicell + Cell + Lign + ALT.m + NDVI + Sex + D.deer + Season*Lign + Sex*D.deer + [RF]	2	– 89.9	179.8	15	209.8	25.8	0.44	0.73

Continuation of **Table A.1.4** (previous page)	**Interaction terms**	**Log ($\mathcal{L}$)**	**REML**	***K***	**AIC**	**ΔAIC**	***Marginal R^2***	***Conditional R^2***
FN = Hunt_season + Season + Hemicell + Cell + Lign + ALT.m + NDVI + Sex + Age.class + Season*Lign + Sex*Age.class + [RF]	2	– 90.2	180.4	19	218.4	34.4	0.48	0.69

Table A.1.5. List of the best generalised linear mixed models (GLMM) obtained to describe the variation in kidney fat index (KFI, %) in red deer *Cervus elaphus* in the Mediterranean environments of the Iberian Peninsula. A stepwise algorithm based on Akaike's Information Criteria (AIC) was run to help in selecting the most important predictors and find the most parsimonious models. Models are ordered according to AIC. Model parsimony increases with decreasing AIC. All GLMM were fit using maximum log-likelihood (ML) estimation: Log ($\mathcal{L}$) = log-likelihood; *K* = number of parameters; ΔAIC = difference in the AIC value of a given model in relation to the best model (on the top). Models that differ by less than 2 AIC units (ΔAIC < 2) were considered to have equivalent support (Burnham and Anderson, 2002). The 'study area' was included in all models as a random factor and is indicated as [RF]. Variable abbreviations are shown in Table A.1.2.

Models: KFI [Generalised Linear Mixed Models: gamma distribution + log link function]	**Interaction terms**	**Log ($\mathcal{L}$)**	***K***	**AIC**	**ΔAIC**
KFI = Hunt_season + Sex + Season + NDVI + Feed.Kg.Deer + Lign + D.deer + log$_e$(E.cervi.lpg) + FN + LAT + Season*NDVI + Season*LAT + D.deer*FN + [RF]	3	– 1009.2	17	2052.4	0.0
KFI = Hunt_season + Sex + Season + NDVI + Feed.Kg.Deer + Lign + D.deer + log$_e$(E.cervi.lpg) + FN + LAT + Season*LAT + D.deer*FN + D.deer*Feed.Kg.Deer + [RF]	3	– 1009.3	17	2052.6	0.2
KFI = Hunt_season + Sex + Season + NDVI + Feed.Kg.Deer + Lign + D.deer + log$_e$(E.cervi.lpg) + FN + LAT + Season*LAT + D.deer*FN + [RF]	2	– 1010.5	16	2052.9	0.5
KFI = Hunt_season + Sex + Season + NDVI + Feed.Kg.Deer + Lign + D.deer + log$_e$(E.cervi.lpg) + FN + LAT + Season*LAT + D.deer*FN + Feed.Kg.Deer*log$_e$(E.cervi.lpg) + [RF]	3	– 1009.9	17	2053.8	1.4
KFI = Hunt_season + Sex + Season + NDVI + Feed.Kg.Deer + Lign + D.deer + log$_e$(E.cervi.lpg) + FN + LAT + Season*NDVI + Season*LAT + D.deer*FN + Sex*Season + [RF]	4	– 1009.2	18	2054.4	2.0
KFI = Hunt_season + Sex + Season + NDVI + Feed.Kg.Deer + Lign + D.deer + log$_e$(E.cervi.lpg) + FN + LAT + Season*LAT + D.deer*FN + Sex*Season + [RF]	3	– 1010.5	17	2054.9	2.5
KFI = Hunt_season + Sex + Season + NDVI + Feed.Kg.Deer + Lign + D.deer + log$_e$(E.cervi.lpg) + FN + LAT + Age.class + Season*NDVI + Season*LAT + D.deer*FN + [RF]	3	– 1008.9	20	2057.8	5.4

