UNIVERSITAT DE BARCELONA

Diet composition and habitat use in red deer (*Cervus elaphus*) populations in the northeastern iberian peninsula: relations with phenotypic plasticity in dental roots, group ethology and stress hormones

Araceli Gort Esteve

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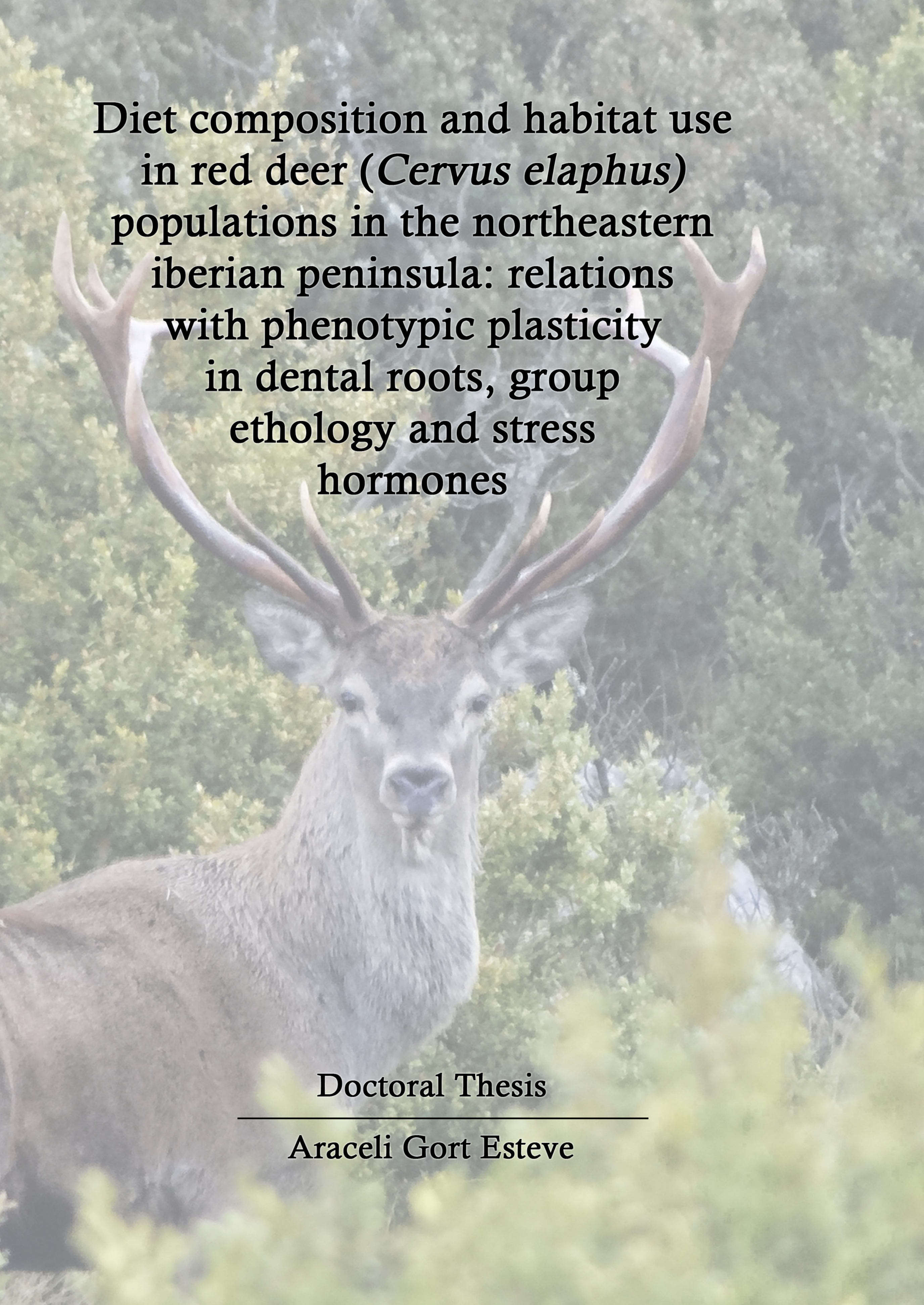

Diet composition and habitat use in red deer (*Cervus elaphus*) populations in the northeastern iberian peninsula: relations with phenotypic plasticity in dental roots, group ethology and stress hormones

Doctoral Thesis

Araceli Gort Esteve

DIET COMPOSITION AND HABITAT USE IN RED DEER (*CERVUS ELAPHUS*) POPULATIONS IN THE NORTHEASTERN IBERIAN PENINSULA: RELATIONS WITH PHENOTYPIC PLASTICITY IN DENTAL ROOTS, GROUP ETHOLOGY AND STRESS HORMONES

Memòria presentada per **Araceli Gort Esteve** per optar al grau de doctora per la Universitat de Barcelona. Doctorat en Ecologia, Ciències Ambientals i Fisiologia Vegetal del Departament de Biologia Evolutiva, Ecologia i Ciències Ambientals.

Araceli Gort Esteve

El **Dr. Joan LLuis Riera Rey**, director i tutor, i **Dr. Jordi Ruiz Olmo**, director, certifiquen la realització de la tesis sota la seva direcció.

Joan Lluis Riera Rey Jordi Ruiz Olmo

Barcelona, 20 de juny de 2023

UNIVERSITAT DE BARCELONA

«…behavioral and ecological studies of freeliving ungulates have only begun to blossom in the last decade, while the last vestiges of wilderness are coming under man's control; the day is in sight when the last natural population of ungulates will become a managed one.»

Geist, V. 1974.
On the relationship of social evolution and ecology
in ungulates. *American zoologist*, 14(1), 205-220.

AGRAÏMENTS

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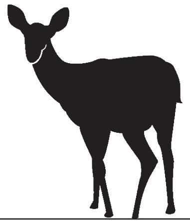
CONTENTS

ABSTRACT



Deer populations are increasing in recent times. One of the main causes is the abandonment of agriculture and extensive livestock farming. This thesis focuses on expanding our knowledge about the biology of red deer (*Cervus elaphus*) on the following aspects: diet composition and habitat use, phenotypic plasticity in dental roots, group ethology, and stress.

First, we determined the composition of the diet and the habitat use in order to help properly manage these populations and conserve the habitats that are newly available to them. The results indicated that the population of Axial Pyrenees were on average more grazers and the one of the Pre-Pyrenees more browsers. We also found that there was no seasonal pattern in the Axial Pyrenees. In the case of the Pre-Pyrenees, the consumption of woody plants was higher for females, which has not been found in the literature. Parallel to this study, we analysed the movements of 7 red deer using GPS tracking collars. The differences found in diet composition and habitat use between the two areas were reflected in their movements. The individuals from the Axial Pyrenees descend in unfavourable seasons and ascend in the favourable ones. These oscillations were due to the low food availability. In the Pre-Pyrenees, we did not observe such altitudinal migration. On the other hand, we found similar results of habitat use in

both studies, confirming that in Axial Pyrenees the main habitat use was as a refuge and in Pre-Pyrenees was for feeding, which was related to the harshness of the climate in the northernmost area.

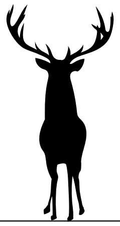
Our second objective was to analyse the influence of several factors, including sex, origin and lineage of deer populations, and type of habitat, on the prevalence of supernumerary roots in molars. The highest prevalence of supernumerary roots found in deciduous teeth was in pm_3 (14%), and in permanent teeth in M_1 (3%). We found significant differences between areas, lineages and geology. We suggest that the high prevalence of supernumerary roots in M_1 may be related to the wear caused by a grazer condition. Thus, the Axial Pyrenean population had the higher prevalence in agreement with a mostly grazing population. Contrarily, we associated a high prevalence in deciduous teeth as a response to a hard diet, a dry climate condition and a harsh weaning period, with the Pre-Pyrenees, which is a browser population, as one of the populations with this higher prevalence. Additionally, we found that individuals that consumed more graminoids and fewer woody species also showed better body condition, which we attributed to a higher nutritional quality of graminoids.

The third objective was pioneering in comparing the size and type of deer groups in five ecologically distinct areas simultaneously. Our results showed that deer group size varied with food availability, with large groups forming in unfavourable periods and small groups forming in favourable periods. On the other hand, the values of mixed group size found during the rutting season are among the lowest reported, and one of the reasons attributed to this is that, being a favourable period, individuals have prioritized feeding over reproduction and have dispersed due to the abundance of food everywhere.

In the last objective focused on hormone stress levels, our results indicated that prior climatic conditions best explained the variations of cortisol within seasons and among years. We also found differences between adult males and the other individuals in cortisol and T3 hormones. We found neither a contribution of nutritional stress to physiological stress nor an effect of the ecotourism influx in this population.

Our results highlight the high adaptability of the red deer to ecosystems differing widely in food quality and availability as well as climate conditions. Food and climate stand out as two of the main factors determining the ecology in these predator-free ecosystems.

INTRODUCTION



Wild ungulate populations have increased in abundance and distribution in most countries over the last 50 years (Gill, 1990). The red deer (*Cervus elaphus*) is a Eurasian species currently found from Europe to Central Asia, including the islands of Corsica, Sardinia, and the Maghreb (Geist, 1998). There have also been some introductions to North Africa. In 2005, the population was counted at 2.4 million (Lovari et al., 2018). This species is the largest herbivore that can be found in the Iberian Peninsula. In Spain, although there are no reliable census data for the entire territory, it is estimated that the population exceeds 300,000 individuals (Carranza, 2017).

The red deer, comes from the area of Kyrgyzstan and northern India. From this area, it expanded to the west (Europe) and to the east (Asia) (Ludt et al., 2004). Geist and McShea (1999) provide a list of subspecies of red deer with a total of 22, among which the Iberian red deer (*Cervus elaphus hispanicus*) was defined as the subspecies present in the Iberian Peninsula. However, studies based on mitochondrial DNA have determined that in Europe, there are only three lineages of *Cervus elaphus* from the refuges of the last glaciation and divide them into western Europe, eastern Europe, and Mediterranean (Ludl et al., 2004; Skog et al., 2009). These studies do not differentiate the Iberian red deer from the rest of the deer subspecies in western Europe. More recent studies provided support for this subspecies based on the analysis of CR sequences (control region sequences) of mitochondrial DNA and nuclear DNA. Using this methodology, two distinct lineages were identified in the Iberian Peninsula: the central-eastern lineage and the south-western lineage (Fernández-García et al., 2014; Carranza et al., 2016). The most recent genetic study carried out in the Iberian Peninsula concluded that there is a hybridization zone in the Pyrenees between the European and the Iberian subspecies (Perez-Gonzalez et al., in press). Externally, the Iberian red deer can be distinguished because adults are brown-reddish with lighter tones on the belly area that continue to the bottom of a short tail, while the non-Iberian red deer has a darker brown coat, a mane in the neck area, and a larger white anal shield (Carranza et al., 2016).

Even though the red deer has always been a fundamental piece in big game, both for its culinary and trophy value, and the antlers of the males are highly prized trophies, its conservation status according to the Red List is of minor concern (Lovari et al., 2018). These values, together with the increase in ecotourism for the observation of wild species, bring economic and social benefits to the populations where this species is found (Garde et al., 2010; Carranza, 2017).

Diet composition and habitat use

Deer are one of the major drivers of vegetation composition and structure (Gill, 1990). Knowing their diet composition is crucial for the conservation of their populations and of the habitats they use. The study of diet is also essential to understand some of their behavioural and physiological changes.

Habitat selection is considered an optimization process that not only involves the availability of food, but also population density, body size, competitors, predators, and topography (Morrison et al., 1998). Deer live in heterogeneous areas characterized by a landscape consisting of a mosaic of different types of forest, meadows, and crops. Some studies show that they prefer to feed in the clearings of the forest rather than the interior, since there is more forage and young trees (Kuijper et al., 2009). There are also some differences in diet preferences between the sexes. For example, in Mediterranean

areas, males ingest more food of lower quality in winter, and the quality of the diet decreases in both sexes in summer (Alvarez, 1999). It has also been found that males feed more at night than females (Clutton-Brock et al., 1982). During the rut period, males select more grasses and dicotyledons which are plants with more cellulose, and fewer shrubs than females and young, who prefer plants with more nitrogen, tannins, and lignin (Miranda et al., 2012). Living in heterogeneous areas allows deer to move during the year following the green wave, since the available food varies seasonally (Pépin et al., 2008a).

Diet and use of habitat also depend on population density. Deer populations at high densities can transform habitats and influence the structure and plant communities (Côté et al., 2004; Gill and Morgan 2009; Martin et al., 2010). In most cases, high deer densities lead to a simplification in the number of species in the area, both plant and animal, since their effect is multi-trophic (Martin et al., 2010). Deer are direct competitors of many species of invertebrates and small vertebrates for plant biomass, although some effects are indirect as they can alter the landscape by changing plant species (Flowerdew and Ellwood, 2001). In the cases that deer cause a decrease in insect populations, it indirectly affects the populations of birds that feed on them. In addition, with high deer densities, bird populations are also affected by increased difficulty in building their nests due to a decrease in leaf mass, as well as predation of some of these nests (Fuller, 2001). Specifically, in a study carried out in forest areas in Spain, red-legged partridge densities decreased as deer densities increased because deer negatively affected the number of hemipterans, one of the main foods of red-legged partridge, and the biomass and height of pastures (Guerrero-Casado et al., 2014). On the other hand, they can also be beneficial for insects that feed on pollen and nectar since they open clearings in the forest and allow species with flowers to grow, which is favourable to them (Stewart, 2001). Osteophagy should be highlighted as a method to obtain natural mineral supplements; males consume antlers after their growing season, and females after parturition. During calving, there is an increase in antler consumption by both sexes (Gambin et al., 2017).

The diet of red deer varies seasonally depending on food availability, and at the same time, food availability depends mostly on climatology. For this reason, significant differences in the feeding of deer populations located in ecologically differentiated areas can be expected.Garin et al. (2001) proposed three feeding patterns for Europe: (i) the oceanic model, with preponderance of the herbaceous and Ericaceae woody plants, (ii) the center-European model, with the alternation of herbaceous and woody plants, and (iii) the Mediterranean model, with a preponderance of woody species all year round. These differences in diet composition between areas could be the triggers for morphological changes and increased variability in phenotypic plasticity within species.

Phenotypic plasticity

Natural selection, resulting from different genotypes and epigenetic factors, is responsible for population divergence and speciation (Flueck and Smith-Flueck, 2011; Price et al., 2023). One of the most well-known examples is Darwin’s finches (Darwin, 1859). But differences between populations can also arise from phenotypic plasticity. Phenotypic plasticity refers to the ability of a given genotype to express different phenotypes in response to environmental inputs in different conditions (West-Eberhard, 2003; Sultan, 2021). This ability is increasingly recognized for its contribution to evolution.

Food availability and variability are some of the primary environmental inputs that could alter an individual's morphology. In the case of food availability, both intraspecific and interspecific competition can play an important role. For example, Wintzer and Motta (2005) studied the phenotypic plasticity of hatchery-reared Florida largemouth wild bass (*Micropterus salmoides floridanus*), which developed morphological changes in their jaw apparatus, including a more fusiform head and elongated jaw structures, during skull development to capture elusive prey, unlike their wild counterparts that fed on inert pellet food. Other studies have demonstrated that diet can affect jaw morphology, such as in the European eel (*Anguilla anguilla*), in which De Meyer et al., (2016) found that hard feeders develop a broader head and a larger adductor mandibulae region, and similarly in cichlid fish (*A. alluaudi*) (Muschick et al., 2011) and in orange spotted sunfish (*Lepomis humilis*) (Hegrenes, 2001). In mammals, studies have shown that hard diets can change biomechanical loading on the postweaning development and structure of the hard palate in rabbits (Menegaz et al., 2009) or increase the length of the anterior portion of the gut in the rodent *A. azarae* (del Valle et al., 2006).

Cervus elaphus is characterized by its ability to adapt to a wide range of woodland-related environments in the northern hemisphere, resulting in a large number of distinct populations and subspecies (Di Stefano and Petronio, 2021).However, we have not found studies that focus on the phenotypic plasticity of morphological traits in this species due to changes in diet. Studies have mainly focused on changes in the phenology of its life history traits, and the influence of climatic variation and food availability on life history traits in deer populations is well documented (Pelaez et al., 2017; Ayotte et al., 2019; Froy et al., 2019; Stenseth et al., 2002). For example, Nussey et al., (2005a and 2005b) found in the deer population of the Isle of Rum significant variation between female deer in their average calving dates and in their individual plastic responses of calving date to autumn rainfall, and they also found a relationship between offspring birth weight and spring temperature. On the other hand, not only does the availability and variability of food affect morphological or life history traits, but it can also affect behavioural aspects, as group ethology.

Group ethology

The most serious damages to the environment caused by ungulates are due to large aggregations. Deer is a gregarious species that lives in hierarchical groups. Group formation is a survival strategy for many species of ungulates, although at the same time, it can generate a cost to the members of the group individually. The pattern found in the literature shows that in spring, summer, and winter, deer's behaviour is based on sexual segregation, while in autumn, during the rut, they form harems (Appleby, 1983; Carranza and de Reyna, 1987; Carranza and Valencia, 1992; Haskell et al., 2010; Carranza, 2017). However, do we always find the same pattern? Does it change between ecologically distinct areas or between subspecies? Or depending on habitat selection?

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The great sexual dimorphism that this species presents is one of the main factors to consider when studying group ethology. Males present antlers and are heavier and more corpulent than females. Antlers are the most distinctive feature between sexes and their formation and development is key in the study of this species. The antlers of deer are formed annually, fall at the beginning of spring and start growing again at the end of this season. *The Cervidae* family contains the only mammals that have not lost the ability to regenerate lost or damaged body parts. Antler growth is closely related to testosterone production and is one of the fastest cases of organogenesis in the animal kingdom (Price et al., 2005). During its growth, it is covered with velvet, and in the summer, when it reaches its maximum growth, the males rub it against the trunks of the trees to remove this velvet and leave the antlers ready for the fights that will occur during the rut. The first antler begins to develop on average between 10 and 11 months of life and is made up of two unbranched trunks or rods (Gaspar-Lopez et al., 2007). Its growth is positively related to the amount of protein in breast milk and negatively to the date of the start of growth (Gaspar-Lopez et al., 2008). From the second year, different types of spikes develop on each trunk. The ones closest to the skull are called fighters; then, we find the central spikes, and in the upper part, called the crown, we find a variable number. Between the ages of 7 and 8, the antler is at its peak of development (Carranza, 2017). On the other hand, its size and complexity are associated with testicular size and sperm velocity, thus the antler of males can indicate its fertility, which may influence a female's decision to mate (Malo et al., 2005). Body and antler growth are highly related to environmental conditions and population density, not so much to genetics, with which deer characteristics can vary greatly between populations (Azorit et al., 2002b; 2003; Kruuk et al., 2002; Rodriguez-Hidalgo et al., 2010). The mineral composition of the antler reflects the composition and quality of their diet (Estevez et al., 2009).

The reproductive strategy of red deer is polygyny, and the mating season, known as the rut, occurs in autumn. The gestation period typically lasts between 235 and 240 days, but females adjust their reproductive timing based on climatic conditions to ensure lactation occurs during the most favourable time (Carranza, 2017). Births usually take place in May and June. High temperatures during gestation are linked to an advanced parturition period, and a high rainfall regime is associated with lower juvenile survival rates and fertility (Stopher et al., 2014). Female red deer have a fertility rate of about 0.69 fawns per year, and they can produce one offspring per year from the age of 3 until death (Santiago and Martín, 1997). Most females have their first offspring at 3 years old and begin the senescence stage at 9. Early reproduction can hasten the onset of senescence (only 5.7% of females continue reproducing after the age of 17) (Nussey et al., 2006). In males, high population density is the primary cause of senescence (Mysterud et al., 2001).

Fawn birth weight is also density-dependent, and fawns born in high-density populations typically weigh more to minimize the risk of mortality due to intraspecific competition during winter (Albon et al., 1983). Additionally, the birth weight of males can impact their future reproductive success, and dominant females with better fitness tend to give birth to a higher percentage of males than females (Kruuk et al., 1999; Bonenfant et al., 2003; Landete-Castillejos et al., 2004). Male fertility can also affect the sex of offspring (Gomendio et al., 2006). The absence of a mother during a fawn's first year of life increases natural mortality rates and can negatively affect their future reproduction and physical condition (Andres et al., 2013).

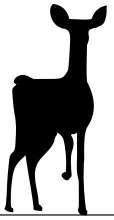
Recent studies have suggested that the maximum life expectancy of wild red deer is up to 19 years for females and 16 years for males(Carranza, 2017). However, in captivity, this value can be higher (Gort-Esteve, et al,. unpublished data). In the study area, hunting is the main cause of mortality, although landslides and heavy snowfalls can also result in a high number of casualties in high mountain areas. In other parts of Europe, red deer is one of the primary prey species of wolves and lynx (Okarma et al., 1997; Okarma, 1984). Chronic stress can also cause mortality, for this reason it is important to know what the main stressors are, as discussed next.

Stress hormones and stressors

Study the individuals stress is essential for the conservation of species. Chronic stress, long-term/prolonged responsiveness of the stress system can reduce individual fitness by turning off growth and development, suppressing the immune system and inhibiting biological functions such as reproduction, or even cause death (Sapolsky, 2000a; Monclús et al., 2017; Möstl and Palme, 2002). Stressors activate the hypothalamic-pituitary-adrenocortical axis (HPA), resulting in the release of glucocorticoid hormones (GC) (cortisol in most mammals) into the bloodstream (Norris 2006; Hadley and Levine, 2007). For this reason, GCs are frequently used to study the stress response (Pacak and Palkovits, 2001). Chronic stress reduces the number of these receptors, resulting in a less efficient feedback signal (De Kloet and Reul. 1987). Glucocorticoid hormones also regulate glucose homeostasis (Kuo et al., 2015). On the other hand, thyroid hormones respond to nutritional deficits or overfeeding conditions, and for this reason are used as food stress indicators (Silva, 2006; Wasser et al., 2010; Douyon and Schteingart, 2002; Kitaysky et al., 2005). These hormones have been identified as a potentially viable index of body condition in ungulates due to their correlation with body fat (Bishop et al., 2009a). In our study, we aim to understand the causes of the stress oscillations throughout the year in a wild red deer population of the Pre-Pyrenees. The main stressors that we identified that can affect our study population were climatology, food availability, social interactions and ecotourism.

To not to cause an additional stress capturing the individuals (Arroyo et al., 2013), we used a non-invasive method. GC, thyroid hormones and their metabolites can be accurately and reliably measured in faeces, which did not require individuals to be captured and handled. (Huber et al., 2003; Touma and Palme, 2005; Wasser et al., 2010; Turpeinen and Hämäläinen, 2013). These hormones can be studied using other methodologies depending on the study objectives. The analysis of GC in serum and saliva is useful for studying acute changes or sharp peaks of stress (Negrao et al., 2004; Hernandez et al., 2014), while long-term stress can be studied by the measurement of GC in hair (Tallo-Parra et al., 2015; Salas et al., 2016).

OBJECTIVES



This thesis focuses on expanding our knowledge about the ecology of red deer with four objectives: (i) diet composition and habitat use, (ii) phenotypic plasticity in dental roots, (iii) group ethology, and (iv) stress. We compared the diet composition in two areas, one in the Axial Pyrenees and one in the Pre-Pyrenees, which differ ecologically. We expected to find differences in the red deer population's diet composition not only between areas, but also between seasons and sexes. In addition, we studied the habitat use by examining whether the proportions of immediate habitats around the capture points predicted diet composition and if they were used for foraging.

Closely related to diet, we compared the prevalence of supernumerary roots in seven populations across the Iberian Peninsula, as there could be phenotypic plasticity related to the hardness of the food in this character. We also analysed its association with the sex, origin, and lineage of deer populations and the type of soil.

Favourable and unfavourable moments during the year, related to climate and food availability, may result in alterations in the group ethology of red deer populations. For this reason, we studied the annual changes in group size and composition in five different ecosystems of the northeaster of the Iberian

Peninsula. We hypothesized that areas of food richness in unfavourable moments could prompt the aggregation of individuals, and contrarily, that abundant and well-distributed food in favourable seasons could result in a dispersion of individuals. We also expected larger groups during the rutting season due to the aggregation of the individuals to the formation of harems.

The last challenge was to determine whether climate, diet, social interactions, and ecotourism caused stress (as measured by cortisol and thyroid hormones in faecal samples) in populations of red deer in the Pre-Pyrenees. We expected diet and ecotourism during the rut to be the main stressors in this population.

By reaching these objectives, we hope to increase knowledge about the biology of the red deer and contribute to the management and conservation of its populations worldwide.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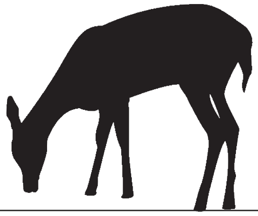
CHAPTER 1: DIET COMPOSITION AND HABITAT USE BY RED DEER IN TWO REWILDED MOUNTAIN AREAS



ABSTRACT

The abandonment of extensive livestock farming has led to an increase in wild populations taking advantage of the vacuum in the trophic level of the habitats previously occupied by herds. Determining the composition of the diet is crucial to understand the level of adaptation of these new populations, as well as the potential impact on the new occupied habitats. The composition of the diet can also determine the type of the habitat use. This study compared the diet composition between two ecologically differentiated mountain Pyrenean areas, as well as between seasons and sexes. Additionally, a possible relationship between the habitats around the capture point and the diet composition was also sought. The results indicated a significant difference between the two areas, with a higher consumption of graminoids in the Axial Pyrenees and woody plants in the Pre-Pyrenees. On the other hand, the results showed that there is no seasonal pattern in the Axial Pyrenees, while it does exist in the Pre-Pyrenees. Significant differences were also found between the consumption of woody plants and herbaceous plants between sexes. In the case of the Pre-Pyrenees, the consumption of woody plants was higher for females, which has not been found in the literature, where it is concluded that

males are more browser. Finally, differences were also found between the two areas in habitat use. In the Axial Pyrenees, the main use of the habitat is for refuge, whereas in the Pre-Pyrenees, it is for feeding, which is related to the harshness of the climate in the northernmost area.

INTRODUCTION

European landscapes were shaped by herbivory of wild megafauna in pre-agricultural times and by livestock and farming ever since. Yet during the last century humans have dramatically accelerated alterations and loss of biodiversity, with habitats becoming disturbance-dependent (Pereira and Navarro 2015). The decline and abandonment of extensive livestock farming and agricultural land has provided an ecological opportunity for wildlife to spread through these “new” habitats (Acevedo et al., 2011). This implies that wildlife may be changing their diet composition and habitat use to adapt to these new opportunities (Svenning et al., 2016; Iacolina et al., 2020). Rewilding is a new methodology to preserve habitats where extensive livestock farming and land agriculture have decreased by returning these managed areas back to the wild, with passive or active actions (Guillson et al., 2011; Lorimer et al., 2015; Pereira and Navarro, 2015; Corlett, 2016; Perino et al., 2019).

The study of diet composition and food preferences is key to know if wildlife is producing significant impacts on habitat and community structure (Zielke et al., 2019). In most of species, as in the case of deer, diet composition varies depending on a wide range of factors such as habitat, season or sex (Garin et al., 2001; Gebert and Verheyden-Tixier, 2001; Krojerová-Prokešová et al., 2010; Azorit et al., 2012c; Miranda et al., 2012a). For that reason, two nearby populations could present large differences in their diet composition. In highly seasonal environments, both plant quality and available biomass may act as dietary constraints (Zweifel-Schielly et al., 2012). For example, in a Mediterranean climate, summer is the most unfavorable season, as drought causes a shortage of food and water. Contrarily, in high mountain climates, the most unfavorable seasons are winter and early spring because of the snow cover and low temperatures, which prevents the animals to reach the food and also reduces the food availability (Álvarez and Ramos, 1991;

Bugalho and Milne, 2003; Suter et al., 2004). In the unfavorable seasons the large herbivore diet composition changes to adapt to poor conditions and the consumption of woody plants is usually increased (Garin 2001; Bugalho and Milne, 2003). These changes in the diet composition are also observed when the forage availability implies seasonal movements between high and low elevations (Zhang et al., 2013). Sex dimorphism in red deer also implies differences in feeding. Due to their smaller size, females cut grass at a lower height than males, and that is a disadvantage for males (Clutton-Brock et al., 1982). Compensating for this, in Mediterranean ecosystems, males tend to be browsers more often than females (Azorit et al., 2012c). The reason is, in part, because their larger size provides them with the ability to reach parts of bushes or trees that females cannot reach (Bugalho et al., 2001). In addition to these different morphological or size capacities, in general it has been shown that males and females select the habitat differently to the improve their feeding efficiency (Stewart et al., 2011), which also implies differences in the diet. In Axial Pyrenees males use higher habitats than females, which implies fewer habitats in which to take refuge, since there are fewer trees, but a greater availability of herbaceous plants, since there are more parts of high mountains (Gort-Esteve et al., unpublished data).

Knowing the relation between diet composition and habitat use is fundamental to improve wildlife management and habitat conservation. Without the intervention of farmland activities, landscape change may create the right conditions for higher wildfire risk, due to fuel accumulation associated with increasing forest and shrub cover (Navarro et al., 2015). This is particularly worrisome in areas with low tree density, such as some Mediterranean areas. In Mediterranean rangelands, the probability of shrub encroachment in the open areas is very high, as shrubs are favored due to seed limitation in tree species by predatory pressure over oak acorns and deficient abiotic conditions, such as poor soils (Acácio et al., 2007). In contrast, in landscapes with high forest density, as high mountain areas, forests can expand, increasing the forest mass and, in addition, reducing habitat heterogeneity (Proença and Pereira 2013). Heterogeneous landscapes with a diverse mosaic of habitats after farmland abandonment can be maintained by an assisted process, such as prescribed fires or wild herbivore reintroduction (Navarro et al., 2015). But it can also be a natural process driven by the comeback of large wild herbivores, which, at the same time, will have a direct impact on the distribution and use of habitats (Vera 2000; Birks 2005). Knowing how wildlife use these habitats is crucial to preserve them. Deer tend to use open areas, like grasslands or forest clearings, to forage, and closed habitats, like scrublands, to rest or take refuge (Borkowski and Ukalska, 2008; Bonnot et al., 2013; Alves et al., 2014; Laguna et al., 2021). Forests provide both food and cover, thus representing an attractive and important habitat for deer populations (Borkowski and Ukalska, 2008). Although in some cases, in protected areas, where human disturbances were highly reduced, deer mainly selected grassland and meadows (Laguna et al., 2021), or like in areas with an agricultural land, where deer also selected grasslands over other habitats (Lande et al., 2014). Consequently, it is thus essential to study the use of habitats by wildlife to apply the correct management at each area.

The goal of this study was to compare diet composition by red deer populations in two nearby mountain areas, the Axial Pyrenees and the Pre-Pyrenees. These areas differ widely in topography, maximum height and altitudinal range, climate and habitat composition. Therefore, we expected them to differ in diet composition. We also expected to find seasonal differences in each area. Specifically, we expected a higher percentage of woody plants in the diet during the hard seasons due to low forage availability: winter (and may be spring) at the Axial Pyrenees and summer (and may be winter) at the Pre-Pyrenees. In addition, we expected differences by sex, with the male diet composition higher in woody species year-round, as males tend to be more browsers than females. Moreover, we assessed if the proportions of immediate habitats around the capture points were could predict or affect the diet composition. An affirmative answer to this question would suggest a habitat selection driven by foraging, whereas a lack of relationship would suggest that other habitat uses may be prominent in driving habitat selection.