Continuation of **Table A.1.5** (previous page)	**Interaction terms**	**Log ($\mathcal{L}$)**	***K***	**AIC**	**ΔAIC**
KFI = Hunt_season + Sex + Season + NDVI + Feed.Kg.Deer + Lign + D.deer + $\log_e$(E.cervi.lpg) + FN + LAT + Season*NDVI + D.deer*FN + [RF]	2	– 1014.4	16	2060.7	8.3
KFI = Hunt_season + Sex + Season + NDVI + Feed.Kg.Deer + Lign + D.deer + $\log_e$(E.cervi.lpg) + FN + LAT + Age.class + Season*NDVI + Season*LAT + D.deer*FN + Sex*Age.class + [RF]	4	– 1008.0	23	2061.9	9.5
KFI = Hunt_season + Sex + Season + NDVI + Feed.Kg.Deer + Lign + D.deer + $\log_e$(E.cervi.lpg) + [RF]	0	– 1019.6	12	2063.1	10.7
KFI = Hunt_season + Sex + Season + NDVI + Lign + D.deer + $\log_e$(E.cervi.lpg) + [RF]	0	– 1020.6	11	2063.2	10.8
KFI = Hunt_season + Sex + Season + NDVI + Feed.Kg.Deer + Lign + D.deer + $\log_e$(E.cervi.lpg) + FN + [RF]	0	– 1019.3	13	2064.6	12.2
KFI = Hunt_season + Sex + Season + NDVI + Feed.Kg.Deer + Lign + D.deer + $\log_e$(E.cervi.lpg) + FN + LAT + [RF]	0	– 1019.3	14	2066.5	14.1

References

Burnham, K.P., Anderson, D.R., 2002. Model Selection and Multi-Model Inference: A Practical Information-Theoretic Approach, second edn. Springer, New York.

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

Nakagawa, S., Schielzeth, H., 2013. A general and simple method for obtaining R^2 from generalized linear mixed-effects models. Methods Ecol. Evol. 4, 133-142.

Appendix 2

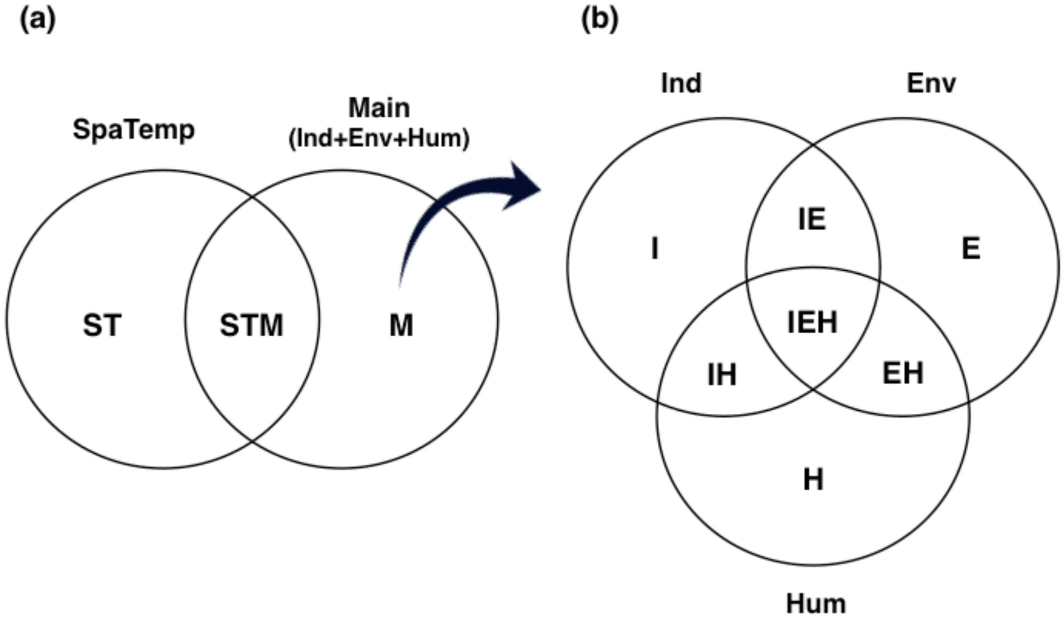
Supplementary material for **Chapter 4**

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

Table A.2.1. Results from forward stepwise analysis for model selection. The model with the lowest AIC score was considered the most parsimonious (highlighted in bold). Variables are presented by order of entry in the stepwise procedure.