METHODS

Study areas

The present study was carried out in two areas in the northeast of the Iberian Peninsula, the National Hunting Reserve of Alt Pallars, in the Axial Pyrenees, and the National Hunting Reserve of Boumort, in the Pre-Pyrenees (Figure 1).

The National Game Reserve of Alt Pallars, in the Axial Pyrenees (42° 31’ 18’’ N, 1° 11’ 18’’ E), covers 81,772 ha and is one of a few high mountain game reserves in Spain, with the highest peak at “Pica d’Estats” with 3,143 m, and a wide altitudinal range (2,243 m, from the minimum altitude, at 900 m). Climate at the area is Atlantic and subalpine, with very cold temperatures and extensive snow cover in winter, which remains into spring, what makes these two seasons the hardest. Rainfall is often over 800-1,000 mm/m^2 per year. Above the treeline, grasslands, rocks and screes dominate the landscape. Fir (*Abies alba*) and black pine (*Pinus uncinata*) forests are found in the subalpine stage, and red pine (*Pinus sylvestris*) and deciduous forests composed mainly by oak (*Quercus petrea*), hazel (*Corylus avellana*), birch (*Betula pendula*), tremble (*Populus tremula*) or ash (*Fraxinus excelsior*), dominating the mid-mountain ecosystem, and forming extensive forests. The mid-mountain landscape is completed by rocky outcrops and extended formations of Pyrenean broom (*Genista balansae*) and grasslands. The global grassland availability within the study area is high. Valley bottoms are the most anthropized, including often managed grasslands and some agricultural fields.

The National Game Reserve of Boumort, in the Pre-Pyrenees (42° 12’ N, 1° 06’ E), is smaller, with 13,097 ha. The maximum altitude is in “Cap de Boumort” with 2,077 meters, with an altitudinal range of 1.206 m (minimum altitude: 871 m). The climate is mainly a Mediterranean mountain continental influenced climate, with usually hot and dry summers, cold winters with snow accumulation and with most rainfall concentrated in spring and autumn (between 500-700 mm/m^2 per year. In this area the hardest seasons are winter and summer. In the highest levels, we find some subalpine grasslands, but the landscape is mainly dominated by mountain black pine forests with bearberry

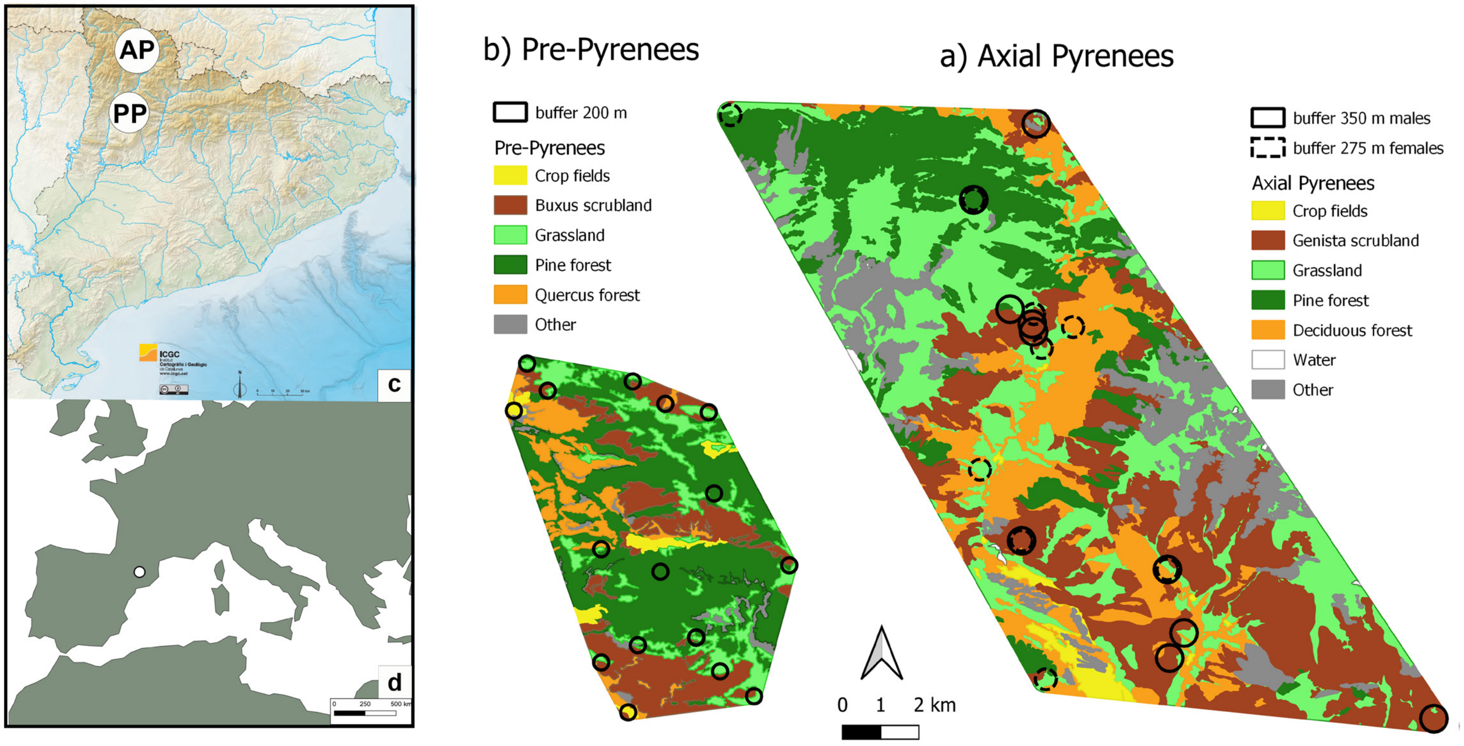


Figure 1. Map of major habitat classes in study areas: a) Axial Pyrenees, b) Pre-Pyrenees). Circles indicate locations of sampled individuals, with buffers used to characterize habitats around locations (see Methods) with the individuals’ buffers and the habitats of each area. b) Study areas in relation to Catalunya, AP: Axial Pyrenees and PP: Pre-Pyrenees (Physical and hypsometric map 2019, Cartographic and Geological Institute of Catalonia). c) Study area in relation to Europe (Land map, version 4.1.0. Made in Natural Earth).

(*Arctostaphylos uva-ursi*) and juniper (*Juniperus communis*) undergrowth and, below, red pine and Pyrenean pine (*Pinus nigra subsp. salzmanii*) forest. At lower elevations we find a mosaic of holm oaks (*Quercus ilex*) forest and large patches of thermophilic bushes. No managed grasslands or agricultural fields are found inside the Reserve. Pastures are poor, and the global grassland availability within the study area is low.

Both these areas have undergone a process of rewilding due to the drastic decrease of the number of extensive livestock and the increase of ungulate wildlife populations (Supplementary Table 1). The loss of farms in the study areas was 95.8% in the Axial Pyrenees and 81.3% in the Pre-Pyrenees from 1992 to 2021 (Register of livestock farms of the Ministry of Climate Action, Food and Rural Agenda, Generalitat de Catalunya, https://agricultura.gencat.cat/ca/serveis/registres-oficials/ramaderia-sanitat-animal/registre-explotacions-ramaderes/).

Sampling methods

Samples were collected 4 times a year, matching seasons, between 2015 and 2020. Samples from autumn, winter and spring, which together cover the hunting season, were of ruminal content, and were provided by hunters. To have samples for every season, some of spring and all summer samples were obtained from faecal pellets, since hunting permits in spring were scant, and hunting is not allowed in summer. Both methods appear to produce comparable estimates of diet composition in wild ungulates (Homolka and Heroldová, 1992). We collected 3 samples per sex, season and reserve, for a total of 48 samples.

Laboratory analysis

Diet composition was determined by the microhistological analysis of epidermal fragments (Bartolomé et al., 1995) as relative frequencies. First, subsamples of wet faeces and rumen contents were lightly ground in a mortar to separate out the epidermal fragments. From each subsample, 10 g were placed in a test-tube with 5 ml of concentrated HNO3 (Crocker, 1959; Stewart, 1967; Chapuis, 1980; Garcia-Gonzalez, 1984). The test tubes were placed for

1 min in a bath of water at 80ºC and then diluted with 200 ml of water. This suspension was passed through 0.5 mm and 0.1 mm filters. The 0.1 to 0.5 mm fraction was dispersed in a 50% aqueous solution of glycerin. Samples of the suspension were spread on glass microscope slides at a density that precluded any significant overlapping of fragments. Finally, cover slips were fixed to the slides with DPX microhistological varnish and left to dry overnight. Three slides were prepared from each subsample. An epidermal collection of the most significant and abundant plants of the study areas were prepared using the same technique.

All the slides were examined under an optical microscope at 100x to 400x magnification. We identified and counted a maximum of 600 fragments in each sample. The taxa identified from the epidermis fragments were grouped into five categories: graminoids, forbs, *Pinus*, *Quercus* and other woody species (Suplementary Table 2). They were grouped in order to increase the potential of the statistical analysis, due some species were hardly represented in the diet.

Statistical Analysis

To model diet data against the factors area, season and sex, we fitted Dirichlet models, which are appropriate for compositional data expressed as proportions, using the R package DirichletReg, version 0.7-0 (Maier, 2021). Model selection with all combinations of factors and interactions was done using the Akaike Information Criterion (AIC), with likelihood ratio tests against the null model to obtain statistical significance. To determine which diet items contributed significantly to the differences detected in the selected model, we compared the model with all diet items against all models lacking one of the items using likelihood ratio tests (LTR). We also analyzed each area separately, with season and sex as candidate explanatory factors. To test for differences in the percentage of herbaceous and woody plants in the diet we used beta regression using R package betareg (Cribari-Neto and Zeileis, 2010).

To explore the relationship between diet composition and habitat composition, we first extracted habitat percentages within individual buffers from the CORINE biotopes map of Catalonia (Vigo et al., 2006) using QGIS

3.26.2 (QGIS.org 2023). These habitats were grouped into five categories in the case of Axial Pyrenees (*Genista balansae* scrubland, Deciduous forest, Pine forest, Other habitats and Grassland) and in six categories in the case of Pre-Pyrenees (*Buxus sempervirens* scrubland, Crop fields, *Quercus ilex* forest, Pine forest, Grassland and Other habitats) (Supplementary Table 3). Habitat composition around sample locations was calculated as the percentage of habitats in a buffer defined for each reserve based on the deer home range obtained from Tellus GPS collars. The movement data was obtained from 4 individuals (2 males and 2 females), one of each sex for each area for at least 1 year. We defined the buffer radius as the average distance traveled daily. This gave us a radius of 350 m for males and 275 m for females in the Axial Pyrenees area, and a radius average of 200 m for both sexes in the Pre-Pyrenees area. We analyzed these data with Redundancy Analysis (RDA), an asymmetrical ordination technique, with habitat composition as the explanatory matrix and diet composition as the dependent matrix. Diet data were first transformed using the centered log-ratio, which is appropriate for compositional data and ensures the RDA, which implicitly uses the Euclidean distance, effectively uses the Aitchison distance on these data. Zero counts, which are not allowed by this transformation, were imputed with the multiplicative simple replacement method using function cmultRepl in the zCompositions R package (Palarea-Albaladejo and Martin-Fernandez 2015). The RDA was performed with function rda in the vegan R package (Oksanen et al., 2022). All the statistical analyses were performed with R 4.2.1 (R Core Team 2022).

RESULTS

There were significant differences in diet composition between areas, but only between seasons in the case of pre-Pyrenees. The main differences were between graminoids, *Pinus* and Other woody species. On the other hand, we found that in Axial Pyrenees were more grazers, and in Pre-Pyrenees more browsers. These differences were also in the habitat use, in the Axial Pyrenees the main function was as a refuge and in Pre-Pyrenees to feed.

The best model for diet composition comprising both areas included only area as a significant variable (Table 1, p = 0.001). The model including area and season also produced a significant model, but less parsimonious than with area alone (AIC = -472 for area only vs. AIC = -464.6 for area*season, Table 1). Differences between areas were due mostly to differences in the proportions of Pinus and Other woody in the diet, with the former more prevalent in the Pre-Pyrenees site and the latter more prevalent in the Axial Pyrenees site (Figure 2 and Table 2).

Model	K	AIC	∆AIC	AIC weight	LL	p-value
area	10	-472.1	0.000	0.946	246.1	0.001080
area*season	40	-464.6	7.523	0.022	272.3	0.000183
area*sex	20	-463.7	8.436	0.014	251.8	0.006641
area+sex	15	-463.6	8.536	0.013	246.8	0.016150
Null	5	-461.8	10.340	0.005	235.9	1

Table 1. Model selection table for the best five models for diet composition with area, season and sex as candidate explanatory variables. The columns represent the number of free parameters (K), the Akaike Information Criterion (AIC), the difference in AIC score between the best model and the model being compared (∆AIC), the relative likelihoods of the models (AIC weights), the log-likelihood (LL) and the p-value for the likelihood ratio tests against the null model.

Model	Deviance	AIC	LRT	p value
Full model	-492.14	-472.14		
Forbs	-492.05	-474.05	0.0907	0.763341
Quercus	-491.57	-473.57	0.5649	0.452287
Graminoids	490.24	-472.24	1.8973	0.168380
Other woody	-487.35	-469.35	4.7863	0.028687
Pinus	-481.46	-463.46	10.6709	0.001088

Table 2. Likelihood ratio tests for models excluding a given diet item compared to the model including all items (Full model) for the most parsimonious global model (both sites), with area as an explanatory variable. AIC is the Akaike information criterion and LRT is the chi-squared distributed likelihood ratio test statistic.

Diet composition in Axial Pyrenees

We could not detect significant differences by season or sex for the diet composition in the Axial Pyrenees, where the best Dirichlet model was the null model (Table 3). Graminoids were the dominant diet item in all seasons, and the consumption of more woody species in winter can be highlighted (Figure 2). Autumn and summer were the seasons with a more varied diet.

Model	K	AIC	ΔAIC	AIC weight	LL	p-value
Null	4	-170.5	0.000	0.947	89.27	-
season	8	-164.3	6.229	0.0421	90.16	0.7777
sex	16	-161.4	9.137	0.010	96.70	0.2490
season+sex	20	-157.1	13.470	0.001	98.54	0.2938
season*sex	32	-138.9	31.600	<0.001	101.50	0.6602

Table 3. Model selection table for the best models for diet composition of Axial Pyrenees, with season and sex as candidate explanatory variables. The columns represent the number of free parameters (K), the Akaike Information Criterion (AIC), the difference in AIC score between the best model and the model being compared (ΔAIC), the relative likelihoods of the models (AIC weights), the log-likelihood (LL) and the p-value for the likelihood ratio tests against the null model.

Diet composition in Pre-Pyrenees

In the Pre-Pyrenees, the best Dirichlet model for the diet composition included the variable season (Table 4, $p = 0.0025$). Adding sex in interaction with season also produced a significant ($p = 0.031$, LRT test against model with season alone), but less parsimonious model as judged by the Akaike Information Criterion (Table 4). Differences among seasons were due to changes in the proportions of graminoids, *Pinus* and Forbs in the diet (Figure 2 and Table 5). Graminoids were more prevalent in the diet in summer and autumn as compared to winter and spring (with significant differences for spring vs. summer, $p = 0.00033$, and for spring vs. autumn, $p = 0.0037$). The mean contribution of Pinus to the diet was greater in spring and summer, but with low support (statistically significant only for summer vs. each of

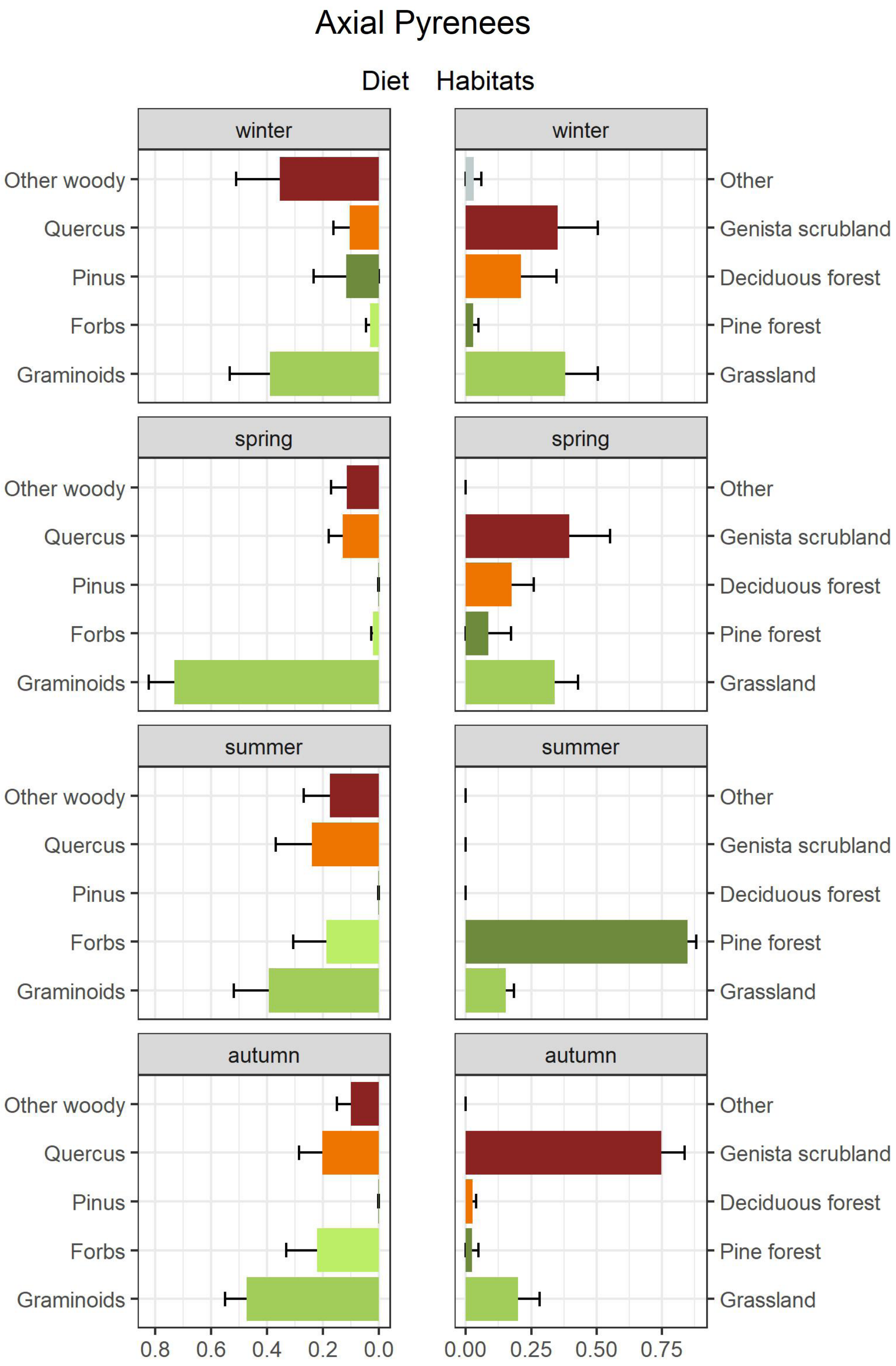


Figure 2.1. Comparison of the diet composition and habitats in Axial Pyrenees (means with standard errors).

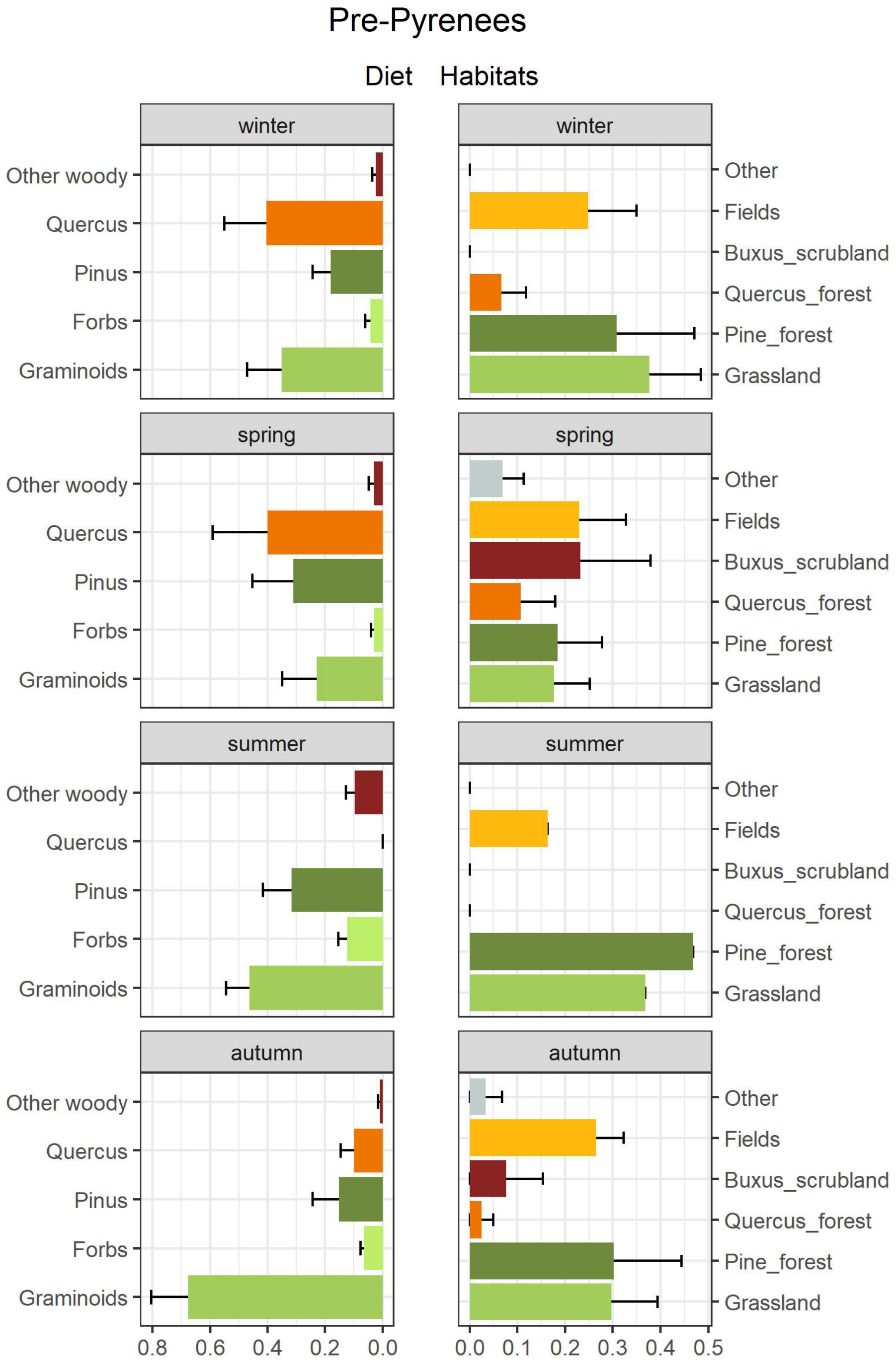


Figure 2.2. Comparison of the diet composition and habitats in Pre-Pyrenees (means with standard errors).

the other seasons, $p < 0.05$). Forbs showed a higher proportion in the diet in summer and autumn ($p = 0.00478$ for spring vs. summer).

Model	K	AIC	ΔAIC	AIC weight	LL	p-value
season	20	-199.8	0.000	0.803	119.9	0.0024
season+sex	25	-195.6	4.215	0.098	122.8	0.0039
Null	5	-194.8	4.996	0.066	102.4	-
season*sex	40	-193.1	6.685	0.028	136.5	0.0006
sex	10	-189.5	10.220	0.005	104.8	0.4438

Table 4. Model selection table for the best models for diet composition of Pre-Pyrenees, with season and sex as candidate explanatory variables. The columns represent the number of free parameters (K), the Akaike Information Criterion (AIC), the difference in AIC score between the best model and the model being compared (ΔAIC), the relative likelihoods of the models (AIC weights), the log-likelihood (LL) and the p-value for the likelihood ratio tests against the null model.

Model	Deviance	AIC	LRT	p value
Full model	-239.77	-199.77		
Quercus	-236.00	-202.00	37.673	0.2877178
Other_woody	-232.92	-198.92	68.487	0.0768802
Forbs	-231.59	-197.59	81.787	0.0424589
Pinus	-230.43	-196.43	93.442	0.0250480
Graminoids	-220.37	-186.37	194.006	0.0002259

Table 5. Likelihood ratio tests for the Pre-Pyrenees model excluding a given diet item compared to the model including all items (Full model) for the most parsimonious global model, with season as an explanatory variable. AIC is the Akaike information criterion and LRT is the chi-squared distributed likelihood ratio test statistic.

Woody vs herbaceous

The best beta regression model for the consumption of woody vs herbaceous species, as judged by the Akaike Information Criterion, included all factors (area, season and sex), and their pairwise interactions (Table 6). The significant differences between areas were due to a higher consumption of woody species in the Pre-Pyrenees (p-value = 0.0275) (Figure 3). The differences in the consumption of woody species between sexes was the main cause of these differences between areas. Specifically, the differences between areas were caused by the differences between the females' diets (p-value < 0.0001) (Figure 3). In the Axial Pyrenees there were not differences in the seasonality of woody consumption in females, but males consumed significantly more woody species in winter and summer than in the other seasons (winter – spring: p-value = 0.0011; winter – autumn: p-value = 0.0044; spring – summer: p-value = 0.0094; summer – autumn: p-value = 0.0261) (Figure 3). In the Pre-Pyrenees both sexes differed significantly in their consumption of woody species. Females consumed more woody species in spring than in summer or autumn (spring – summer: p-value < 0.0001; spring – autumn: p-value = 0.0090), whereas males consumed less woody species in autumn than in any of the other seasons (winter – autumn: p-value = 0.0003; spring – autumn: p-value = 0.0005; summer – autumn: p-value = 0.0085) (Figure 3).

Model	K	**AIC**	**ΔAIC**	**AIC weight**	**LL**	**p-value**
Full2	13	-14.500	0.00	0.934000	21.30	<0.001
Full3	16	-9.100	5.43	0.062000	21.50	<0.001
area*sex+season	7	-2.520	12.00	0.002310	9.26	0.00211
area*season	4	-0.755	13.80	0.000958	5.38	0.0169
area+sex*season	9	1.210	15.70	0.000359	9.40	0.00881

Table 6. Model selection table for the best models for diet composition of woody and herbaceous species of Pre-Pyrenees, with area, season and sex as candidate explanatory variables. The columns represent the number of free parameters (K), the Akaike Information Criterion (AIC), the difference in AIC score between the best model and the model being compared (ΔAIC), the relative likelihoods of the models (AIC weights), the log-likelihood (LL) and the p-value for the likelihood ratio tests against the null model. Model Full2 includes all factors and pairwise interactions; model Full3 is Full2 plus the three-way interaction.

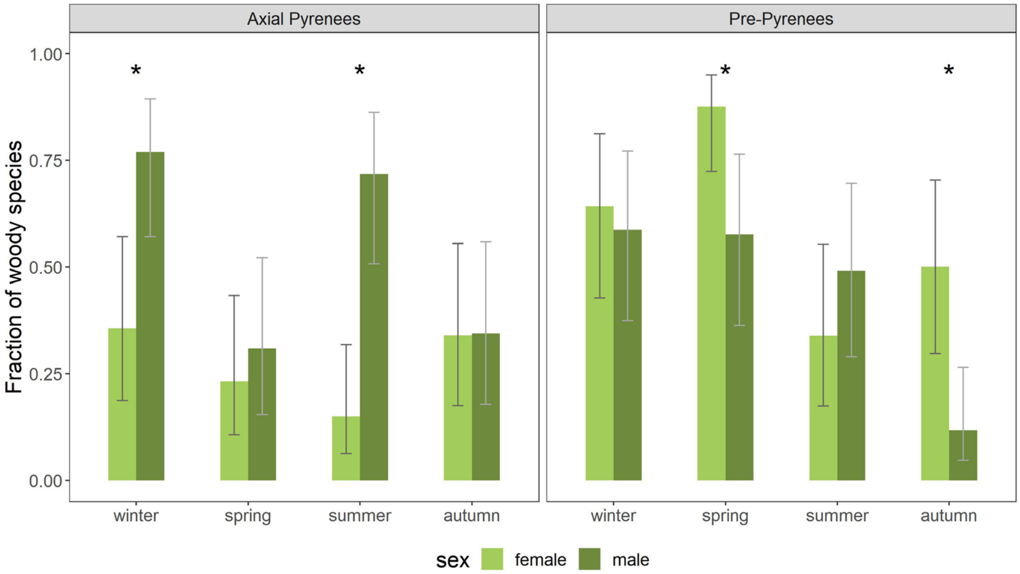


Figure 3. Mean fraction of woody species in the diet composition of the deer populations at the two study areas (Axial Pyrenees and Pre-Pyrenees), by sex and season. Mean expected values and 95% confidence intervals are from predictions obtained from the best beta regression model (Table 6). Asterisks indicate statistically significant differences between sexes within area and season.

Habitat use

The relationship between diet composition and habitat composition differed between areas. In the Axial Pyrenees, the species found in the diet and the dominant species in the surrounding habitats, around the capture points, were not related, as the RDA analysis showed (permutation test, p = 0.479; Figure 4). In contrast, we found a strong relationship in the Pre-Pyrenees (permutation test, p = 0.003; Figure 4).In this area, the two more abundant groups in the diet, graminoids and *Pinus*, were directly related with the two more abundant habitats, grasslands and *Pinus* forests.

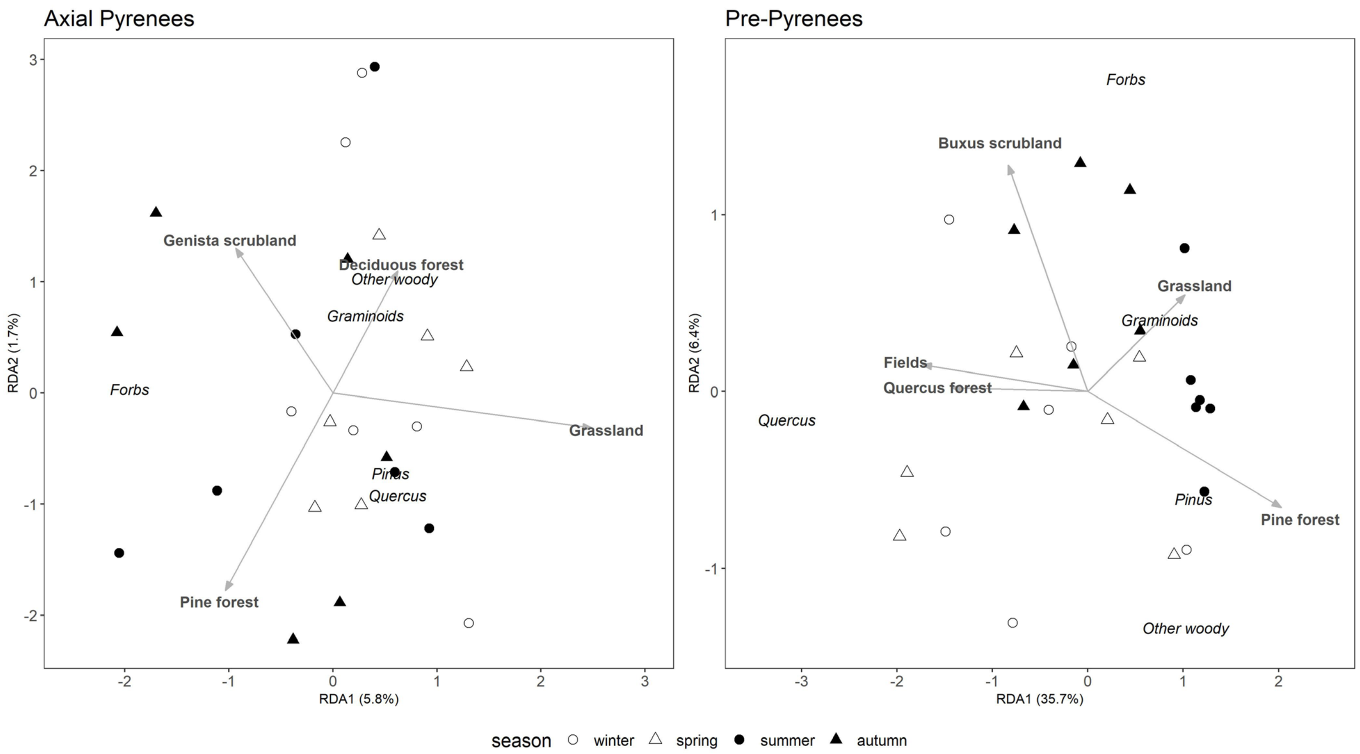


Figure 4. Redundancy analysis of diet composition and use of habitat in the Axial Pyrenees and Pre-Pyrenees study areas. Arrows and bold labels show biplot scores for the explanatory habitat variables, while labels in italics are ordination scores for the response matrix (i.e., diet composition). Dots show individual deer, with symbols indicating the season that the sample comes from. Percentages in the axis labels show the percentage of total variance explained by each (cumulative 42.1%; 48.8% for all four canonical axes).

DISCUSSION

A specific diet for each area

There were differences in the diet composition between both areas, as we hypothesized. The consumption of other woody stood out in the Axial Pyrenees, mainly because of the high consumption of *Calluna vulgaris*. In study area of the Pre-Pyrenees, the availability of habitats with this species was null (Vigo et al., 2006), and no species of this family was abundant. *Calluna sp*. has been found in the diet of red deer in other studies as in the case of Isle of Rum Island. Virtanen et al., (2002) study the effects of deer on the vegetation of this Isle, and indicates that high deer densities within an unculled food-limited population were however associated with negligible effects on plants in *Calluna-Molinia* wet heath vegetation. This might also be our case. A preference for *Calluna sp.* has also been found for another cervid, the roe deer (*Capreolus capreolus*), in autumn in Poland (Siuda et al., 1969). And, also, in chamois (*Rupicapra rupicapra*) (Villamuelas et al., 2016; Espunyes et al., 2019), which shares the niche with red deer in this area, which gives rise to thinking about interspecies competition. On the other hand, another difference between these areas was in the consumption of *Pinus*, which was higher in the Pre-Pyrenees. This high consumption of *Pinus* was also found in a study of the diet composition of red deer in the western Axial Pyrenees (Garin et al., 2001). In the case of the Pre-Pyrenees, it could be, as in the case of Garin's study, that there was a high consumption of *Pinus* because the availability of more nutritious food was limited in the area. This may also explain our finding that in the Pre-Pyrenees red deer were more browsers than in the Axial Pyrenees. It also fits with the three feeding patterns proposed by Garin et al., (2001) for Europe. In this scheme, the Axial Pyrenees would fit between the oceanic, with preponderance of the herbaceous and *Ericaceae* woody plants, and the center-European model, with the alternation of herbaceous and woody plants, whereas the Pre-Pyrenees would better fit the Mediterranean model, with a preponderance of woody species all year round.

Seasonality in diet composition

In contrast with most studies on diet in red deer populations, we did not find a significant clear seasonal trend in the case of Axial Pyrenees. Graminoids were the main group consumed during all seasons, in this area there were more habitats with availability of herbaceous plants and graminoids. In this area the hardest seasons to find food were winter and spring. In winter, the diet composition was based on graminoids and other woody species. During this season the snow depth prevents deer from feeding at higher altitudes, so they have to move to lower areas with higher food availability where they find grasslands with no or little snow cover (Gilbert et al., 2017; Gort -Esteve et al., unpublished data). Other habitats where they found food in winter were deciduous forests, where deer can feed on the fallen leaves, as sika deer (*Cervus nippon*) do under food limitation (Takahashi and Kaji 2001). This was also the season when the most Pinus was consumed, in agreement with other studies that also show increased consumption of pine and other woody species in hard seasons with poor forage quality (Garin 2001; Bugalho and Milne 2003). In this area spring was an unfavorable season, which could be called "late winter". During these months the snow was still found in many habitats, especially in the higher ones, but in some others, like valley bottoms, it was starting to melt. For this reason, it could be that the consumption of graminoids increased and that of woody species decreased. In summer and autumn, although graminoids continue to dominate, the composition of the diet was more varied due to the absence of snow. Also noteworthy is the increased the consumption of *Quercus*, since in this area the dominant oak species (*Quercus petraea*) is deciduous.

In the Pre-Pyrenees there were significant differences between seasons. In both winter and spring, the diet groups that dominated the diet composition were graminoids, *Quercus* and *Pinus*. In the Pre-Pyrenees, the species of *Quercus* that dominated was *Quercus ilex*, a perennial one. There could be two reasons why red deer in this area prefer *Quercus* in winter and spring. One reason is that this species begins to sprout in later winter. For this reason, *Quercus* may have more tender shoots in these seasons and leaves will be generally more palatable (Miranda et al., 2015; Petersson et al., 2019). The consumption of *Quercus* and *Pinus* leaves could be due to higher protein and digestibility levels reported for leaves compared with graminoid stems, depending on the maturity of their tissues (Aldous, 1945, Blair and Epps, 1967, Short et al., 1972). In summer, there were mainly two groups that dominated the diet composition, graminoids and *Pinus*. In this dry season, the percentage that was consumed from *Quercus* was replaced by *Pinus*, probably because the dry thorns of *Quercus ilex* leaves are excessively unpalatable. Contrarily to most studies, where pine is consumed almost exclusively in winter, in this area it is consumed also in summer. The reason could be that graminoid species are also very dry, as has been suggested for other areas (Homolka, 1993; Garin et al., 2001). In autumn, the diet composition was more uniform than in other seasons, being most of the diet composition graminoids, possibly because the increase of rainfall in this season makes these species regrow and become more palatable (Bugalho and Milne, 2003), at a time when tender shoots of other species are less abundant.

Differences between sexes

We expected males to be more browsers than females in both areas, as found in most of studies (Clutton-Brock et al., 1982; Bugalho et al., 2001; Azorit et al., 2012c, Miranda et al., 2012b; Zlatanova et al., 2019). This expectation was upheld in the Axial Pyrenees area (in winter and summer), but not in the Pre-Pyrenees area, where females tended to be more browsers than males (with statistically significant differences between spring and other seasons). The fact that the males were more browsers was in agreement with the Jarman Bell principle (Demment and Van Soest, 1985), which predicts that larger animals can subsist on lower quality bulk food. This principle is based on the fact that males have a larger rumino-reticular volume than females, which makes them more efficient at assimilating fibers, and have lower relative metabolic requirements (Miranda et al., 2012b). Additionally, we also expected more differences between sexes in the Axial Pyrenees due to the differences in their seasonal movements. Monitoring data and the GPS movement analysis strongly suggest that males and females select habitats differently in each area, with populations spatially segregated by sex throughout most of the year (with the exception of the rut) in the Axial Pyrenees, but not in the Pre-Pyrenees (Gort-Esteve et al., unpublished data). In Axial Pyrenees, it seems that males made greater movements during the year than females, having a home range up to 7 times greater, which would explain their differences in the consumption of woody and herbaceous plants between seasons.

The finding that females were more browsers than males in the Pre-Pyrenees area was unexpected, and we have not been able to find a similar observation in the published literature. A possible explanation for this observation was the lower quality of forage in the study area. When resources are scarce small animals have an advantage because they need less food (Case, 1978). At any rate, our results emphasize the high plasticity of this species in terms of diet.

Relation between diet and habitat use

Studies concur in that deer use shelter habitats for refuging or resting and open areas mainly to feed (Matsubayashi et al., 2003; Bonnot et al., 2013; Laguna et al., 2021). Our results show that habitat and diet composition were significantly related at one of the areas (Pre-Pyrenees) but not at the other (Axial Pyrenees). This contrasting result suggests that there may be differences in the drivers of habitat selection between the two areas, with foraging a stronger driver in the Pre-Pyrenees, which might be explained by the composition and configuration of the landscape at each of the areas, and the role those specific habitats play as providers of forage or of places for resting and taking refuge.

In the Axial Pyrenees, winter was the only season when the diet and the habitat composition were related, with more than 50% of the home range composed of habitats dominated by species found in the diet (Figure 2). The most abundant habitats in deer winter home range were grasslands and scrubs of *Genista balansae*. Grasslands are congruent with the most abundant group in diet composition, graminoids. There was a high percentage of scrubs of *Genista balansae* in all seasons expect on summer, yet we never found this species in the diet composition, possibly because of its toxicity (Agelet and Valles 2003). Scrubs of *Genista balansae* could instead be used for resting or as a refuge, as this specie in this area had a high height that could protect deer form wind and without letting the snow penetrate inside it creating the effect of a cave. Similarly, in summer pineforest were abundant in the habitat composition, even though *Pinus* was the only diet group that was not consumed. We hypothesize that, similarly to the scrubs of *Genista balansae*, the pine forest was used as a refuge, but in this case from high temperatures, while deer possibly fed on species from crown gap areas, as Matsubayashi et al., (2003) observed for lesser mouse-deer (*Tragulus javanicus*) deer in Southeast Asia. Unlike most studies, which show that deer prefer habitats where they can both take refuge and forage (Borkowski and Ukalska 2008; Alves et al., 2014), in the Axial Pyrenees it seems that the main habitat is used mostly as refuge, perhaps because those dual-function habitats are not available. In contrast, in the Pre-Pyrenees we found that scrubs of boxwood, *Buxus sempervirens*, were abundant within the habitat composition in each season. The reason could be that this habitat is very suitable for deer, as it combines protection and food. Deer can then take refuge and rest under boxwood, and feed in patches of grassland interspersed among scrub patches (Aymerich et al., 2006). Boxwood did not appear in the diet composition due to its toxicity (Kuhns and Sydnor, 1976; Široká et al., 2016). On the other hand, we also found grasslands and pine forest, in accordance with the amounts of gaminoides and *Pinus* that we found in the diet composition. For these reasons, in this area it seems that the main habitat is used mostly for feed.

CONCLUSIONS

Red deer had a high flexibility and adaptability in their diet. In two areas as differentiated as those of this work we have been able to check how the composition of the diet fits perfectly. The contrast between the diet composition of each area provided us with key information to evaluate the possible impact of red deer populations on the different habitats. The high consumption of woody species confirmed that these populations could play a role on woody species control, contributing to the maintenance of open areas such as clearings or grasslands. On the other hand, the consumption of herbaceous species gave us reason to think that they could be occupying part of the trophic niche of the domestic herds of grazers (cattle and sheep) that have disappeared. Not only diet, but they also adapted habitat use. In more unfavorable climatological conditions, such as those of the Axial-Pyrenees, the main use of the habitat is for refuge, while in more favorable conditions, such as those of the Pre-Pyrenees, the main use of the habitat is for feeding.

SUPPLEMENTARY MATERIAL

Area	2015	2016	2017	2018	2019	2020
Axial Pyrenees	5.60	6.70	5.21	3.18	3.65	6.70
Pre-Pyrenees	3.85	3.67	5.33	6.63	7.35	5.66

Supplementary Table 1. Density of red deer in each study area (average individuals/km^2) estimated using the distance sampling methodology. Data obtained through nocturnal transects with a vehicle using 2 spotlights per vehicle by the Dept. of Agriculture, Livestock, Fisheries and Food, Generalitat de Catalunya, Spain.

Group	Species	% Axial Pyrenees	% Pre-Pyrenees
Other woody	*Calluna vulgaris*	13.76	0
Other woody	*Cistus sp*	0	0.03
Other woody	*Genista balansae*	1.93	0.19
Other woody	*Genista cinerea*	0	0.01
Other woody	*Hippocrepis comosa*	0	0.06
Other woody	*Juniperus communis*	4.68	2.32
Other woody	*Ononis tridentata*	0	0.01
Other woody	*Prunus sp*	0	0.05
Other woody	*Prunus sp*	0.01	0

Other woody	*Thymus sp*	0	0.18
Other woody	*Other woody*	0.96	0
	Total Other woody	***21.34***	***2.85***
Quercus	*Quercus ilex*	0	37.83
Quercus	*Quercus petraea*	17.61	0.33
	TotalQuercus	***17.61***	***38.16***
Pinus	*Pinus sp*	3.3	20.31
	TotalPinus	***3.3***	***20.31***
Forbs	*Brassicaceae*	2.21	0.03
Forbs	*Carex sp*	0.57	1.64
Forbs	*Cruciata glabra*	0	0.17
Forbs	*Medicago suffruticosa*	0	0.01
Forbs	*Rubia peregrina*	0.07	0.1
Forbs	*Sedum sp*	0.06	0
Forbs	*Solanum nigrum*	0	0.22
Forbs	*Valeriana montana*	0.16	0
Forbs	*Verbascum sp*	1.23	0.1

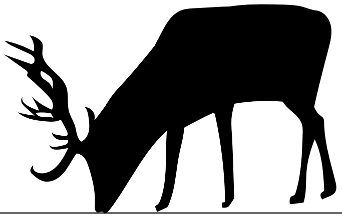
Forbs	*Vicia pyrenaica*	0.14	0
Forbs	*Other forbs*	4.17	3.54
	Total Forbs	***8.6***	***5.81***
Graminoids	*Aegilops geniculata*	0	1.11
Graminoids	*Avenula pratensis agg.*	0	0.07
Graminoids	*Bromus hordeaceus*	0	0.07
Graminoids	*Hordeum murinum*	0	0.06
Graminoids	*Lolium sp*	0	0.02
Graminoids	*Poa pratensis*	0	1.32
Graminoids	*Poa trivialis*	0	0.09
Graminoids	*Trisetum flavescens*	0	0.13
Graminoids	*Other Graminoids*	49.14	29.99
	Total Graminoids	***49.14***	***32.86***

Supplementary Table 2. Percentage of diet items, identified to the finest possible taxonomic level, in the diet composition of sampled deer in the Axial Pyrenees and Pre-Pyrenees study areas. "Group" indicates aggregated classes used in the analyses.

Axial Pyrenees	
Genista scrubland	31t and 31u
Deciduous forest	31ac, 41e, 41h, 41i2, 41r and 41s
Pine forest	42af, 42f, 42g, 42l and 42p
Other	31c, 31f and 37d
Grassland	35b, 35h, 36e, 36f2, 36g, 36u, 38b and 38d1
Pre-Pyrenees	
Other	32u, 32w and 62a
Buxus scrubland	32aa
Fields	82c and 87a
Quercus forest	41f, 41k and 45f
Pine forest	42i, 42j, 42k, 42m and 42t
Grassland	41f, 41k and 45f

Supplementary Table 3. Categories and habitats extracted with the individual buffers from a CORINE map (Vigo et al., 2006).

CHAPTER 2: ASSESSING SUPERNUMERARY ROOTS OCCURRENCE AS A POSSIBLE ADAPTATION ENHANCING TEETH PERFORMANCE IN MEDITERRANEAN DEER POPULATIONS



Gort-Esteve, A., Riera, J. L., Ruiz-Olmo, J., Carrasco, R., Garcia del Rincon, A., and Azorit, C., 2022. Assessing supernumerary root occurrence as a possible adaptation enhancing teeth performance in Mediterranean deer populations. *Canadian Journal of Zoology*, 100(6), 334-344

ABSTRACT

Teeth root morphology and integrity are essential to provide appropriate attachment, allowing for continuous and functional movement, with implications for adequate food processing, animal performance and longevity. We studied the occurrence of supernumerary roots in mandibular molariform teeth of red deer (*Cervus elaphus* Linnaeus, 1758) from seven separate populations spanning a range of latitudes in the Iberian Peninsula. We analyzed the influence of several factors, including sex, origin (native vs. reintroduced), lineage and habitat to assess extra root prevalence variations. The highest prevalence of supernumerary roots in deciduous teeth was found in pm_3 (14%) and in permanent teeth in M_1 (3%). We found significant differences between areas, lineages and soil type; however, no significant relationships were found with the origin, or with the sex of individuals. We

speculate that the high prevalence of supernumerary roots in M_1 might be related to increased wear in grazers. Furthermore, we suggest that this high prevalence in deciduous teeth might be associated with a hard diet, dry climatic conditions and a harsh weaning period, which suggest supernumerary roots might have adaptive value.

INTRODUCTION

Across the evolution of mammals, numerous morphological and functional changes in dentition and tooth structure made possible the adaptation of the craniodental system to a wide variety of food types, allowing for an efficient processing of food (Berkovitz et al., 2018). In ruminants, such as cervids, one of these changes in teeth involved the morphology of the occlusal surface of molariform pieces. Both premolars and molars are not just covered in enamel, but the enamel, dentin and cement layers are exposed in a so-called selenodont design (Dorit et al., 1991). Abrasive ruminant diets are known to wear down teeth, and this type of morphological structure appears to improve their durability and functionality (Fortelius, 1985; Kaiser et al., 2013). However, roots are also considered to be very important structures for the performance of ruminant dentition. The need to maintain a precise dental occlusion entailed modifications in the craniodental system, as well as the presence of a tooth-support system consisting of the gingiva, periodontal ligament, alveolar bone and cementum, which covers the root dentine surface of all teeth (Berkovitz and Shellis, 2018). Additionally, a wear compensation mechanism in dental roots of ruminants has been recently found, showing the root as an important structure in modulating crown height losses caused by dietary abrasives (Ackerman et al., 2021). Therefore, root morphology and integrity are essential to provide appropriate teeth attachment allowing for continuous and functional movement, with implications for adequate food processing, animal performance and longevity.

All teeth generally have a definite morphology and fixed number of roots. However, extra roots have been described in mammals, a condition defined by the presence of more roots than expected (Kannan and Santharam, 2002; DeLong and Burkhart, 2013). In wild ruminants, the presence of extra roots has been occasionally reported in mandibular premolars of white-tailed deer (*Odocoileus virginianus* Zimmermann, 1780) as a congenital defect (Mech et al., 1970), and in the first lower molar (M_1) of Iberian red deer (*Cervus elaphus hispanicus* Hilzheimer, 1909) as developmental anomalies involving the tooth root morphology (Azorit et al., 2002d; Azorit et al., 2012a). Disturbances to the Hertwig's epithelial root sheath forming the root have been reported as a possible cause (Kannan and Santharam, 2002; Zeichner-David et al., 2003; Luan and Diekwisch, 2006; Kumakami-Sakano et al., 2014), but no other causes of this developmental anomaly have been identified, except for external pressure, trauma and metabolic dysfunction during root development, as suggested by DeLong and Burkhart (2013). Importantly, variations in the prevalence of extra roots in deer have not been related to genetic or environmental factors, both of which could influence the occurrence of this anomaly. In human teeth, however, variations in in the prevalence of extra roots across populations have been reported (Thomas et al., 2016), which leads to infer some genotypic implication (Ferraz and Pécora, 1992; Ahmed et al., 2007).

Also noteworthy is the influence of environmental factors. A deficient dental support system, coincident with a high prevalence of periodontal diseases, has been detected in deer living on acidic soils in a Mediterranean ecosystem during periods of drought and environmental harshness (Azorit et al., 2012b). Acidic soil conditions can contribute to descaling the root cementum, particularly acellular extrinsic fiber cementum and cellular and acellular intrinsic fiber cementum, which are important tissues of the tooth support system and play an important role in tooth attachment and movement (Bosshardt et al., 1997; Berkovitz, 2018). Vegetation is also important for the evolution of ruminant dentition. In the case of red deer (*Cervus elaphus* Linnaeus, 1758), the type of feeding, i.e. grazers vs. browsers, implies a different level of tooth wear. Graminoids contain more phytoliths (silica) than other species. In addition, plants growing in open and dry environments tend to have more grit and soil particles adhered to their surfaces. Both these suggest that grazers may ingest more abrasive matter than browsers (Ozaki et al., 2010; Kubo and Yamada, 2014). Because of sexual dimorphism, feeding habits also vary among sexes (Carranza, 2017). Females are more frequently grazers than males, because they are able to reach short grass (Clutton-Brock et al., 1982; Azorit et al., 2012c). On the other hand, males tend to be browsers more often than females, in part because their larger size provides them with the ability to reach parts of bushes or trees that the females cannot reach (Bugalho et al., 2001).

In this work we study the occurrence of supernumerary roots in mandibular molariform teeth of red deer from seven separate populations in the Iberian Peninsula that differed in their habitats (as determined by climatology, topography and vegetation types), as well as in their lineages and origin (native vs. reintroduced). Although today red deer can be found throughout the Iberian Peninsula, their range had decreased dramatically at the beginning of the 20th century, when Cabrera (1911) found them restricted to the SW. of the Peninsula (Doñana, Sierra Norte of Madrid, Sierra Morena and Montes of Toledo), with two exceptions: Monte del Pardo in Madrid and Riofrío in Segovia (Soriguer et al., 1994; Carranza et al., 2016). Although the subspecies of red deer autochthonous to the Iberian Peninsula is the Iberian deer introductions, reintroductions and range expansion during the last century have resulted in populations of red deer with a high percentage of hybrids or individuals from another deer subspecies, especially in the High Pyrenees (Queirós et al., 2020; Pérez-González et al., in press). In Europe there are three lineages of red deer resulting from isolation in different refugia during the last glaciation, namely, the western European lineage, the eastern European lineage and the Mediterranean lineage (Ludt et al., 2004; Skog et al., 2009). Deer size morphology and genetics suggest that the deer population from the High Pyrenees comes from a natural expansion of the western European lineage, (*Cervus elaphus hippelaphus* Erxleben, 1777) (Geinst and McShea 1999; Fernández-García et al., 2014; Carranza et al., 2016; Pérez-González et al., in pres).

Here we show striking patterns of extra root prevalence across populations, and explore their association with sex, with the origin and lineage of deer populations, and with habitat type, and suggest supernumerary roots as a possible adaptation enhancing teeth performance in Mediterranean ecosystems. Firstly, we analyze prevalence variations in extra roots in mandibular molariform teeth across populations. Secondly, we determine if variations in the occurrence of supernumerary roots are statistically related to differences with the lineage (Iberian vs. western European), origin (native vs. reintroduced), environmental conditions across populations and sex. We expect differences in prevalence associated to the lineage of the populations, which would suggest genetic differences. We also hypothesize differences linked to environmental conditions. In particular, we expect a higher prevalence of supernumerary roots in habitats with siliceous, acidic soils, which are poor in nutrients and calcium, as an adaptation reinforcing the dental system to provide a more long-lasting tooth attachment. Finally, we also hypothesize a higher prevalence in females because they tend to be grazers more often than males.

METHODS

Study areas and deer populations

We studied the mandibles of 2.933 red deer from High Pyrenees (43), Prepyrenees (108), Sierra Morena (2.673), Sierra de Cazorla (11), Quintos de Mora (33), Cádiz (14) and Doñana (46) (Figure 1 and Table 1). These deer populations are isolated from one another. Specimens were hunted between 1990 and 2019 during regular sport hunting and management culling in accordance with local regulated hunting plans at each area. Deer populations lineage and origin, together with location soil type and ecology, with strong climatic contrasts, differed across these areas (Table 1).

In the High Pyrenees, samples were collected in the National Hunting Reserve of Alt Pallars. This is the population that is located further north and at the highest altitude. Morphological differences suggest that deer in this population are not native, but originated from natural migrations or introductions from France, and, unlike at the other areas considered here, could belong to the western European lineage, or could be a hybrid (Geinst and McShea 1999; Fernández-García et al., 2014; Carranza et al., 2016; Queirós et al., 2020; Pérez-González et al., in preparation).

In both the Prepyrenees (National Hunting Reserve of Boumort), and Cazorla (Sierra de Cazorla, Segura las Villas Natural Park), deer were reintroduced (in the 1980s and between 1949 and 1964, respectively), using

	Area (km^2)	**Max. altitude (m above sea level)**	**Habitat**	**pm^2**	**pm^3**	**pm^4**	**M^1**	**M^2**	**M^3**	**PM^2**	**PM^3**	**PM^4**
High Pyrenees	817.72	3,144	Alpine ecosystem in the highest parts of the mountains (absence of trees, dominance of grasslands rocks and screes). Subalpine ecosystem with fir (*Abies alba* Mill.) and black pine (*Pinus uncinata* Mill.). Mid-mountain ecosystem with Scots pine (*Pinus sylvestris* L.). Ecosystem most anthropized in bottom of the valleys, with some oaks forests.	0	0	0	23.81	0	0	0	0	0
Prepyrenees	130.97	2,077	Pine forests, scrubs of *Buxus* (L.) and some open areas with mountain meadows	0	21.74	4.35	1.04	0	0	0	0	0
Quintos de Mora	68.64	1,2	Mainly sclerophyllous vegetation, with occasional stands of pine and Pyrenean oak (*Quercus pyrenaica* Willd.)	0	0	0	3.03	0	0	0	0	0

Sierra Morena	2241.00	1,291	Plant communities typical of the siliceous soils and Mediterranean ecosystems; savannah-like landscapes of the Iberian Peninsula known as ‘‘dehesas’’ or “montados” are a dominant part of the agroforestry system	0	12.89	1.50	2.09	0.05	0.06	0	0.11	0.11
Cazorla	687.00	2,1	Plant communities typical of Mediterranean ecosystems with a greater abundance of pine forests and some “dehesas”	0	37.50	12.50	0	0	0	0	0	0
Doñana	537.09	40	There are three characteristic habitats: Dunes (xerophytic scrubs); Marsh (typical vegetation of wetlands); Scrubs (thick scrub of heterogeneous composition with oaks, pine and juniper scrub (*Juniperus communis*, L.)	0	37.84	2.70	0	0	0	0	0	0
Cádiz	1677.67	1,092	It is the world largest cork oak (*Quercus suber*, L.) forest. Under particular conditions, a unique vegetation, the laurisilva	0	0	0	0	0	0	0	0	0

Table 1. Characteristics of the habitats and prevalence (as percentage) of supernumerary roots in each tooth by area.

individuals from Quintos de Mora National Reserve (central Spain) and other southern areas, such as Lugar Nuevo Reserve (Sierra de Andújar Natural Park, in Sierra Morena) (Arenzana et al., 1965; Marco et al., 1995). Consequently, both the Prepyrenees and Cazorla harbour non-native populations of the Iberian subspecies lineage. In the Cádiz study area (Los Alcornocales Natural Park) the deer population was also reintroduced from native populations of Iberian deer from southern Spain (Soriguer et al., 1994; Carranza et al., 2016). However, this area differs strongly from Boumort and Cazorla in that Cádiz is located much further south, at a lower mean altitude with a coastal (not continental) climate, and the habitat is mainly a humid oak forest.

Native Iberian deer are also found in Quintos de Mora (in the heart of the Montes de Toledo), in Sierra Morena Range (mostly sampled at the Sierra de Andújar Natural Park), and in Doñana Natural Park (Soriguer et al., 1994; Carranza et al., 2016).

These areas also differ strongly in their climate, soil type and dominant habitats (Rivas and Gandullo 1987). The Pyrenean population, with an Atlantic-influenced highland climate, has mainly alpine and subalpine habitats on mostly siliceous soils. Prepyrenees and Cazorla have a continental Mediterranean mountain climate and vegetation there is dominated by pine forests, mainly on calcareous soils. Quintos de Mora and Sierra Morena are under the same climate but, being at lower altitudes, are dominated by plant communities typical of Mediterranean siliceous soils (Aguilar et al., 1987). Finally, the climate at the two southernmost areas, Doñana and Cádiz, is Atlantic-influenced Coastal Mediterranean, but humidity is much higher in Cádiz than in Doñana (Bellinfante-Crocci 1997; Mudarra 1989).

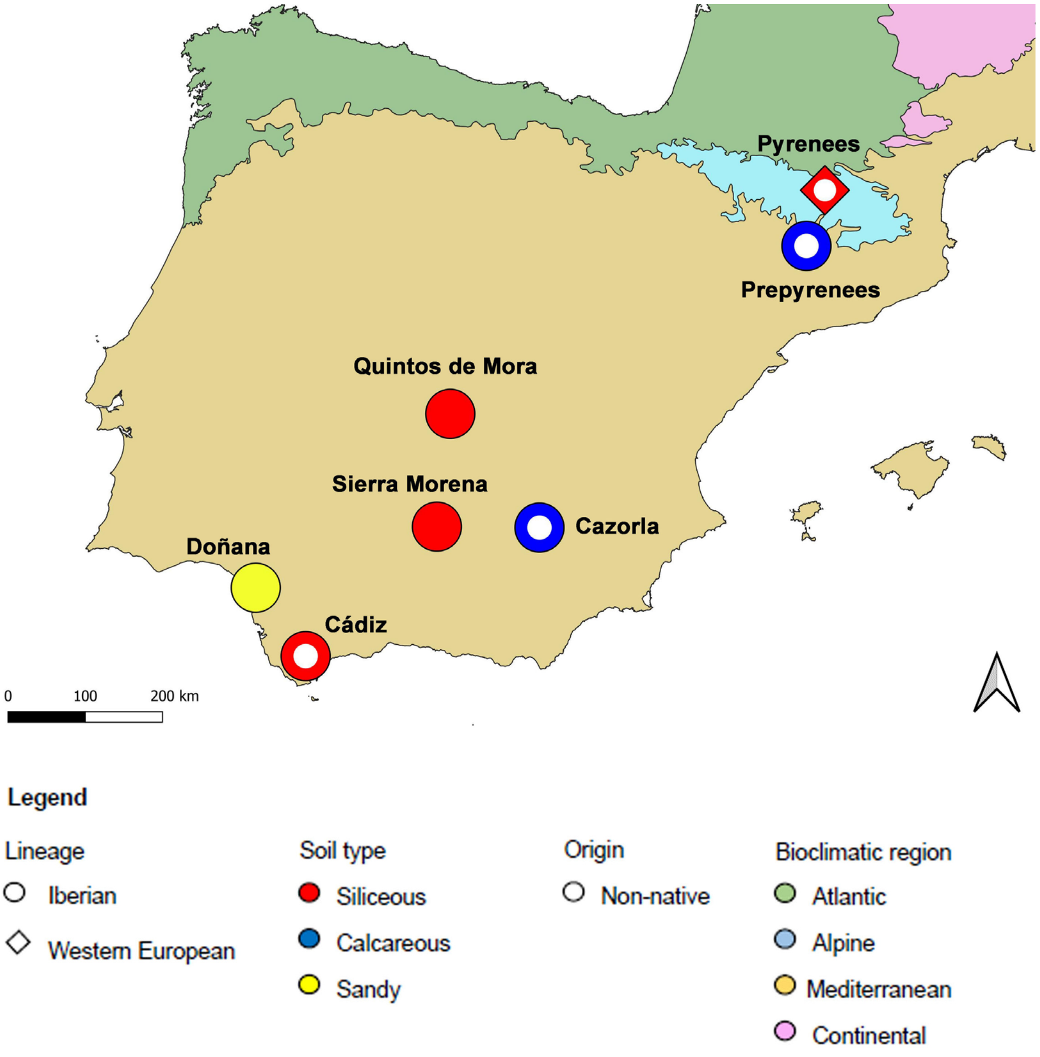


Figure 1. Locations of study red deer (*Cervus elaphus*) populations showing their lineage and origin, area soil type and bioclimatic region (EPSG:25831 - ETRS89 / UTM zone 31N). Based map of Biogeographical regions, Europe 2016, version 1 (https://sdi.eea.europa.eu/catalogue/biodiversity/eng/catalog.search#/metadata/c6d27566-e699-4d58-a132-bbe3fe01491b)

Assessing supernumerary roots occurrence by teeth and age

The dentition of red deer consists of 3 lower incisors and 1 lower incisiform canine, 1 upper canine, 3 lower and 3 upper premolars and 3 upper and 3 lower molars. In the Iberian red deer, deciduous premolars (pm_2, pm_3 and pm_4) are replaced by permanent premolars (PM_2, PM_3 and PM_4) at 27-30 months of age (Azorit et al., 2002a; Azorit 2011). The terminology followed for individual teeth is that of Riney (1951), in which the three normally occurring deciduous premolars are designated pm_2, pm_3, and pm_4, with pm_1 having been lost during evolution. Molars and most premolars, except for deciduous third premolars, usually have two roots.