Step predictors/Models	AIC	ΔAIC
[Null model] = 1 + Study_area (random factor; [RF])	3208.8	130.9
[RF] + Hunt_season	3198.1	120.2
[RF] + Hunt_season + TEMPavg_24h	3195.5	117.6
[RF] + Hunt_season + TEMPavg_24h + LAT	3190.0	112.1
[RF] + Hunt_season + TEMPavg_24h + LAT + WULAI	3184.5	106.6
[RF] + Hunt_season + TEMPavg_24h + LAT + WULAI + SUPPL	3173.6	95.7
[RF] + Hunt_season + TEMPavg_24h + LAT + WULAI + SUPPL + Season	3149.1	71.2
[RF] + Hunt_season + TEMPavg_24h + LAT + WULAI + SUPPL + Season + Age_class	3120.3	42.4
[RF] + Hunt_season + TEMPavg_24h + LAT + WULAI + SUPPL + Season + Age_class + MASS_HUNT	3112.3	34.4
[RF] + Hunt_season + TEMPavg_24h + LAT + WULAI + SUPPL + Season + Age_class + MASS_HUNT + D_deer	3111.8	33.9
[RF] + Hunt_season + TEMPavg_24h + LAT + WULAI + SUPPL + Season + Age_class + MASS_HUNT + D_deer + SUPPL*D_deer	3106.2	28.3
[RF] + Hunt_season + TEMPavg_24h + LAT + WULAI + SUPPL + Season + Age_class + MASS_HUNT + D_deer + SUPPL*D_deer + PRECIP_m1	3107.5	29.6
[RF] + Hunt_season + TEMPavg_24h + LAT + WULAI + SUPPL + Season + Age_class + MASS_HUNT + D_deer + SUPPL*D_deer + PRECIP_m1 + Season*TEMPavg_24h	3101.6	23.7
[RF] + Hunt_season + TEMPavg_24h + LAT + WULAI + SUPPL + Season + Age_class + MASS_HUNT + D_deer + SUPPL*D_deer + PRECIP_m1 + Season*TEMPavg_24h + Sex	3097.6	19.7
[RF] + Hunt_season + TEMPavg_24h + LAT + WULAI + SUPPL + Season + Age_class + MASS_HUNT + D_deer + SUPPL*D_deer + PRECIP_m1 + Season*TEMPavg_24h + Sex + Sex*Age_class	**3077.9**	**0**

Fig. A.2.1. Scheme illustrating the partition of variation explained by the final model in a two-step procedure: (a) determination of the amount of variation of the final model that was explained independently and jointly by the 'spatio-temporal' component in relation to the remaining set of variables, and (b) calculation of the variation explained by the individual (Ind), environmental (Env) and human (Hum) components, and their intersections. See the text below for details.



In the first step (a), 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 (*sensu* Nakagawa and Schielzeth, 2013). The R^2 values obtained for each model were subjected to the following subtraction operations:

- ST = (SpaTemp+Main) – (Main), which corresponds to the amount of variation explained exclusively (*i.e.*, independently of other components) by the 'spatio-temporal' component;
- M = (SpaTemp+Main) – (SpaTemp), which refers to the amount of variation explained exclusively by the 'main' components;
- STM = (SpaTemp+Main) – ST – M, which corresponds to the amount of variation attributable to the intersection of two components.

In the second step (b), 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 (*sensu* Nakagawa and Schielzeth, 2013). The following subtraction rules between the R^2 values were applied to separate out the fractions of variation explained exclusively by each component, as well as by their intersections:

- I = (Ind+Env+Hum) – (Env+Hum), which corresponds to the amount of variation explained exclusively by the individual component;
- E = (Ind+Env+Hum) – (Ind+Hum), which corresponds to the amount of variation explained exclusively by the environmental component;
- H = (Ind+Env+Hum) – (Ind+Env), which corresponds to the amount of variation explained exclusively by the human component;
- IE = (Ind+Env+Hum) – Hum – I – E, which refers to the amount of variation explained attributable to the intersection between the individual and environmental components;
- IH = (Ind+Env+Hum) – Env – I – H, which refers to the amount of variation explained attributable to the intersection between the individual and human components;
- EH = (Ind+Env+Hum) – Ind – E – H, which refers to the amount of variation explained attributable to the intersection between the environmental and human components;
- IEH = (Ind+Env+Hum) – E – H – I – EH – IH – EI, which refers to the amount of variation explained attributable to the intersection between the individual, environmental and human components together.

References

Nakagawa, S., Schielzeth, H., 2013. A general and simple method for obtaining R^2 from generalized linear mixed-effects models. Methods Ecol. Evol. 4, 133-142.