The mandibles were examined in the laboratory for the presence of supernumerary roots by direct observation of the gum line, between tooth and bone, where the identification of extra roots is easy and clear, in one of the hemimandibles (Figure 2). In case of doubt, the presence of a supernumerary root was verified after root extraction.

For the purpose of analyses, we considered four groups of teeth based on previous studies of age and dental replacement patterns in our study areas (Azorit et al., 2002a; Azorit 2011). In the first group, we tallied supernumerary roots in pm_2, pm_3 and pm_4, and took into account only individuals younger than 2.5 years, with deciduous teeth still not replaced. The second group included the study of M_1 in individuals older than 6 months, in which M_1 was completely emerged. The third group included M_2, and took into account only individuals older than 1.5 years. The fourth group included M_3, PM_2, PM_3 and PM_4, and considered only adult individuals, older than 2.5 years, with erupted permanent premolars (Azorit et al., 2002c; Azorit 2011).

Statistical analysis

Prevalence of root anomalies was modeled with binomial logistic regression followed by pairwise z-score comparisons with p-value adjustments using the Tukey method as needed. Our goals were, firstly, to obtain estimates of prevalence with 95% confidence intervals for each study area and, secondly, to test for differences associated with potential explanatory factors. Models were fitted separately for each of the three groups defined above, based on dentition and age, and the significance of terms was evaluated with likelihood ratio tests on nested models. Pairwise comparisons were performed using the R package emmeans (Lenth, 2019). Differences with origin, lineage, soil type and sex of deer populations were tested, when possible, with logistic mixed models with area as a random factor. These were fitted with function glmer in the lme4 package (Bates et al., 2015). All statistical analysis were done with R version 3.6.1 (R Core Team 2019).

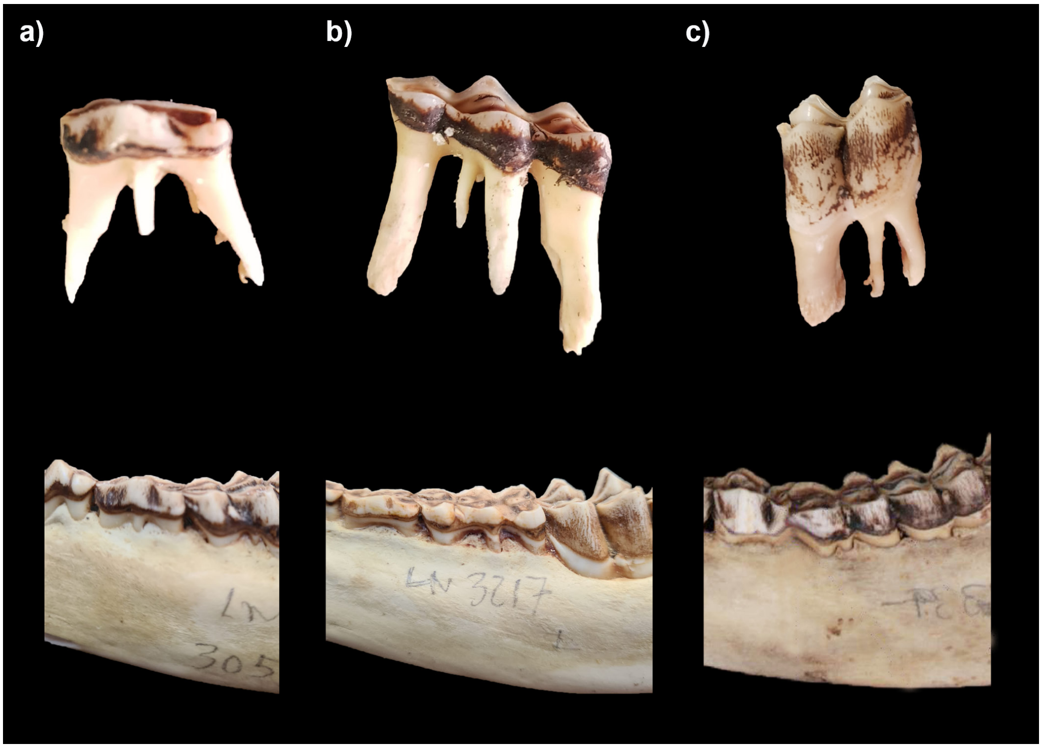


Figure 2. Photographs of supernumerary roots in each tooth and in the mandible: a) supernumerary root in pm^3, b) supernumerary root in pm^4, c) supernumerary root in M^1.

RESULTS

Across areas, the highest prevalence of supernumerary roots was found in deciduous premolar teeth, mostly in pm$_3$ (pm$_3$ = 14.08%, pm$_4$ = 1.72%). Among the permanent teeth, the highest prevalence was found in M$_1$ (2.33%), while it was very low in M$_2$, PM$_3$ and PM$_4$, and limited to Sierra Morena

(M_2 = 0.04%; PM_3 = 0.10%; PM_4 = 0.10%), where sample sizes were substantially larger (n = 1806) than at all other areas. We did not detect supernumerary roots in pm_2, M_3 or PM_2 (Figure 3 and Table 1).

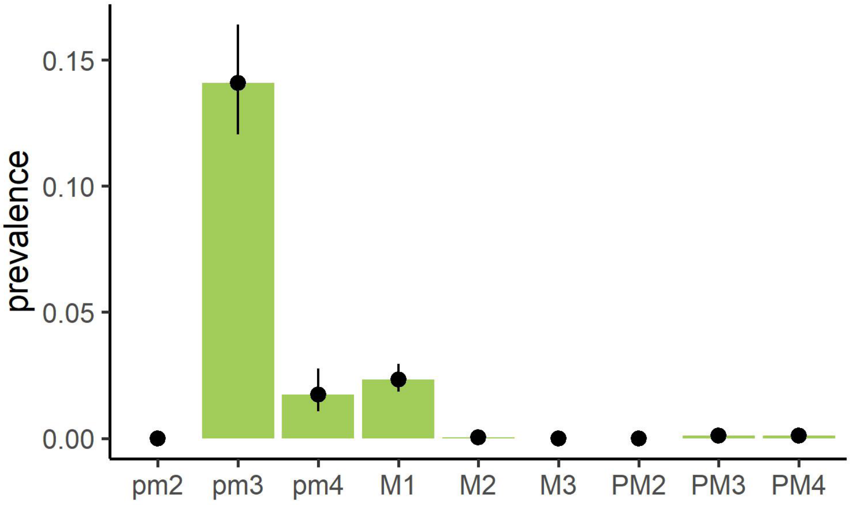


Figure 3. Prevalence (with 95% confidence intervals) of supernumerary roots by tooth

Supernumerary roots in the deciduous dentition were found in Prepyrenees, Cazorla, Sierra Morena and Doñana, with significant differences only between Doñana vs. Sierra Morena (z = 3.835, p = 0.0007). Among all areas with presence of supernumerary roots in deciduous premolar teeth, Sierra Morena had the lowest prevalence (Sierra Morena = 13.6%), and it was remarkably high in the other areas (Prepyrenees = 23.9%, Cazorla = 37.5%, Doñana = 37.8%; but note small sample size and wide confidence intervals (Figure. 4). No deciduous premolars with supernumerary roots were found in High Pyrenees, Quintos de Mora or Cádiz.

In contrast, supernumerary roots in M_1 were found with high prevalence in the High Pyrenees (23.8%), and with lower prevalence in Prepyrenees (1%), Sierra Morena (2.1%) and Quintos de Mora (3%). Significant differences were found between High Pyrenees and Sierra Morena (z = 6.933, $p < 0.0001$) and High Pyrenees and Prepyrenees (z = 3.173, p = 0.0083). No supernumerary roots were found in M_1 in Cazorla, Cádiz or Doñana.

Factors that vary across areas (i.e., origin, lineage, soil type and sex) are difficult to test with only seven areas, yet some patterns are suggested by the data.

Firstly, the lineages strongly differed. The only area with the western European lineage (High Pyrenees) showed the largest prevalence of supernumerary roots in M_1 compared to the other areas (Chi-square = 12.804, df = 1, p = 0.0003458). At the same time, not a single occurrence of supernumerary roots was associated with any deciduous premolar teeth in specimens from this area.

Origin of population (native vs. introduced) was not found to be associated to any differences in prevalence.

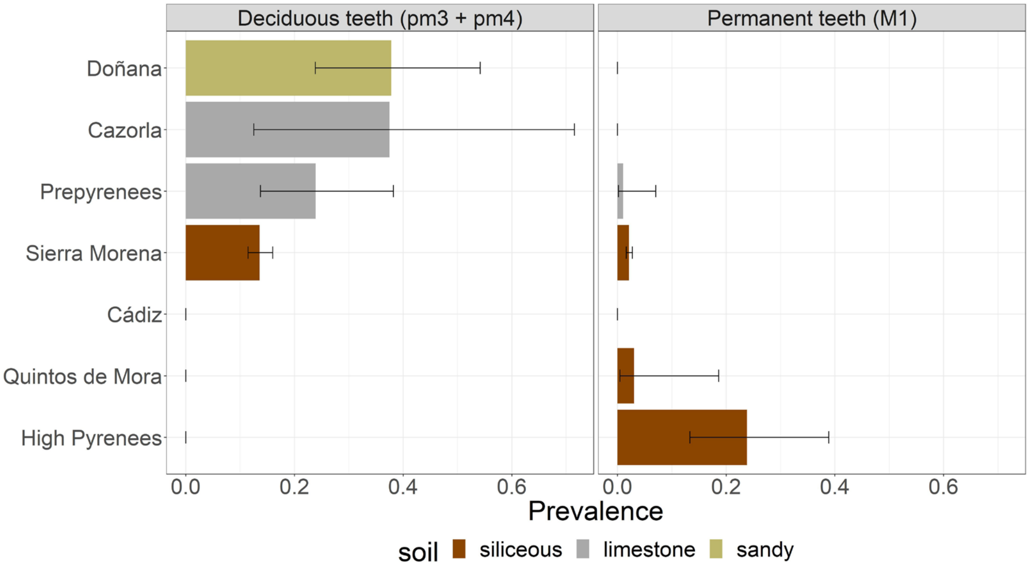


Figure 4. Prevalence (with 95% confidence intervals) of supernumerary roots in permanent dentition (M^1) and deciduous dentition (pm^2 and pm^3) by area and soil type

The soil type was a significant factor for deciduous teeth (Chi-square = 11.486, df = 2, p = 0.003206), with an increasing trend from siliceous (13% prevalence in Sierra Morena, none in High Pyrenees, Quintos de Mora and Cádiz), to limestone (26% in Prepyrenees, 38% in Cazorla), to sandy (37.8% in Doñana) (Figure 4).

No significant differences were found in the prevalence of supernumerary roots with sex, although it was consistently higher in males than in females (deciduous teeth: males = 17%, females = 13.0%; M_1: males = 2.7%, females = 2%).

DISCUSSION

We found a high prevalence of supernumerary roots, exceeding more than the 20% in some cases, varying non-randomly between areas and with contrasting patterns between the deciduous and permanent dentition. Published studies on the prevalence of supernumerary roots in extant mammalian populations are scant, and mainly based on humans and carnivores. For example, in humans, a prevalence of 0.7% was found among 1000 adults in Saudi Arabia (Vani et al., 2016), of 0.68% among 800 individuals in Germany (Schäfer et al., 2009), and of 5.6% among 124 Indian children (Srivathsa, 2015). In the case of carnivores, a prevalence of supernumerary roots of 39.1% has been reported for the Eurasian lynx (Lynx lynx Linnaeus, 1758) (Pettersson, 2010). Herbivore studies however, have either focused on the identification of other dental modifications and anomalies (Mech et al., 1970; Azorit et al., 2002a; Gisburne and Feldhamer, 2005; Martin, 2007), or report chance findings in jaw disease studies (Azorit et al., 2012a). This study is, to our knowledge, the first one that looks at the prevalence of this modification in an extant herbivore species, i.e. the red deer. Furthermore, the strong, non-random patterns that we have found suggest that the prevalence of supernumerary roots could be maintained because of its hypothesized adaptive value.

Differences in the prevalence of supernumerary roots in the studied populations suggest a distinction between two main groups that differ in both ecosystem type and red deer lineage: High Pyrenees (a), where the main ecosystem is high mountain with Atlantic and eurosiberian influence and vegetation, and deer possibly come from the western European lineage (Pérez-González et al., in press), and the remaining six areas (b), where the main ecosystem is Mediterranean and deer belong to the Iberian lineage. In High Pyrenees, we found a high prevalence of supernumerary roots in permanent teeth, particularly in M_1, and we could not find any individuals with supernumerary roots in deciduous premolar teeth. In contrast, in the other areas (i.e., Prepyrenees, Cazorla, Sierra Morena, Quintos de Mora, Cádiz, and Doñana) the prevalence of supernumerary roots in permanent teeth was very low or zero in some areas, whereas the prevalence of supernumerary roots in deciduous teeth was high, particularly in pm_3. We can speculate on both genetic and environmental causes for the observed differences.

The difference in the prevalence of supernumerary roots between the High Pyrenees population and the other populations could be associated with lineage, which is the main cause in other studies with other mammal species (Hauer, 2002; Ota et al., 2009; Iurino and Sardella, 2015). The population of High Pyrenees originated in a migration of French deer, whereas the populations of the other areas are either native of Iberian deer or originated in reintroductions with Iberian deer (Soriguer et al., 1994; Carranza et al., 2016; Queirós et al., 2020). Deer from these two lineages can be easily differentiated phenotypically. Individuals from High Pyrenees population are bigger, and they appear to either belong to the Western European subspecies or be hybrids (Pérez-González et al., in press). Our results suggest that differences in the prevalence of supernumerary roots could be an adaptive response to hard environments, either through phenotypic plasticity or as a result of genetic selection. In the literature there are other examples where the presence of supernumerary roots is attributed to hard environments and diets, i.e. the population analysis of abnormal teeth in wolf (*Canis lupus* Linnaeus, 1758) in Turkey (Ay and Albayrak 2019) or in Eurasian otter (*Lutra lutra* Linnaeus, 1758) in Germany (Hauer 2002). On the other hand, other mammal studies propose a genetic cause involving the process of the root morphogenesis and the Hertwig's epithelial root sheath (Horwitz and Davidovitz, 1992; Hauer, 2002; Ota et al., 2009; Iurino and Sardella, 2015), while this modification has gone unnoticed in some studies (Pasda, 2006; photo of individual 64 and 171, pg. 134).

The presence of extra roots could be advantageous under some environmental conditions. The dissimilarities between the ecosystem of High Pyrenees and the others give rise to an enormous variance in the diet of deer in each area. In the high mountain ecosystem, in High Pyrenees, the diet is composed mainly of graminoids and, for that reason, deer in this area are considered more grazers than browsers (Garin et al., 2001; Gort-Esteve in press). Grazers ingest more abrasive matter and phytoliths than browsers, and that is one of the main factors influencing molar wear (Kubo and Yamada 2014). The presence of high wear implies a weakening in the tooth, and supernumerary roots could help strengthen it. If this were the case, the presence of supernumerary roots would imply greater survival, since wear is related to the loss of tooth and chewing functionality, which is positively associated to senescence in large herbivores (Carranza et al., 2004; Gaillard et al., 2015). Since the sequence of tooth eruption indicates the sequence of wear (Brown and Chapman, 1991; Azorit et al., 2003a, 2003b), and since the first molar is the first permanent tooth to erupt, this is where we would expect most wear. This could be the cause of the high prevalence of supernumerary roots in the first molar. Ozaki et al., (2010) found a positive correlation between the wear rate of M_1 and the consumption of graminoids in wild sika deer (*Cervus nippon* Temminck, 1838), which supports our contention that M_1 has a high prevalence of supernumerary roots because it wears out more. In addition, they relate M_1 and M_3 durability to life span, and conclude that M_3 is more important because it is stronger and has less wear than M_1 (Ozaki et al., 2010). Furthermore, the High Pyrenees is the area with the harshest winter, with a thicker and more persistent layer of snow (Meteorological Service of Catalonia). The greater difficulty of finding food in winter due to the snow cover may involves compensatory overgrazing in spring and summer, which would contribute to rapid tooth wear, as seen in reindeer (*Rangifer tarandus* Linnaeus, 1758) (Skogland, 1988). For these reasons, deer in this area might have less durable M_1, and this might be compensated for by both the highly durable M_3 (Solounias et al., 1994), and by supernumerary roots, which would explain their higher prevalence in High Pyrenees.

In contrast to the High Pyrenees, the prevalence of supernumerary roots in deciduous premolar teeth is remarkably high in populations living in Mediterranean ecosystems. The third deciduous premolar had the highest prevalence. The lower prevalence in the fourth deciduous premolar could be due to the fact that it is the first to be replaced. In addition, pm_4 already has one more root than pm_2 and pm_3, and with three roots there might be little room for another one. The absence of supernumerary roots in pm_2 might be linked to a loss of functionality, which would imply less pressure for strong attachment, similarly to what happened to the first deciduous premolar, whose loss of functionality probably explains its evolutionary disappearance (Riney, 1951). Selective pressures may act against those premolars that likely do not contribute substantially to increasing fitness and survival (Jung et al., 2016). In contrast, having supernumerary roots in pm_3 could be an advantage, as it might better anchor the tooth to the mandible, especially in the areas where forage after weaning is hard. Weaning is a key event for deer survival (Hanson 1958; Milne et al., 1987; Pollard et al., 1998; Pollard and Littlejohn 2000). In the strongly seasonal Mediterranean southern ecosystems, weaning usually coincides with a period of hard forage in autumn, with a diet richer in acorns or browse (Bugalho et al., 2001; Bugalho and Milne, 2003; Azorit et al., 2012c). Thus, in this ecosystem, where hard forage compromises dental stability, calves might benefit from having strong deciduous teeth. By allowing appropriate teeth attachment and continuous and functional movement, supernumerary roots in deciduous teeth could have implications for adequate food processing, animal performance and even increased survival.

However, supernumerary roots in deciduous premolar teeth appears to have higher prevalence in Prepyrenees, Cazorla and Doñana than in Sierra Morena, and none could be found in Quintos de Mora or Cádiz. Both Prepyrenees and Cazorla are located at a higher elevation, where the dominant tree is pine (*Pinus* sp. L.), whereas Sierra Morena and Quintos de Mora are at lower elevations, with a vegetation is dominated by oaks (*Quercus* sp. L.), and shrubs (Rodríguez-Berrocal, 1978; Alvarez and Ramos, 1991; Miranda et al., 2012). Pine needles and bark are harder and more difficult to chew through than the same parts in oaks and other shrubs. In the case of Doñana, deer are found mainly in the scrub and dune ecosystems (Rogers and Myers, 1980; Braza and Alvarez, 1987), where the conditions, i.e. sandy soil, arid climate and strong seasonality, produce a hard vegetation, and it could be the predisposing factor for the high prevalence of supernumerary roots in this area. In contrast, the climate in the area where the Cádiz population lives is very humid, and deer live on a softer diet (San José et al., 1997), which implies low wear and could explain the low prevalence of supernumerary roots in this area. Kaiser and Schulz (2006) found that teeth wear, low reliefs and blunt cusps are negatively correlated with precipitation, water balance and humidity, as they make the vegetation softer (Kaiser and Schulz, 2006; Ozaki et al., 2010). For that reason, we suggest that the higher prevalence of supernumerary roots in Prepyrenees, Cazorla and Doñana, could be due to their harder forage. Kangas et al., (2017) studied the shape of the mandible in moose, and found that its morphometric variation corresponded to an adaptive response to the environment and diet, with no or only a weak association with genetic population structure.

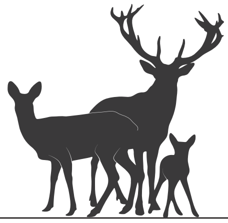
The hypothesis of a higher prevalence of supernumerary roots in areas with siliceous soils as an adaptation reinforcing the dental system in response to low nutrients and calcium and the acidic pH of this soil type (Azorit et al., 2012c; Berkovitz and Shellis, 2018), was not supported by our results. In High Pyrenees, where there is a high prevalence of supernumerary roots in permanent teeth, the soil is siliceous, but there is also siliceous soil in Sierra Morena, Quintos de Mora and Cádiz, and yet the prevalence of supernumerary roots in M_1 is very low or zero. In the case of deciduous teeth, the highest prevalence of supernumerary roots is in Doñana, which has a sandy soil, but the other two high values of prevalence are in Prepyrenees and Cazorla, where the soil is calcareous.

Our results also did not support the hypothesis we posed regarding sex differences, i.e. that the prevalence of supernumerary roots would be higher in females than in males. Nonetheless, we found a marginal difference in the prevalence of supernumerary roots in deciduous teeth, i.e., higher in males than in females, that was consistent across populations. This minor difference could be attributed to the fact that there is greater dental wear in stags than in hinds (Azorit et al., 2003b), and males might therefore benefit from stronger deciduous teeth to avoid compromising the dental stability, as discussed for juveniles in Mediterranean ecosystems (Azorit et al., 2012c).

CONCLUSIONS

Dental anomalies have been relatively well studied in some mammalian groups, perhaps due to interest in the specialization of their dentition and the functional role teeth play in determining fitness and survival (Jung et al., 2016). The presence of supernumerary roots has been hardly studied, yet the discovery of differential prevalence among populations would allow us to make inferences about links with their environments, lineages or origins. Deepening this approach can open up new possibilities in zooarchaeological, paleontological, wildlife and evolutionary research.

CHAPTER 3: SEASONAL DYNAMICS OF GROUP SIZE AND TYPE IN WILD RED DEER ACROSS ECOLOGICAL GRADIENTS:HOW TO INCREASE FORAGING EFFICIENCY?



ABSTRACT

The dynamics of group formation are influenced by various factors, such as food availability, interspecific competition, and predation, among others. These factors differ between areas, which is why different patterns of individual aggregation can be observed. Additionally, these factors also vary throughout the year, especially in relation to changes between favourable and unfavourable seasons and areas. The type of group can also vary depending on the topography, habitat types, and the factors described above. This study is pioneering in comparing the size and type of deer groups in five ecologically distinct areas simultaneously, representative of a wide range of ecological gradients. According to precedent works, we expected large, mixed groups to be more frequent in unfavourable seasons due to the aggregation of individuals in areas with abundant food or shelter. Conversely, in favourable seasons, we would expect individuals to disperse, resulting in smaller group sizes. Considering that deer form harems during the rutting season, we also expected large and mixed groups with a higher proportion of females during this period. Our results have shown that deer group varied according to food and resources availability, with large groups forming in unfavourable periods

and small groups forming in favourable periods, as hypnotized. Nevertheless, the large groups during unfavourable periods have not been mixed, but rather segregated by sex. On the other hand, the mixed group size found during the rutting season are among the lowest reported, and one of the reasons attributed to this is that, being a favourable period, individuals have prioritized feeding over reproduction and have dispersed due to the abundance of food everywhere. The repercussions of this are discussed.

INTRODUCTION

Many species are gregarious and form groups with simple or complex social relationships. Being part of a group directly influences individual fitness in aspects such as pathogen transmission, predation risk or reproductive success (Geist, 1974; Clutton-Brock et al., 1982; Schauber et al., 2007; Pépin and Gerard, 2008b; Vander Wal et al., 2013; Creel et al., 2014; Brennan et al., 2015). For most large mammal herbivore species group formation is a survival strategy that carries both benefits and costs, therefore generating trade-offs. A numerous group reduces the risk of being predated, since it increases the number of warden individuals, but, at the same time, a high number of individuals increase the detectability for the predator (Vine, 1971; Alcock, 1998; Barja et al., 2008). On the other hand, increased vigilance may cause a large reduction in foraging time (Creel, et al. 2014). Therefore, the predation risk is an important factor that influences the group size (Hebblewhite and Pletscher, 2002). For example, Jedrzejewski (2006) determined that deer groups vary seasonally, increasing the number of individuals in winter because of hunting. Another trade-off is between the ease of finding food for a large group, since a large number of individuals cover a larger search area, and the increase of intragroup competition (Hobbs et al., 1996; Fortin et al., 2004; Thaker et al., 2010). These trade-offs imply some changes in the size and composition of groups during the annual cycle.

Environmental factors (especially those changing over time) can also provoke variation of group size, but studies of group size and social organization in wild ungulates in response to environmental conditions are limited (Carranza and Valencia, 1992). An environmental factor which has been shown to condition the size of groups is the thickness of the snow cover and low temperatures (including frozen environments). The formation of larger groups in winters with a deep snow cover or a frozen environment as a behavioural strategy to reduce energetic costs to find food, predation risk, or both, has been studied in species such as boreal woodland caribou and red deer (Jedrzejewski et al., 1992; Jung et al., 2019).

Food availability, directly related to climatological seasonality and ecological gradients, is another cause of intra-annual and spatial variation in group size. The competition for scarce resources and the aggregation at foraging sites are factors that contribute to the modification of group size (Elmhagen et al., 2014), and resource availability has been shown to produce seasonal differences in group size both in herbivores and in carnivores (Ramesh et al., 2012; Elmhagen et al., 2014). The habitat type also affects food availability, and therefore, group size (Borkowski and Furubayashi, 1998; Lingle, 2003), and several studies have shown that groups are larger in open habitats, which tend to provide better foraging grounds (Hirth, 1977; Pays et al., 2007; Brennan et al., 2015)

Two hypotheses explain the aggregation of species depending on the availability of food: the resource dispersion hypothesis and the conspecific attraction hypothesis. The resource dispersion hypothesis suggests that where resources are dispersed, heterogeneous, and rich enough, multiple individuals can collapse into groups that share the same space at little cost to each other (Johnson et al., 2002; Macdonald and Johnson, 2015). On the other hand, the conspecific attraction hypothesis proposes that individual space use is dependent on the distribution of conspecifics rather than on the location of resource patches, because the presence of conspecifics allows a recognition of suitable habitat or other social benefits such as mating success or defence against predation (Ray and Smith, 1991; Peignier et al., 2019). Using the social information to increase foraging opportunities is a studied strategy in some ungulate species (Kao et al., 2014; Merkle et al., 2015; Webber and Vander Wal, 2021).

Sex is another factor that must be considered in a group size study. The sexual dimorphism in ungulates implies, not only variability in group size seasonally, but also differences in group composition. As in most ungulate populations, red deer hinds and stags were segregated most of the year, hinds spend 80-90% of its time without adult stags (Clutton-Brock et al., 1982). Red deer form sex-specific groups outside the breeding season, and mixed groups during the rut, since its reproduction strategy is polygyny with harem formation (Carranza, 2017). This segregation is due to the sexual dimorphism of this species. There are many hypotheses of how dimorphism could affect group formation, from the result of aggression between sexes (Weckerly, 2001; Weckerly et al., 2001), to the improve of feeding efficiency (Stewart et al., 2011), or the predation hypothesis, which predicts a bigger segregation with lower predation risk (Bowyer, 2004).

In this study, we analysed red deer group size and composition in wild populations living in five contrasting ecosystems along an environmental gradient, from high mountains (colder, with higher precipitations and vegetal production, and snow) to Mediterranean lowlands (warmer, drier and normally no snow). This is, to our knowledge, the first study to examine the temporal dynamics in red deer group size and composition in populations of the same species with several different ecological conditions at the same seasons. Our hypothesis was that we would find more large, mixed groups in unfavourable seasons and smaller, sex-specific groups in favourable seasons. We expected large, mixed groups to be more frequent in unfavourable seasons due to the aggregation of individuals in areas of food richness. Contrarily, in the favourable seasons, with food more abundantly and homogenously distributed across the territory, we would expect individuals to disperse and group size to decline. In addition, we expected to find large mixed groups with a higher proportion of females in autumn considering it is the rut time and red deer form harems.

METHODS

STUDY AREAS

The study was conducted in five ecologically differentiated areas, representing the ecological gradient, that vary in climate, topography and vegetation (Figure 1). Even though they are separated by no more than 200 km, their altitudes and climates are very contrasting and, as a consequence, there are contrasted habitats and they do not have the same unfavourable and favourable seasons (Supplementary Table 1).

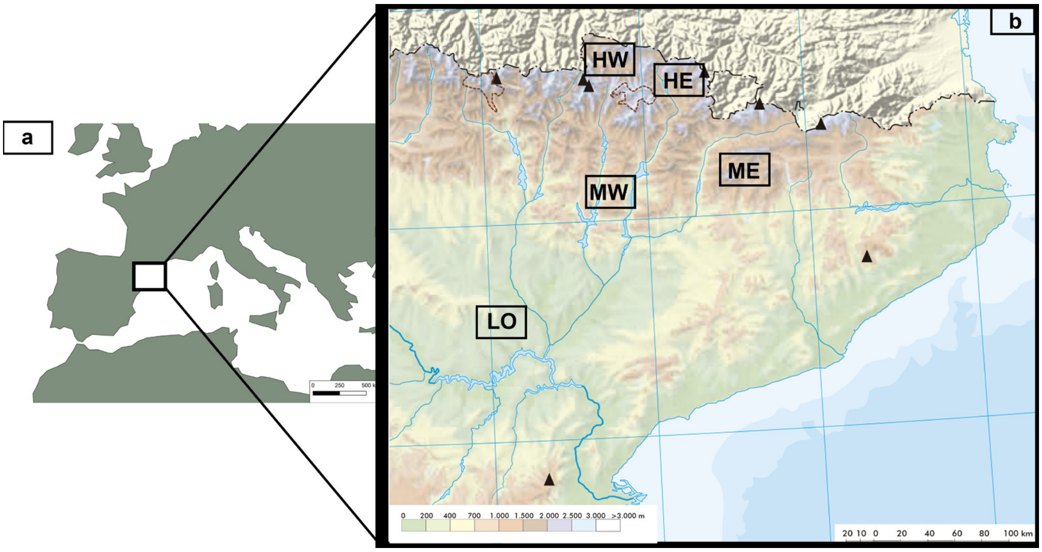


Figure 1. Map of study areas: a) Study area in relation to Europe (Land map, version 4.1.0. Made in Natural Earth); b) Study areas in relation to north-east Spain, HW: High-West; HE: High-east; MW: Mid-West; ME: Mid-East; LO: Lowland (Physical map 2022, National Geographic Institute of Spain).

The High-East and the High-West areas are both in the high Pyrenees, within the National Hunting Reserve of Alt Pallars and within the Controlled hunting area of Baish i Mig Aran, respectively. The climate at both areas is montane and subalpine (with oceanic influence), with temperate summers and a large snow cover in winter and spring, presenting cold winters, temperate summers and high precipitations. However, differ in Atlantic influence. In particular, the Hight-East area has a Mediterranean subalpine and montane climate with Atlantic influences, while the High-West area is under an Atlantic subalpine and montane climate. In the High-East area the highest peak is 2.947 meters, with many peaks of more than 2.000 meters, while in the High-West area the maximum altitude is around 1.721 meters. Above the treeline, grasslands, rocks and screes dominate the landscape. Fir (*Abies alba*) and black pine (*Pinus uncinata*) forests are found in the subalpine stage, and red pine (*Pinus sylvestris*) and deciduous forests composed mainly by oak (*Quercus petrea*), hazel (*Corylus avellana*), birch (*Betula pendula*), tremble

(*Populus tremula*) or ash (*Fraxinus excelsior*), dominating the mid-mountain ecosystem, and forming extensive forests. The mid-mountain landscape is completed by rocky outcrops and extended formations of Pyrenean broom (*Genista balansae*) and grasslands. The global grassland availability within the study area is high. Valley bottoms are the most anthropized, including often managed grasslands and some agricultural fields. The genetic composition of red deer in these areas was associated with that of European non-Iberian red deer populations (Pérez-González et al. 2023, in press).

The Mid-East and Mid-West areas are more contrasting than the northern areas. They are located in the National Hunting Reserve of Cadí and Boumort, respectively. The climate in these areas is a transition between the northern areas with the Mediterranean, with also sub-Mediterranean areas, but in the case of the Mid-East area, it is heavily influenced by an Atlantic climate. The maximum elevation is around 1.830 meters in the Mid-East area, and 2.075 meters in the Mid-West area. In the latter, there are more deciduous forest in the lowest stages, with mosaics of fir and black pine forests, and there is an alpine stage, with extensive zones of grasslands. Contrarily, in the Mid-West area, the current vegetation represents practically all the altitudinal stages except the alpine one. Sorted from lowest to highest, we find holm oaks forests (*Quercus ilex*) and buxus scrublands (*Buxus sempervivens*), pine forests (*Pinus nigra* and *Pinus sylvestris*) and, in the higher stages, black pine forest (*Pinus uncinata*) and grasslands. The genetic composition of red deer in these areas was associated with the Iberian red deer subspecies (Pérez-González et al. 2023, in press).

Finally, the Lowland area is located near the town of Fraga. It is characterized by an extreme Mediterranean climate with a large seasonal thermal oscillation, from –10 degrees Celsius in winter to 40 degrees Celsius in summer. Also noteworthy is the dry rainfall regime, with an average of 350 mm annually. The highest elevation reaches 394 m. Most of the area is plain with fields of cereals and steppes, and forests of Pinus halepensis. The genetic composition of red deer in this area was associated with the Iberian red deer subspecies (Carranza et al., 2016; Queirós et al., 2020).

Data collection

We observed a total of 3,169 groups. To qualify as a group, we required that members be involved in one or more similar or coordinated activities such as movement in the same direction or in coordination (Carranza and Valencia 1992).

To obtain the data we made diurnal and nocturnal transects. These transects were made by direct observations in secondary and primary roads selected to cross the zones with higher density of red deer, or in points with a wide view in areas without roads. These transects or points were done four times a year (summer, autumn, winter and spring) between 2016 and 2019, although not uniformly at all areas (Table 1). Summer matched the observation of the first fawns and autumn coincided with the rut. Winter was the coldest time, but especially hard in mountain areas, where the harshness brought about by the snow cover and frozen surface extended into early and mid-spring. In contrast, summer was considered a hard season in the Lowland are due to high temperatures and lack of water and food (Table 1).

Data analysis

To model group size, we used all the observed groups (n = 3.169), but to properly model group composition, only groups with all individuals identified to age (juvenile or adult) and sex (for adults) were included in the analyses (n = 2.855). In addition, we did not include fawns in the analysis, as they do not make decisions about which group to join or where to go, which their mothers do. Therefore, the analysis only contained the identified individuals older than 1 year. Deer groups were then classified into three types: mixed group, which consisted of individuals of both sexes; female groups, where individuals > 1 year old were all females; and male groups, formed by >1 year old males only.

Group size was modelled following Niwa (2003, 2004) as a discrete logarithmic distribution with probability mass function defined as:

where c = (1 – 1/Np) is the shape parameter, expressed here in terms of Np, which is the mean experimented group size. While the average group size (N) describes the outsiders' viewpoint (how big is on average a group), the experimented group size (Np) describes the group members' viewpoint (how big is on average the group that an individual belongs to) (Carranza and

Valencia 1992; Reiczigel et al. 2008). Thus, this one-parameter distribution, which is simple and has been shown to provide a good fit to group size distribution for a variety of species (Griesser et at., 2011; Ma et al., 2011), has the added advantage that it simultaneously yields estimates of N and Np, which are linked in the model. Models were fitted by maximum likelihood using function vgam with family logff in the R package VGAM (Yee, 2010).

$$f(N) = \frac{1}{\log(N_p)} N^{-1} (1 - \frac{1}{N_p})^N$$

We fitted two different models to our data. The first model included the variables area and season and used the full data set, while the second used these two variables plus group type (i.e. group sex composition) and used the reduced data set. In each case, we fitted separate models for the High-West area (with no winter data) and for the remaining areas (with data for all four seasons). The statistical significance of each factor is shown as analysis of deviance tables with marginal (type II) likelihood ratio tests.To test for differences between seasons, we did pairwise comparisons by refitting the model for each pair of seasons while holding area fixed (due to significant interaction between area and season). Statistical significance was obtained from likelihood ratio tests, with p-values later adjusted by the Benjamini & Hochberg (1995) method to correct the false discovery rate.All statistical analyses were done with the R statistical package, version 3.6.1.

RESULTS

Mean group size, N, was 2.74 and mean experimented group size, Np, was 5.97 across all areas and seasons, with maximum observed group size between 14 and 19 in all areas except for High-West, where it reached 75 individuals in one group. The relative frequency of groups of each size changed between areas and seasons (Figure 2). In analysing the full data set (i.e. without regard for group composition), we found that the logarithmic model generally fitted well the observed frequency distributions (Supplementary Figure 1 and 2), although in several cases, and most notably in spring, the observed frequency of two-member groups was higher that of solitary individuals.

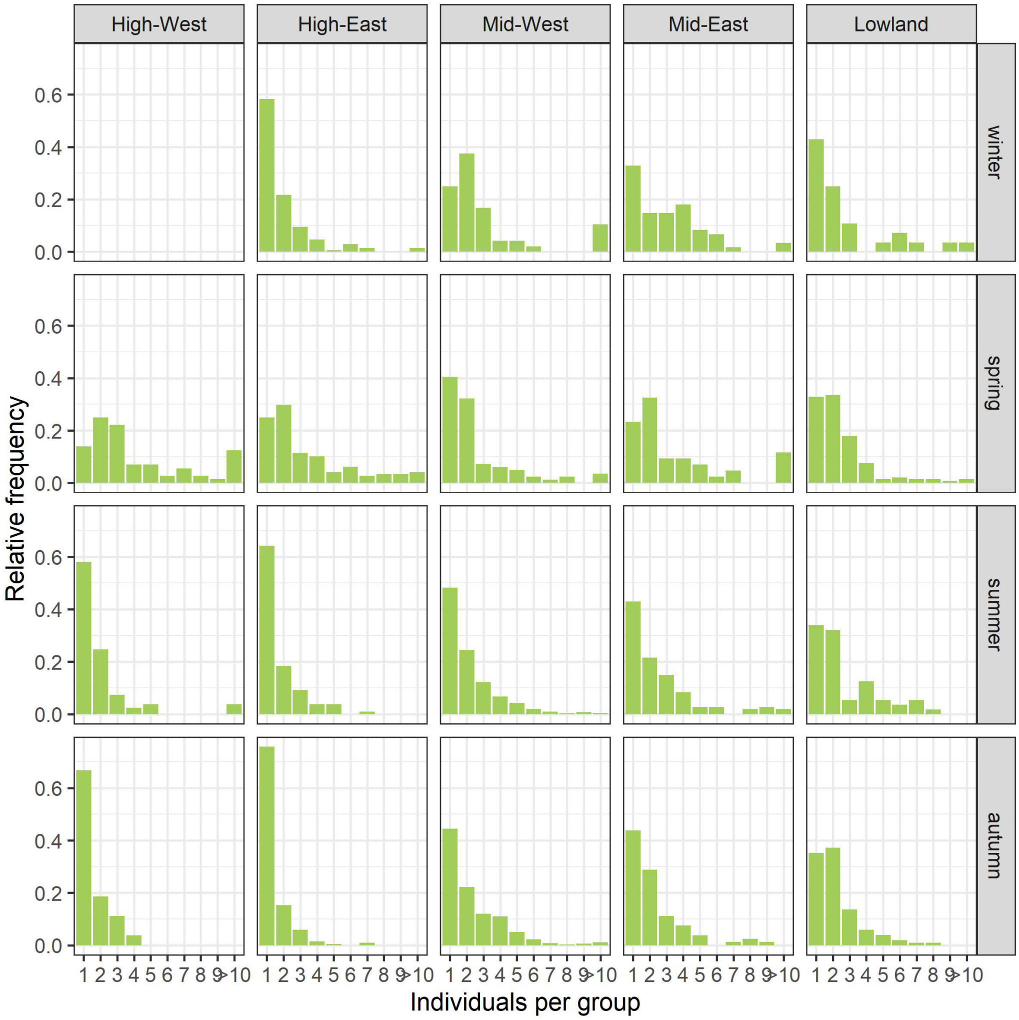


Figure 2. Group size relative frequencies by area and season.

We found differences in group size distribution across seasons and areas. Season was significant in the model for High-West area alone and in interaction with area in the model for the four areas that had data for all four seasons (Table 1). A significant interaction between area and season indicates that there was not a common seasonal pattern across areas. Pairwise comparisons among seasons are shown in Table 2. Both northern areas and the Mid-East area, however, followed a similar pattern, with a clear peak in average group size in spring and with the lowest values in autumn (Figure 3, top row). In the Mid-West area, the biggest average group size occurred in winter and the smaller in summer. In the Lowland area, differences between seasons were less clear, although the biggest average group sizes occurred in winter and summer, and the smaller ones in autumn.

High-West : season	**Df**	**LogLik**	**Pr(>Chi)**
Season	2	-365.95	1.987e-12
Without High-West: area + season	**Df**	**LogLik**	**Pr(>Chi)**
Area	3	-4007.7	1.173e-10
Season	3	-4018.8	1.992e-15
Area:Season	9	-3983.1	8.797e-09

Table 1. Analysis of deviance tables for models using all groups and testing differences between seasons and areas. Separate models are presented for the High-West area (no winter data) against factor Season, and for the remaining areas against factors Area and Season. Type II (marginal) likelihood ratio tests compare each model against the same model without the focal factor. The difference in number of parameters between the compared models, the log-likelihood of the simpler model and the p-value for the chi-square test are shown.

	winter vs spring	**winter vs summer**	**winter vs autumn**	**spring vs summer**	**spring vs autumn**	**summer vs autumn**
High_West				1.09e-08	3.33e-07	5.29e-02
High_East	9.06e-08	1.50e-01	8.45e-06	5.02e-08	1.74e-20	2.04e-02
Mid_West	0.23	0.01	0.03	0.07	0.30	0.12
Mid_East	0.37	0.31	0.10	0.07	0.02	0.37
Lowland	0.72	0.83	0.72	0.72	0.72	0.72

Table 2. Multiple comparisons analysis for seasons in each area. The testused for the p-value was the Chi-square test with the adjust of p-values with the Benjamini and Hochberg (1995) method.

Models including group type (male only, female only and mixed) showed reasonably good fit, but poorer than that of models with the full dataset, as expected due to smaller sample size and higher model complexity. Group

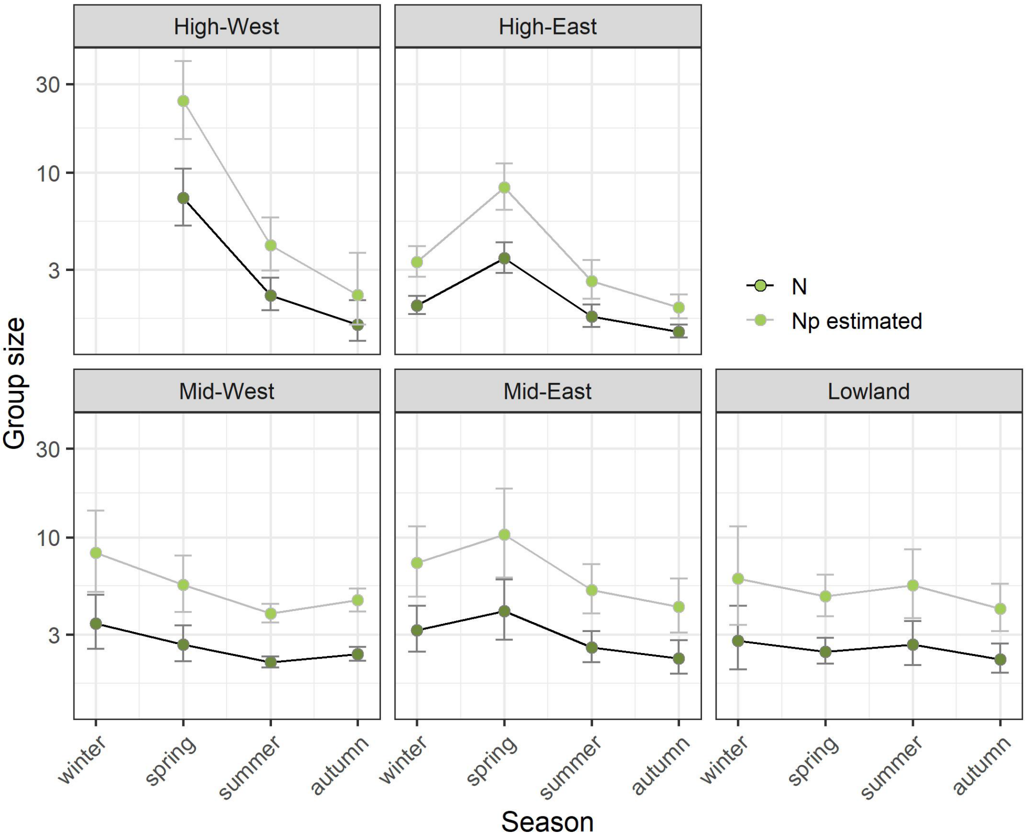


Figure 3. The average group size (N, black) and the average experimented group size (Np, grey) for each area as estimated from the fitted models, with 95% confidence intervals.

type was significant in the model for High-West, with no interaction with season (Figure 4 and Table 3). There were strong differences in mean group size by group type, with much larger values for mixed groups at all seasons, and smaller mean group size in male-only groups. In the model without the High-West areaall three variables were significant (area: p = 5.221e-06; season: p = 1.264e-15; and type: p = < 2.2e-16), but also the interaction between area and season (p = 3.876e-05), and type and season (p = 0.007222), indicating complex responses to seasons among areas and group types (Table 4). Again, mixed groups were larger on average, while male groups showed the smaller average group size, most notably in autumn at all areas. In general, female-only and mixed groups showed more concordant seasonal patterns and distinct

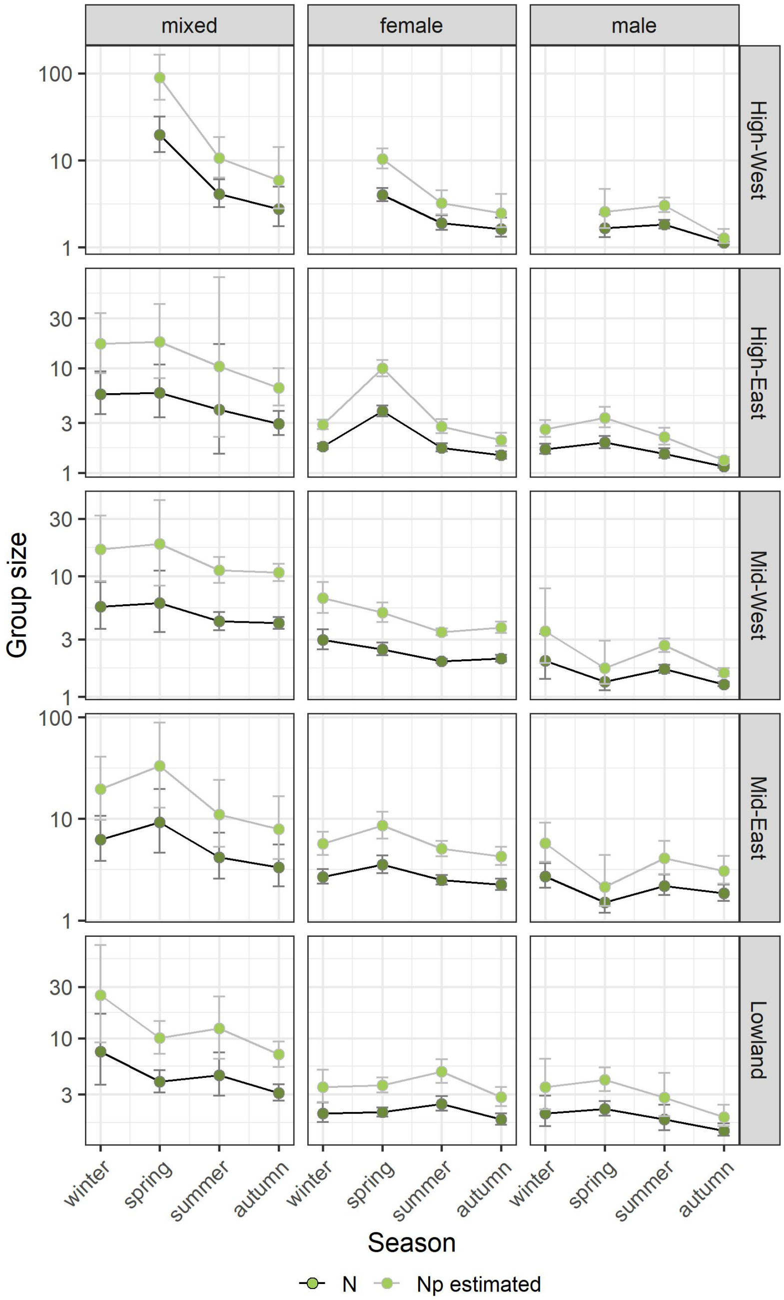


Figure 4. The average group size (N, black) and the experimented group size (Np, grey) for each area as estimated by the fitted models that included the variable type (mixed sex, female only and male only), with 95% confidence intervals.

High-West : season + type	**Df**	**LogLik**	**Pr(>Chi)**
Season	2	-333.01	9.460e-05
Type	2	-339.01	2.349e-07
Season:Type	4	-323.74	0.2832
Without High-West :area + season + type	**Df**	**LogLik**	**Pr(>Chi)**
Area	3	-3851.8	5.221e-06
Season	3	-3882.3	1.264e-15
Type	2	-3955.2	< 2.2e-16
Area:Season	9	-3835.0	3.876e-05
Area:Type	6	-3821.3	0.192470
Season:Type	6	-3825.8	0.007222
Area:Season:Type	18	-3817.0	0.945687

Table 3. Analysis of deviance tables for models using all groups and testing differences between areas, seasons and group types. Separate models are presented for the High-West area (no winter data) against factor Season and Group Type, and for the remaining areas against factors Area, Season and Group Type. Type II (marginal) likelihood ratio tests compare each model against the same model without the focal factor. The difference in number of parameters between the compared models, the log-likelihood of the simpler model and the p-value for the chi-square test are shown

	High_West	**High_East**	**Mid_West**	**Mid_East**	**Lowland**
mixed vs male	3.9948e-15	1.3964e-14	1.5275e-32	0.0003	6.9276e-05
mixed vs female	4.5223e-06	5.2209e-04	1.8778e-19	0.0007	1.2356e-05
female vs male	3.7067e-05	1.8061e-12	1.9757e-10	0.1886	4.5959e-01

Table 4. Multiple Comparisons analysis for seasons in Axial Pyrenees and Pre-Pyrenees. The test used for the p-value was the chi-square test with the adjust of p-values with the Benjamini and Hochberg (1995).

from those of male-only groups. In mixed groups, average group size was larger in winter and spring, and smaller in autumn. The larger female groups occurred in spring in the northern areas, but also in winter in the Mid-West area. The larger average group size of female groups in the Lowland area occurred in summer. In both female-only and male-only groups, mean group size decreased in autumn. The biggest male groups were in winter and spring.

We also analysed the proportion of individuals that were in mixed groups (Figure 5). The lowest proportion of individuals in mixed groups was found in the northernmost area (High-East), while the southernmost area (Lowland) presented the highest proportion. Curiously, despite having the minimum and the maximum proportions, these areas followed the same seasonal structure, with the highest proportion of individuals in mixed groups in autumn, and declining proportions through winter, spring and summer. A similar pattern was found in the Mid-West area. In contrast, the High-West area and the Mid-East area had a higher proportion of mixed groups in winter and spring, and lower in summer and autumn.The proportion of females in mixed groups was higher than the one of males in all seasons and areas.

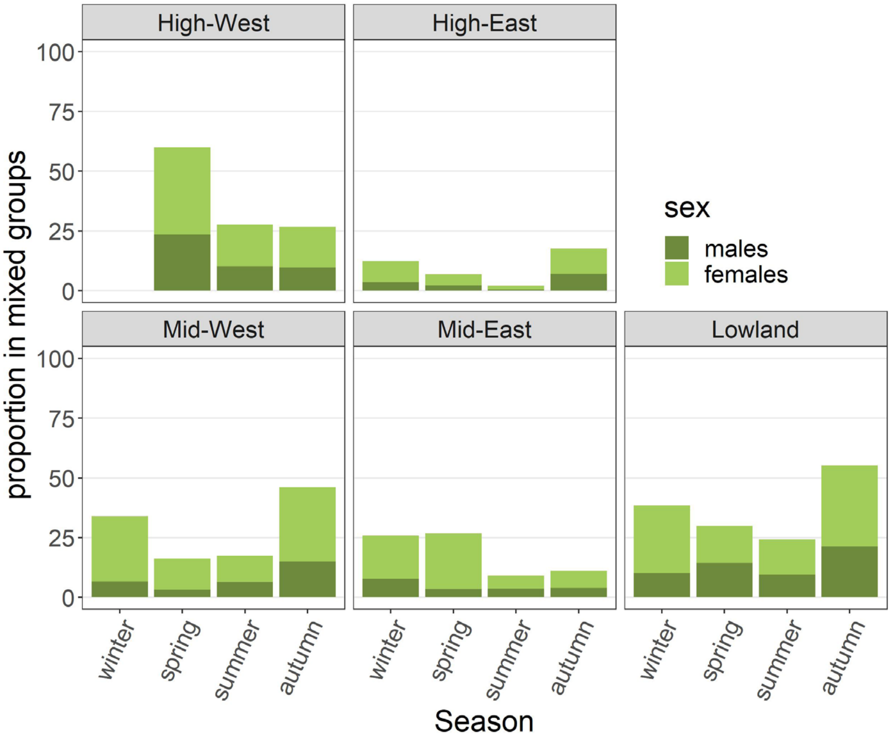


Figure 5. The proportion of individuals in mixed groups for each area as estimated by the fitted models, and the proportion of males and the proportion of males and females in these groups.

DISCUSSION

This is, to our knowledge, the first study that analyses differences in group size and composition in wild populations of red deer in different ecological areas across seasons. Due to the ecological contrasts between the five studied areas, favourable and unfavourable seasons differed between them. These conditions changed according to the climatology and food availability and dispersion of each area. We found large mixed groups in unfavourable moments and small, sex-specific groups in favourable moments in almost all areas and seasons. Considering that deer form harems, we expected to find larger mixed groups with a higher proportion of females during the rut, but this has not been the case in all areas.

GROUP SIZE

In the high mountain areas (High-East and High-West), and in the Mid-East area, the unfavourable seasons were winter and spring. Although we have no data for winter in the High-West area, we found mean group size to be consistently higher in spring and (in High-East and Mid-East) in winter. In these seasons, especially in spring, food is only available in some grasslands without snow or with a thin snow cover. Despite being also at high altitude and under a mountain climate, the Mid-West area does not receive as much snowfall as the high mountain areas. Still, the low temperatures and the snow depth during winter make this season the most unfavourable, and it is when the largest mean group size was found. In the Lowland area, the most unfavourable seasons were winter (because of low temperatures and reduced food availability) and, unlike the other areas, summer due to the drought, and again the largest mean group sizes were found in these two seasons. Presumably, in the unfavourable seasons red deer aggregate in the patches with better forage availability, especially grasslands in the mountain areas and agricultural zones in the Lowlands (Jedrzejewski et al., 1992; Jung et al., 2019). Other species use the same strategy in unfavourable seasons. Ramesh et al. (2012) found that sambar (*Rusa unicolor*) formed groups with large aggregations near swampy grasslands and burnt areas to increase foraging efficiency. Some studies of caribous (*Rangifer tarandus caribou*) showed that foraging efficiency of individuals in larger groups may be enhanced because of the ease to locate food and establish and maintain snow-free feeding craters (Jung et al., 2019; Webber et al., 2021; Webber and Vander Wal, 2021). Interestingly, Jung et al. (2019) found smaller groups of caribou in the same season in other areas and suggested that, under different circumstances, smaller groups might also decrease intraspecific competition at a time of limited forage resources. This is not the only reported case of small groups in unfavourable moments. Other species, like the blue sheep, broke into smaller foraging groups when patchily distributed and low quality forage did not support big groups (Oli and Rogers, 1996).

On the other hand, during favourable seasons food availability was higher and more distributed across the territory, mainly because of the greater rainfall and the sprouting of most plants. Accordingly, we expected individuals to disperse and to benefit from being in smaller groups. This was also upheld by our data. Other studies have also found mean group size to decrease in favourable conditions. In the case of caribou in areas with deep snow cover in winter, group size decreased as snow depth decreased in late winter (Jung et al., 2019). In contrast, in the southern Iberian Peninsula, Carranza and Valencia (1992) found larger groups of red deer in winter and spring, which in this area were considered favourable seasons, and argued that this was due to a higher use of open habitats in these seasons, since in the summer in these habitats there is not such an abundance of herbaceous plants. Another case of large groups of red deer in favourable seasons was found in the Isle of Rum, where groups were larger in summer, when food availability was higher and intraspecific competition decreased (Clutton-Brock et al., 1982). All these studies and other with similar results (Hirth, 1977; Miura, 1983) argued that red deer group size increases with decreasing plant cover and increasing predation risk. In these areas in the favourable seasons, forage was concentrated in open areas, while in our case, food waswidely distributed in the favourable seasons, which may explain the contrasting dynamics.

Seasonal changes in deer group size could be also related to the trade-off between the availability of foraging opportunities and predation risk (Cherry et al., 2015; Webber et al., 2021). The patterns of variation of group size in our populations, larger groups in unfavourable seasons and lower in favourable ones, appear to be similar to what Jedrzejewski et al. (2006). They found this pattern in a deciduous mixed-lowland forest in the northeasterPoland, but they attributed this size changes to human hunting, followed by a corn crop and in the third place to snow cover. Despite following a similar pattern, our group sizes, both in unfavourable and in favourable seasons, were much lower. The reason of these lower values could be because predation and hunting pressure was null or lower in our areas. Deer did not have any natural predators in our study areas. On the other hand, being mostly public hunting reserves, this activity was highly regulated, with only a few drives conducted per year, and a major part of hunting permits being issued for stalking, which did not cause significant disturbance to the local wildlife.Therefore, we could consider that in our deer populations the main driver of group size was food availability.

Group composition

We expected a larger proportion of individuals in mixed-sex groups in unfavourable seasons due to the aggregation of individuals in zones of greater food availability. In contrast,we expected to find a higher proportion of individuals in sex-specific groups in favourable seasons except for the rut season, in autumn, which we discuss in the next section. This hypothesis was only fulfilled in the spring of High-West. It was the only unfavourable season in which the proportion of individuals in mixed groups was higher than 50% (Figure 5). Although we found the largest groups in unfavourable times in most cases, these groups were sex specific.A large literature shows sex-segregation in *Cervidae*, which tend to only form mixed groups during the reproduction period, while the rest of the year adult females are mostly in family groups, with subadults and fawns, and males tend to form separate groups (Bowyer, 1984; Bowyer et al., 1996; Main and Coblentz, 1996; Thirgood, 1996; Apollonio et al., 1998; McShea et al., 2001; Villerette et al., 2006). Other authors have described that female and male groups stayed apart in zones with high food availability (Clutton-Brock et al., 1982; Carranza, 2017). This could be our case, despite aggregating in large groups in zones with more food availability in unfavourable seasons, they did it in groups of separate sexes. In other studies, found that the aggregation of individuals in mixed-sex groups was in low-density populations as an antipredator strategy, like in bighorn rams and ewes (*Ovis canadensis*) (Meldrum and Ruckstuhl, 2009).The fact that there was no predatory pressure may also be a possible explanation of not aggregate in mixed groups in our populations.

On the other hand, we found a decrease in the proportion of individuals in mixed groups in most areas in summer. This occurred because hinds tend to aggregate with other females and their new-borns. This may also explain the case of Lowland areas, where summer was an unfavourable season but it was also the time of births.

Another exceptional case, was the proportion of individuals in mixed groups in the High-East area, which was below 20% in all seasons (Figure 5). We speculate that it was more difficult to form mixed groups in this area due to the altitudinal spatial sexual segregation. We know, through a tracking study with GPS collars and observational monitoring (unpublished data) that males in this area tend to use higher habitats than females most of the year. Another case of spatial sexual segregation in altitude by red deer was in a Mediterranean-type mountainous environment in central Portugal, where Alves et al. (2013) found that males used lower altitudes closer to agricultural fields and females preferred to stay at higher altitudes, selecting more protected areas. This case was the opposite pattern to what we found. Other studies have also found this spatial sexual segregation in ungulates, and posited a variety of hypotheses to explain it, including the indirect competition hypothesis, the nutritional needs hypothesis, the activity budget hypothesis, the weather sensitivity hypothesis and the predation risk hypothesis (Ruckstuhl and Neuhaus, 2002; Yearsley and Pérez-Barbería, 2005; Loe et al., 2006). In our case, we could suggest several hypotheses. Firstly, the nutritional needs hypothesis, males moved to alpine grasslands at higher altitudes because there was a greater food source compared to lower areas dominated by broom scrublands (*Genista balansae*) and forests. Secondly, the predation risk hypothesis,it could be that the instinct of females to protect the fawns leads them to stayed in lower areas where there are more covert habitats, such as forests and scrublands, and more comfortable situations.

Mixed groups in the rutting season

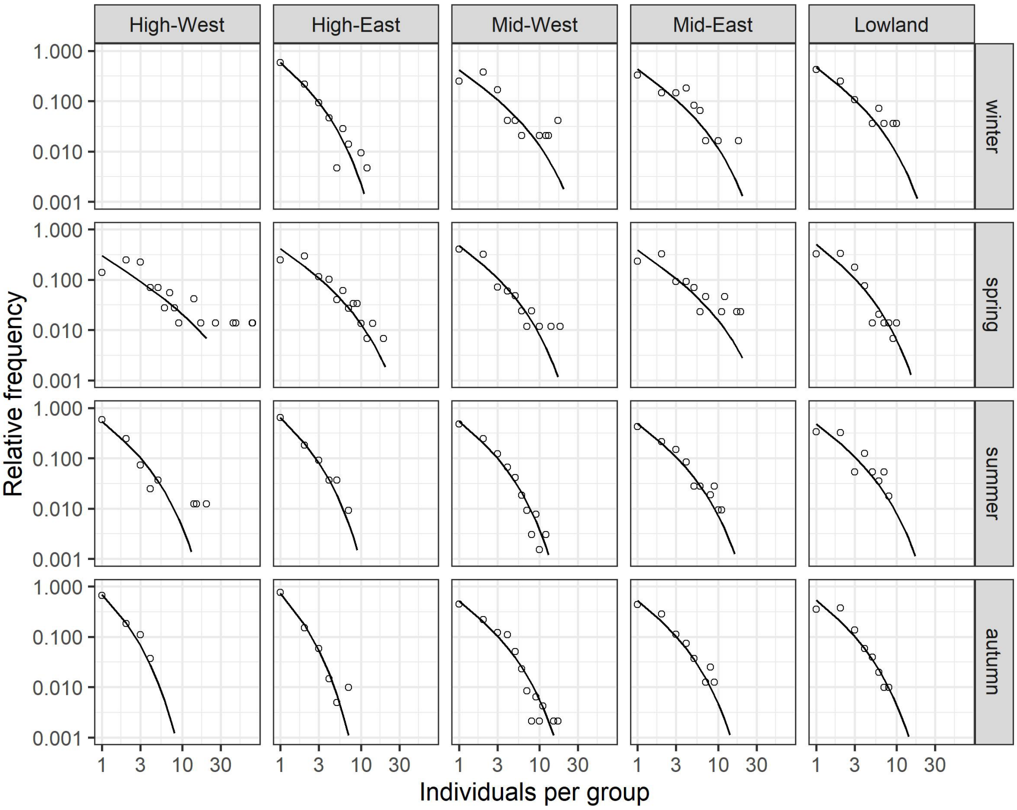
Deer is a polygynous species that forms harems during the reproduction time, the rut (Clutton-Brock et al 1982; Carranza 2017). For that reason, we expected harems when the rut took place in our study areas, in autumn. The females' proportion in mixed groups was higher all seasons in all areas. Therefore, by this fact we cannot differentiate the harems (Figure 5). Yet mean group size during the rut was the smallest of the year in almost all areas (Figure 3). We based our hypothesis on the mean group size of the harems found in the Iberian Peninsula, where the outside mean was 6.11 and the inside mean = 15.67 (Carranza and Valencia, 1992). Despite this, our results were more similar to those found in northern Europe, as in the case of the Isle of Rum, where the mean harem size was 3.4 (Clutton-Brock et al., 1982). We speculated that the cause of the formation of large harems in the southern Iberian Peninsula could be the aggregation of females where the food is available, because the dry climate, which makes it easier for males to maintain these large harems (Carranza and Valencia, 1992). On the other hand, Bonenfant et al. (2004) found a lower group size of harems in France, as in other northern areas, and explained this by the low density of deer, the variation in the sex ratio and the high forest cover. Our data did not include the density or sex ratio of our populations, but our study areas also had a high forest cover, and this could partially explain the small mean harem size we observed. Another, admittedly far-fetched but intriguing hypothesis could be the formation of leks instead of harems. There are few documented cases of ungulates that form leks instead of harems, including fallow deer and antelope (Bro-Jørgensen, 2002; Apollonio et al., 2003). A lek is a clump of male territories that females visit only for mating, and it is quite rare a mating system among mammals (Apollonio et al., 2003). Lekking typically occurs in species where females live in large, unstable groups and where males cannot effectively defend their harems (Clutton-Brock et al., 1993). In our cases, we did not have large groups and we could not know if they were stable, we need a specific study in which we could recognize the individuals to prove that theory.

Furthermore, the proportion of individuals in mixed groups was only higher in the rut compared to the others in the case of high-east, Mid-West and Lowland, but not in High-West or Mid-East (Figure 5). Despite this, the only areas in which this proportion of individuals in mixed groups reached almost 50% were the cases of Mid-West and Lowland, in the other areas was below 30%. We suggest that these results could be explained by the fact that, in all our study areas, autumn was a favourable season, and deer must have been more homogeneously distributed over the territory, which would have made it less likely for the two sexes to aggregate in the same area and form mixed groups and for this reason smaller harems should be observed.

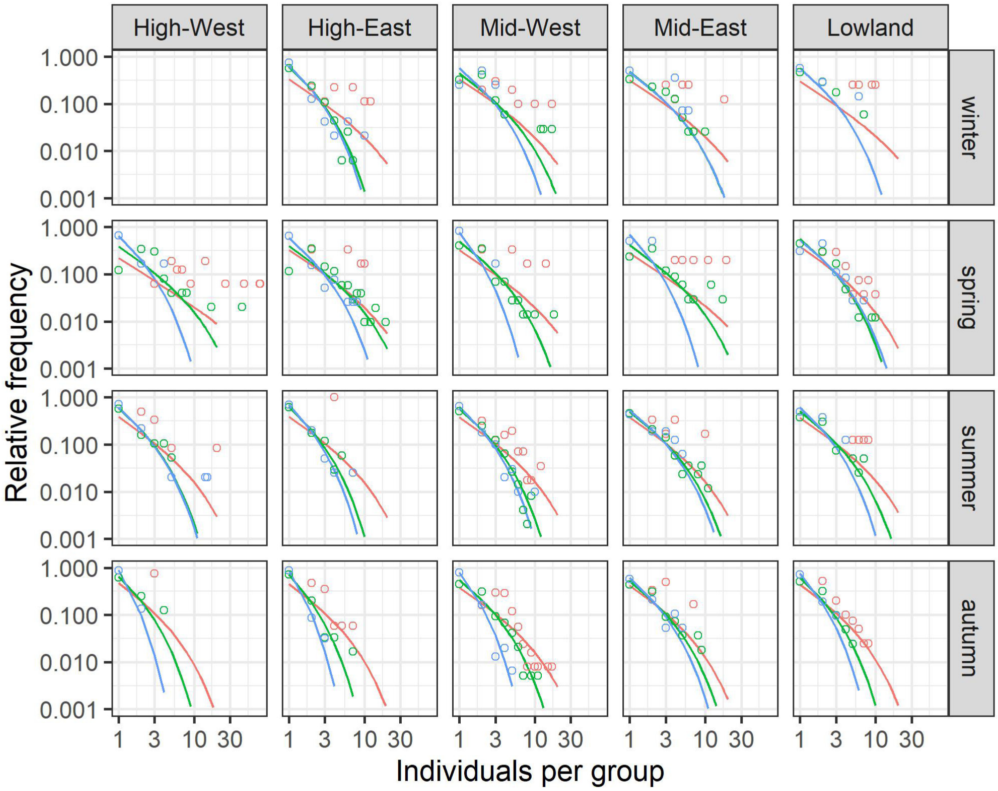
CONCLUSIONS

The availability of food was one of the most important factors influencing the dynamics of group size and composition, and across gradients. Even, in the case of our populations, it dominates over reproduction. In future studies the influence of density and sex ratio should be tested. According to our results, we expect significant changes in the group dynamics of our populations in the coming decades, as climate change will alter favourable and unfavourable seasons.

SUPPLEMENTARY MATERIAL



Supplementary Figure 1. Model fit to group size distributions by area and season. Points are observed relative frequency values and solid lines are fits to a logithmic distribution from a joint model with area, season and their interaction. Note logarithmic y scale.

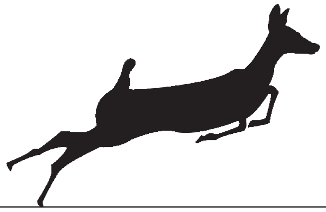


Supplementary Figure 2. Model fit to group size distributions by area, season and type. Points are observed relative frequency values and solid lines are fits to a logithmic distribution from a joint model with area, season, type and their interaction. Note logarithmic y scale

	Winter	Spring	Summer	Autumn
High-east	**Unfavourable**	**Unfavourable**	**Favourable**	**Favourable**
	2017: 129 / 129 2018: 84 / 84	2017: 118 / 118 2018: 30 /30	2016: 61 /61 2017: 21 /20 2018: 28 /28	2017: 144 /144 2018: 60 /59
High-West	**Unfavourable**	**Unfavourable**	**Favourable**	**Favourable**
	n.a.	2018: 77 / 73	2019: 81 / 81	2018: 29 / 27
Mid-East	**Unfavourable**	**Unfavourable**	**Favourable**	**Favourable**
	2016: 20 / 16 2017: 40 / n.a. 2018: 65 / 48	2018: 48 / 45	2016: 43 / 21 2017: 16 / 15 2018: 56 / 54 2019: 25 / 22	2016: 30 / n.a. 2017: 51 / n.a. 2018: 85 / 80
Mid-West	**Unfavourable**	**Favourable**	**Favourable**	**Favourable**
	2018: 40 / 40 2019: 9 / 9	2017: 69 / 63 2018: 25 / 25	2016: 125 / 119 2017: 320 / 293 2018: 285 / 271	2016: 103 /102 2017: 261 / 247 2018: 140 /138
Lowland	**Unfavourable**	**Favourable**	**Unfavourable**	**Favourable**
	2017: 18 / 10 2018: 21 / 20	2016: 82 / 73 2017: 31 / 26 2018: 80 / 71	2017: 43 / 33 2018: 18 / 14 2019: 29 /24	2016: 79 / 76 2017: 50 / 46

Supplementary Table 1. Favourable and unfavourable seasons in each area, with sampling years and total number of groups and number of groups with individuals identified to sex. (n.a.): not available.

CHAPTER 4: FAECAL CORTISOL LEVELS IN WILD RED DEER POPULATION ARE BEST EXPLAINED BY PRIOR CLIMATOLOGICAL CONDITIONS



ABSTRACT

The responsiveness of the stress system to stressors is crucial to survive and adapt to new environmental changes without decreasing the individual welfare. We analysed the influence of prior weather conditions, seasonality, sex, influx of ecotourism and nutrition on stress levels of a wild population of red deer in a Mediterranean hunting reserve in the Pre-Pyrenees (Spain). We used faecal cortisol metabolites as a proxy to physiological and psychological stress and faecal T3 metabolites as an indicator of nutritional stress. Faecal analyses were chosen because it is a non-invasive technique that does not alter individual's behaviour, and for ease of analysis in a wild population, which involves added difficulty for lack of a controlled environment. Our results indicate that prior climatic conditions, rather than just seasonality, best explained the variations of these hormones within seasons and among years. On the other hand, the results showed that high levels of the cortisol hormone did not involve low levels of the T3 hormone, this could be a sign of a non-chronic nutritional stress in this population. We also find differences between adult males and the other individuals in both hormones, possibly due to the different implications and necessities of males and females and adults

and juveniles. Finally, we could not find an effect of the ecotourism influx on cortisol levels, suggesting that this deer population tolerates well the presence of tourists during the rut season.

INTRODUCTION

Stress is defined as a state of threatened homeostasis which induces physiological and behavioural responses mediated by the release of glucocorticoid hormones (GC), cortisol in most mammals (Greenberg et al., 2002; Cockrem, 2013). For this reason, GC are frequently used to study the stress response (Pacak and Palkovits, 2001). Stress evolved in vertebrates as an adaptive endocrine response that increases survival, as it helps individuals cope with noxious stimuli or stressors (Pacak and Palkovits, 2001; Möstl and Palme, 2002). However, under chronic stress, prolonged responsiveness of the stress system can reduce individual fitness by turning off growth and development, suppressing the immune system and inhibiting biological functions such as reproduction (Sapolsky et al.,2000b; Möstl and Palme, 2002). For this reason, the study of stress is crucial for the conservation of wild populations.

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

In the strongly seasonal Mediterranean climate, food availability, and consequently nutritional stress, may also be expected to vary seasonally. Periods of starvation may be brief and cause no apparent physiological impacts, or they may be moderate and affect reproduction, growth, or immune responses; in the extreme, they can be life-threatening (McCue et al., 2017). Starvation periods imply changes in the levels of thyroid hormones, rapidly suppressing triiodothyronine levels (Blake et al., 1991; Flier et al., 2000). T3 and T4 respond to nutritional deficits by lowering metabolism and allowing the body to conserve energy during a nutritional emergency (Silva 2006; Wasser et al., 2010). Conversely, under overfeeding conditions, levels of thyroid hormones increase (Douyon and Schteingart, 2002; Kitaysky et al., 2005). Accordingly, thyroid hormones have been identified as a potentially viable index of body condition in ungulates due to their correlation with body fat (Bishop et al., 2009b). Therefore, these hormones can help us to discriminate between nutritional stress and other sources of stress.

Social interactions, such as courtship and copula, may also increase stress levels that help individuals cope with social challenges through behavioural responses (Broom and Johnson, 1993). Males and females have distinct life history traits regulated by hormones, some of which are related to the stress response. For example, prolonged or chronic stress results in suppressed gonadotrophin secretion and inhibition of reproduction (Tilbrook, Turner and Clarke, 2000). The mating period is stressful for many species, especially when it implies competition for mates (Vera, Zenuto and Antenucci, 2013). In species with harem mating systems, including the red deer (Cervus elaphus), males may be under particularly stressful conditions, since they must fight other males to keep their harems and increase their reproductive success (Bonenfant et al., 2004; Pavitt et al., 2015). In females, stress varies across the ovulatory cycle and during gestation and lactation (Ziegler, Scheffler and Snowdon, 1995; Pavitt et al., 2016). Stress levels of both sexes may also be related to the social status of the individual (Bartos et al., 2010).

Anthropogenic activities may also be responsible for increased stress levels in wild populations. While rapid increases in ecotourism over the last decades have come with various positive impacts, including on the local economies and through financial contributions to environmental causes (Higginbottom, Northrope and Green, 2001; Ballantyne, Packer and Falk, 2011), ecotourism activities may also pose a threat for wild animal populations. Their effects range from short-term impacts, such as changes in physiology and/or behaviour of individuals, to long term effects, such as increased mortality or reduced breeding success of entire populations (Burns and Howard, 2003; Higginbottom and Scott, 2004). In ungulates, anthropogenic stress has been shown to cause behavioural changes, such as an increase in vigilant individuals, especially in highly sensitive species (Borkowski 2001; Pelletier, 2006; Jayakody et al., 2008; Wasser et al., 2011). Several studies, however, provide evidence that ungulates may tolerate or acclimate to the presence of humans (Sibbald et al., 2011).

Here, we present a joint analysis of stress hormones and nutrition-related hormones in a free-ranging population of Iberian red deer (*Cervus elaphus hispanicus*). Our goal was to evaluate the influence of prior weather conditions, seasonality, nutrition, sex and influx of ecotourism on stress levels. To address this goal, we collected faecal samples that we analysed for cortisol metabolites (FCM) as a proxy of physiological and psychological stress, and T3/T4 metabolites (FT3M) as a proxy of nutritional stress. Samples were collected at two different locations differing in their ecotourism loads and at different time points of the year along five consecutive years. We expected to find a strong seasonal pattern, with higher levels of FCM in periods with high temperatures and low precipitation (summer), and in periods with low temperatures and extensive snow cover (winter), due to the direct physiological impacts of adverse weather conditions. We also expected to find high nutritional stress due to the reduced availability of high-quality food (producing low FT3M levels) in the same periods. As a consequence, we expected to find high FCM levels associated with low FT3M levels in summer and winter. We further hypothesized that deer would have higher levels of stress during the rutting period due to mating-associated stress, and that stags should have higher levels of FGC than hinds and juveniles during the rut because stags have to fight to secure their harem and hence their offspring. Finally, we expected to find higher levels of stress in the high ecotourism location than in the low ecotourism location due to the added anthropogenic stress.

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

METHODS

Study area and sampling design

The National Game Reserve of Boumort, located in the Prepyrenees (Figure 1), covers a surface area of 13,097 ha and has a maximum altitude of 2,077 metres. Its climate is supramediterranean (Rivas and Gandullo, 1987), with hot and dry summers, cold winters with snow accumulation and most rainfall concentrated in spring and autumn (Supplementary Figure 1). It is inhabited by the Iberian deer subspecies (Pérez-González et al., in press), which differs from the nominal subspecies (*C. e. elaphus*) due to its smaller size, grey coloration and smaller skull (Carranza, 2017). The red deer population density is about 4 ind/km^2 (annual census data; Joan Curià, personal communication). Low intensity hunting is allowed from September through March, mostly by stalking.

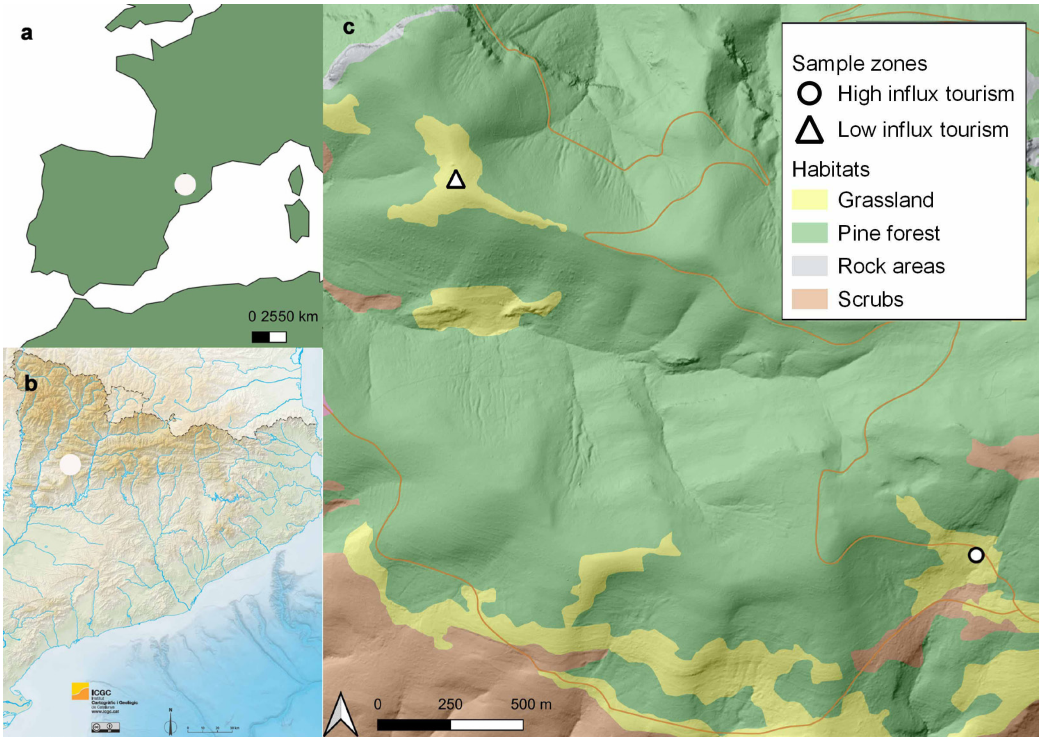


Figure 1. a) Map of the sample zones (Base map: MET 2x2 of the Cartographic and Geological Institute of Catalonia (ICGC), license CC BY 4.0; Habitat's map: Carreras and Ferré 2014). b) Study area in relation to Europe (Land map, version 4.1.0. Made in Natural Earth)

To evaluate FCM patterns related to climatic variation, we sampled the two study sites for five consecutive years coincident with important events during the red deer annual cycle: summer, the highest temperature period (in 2016 and 2017), autumn, the rut period (2016, 2017 and 2020), winter, the lowest temperature and snow period (2016 – 2017 and 2020 – 2021) and spring, the period after the lowest temperatures (2017, 2018, 2019, 2020 and 2021). Although our initial design was to sample each season in two different years, extensive snow cover over two winters and the recognition of high interannual variability (see results) prompted us to prolong sample collection until the winter of 2020-2021.

In order to assess the effects of ecotourism on FCM levels, we selected two sampling zones within the study area, one easily accessible and with a high influx of ecotourists, the other more isolated, with a low influx of ecotourists. These two study zones were 2.2 km apart and had similar habitats (Figure 1). The influx of visitors was higher in summer and extremely high in autumn, due to the rut (Supplementary Figure 2).

Samples were collected along a fixed transect at each of the two zones. In each sampling occasion, faeces of two sizes were collected as a proxy for the sex and size of the individual, with large faeces (> 2 cm) assumed to belong to stags, and smaller faeces (< 1.5 cm) assumed to belong to either hinds or juveniles. Sample pellets were collected in separate pellet groups in order to ensure the maximum independence between the sampled individuals in transects of 150 m.. We collected a total of 147 samples. Every sample contained at least 5 pellets. Until the winter of 2016 - 2017, we only collected 8 samples seasonally, two per size and zone, then we proceeded to collect 4 samples per size and zone to maximise the power of the statistical analyses. An exception was the early winter of 2020 – 2021, when the snow cover prevented us from reaching the sampling areas on all sampling days and we could only obtain 6 samples in the high ecotourism zone (4 small and 2 big), and 5 in the low ecotourism zone (3 small and 2 big). Samples were collected fresh in the field, of the previous hours, and frozen at -20ºC until analysis.

Steroid extraction and hormone analysis

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

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

Statistical analysis

FCM and FT3M data were modelled with linear mixed effects models. Climate effects were included in two ways: (1) by including season as a fixed factor to test for a seasonal pattern, with year as a random effect on the intercept to account for interannual variability, and (2) by including climate-related variables to test for direct responses to prior weather conditions (see below), with sample collection date as a random effect on the intercept. The candidate climate variables were solar irradiance, minimum, average and maximum daily temperature, and the daily precipitation aggregated over a given period as averages or, in the case of precipitation, the sum over the period. Climate data were obtained for the Climatological Station of Talarn (421219N - 005155E, altitude: 807 m) run by the State Meteorological Agency of Spain (https://www.aemet.es/en/datos_abiertos/AEMET_OpenData) located 19,5 km from the sampling zones (Supplementary Figure 1). Pellet size (a proxy for sex) and ecotourism (high vs. low) were included in all models as fixed effects. In addition, FT3M was added as a candidate predictor for FCM under the assumption that nutritional stress also produces an increase in cortisol (i.e. contributes to physiological stress as measured by FCM). After inspection of model residuals, cortisol metabolite values were cube-root transformed to meet normality and homoscedasticity assumptions.

To choose the best set of climate predictors and the aggregation period prior to sampling dates, we explored model fits for aggregation periods ranging from 1 to 31 days. For each of these periods, we built the most parsimonious model by stepwise backward selection, starting with a full model that included all main fixed terms and their pairwise interactions. For model selection, we used the Akaike Information Criterion (AIC) as a measure of model fit penalised by model complexity. To build the final models, we chose the period of aggregation that yielded the lowest AIC.

To test for model terms in the final selected models, we used likelihood ratio tests for marginal tests (i.e. for comparing models with and without the target variable plus all other variables in the model). To visualise model fit to explanatory variables we calculated model predictions for focal variables while holding non-focal variables at their mean values and factors at their reference level. To visualise model predictions based on climate variables for the duration of the study period, we calculated predictions as before using the selected climate variables and, to facilitate comparisons of observations to model predictions, we built 95% confidence intervals around population predictions (i.e., accounting only for fixed effects) using code provided by Ben Bolker (http://bbolker.github.io/mixedmodels-misc/glmmFAQ.html). Goodness of fit was measured as marginal (i.e., explained by fixed factors alone) and conditional (i.e. explained by both fixed and random factors) pseudo R2 using function r.squaredGLMM in R package MuMin (Barton, 2022). All statistical analyses were done with the R statistical package, version 4.2.1 using function lme in the nlme package to fit the mixed models.

RESULTS

Mixed models for FCM including season and models with climate-related variables showed similar explanatory power as assessed by AIC (model with season: -79.665, model with climate variables: -88.099). However, the model with season and year as random effect concentrated variability in the random effect, leaving the fixed factor season as non-significant, strongly suggesting that whatever seasonal pattern was in the data was obscured by high interannual and intraseasonal variability. Thus, the model carried little information as to the drivers of FCM levels, and was disregarded in favour of the models with climate-related variables.

In the process of selecting the most adequate antecedent period to integrate climatic variables, the best model (as per AIC) was obtained for a period of 13 days prior to sample collection (Supplementary Figure 3). This model included solar irradiance, average minimum daily temperature and FT3M. In all models (between 1 and the 31 days prior to sample collection), FT3M and solar irradiance were always the best predictors. For clarity of exposition, we refitted this model to include all our hypothesized target factors, i.e. to include also pellet size and tourism, which were not significant (Table 1). This model showed high goodness-of-fit (marginal R2: 0.59, conditional R2: 0.66) to mean levels across days and experimental conditions (as defined by fixed effects), while still showing high within-day variability among individuals under the same conditions (Figure 2).

Term	Estimate	Std. Error	df	t value	p-value
a) model with FT3M					
Intercept	0.373	0.102	43.2	3.655	0.00069
solar irradiance	0.130	0.013	25.72	9.827	<0.00001
min. temperature	-0.023	0.005	25.6	-4.733	0.00007
FT3M	0.020	0.006	140.3	3.420	0.00082
pellet size	-0.021	0.013	130.3	-1.628	0.106
ecotourism influx	0.004	0.013	128.0	0.358	0.721
b) model without FT3M					
Intercept	0.548	0.090	28.8	6.077	<0.00001
solar irradiance	0.129	0.013	26.3	9.590	<0.00001
min. temperature	-0.020	0.004	25.0	-4.114	0.00037
pellet size	-0.31	0.014	128.7	-2.257	0.0257
ecotourism influx	0.000	0.014	128.5	0.205	0.838

Table 1. Mixed effects models for faecal cortisol metabolites in red deer (*Cervus elaphushispanicus*) from Boumort Game Reserve. Mixed effects models for faecal cortisol metabolites as a function of climate variables and the *a priori* fixed effects (i.e. tourism influx and pellet size). Model (a) includes faecal triiodothyronine metabolites (FT3M) as a predictor, (b) excludes it. Column "df" gives degrees of freedom with the Satterthwaite correction.

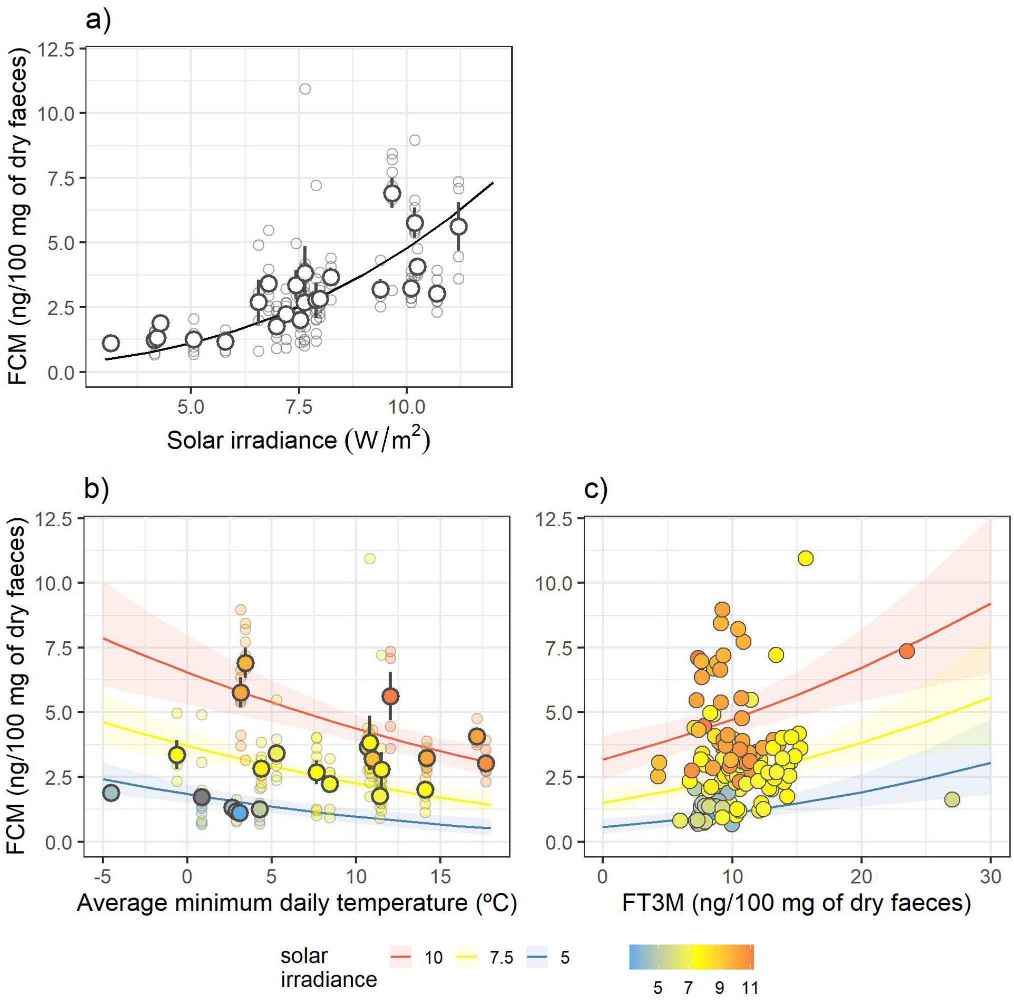


Figure 2. Observed FCM levels and mixed model fit against (a) solar irradiance, (b) average minimum daily temperature, and (c) FT3M levels. Lines are mean expected values from model (a) in Table 1. Colours in (b) and (c) indicate varying levels of solar irradiance. Ribbons show 95% confidence intervals around mean expected values. Grey dots are individual sample values; white dots are mean daily sample values, with error bars indicating 95% confidence intervals).

In agreement with our hypothesis that the highest stress levels would occur in periods of adverse weather conditions, we found that levels of FCM increased both with both higher solar irradiance (Figure 2(a)) and lower temperatures (Figure 2(b)). Model predictions for the entire study period show clearly how a simple seasonal trend (for which minimum temperature is a proxy in this model) is insufficient to capture the strong interannual variability shown by the data, whereas the inclusion of solar irradiance in the model helps correctly predict mean FCM levels than vary strongly across years, as can be seen, for example, in the large differences in observed FCM levels towards the end of winter of 2019 and 2021 (Supplementary Figure 4).

Contrary to our hypothesis, FCM levels increased with FT3M levels, although with a small effect size (Figure 2(c)). Pellet size had a significant albeit slight effect on FCM, but only when FT3M was not included as a predictor (Table 1). Contrary to what we expected, FCM levels were lower in big pellets (i.e., stags) than in small pellets (i.e., hinds and juveniles).

The best FT3M model included mean climatological variables over 2 days prior to sample collection (Supplementary Figure 5). The selected predictors included the interaction between solar irradiance and average minimum daily temperature, plus daily precipitation and pellet size (Table 2). In this case, also in agreement with our hypothesis that the highest nutritional stress levels would occur in periods of adverse weather conditions, we found that

Term	**Estimate**	**Std. Error**	**df**	**t value**	**p-value**
Intercept	4.787	1.463	22.9	3.270	0.003
solar irradiance	0.495	0.180	21.89	2.757	0.011
min. temperature	0.611	0.164	19.8	3.724	0.001
precipitation	0.160	0.056	17.6	2.852	0.010
pellet size	-0.491	0.185	124.4	-2.650	0.009
ecotourism influx	-0.089	0.185	124.3	-0.479	0.632
min. temperature × solar irradiance	0.057	0.019	20.1	-2.948	0.008

Table 2. Mixed effects models for faecal triiodothyronine metabolites in red deer (*Cervus elaphushispanicus*) from Boumort Game Reserve. Mixed effects model for faecal triiodothyronine metabolites as a function of climate variables and the *a priori* fixed effects (i.e. tourism influx and pellet size). Column "df" gives degrees of freedom with the Satterthwaite correction.

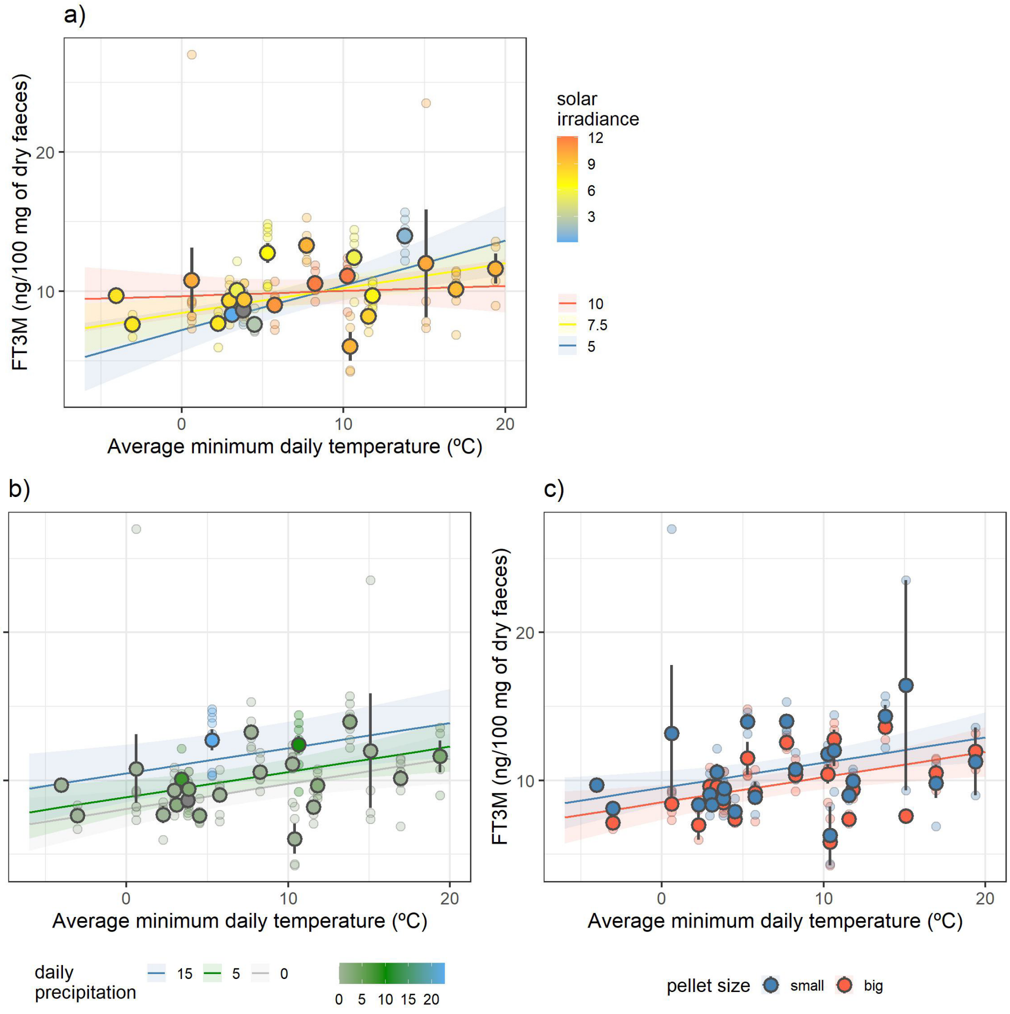


Figure 3. Observed FT3M levels and mixed model fit against (a) the minimum daily average temperature, (b) the daily precipitation, and (c) the pellet size. Lines are mean expected values from model in Table 2. Colours in (a) indicate varying levels of solar irradiance, in (b) varying levels of daily precipitation and in (c) the type of pellet size. Ribbons show 95% confidence intervals around mean expected values. Grey dots are individual sample values; white dots are mean daily sample values, with error bars indicating 95% confidence intervals).

levels of FT3M decreased with lower temperatures (Figure 3(a)) and lower precipitation (Figure 3(b)). On the other hand, FT3M levels were lower in big pellets than in small pellets (Figure 3(c)). As in the case of FCM, we did not find significant differences between the high and the low influx of ecotourism zones (Table 2, Figure 3). Model goodness of fit, as measured by pseudo-R2, was lower for the FT3M than for FCM (marginal R2: 0.27, conditional R2: 0.39) (Supplementary Figure 6 and Figure 7).

DISCUSSION

FCM LEVELS DEPEND ON CLIMATOLOGICAL PRIOR CONDITIONS, RATHER THAN ON A CLEAR SEASONAL PATTERN

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

Our best model includes two climatic variables, average daily minimum temperature and solar irradiance. Both vary seasonally, with solar irradiance varying more irregularly among years and on the short term (Supplementary Figure 1). Accordingly, FCM levels in the modelled data appear to increase in the summer months and decrease in the winter months (Supplementary Figure 4), partly supporting our expectation of higher stress levels under the harsh conditions of the summer. Yet there is substantial between-years and within-season variability that appears to be related with short term (two weeks) prior climate conditions. Wingfield (2013) already points to the importance of short-term disturbances (what he terms abiotic direct labile perturbation factors) as triggers of glucocorticoid hormone secretion, presumably as an adaptive response to prime the organism to cope with changing conditions or to accompany behavioural responses.

Our climatological data are limited, and the selected variables probably function here as a proxy for a more complex set of weather conditions with direct impacts on organisms. Still, solar radiation is directly linked to perceived temperature under the sun, and thus consistent with control of energy homeostasis, which is the main role of GC in mammals (Roldan and Herzig, 2015). Similarly, minimum temperature enters the model with a negative coefficient. This means that high FCM values tend to be higher either at low temperatures with relatively high solar irradiance (i.e. on cold, sunny days in the winter or early spring) or at high solar irradiance in the summer. Huber et al., (2003) also attributed an increase in stress levels to harsh environmental conditions and, as in our population, also found a significant negative relationship between minimum ambient temperature and FCM. It is noteworthy that precipitation was not selected as a predictor in the model. Other climate-related measures might provide a more direct explanation for the link between abiotic conditions and FCM levels. For example, periods of combined low solar irradiance, low temperatures and high wind might be especially harsh.

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

HIGHER NUTRITIONAL STRESS WAS RELATED TO FCM LEVELS AND CLIMATOLOGY

Besides its role on energy homeostasis, GC also plays a key role in the regulation of mammalian glucose. The major effect of GC on glucose homeostasis under stress is to preserve plasma glucose, which is essential to reach maximal brain function (Kuo et al., 2015). Thus, one of the first responses following an immediate stressor is to mobilise glucose (Sapolsky et al., 2000b). The elevated levels of GC might be most functional if associated with elevated T3, so the resultant elevated metabolism can make best use of the increased availability of glucose (Wasser et al., 2017; Mondol, Booth and Wasser, 2020). This may explain why, and contrary to our hypothesis, we found a statistically significant positive relation between FCM and FT3M. On the other hand, T3 function is crucial to survive starvation. During long periods of food deprivation, levels of GC increase while T3 hormones decrease (Douyon and Schteingart, 2002; Kitaysky et al., 2005). Chronically elevated GC might deplete internal reserves, making it important to reduce T3 and associated metabolism to prevent the body from using all its remaining reserves (Wasser et al., 2017; Mondol et al., 2020). This suggests that the deer population in this study did not endure sustained periods of chronic nutritional stress.

The decrease of thyroid hormones in periods of food deprivation has been shown experimentally by Bishop et al., (2009b) in two populations of mule deer, one being the control and the other receiving food supplementation in winter. In that study, the population without the food supplementation had lower levels of thyroid hormones and a weaker physical condition with less body fat. In our study, FT3M levels decreased as temperatures decreased, which suggest higher nutritional stress in during the snow period (Figure 3(a) and 3(b)). The snow cover prevents deer from feeding, because it hinders foraging. In addition, the quality of the red deer diet potentially decreases in both summer and winter as they increase consumption of woody plants due to high temperatures and dry conditions in the summer, and snow cover in winter (Garin, 2001; Bugalho and Milne, 2003). A study of red deer in New Zealand found a seasonal pattern in thyroid hormones, decreasing in winter and increasing in spring and summer (Shi and Barrell, 1992). In contrast, in white-tailed deer in southern Texas, lower concentrations of the thyroid hormone were found in summer, which appears to be the harshest feeding period under this hot, arid climate (Chin and Brown, 1984). Yet, contrary to our expectations, we did not find high FCM levels or low FT3M coinciding with the dry summer period, only with low temperatures. This suggests that harsh winters could lead to chronic stress in this population.

MEAN HORMONE CONCENTRATIONS TENDED TO BE HIGHER IN HINDS AND JUVENILES THAN IN STAGS

Differences in the biological cycle of males and females during the year could cause dissimilarities in the levels of stress between the two sexes. The results of our study show significant differences between big pellets (i.e.

stags), and small pellets (i.e., hinds and juveniles) in FCM levels (in the model without FT3M), and in FT3M levels. For both hormones, although more clearly in the case of FT3M (Figure 3(c)), mean concentrations tended to be higher in hinds and juveniles than in stags throughout the year. This suggests that stags suffered more from nutritional stress, although we acknowledge the limitations of our data, which uses only proxies for sex and age based on pellet size. For future studies with wild populations, it will be important to analyse sex in the faecal samples using genetic markers. Together with pellet size, this may help ascertain sex and age class (juvenile vs. adult) from pellet data only.

Large individual variance in cortisol levels in red deer hinds related to reproductive status has been reported before (Geraghty and Kaufer, 2015; Pavitt et al., 2016). Stress is expected to increase throughout spring and peak in May–June, during the calving season, coinciding with a peak of progesterone, which is a cortisol precursor. Other studies on mammals show higher levels of cortisol during the reproductive season (Pavitt et al., 2015). In wild red deer stags of the isle of Rum, cortisol concentration was higher coinciding with the rutting season (Pavitt et al., 2015). In contrast, in a population of captive Iberian red deer stags, stress peaked in spring, allegedly due to the increase in voluntary feed intake, while the lowest levels occurred after the mating season, possibly because of the inhibitory effect of testosterone on cortisol release (Gaspar-López et al., 2010). Testosterone can inhibit cortisol release by influencing steroidogenic pathways involved in the synthesis of cortisol in the adrenal cortex. This hormone can inhibit cortisol also by competing with it for binding sites on carrier proteins such as corticosteroid binding globulin and plasma albumin (Ingram et al., 1999). This inhibition process might explain our finding of generally lower levels of cortisol in stags, which would mean that differences in hormone levels do not translate into differences in stress levels. Sex dimorphism in red deer also implies differences in feeding. Females cut grass at a lower height than males, which therefore tend to be browsers more often than females (Clutton-Brock et al., 1982; Azorit et al., 2012c) in part because their larger size provides them with the ability to reach parts of bushes or trees that the females cannot reach (Bugalho, Milne and Racey, 2001). Due to these differences in diet, it has been shown that females are better able than males to select diets that satisfy their nutritional requirements (Clutton-Brock et al., 1982; Illius and Gordon, 1987). Other authors also relate a small size to a better-quality diet, because the metabolic requirement to gut capacity ratio increases with decreasing body size. Moreover, small ruminants are less able to exploit lower quality forage than larger ones, for that their diet tends to be of better quality (Demment and Van Soest, 1985; Gebert and Verheyden-Tixier, 2001). This may partially explain why we find higher levels of FT3M in females and juveniles.

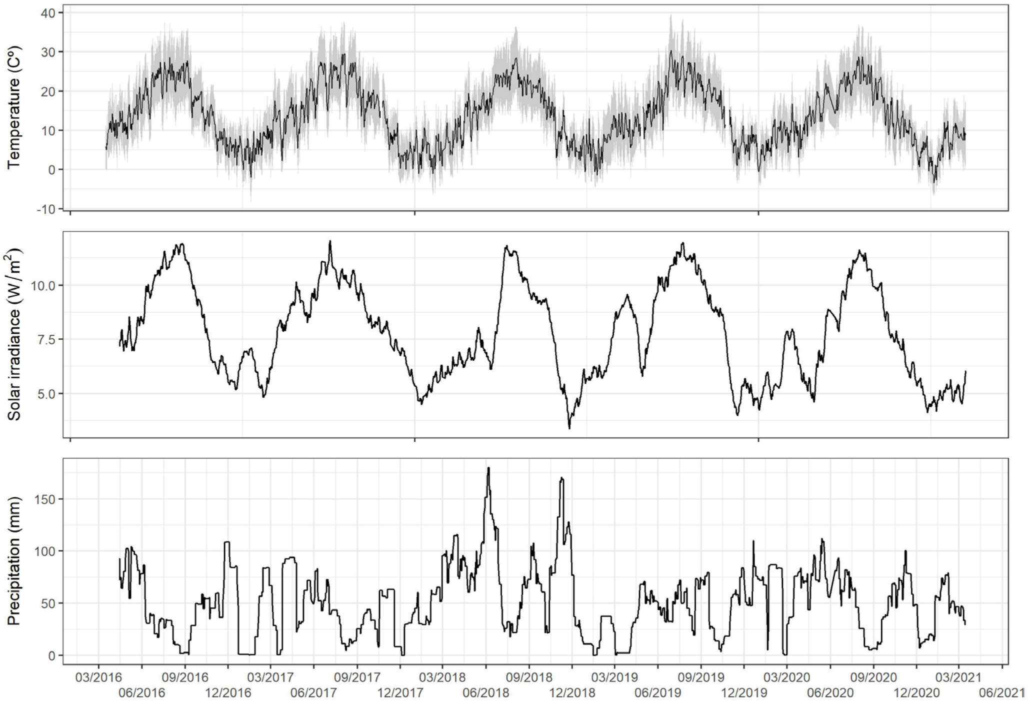
NO EVIDENCE OF ANTHROPOGENIC STRESS

Over the last decades, ecotourism has become ever more popular in Catalonia, with rural tourism increasing from 176,600 travellers in 2003 to 485,200 in 2018, a growth of over 60% in fifteen years (Statistical Institute of Catalonia 2020). The study area also attracts ecotourists, in particular to watch red deer during the rut (Supplementary Figure 4). Yet, contrary to our expectations, ecotourism did not appear to be significantly associated with increased levels of FCM. Concentrations of FCM did not differ between the high and low ecotourism zones, nor were they significantly higher in the months with high influx of tourists, i.e., August, and the rut period, September and October. A possible explanation is that animals have become habituated to the presence of watchers or take refuge in the zones with less ecotourism influx. This agrees with the apparent lack of response to the disturbance caused by hill walkers in Scotland deer (Sibbald et al., 2011), and with the tolerant behaviour to ecotourism by the Sika deer in the Tanzawa Mountains, Japan, where the human population is extremely dense (Borkowski, 2001). In contrast, Jayakody et al., (2008) found differences in red deer behaviour in two zones with contrasting numbers of visitors. In the recreational and hunting season in the Scottish Highlands they observed a higher frequency of individuals with a vigilant behaviour, which implies a decrease in the feeding time, in the zone with more visitors and in the hunting season. In our study area, driven hunting was practised during the hunting period, but only occasionally, no more than two or three times per year, and did not coincide with the sampling periods.

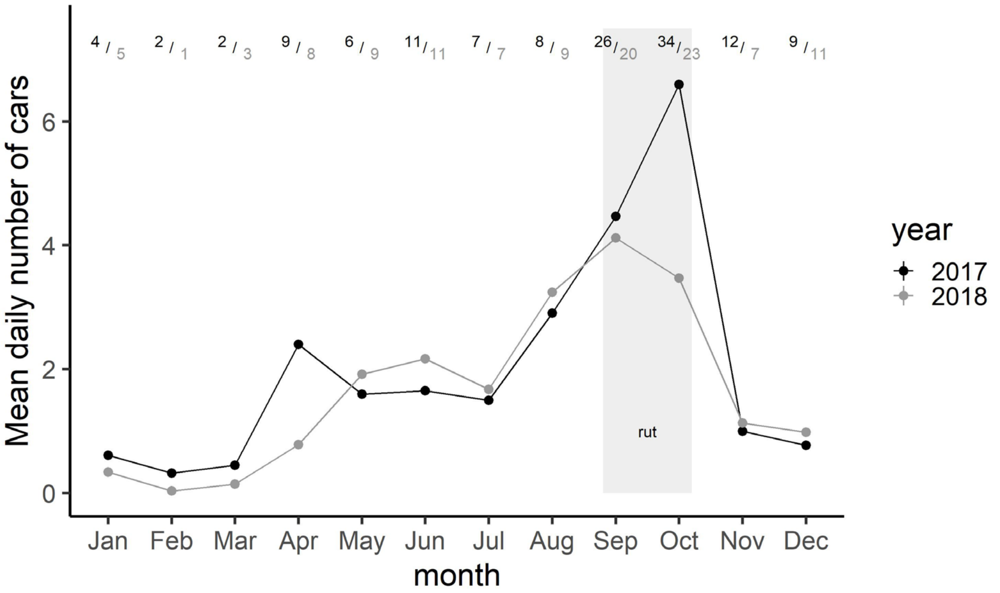
CONCLUSIONS

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

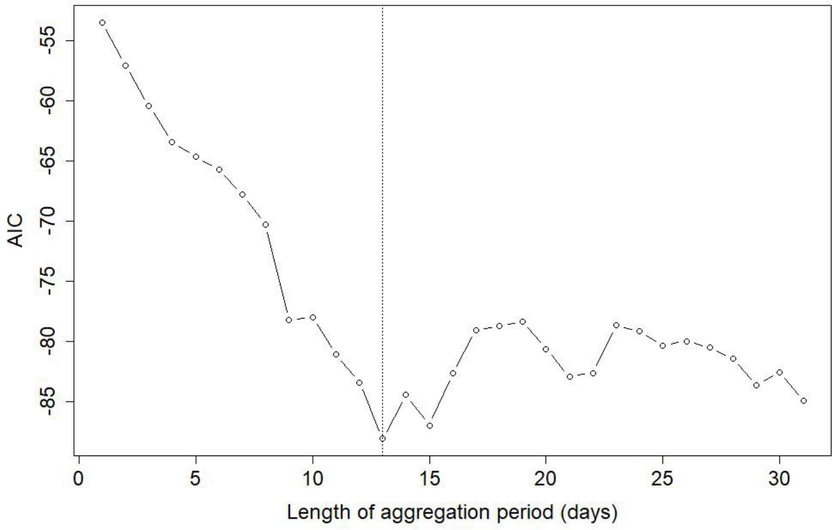
SUPPLEMENTARY MATERIAL



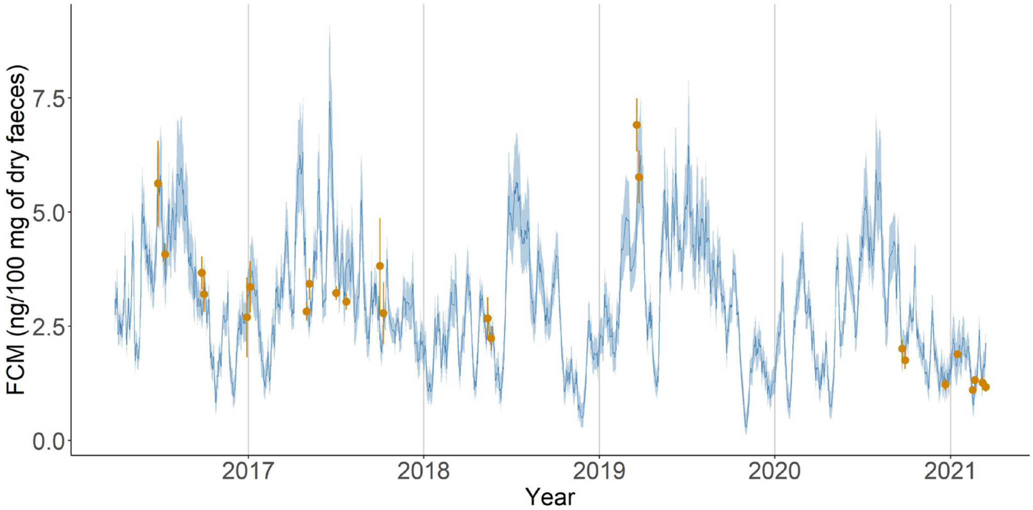
Supplementary Figure 1. Mean daily average temperature, solar irradiance and precipitation for the study period (2016 to 2021). The light grey ribbon for temperature depicts the maximum and the minimum daily temperature. Data from the State Meteorological Agency of Spain, Climatological Station of Talarn (421219N - 005155E, altitude: 807 m).



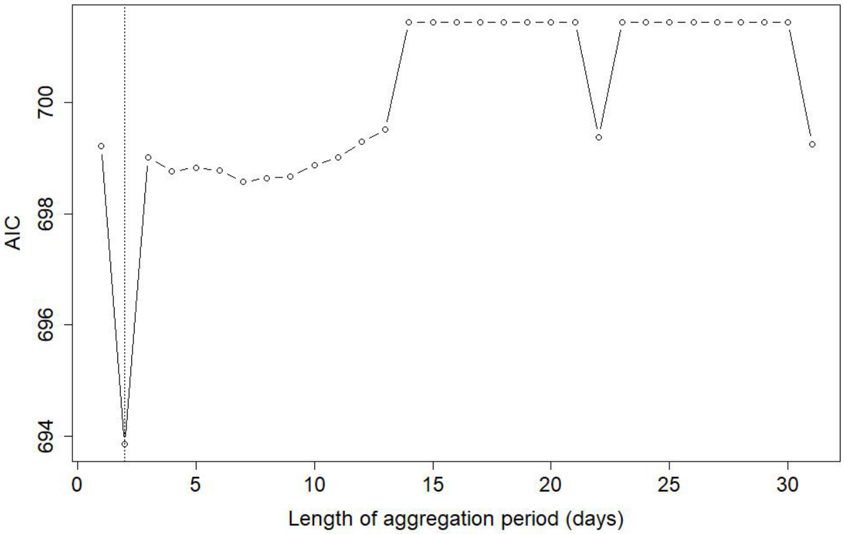
Supp1ementary Figure 2. Mean daily number of cars that entered Boumort Hunting Reserve per month in 2017 and 2018, showing peak affluence during the rut season (shown by grey rectangle). The top row gives the maximum daily number of cars per month and year.



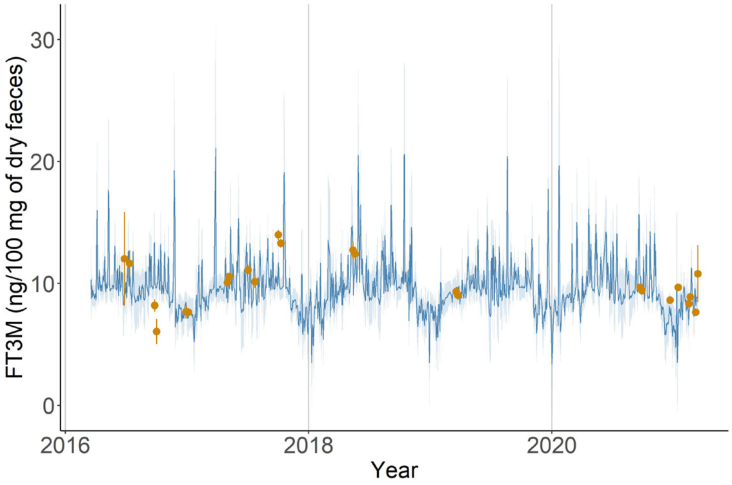
Supplementary Figure 3. AIC of the faecal glucocorticoid metabolites (FCM) model for aggregation periods from 1 to 31 days.



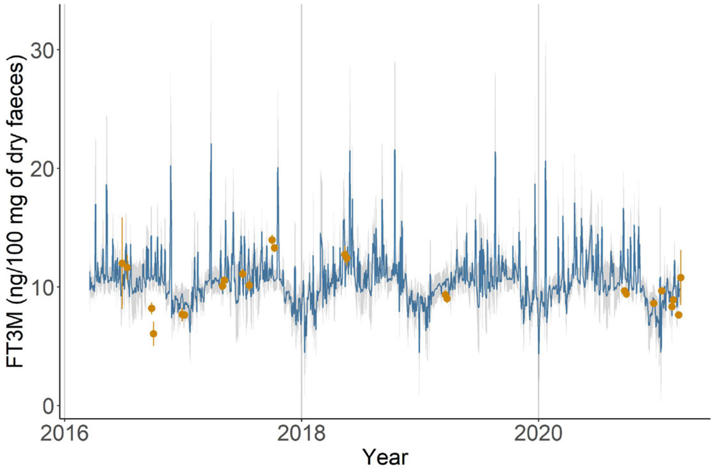
Supplementary Figure 4. Model predictions and observed faecal cortisol metabolites values (FCM). The solid line shows the predicted mean levels based on a moving average of the climatological variables over the prior 13 days. The ribbon shows 95% confidence intervals around the expected mean. Solid symbols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



Supplementary Figure 5. AIC of the faecal triiodothyronine metabolites (FT3M) model for aggregation periods from 1 to 31 days.

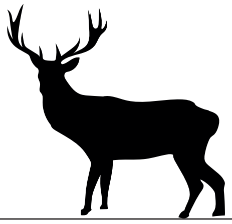


Supplementary Figure 6. Model predictions and observed faecal triiodothyronine metabolites values (FT3M) with big pellets. The solid line shows the predicted mean levels based on a moving average of the climatological variables over the prior 2 days. The ribbon shows 95% confidence intervals around the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



Supplementary Figure 7. Model predictions and observed faecal triiodothyronine metabolites values (FT3M) with small pellets. The solid line shows the predicted mean levels based on a moving average of the climatological variables over the prior 2 days. The ribbon shows 95% confidence intervals around the expected mean. Solid symbols with error bars are mean daily observed values with 95% confidence intervals.

GENERAL DISCUSSION



The present interdisciplinary project focused on red deer biology has allowed us to understand the extensive adaptability of this species to the different characteristics offered by each inhabited area. The study on diet and habitat use (Chapter 1) has enabled us to explain aspects of phenotypic plasticity (Chapter 2) and group ethology (Chapter 3) in this species. However, in Chapter 4, we have found that climate is one of the main factors influencing stress in red deer, and not and not the lack of food. Additionally, two parallel studies have been conducted, one on movement patterns and the other on physical condition and morphometrics, which have also been directly related to diet, as we will see below.

THE DIET CHANGES IN DIFFERENT HABITATS: THE ROLE OF THE MOVEMENTS AND HABITAT USE

Food is a key element for the species. Understanding of why they eat one or other species (diet), is of main concern. The differences in diet composition between the two studied areas (Chapter 1) were in part explained by the annual altitudinal movement patterns. In addition to the studies presented in each chapter of this thesis, a study of red deer movements was conducted using

GPS tracking collars (Table 1), using Followit transmitters (https://www.followit.se/wildlife/). In the case of the Axial Pyrenees, the movements of 2 individuals were tracked (Figure 1), while in the Pre-Pyrenees, 5 individuals were tracked (Figure 2). Our study allowed us to understand spatial and temporal movement patterns, as the altitudinal ones, and its relationships with the feeding habits. The individuals from the Axial Pyrenees, descended to the lowlands (at the bottom valleys) from October-November to the early months of spring (March-April), and ascended to the highlands (alpine and subalpine ecosystems) from April-May to the Autumn (Figure 3). These oscillations are due to the search for food during the most unfavourable months due to low temperatures and snow, as explained in Chapter 1. For this reason, there was no a definite seasonal pattern in diet composition in this area: the graminoids dominated the diet throughout the year, the studied individuals undertook these altitudinal movements in search of the better food resources. Gilbert et al., (2017) also showed variations in seasonal movements of Sitka black-tailed deer (*Odocoileus hemionus sitkensis*) in Alaska due to harsh weather conditions, particularly snow. Studies involving ungulates inhabiting areas with harsh weather conditions, shown a shift in habitat selection during these unfavourable periods has been observed, with a preference for mature forests as a refuge from the snow or habitats where high-quality food can be found (Schoen and Kirchhoff, 1985; Hundertmark et al., 1990; Mcloughlin et al., 2010; Gilbert et al., 2017). This phenomenon has been referred to as "green wave surfing" (Mysterud et al., 2017). Pépin and Gerard (2008b) defined three strategies of altitudinal migrations (i.e., non-, downward-, and upward-migrants). Some species that inhabit mountain areas are generally downward migrants, such as red deer (*Cervus elaphus*), Rocky Mountain elk (*Cervus canadensis nelsoni*) and mule deer (*Odocoileus hemionus*), moving from high-elevation summer ranges to low-elevation winter ranges due to deep snow accumulations and cold at high elevations during winter (Georgii and Schröder, 1983; Gates et al., 2005; Ager et al., 2003; D'Eon and Serrouya, 2005). This would be the case for red deer populations in the Axial Pyrenees. On the other hand, in the case of the Pre-Pyrenees (also including high mountains and an altitudinal stratification of the vegetation), we could not confirm the existence of such altitudinal migrations

(Figure 3). Other studies have shown that red deer populations do not undertake altitudinal migrations, especially when food availability is assured within their home range throughout the year, either through supplemental feeding or a less severe climate compared to other regions (Georgii, 1980; Schmidt, 1992). This seems to be the case of deer living at the Pre-Pyrenees. The differences in strategies between the two study areas are closely related to the habitat use

Individual	**Area**	**Sex**	**Start date**	**End date**	**Nº days**	**Kernel 95 (km^2)**
FadBO01	RNCB	Female	21/09/2015	07/11/2016	413	2.22
MjuBO01	RNCB	Male	19/06/2018	08/11/2018	142	27.34
MadBO01			06/02/2020	20/02/2021	380	
FadBO02	RNCB	Female	10/02/2020	30/05/2020	110	1.28
MadBO02	RNCB	Male	17/06/2021	07/10/2021	112	42.44
FadBO03	RNCB	Female	13/05/2021	07/10/2021	130	1.79
FadAP01	RNCAP	Female	07/04/2015	09/04/2016	368	9.76
MadAP01	RNCAP	Male	07/03/2018	11/05/2019	430	82.74

Table 1. Captured individuals, area, sex, follow-up period and kernel 95. The study areas were the National Hunting Reserve of Alt Pallars (RNCAP) and the National Hunting Reserve of Boumort (RNCB). The kernel was calculated by the function “kernelUD” of the Package adehablitat (Calenge, 2011) in R 4.2.1 (R Core Team 2022). The Utilization Distribution (UD) is the bivariate function giving the probability density that an animal is found at a point according to its geographical coordinates. Using this model, one can define the home range as the minimum area in which an animal has some specified probability of being located. The functions used here correspond to the approach described in Worton (1995). The two data sets MjuBO01 and MadBO01, correspond to two tracking periods of the same individual. This individual was captured in June 2018, but only a few data GPS locations were obtained due to an error with the drop-off system of the GPS collar, which was activated in November of the same year, causing the collar to detach from the deer’s neck. The same individual was recaptured in 2020, but the collar stopped transmitting a few months after its placement. Therefore, we have two data periods for the same male, the first corresponding to a young age and the second corresponding to the adult age of the deer.

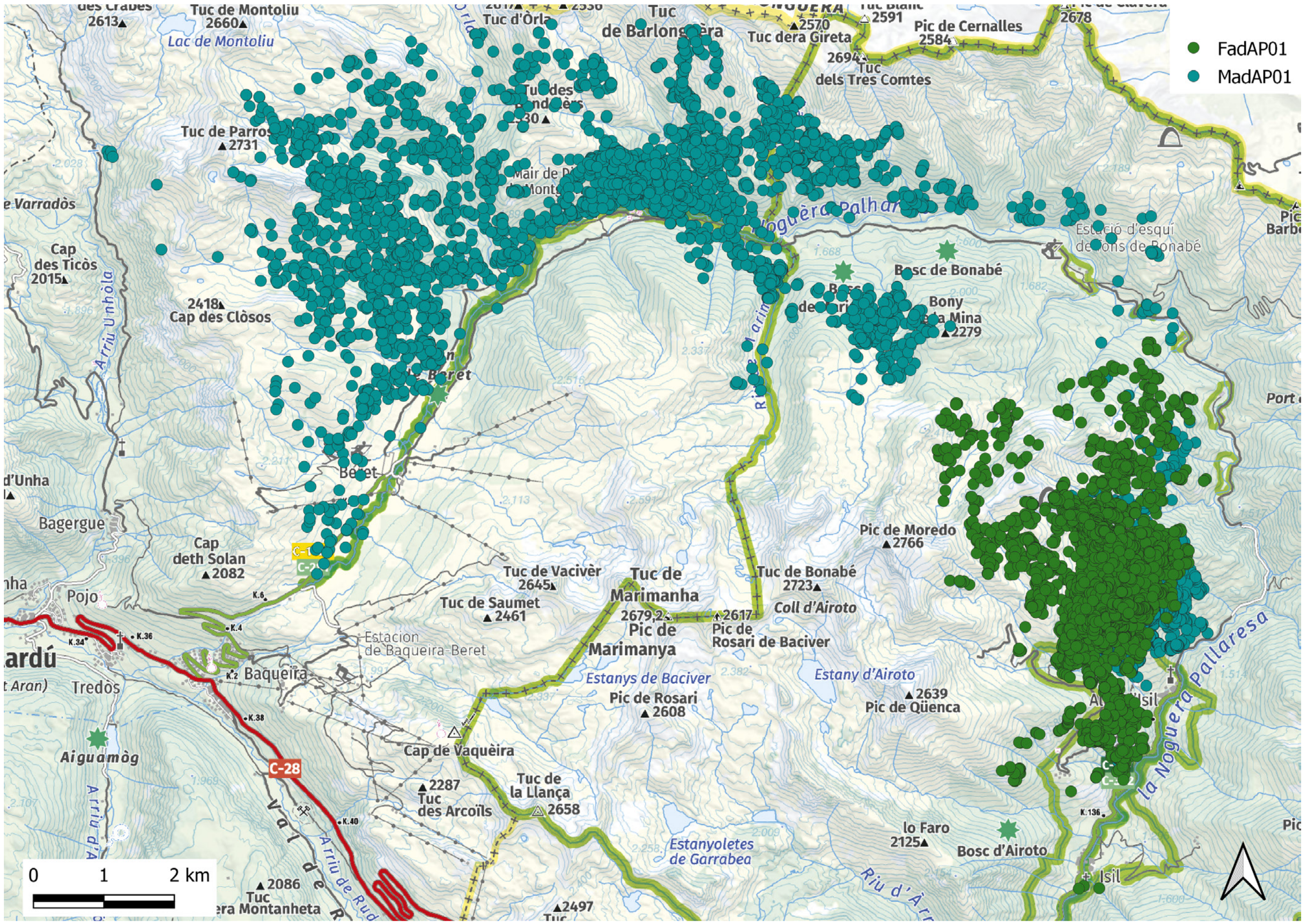


Figure 1. Map of the GPS locations of the individuals of the Axial Pyrenees. Base map: Topographic map 1:100,000 of the Cartographic and Geological Institute of Catalonia (ICGC), license CC BY 4.0.

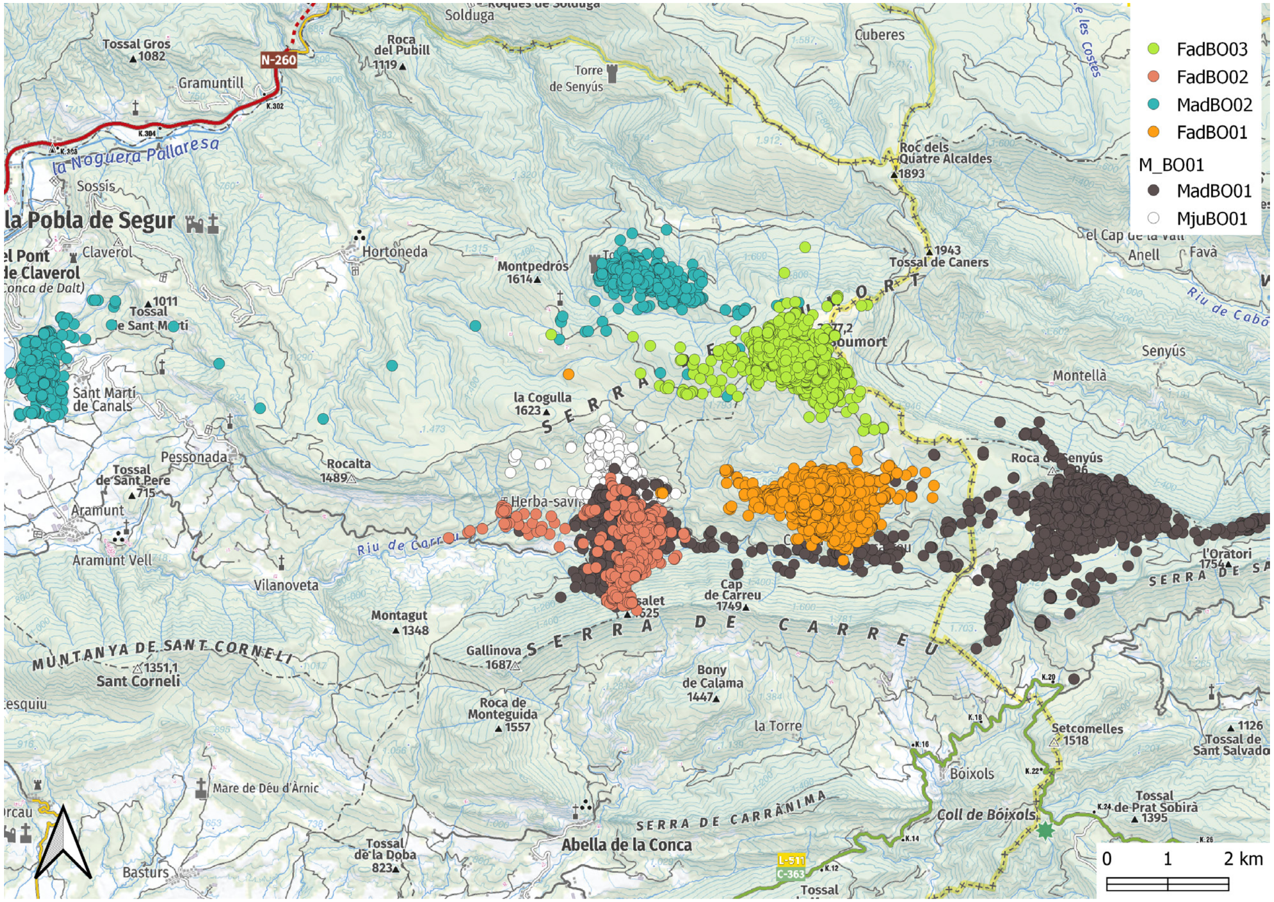


Figure 2 Map of the GPS locations of the individuals of the Pre-Pyrenees. Base map: Topographic map 1:100,000 of the Cartographic and Geological Institute of Catalonia (ICGC), license CC BY 4.0.

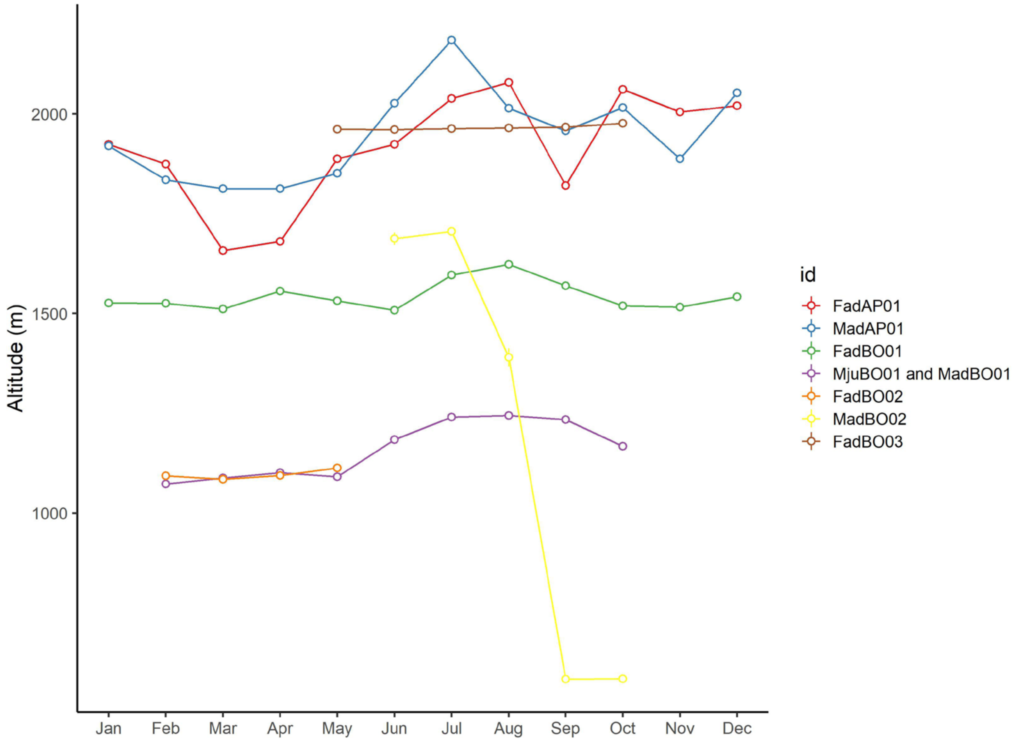


Figure 3. Average monthly altitude of the GPS locations of each of the individuals.

discussed in Chapter 1. In the Axial Pyrenees, the use of the habitat was drove both for refuge and food (feeding areas were more often open areas with a low refuge value), while in the Pre-Pyrenees, the habitat use was mainly drove by feeding needs (these areas providing also enough shelter), reflecting the harsher conditions in the Axial Pyrenees.

Figures 4 and 5 illustrate the habitat use by deer from in the Axial Pyrenees with the categories and habitats extracted from a CORINE map (Vigo et al., 2006) (Supplementary Table 1). Although these results were based on few individuals (but with an enormous number of independent positions), they were very similar to those found by means of other methods, as described in Chapter 1: in this area there was a predominant use of Pyrenean broom (Genista balansae) scrubland and grasslands, as primary habitats. As explained in Chapter 1, we could attribute the use of these grasslands for feeding, as graminoids were the main component of their diet (but not providing any refuge), and the use of Broom scrubland as refuge, especially from the snow, wind and cold, due to its height and special structure (but never was found within the eaten species). Additionally, the use of pine forests in summertime, which here attributed to refuge from high temperatures, was also highlighted.

In the Pre-Pyrenees area (Figures 6 and 7) with the categories and habitats extracted from a CORINE map (Vigo et al., 2006) (Supplementary Table 1)., there was a different pattern, with a clear tendency to use common box (*Buxus sempervirens*) scrubland, as seen in Chapter 1. In this case, its use can be attributed to the dual nature of this habitat as both a refuge and a feeding area. The use of grasslands for feeding is also described in Chapter 1, but in this case, it seems that males obtained more graminoids from the crop fields. On the other hand, the most significant difference between the results of this study and those of Chapter 1 was the low use of pine forests and the higher use of other habitats by males in the Pre-Pyrenean area. In Chapter 1, pine forests were one of the main habitats, whereas in this study, they were among the least used habitats. Among the other habitat's category, males predominantly used cliffs and ledges, which were hardly utilized in Chapter 1. One reason explaining these differences was that the some of the studied males were young individuals, and therefore they may not have established their territories yet. The figure 2 shows how these two males moved between different areas, and we could even say that male MadBO02 underwent a small migration. These movements could be attributed to juvenile dispersal. This type of dispersal has been described not only in red deer but also in many other species (Dobson, 1982; Wolff, 1993; Lawson Handley and Perrin, 2007; Jarnemo, 2011).

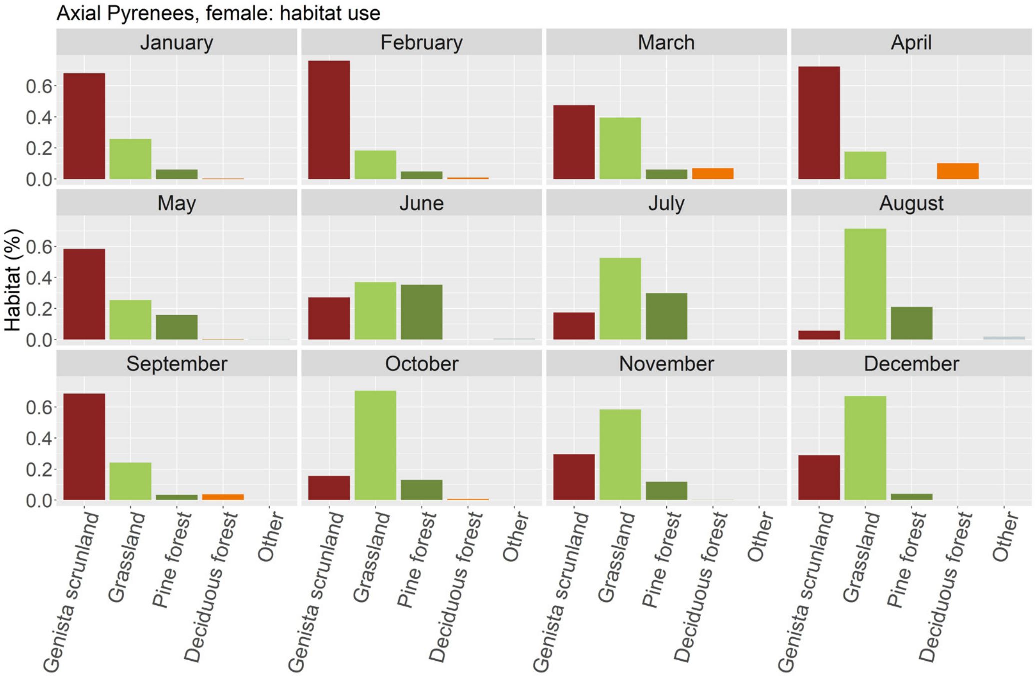


Figure 4. Habitat use by the individual FadAP01 from the Axial Pyrenees.

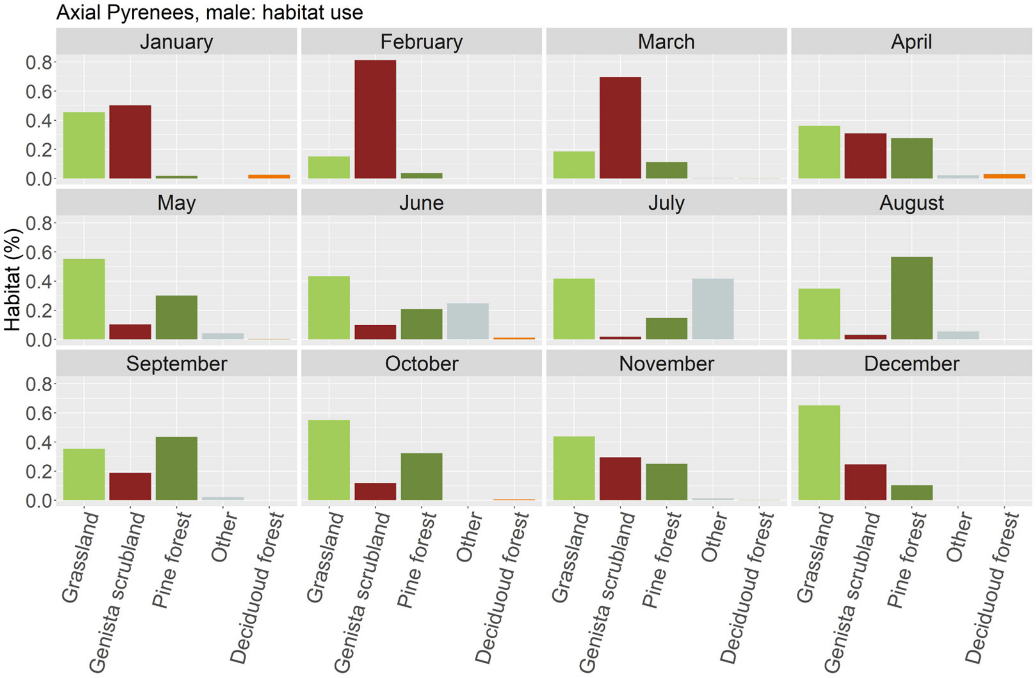


Figure 5. Habitat use by the individual MadAP01 from the Axial Pyrenees.

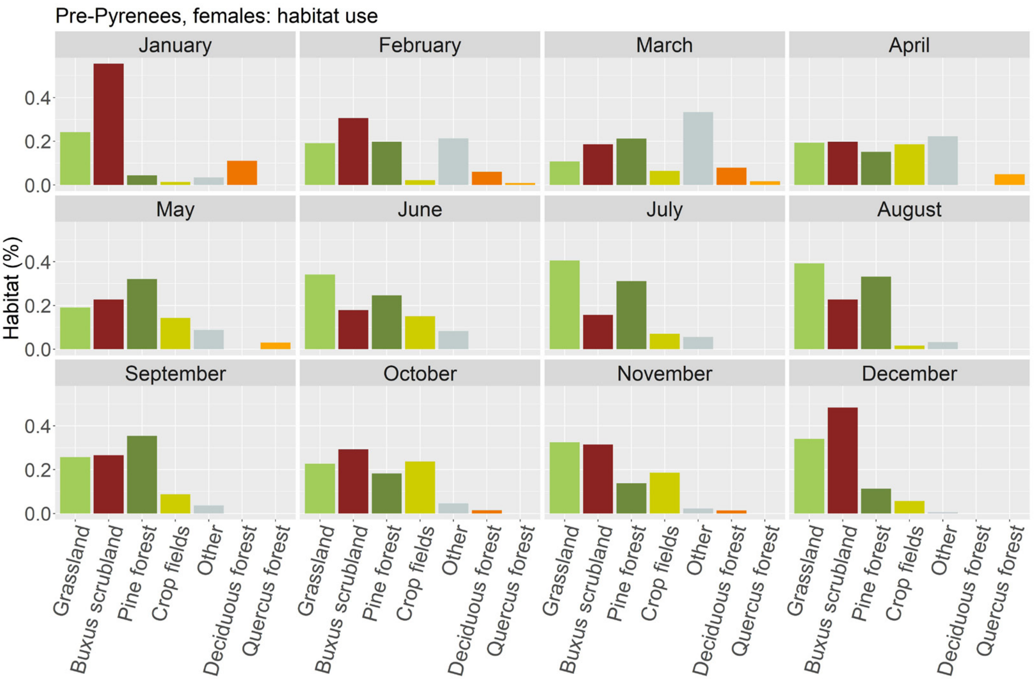


Figure 6. Habitat use by the female individuals from the Pre-Pyrenees.

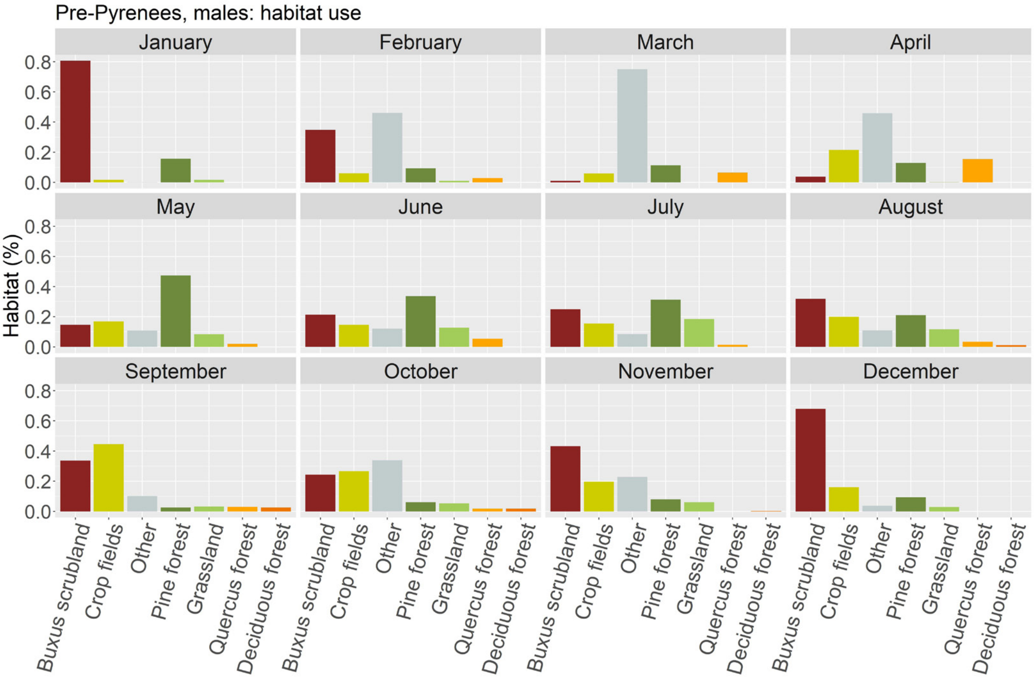


Figure 7. Habitat use by the female individuals from the Pre-Pyrenees.

Influence of diet on phenotypic plasticity

The conclusions of Chapter 2 regarding the reasons for the high prevalence of supernumerary roots align perfectly with the results of Chapter 1. On the one hand, we found a high prevalence of supernumerary roots in the permanent molar M_1 (23.81%, Table 1, Chapter 2) of individuals from the Axial Pyrenees, which we related to the wear caused by a grazing condition. This condition is confirmed in Chapter 1, where it was found that individuals in this area are more grazers, with graminoids being the main component of their diet. However, we should not forget that there could be other possible causes, such as jaw biomechanics or genetics, as individuals in this area do not belong to the Iberian subspecies (Perez-Gonzalez et al., in press).

On the other hand, there was a high prevalence of deciduous teeth, pm_3 and pm_4 (26.09%, Table 1, Chapter 2), in the Pre-Pyrenees area. In this case, we speculated that it was an adaptive response to a hard diet, dry conditions, and a harsh weaning period. Regarding the possible explanation of a hard diet, the results of Chapter 1 showed that the population in the Pre-Pyrenees were more browsers. Moreover, the high consumption of Pinus and Quercus ilex confirms the presence of a hard diet, as the leaves of these species are harder and more difficult to chew compared to those of oaks and other shrubs.

In addition to being related to phenotypic plasticity, the composition of the diet can also affect the growth and development of individuals, modifying their body size and even their individual condition. The body condition of each individual can be calculated using the Kidney Fat Index (KFI) (Santos et al., 2013). This value is correlated with the total fat of the individual and is calculated as:

$$KFI=(FW/KW) \times 100$$

where FW is the weight of the perirenal fat and KW is the weight of the kidney.

During this project, samples of kidneys and perirenal fat were collected to study the conditions of individuals in the Axial Pyrenees and Pre-Pyrenees areas. Additionally, we collected morphometric information (Supplementary material 1, morphological variables). In total, 42 individuals were sampled in the Axial Pyrenees (30 males, 12 females, and 6 unidentified) and 57 in

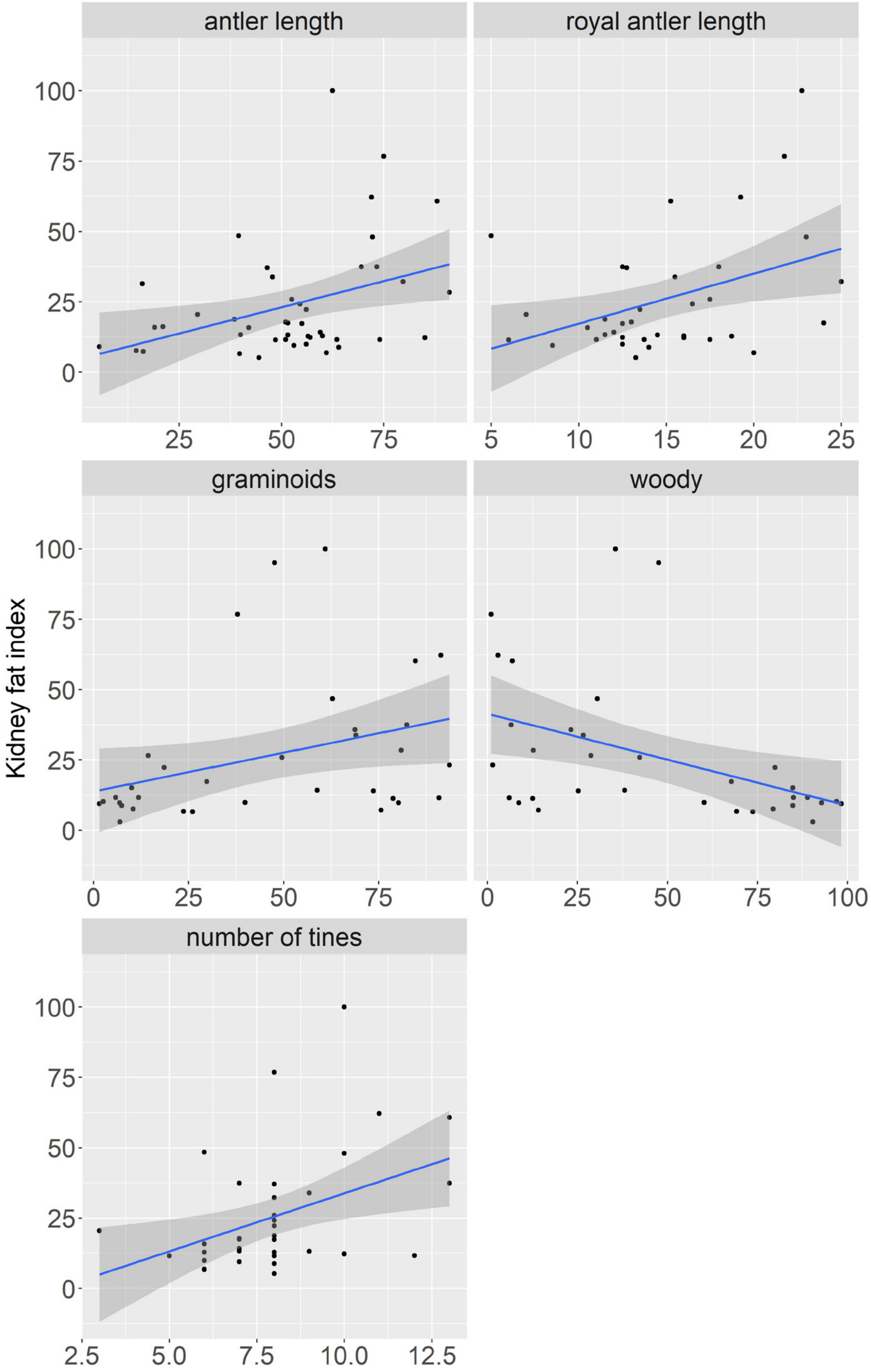


Figure 8. Correlation between the of thesignificant morphological variables (length in centimetres) and the percentage of diet items to the kidney fat index.

Pre-Pyrenees (22 males, 35 females, and 1 unidentified). These sampled individuals were the same that we collected the stomach content for the study of the Chapter 1. The KFI index showed a significant correlation with antler length, royal antler length, number of tines, percentage of graminoids, and percentage of woody species (Table 2 and Figure 8). The correlation was positive for all variables except the percentage of woody species. Therefore, individuals with higher antler length, higher royal antler length, and more tines exhibited better physical condition than the rest. Additionally, individuals that consumed more graminoids and fewer woody species also showed better body condition. Thus, according to the results of Chapter 1, with higher consumption of graminoids in the Axial Pyrenees and more woody species in the Pre-Pyrenees, individuals in the former area should have a better individual condition. The average KFI in the Axial Pyrenees was 28.03%, while in the Pyrenees it was 20.58%, indicating that, as expected, individuals in the Axial Pyrenees had a better individual condition than those in the Pre-Pyrenees. These differences are mainly due to the higher nutritional quality of graminoids, which have lower levels of lignin and higher nitrogen levels compared to woody species (Bartolomé et al., 1998; Santos et al., 2018; Bartolomé et al., 2019). For this reason, another methodology used as an indicator of individual condition is faecal nitrogen (Leslie et al., 2008). Moreover, due to these nutritional differences, the seasons when deer consume more woody species, being more browsers,

	t	**df**	**p-value**	**cor**
antler length	2.5967	42	0.01292	0.3719326
royal antler length	2.5681	34	0.01479	0.4030598
number of tines	2.6926	35	0.0108	0.4142513
graminoids	2.0481	31	0.04911	0.3452258
woody	-2.6977	31	0.01119	-0.4360297

Table 2. Correlation between the significant morphological variables and the KFI index. Test for association/correlation between paired samples with the Pearson method (t). Package stats of 4.2.1 in R 4.2.1 (R Core Team 2022).

are the most unfavourable for them (Staines and Crisp, 1978; Cook et al., 2013). There are studies showing seasonal differences in the consumption of herbaceous plants and woody species, with higher consumption of woody species in winter and spring in northern areas (Klein, 1985; Fløjgaard et al., 2017), and in summer in southern areas (Bugalho and Milne, 2003).

Are changes in group size and composition related to diet composition or quality, or to food availability?

The results of Chapter 3 showed temporal and inter-areas changes in group size and composition. Such changes were linked to different strategies to increase foraging efficiency and according to food availability. Due to climatic differences between the Axial Pyrenees and the Pre-Pyrenees, food availability and distribution also varied between them. Therefore, we found significant differences in group size and composition between the studied areas (Table 3).

	Df	**LogLik**	**Pr(>Chi)**
Area	1	2758.3	-0.002308
Season	3	-2803.0	< 2.2e-16
Type	2	-2861.8	< 2.2e-16
Area:Season	3	-2753.7	7.432e-05
Area:Type	2	-2743.2	0.661699
Season:Type	6	-2752.8	0.002908
Area:Season:Type	6	-2742.8	0.943391

Table 3. Analysis of deviance tables for models using all groups and testing differences between areas, seasons and group types. Type II (marginal) likelihood ratio tests compare each model against the same model without the focal factor. The difference in number of parameters between the compared models, the log-likelihood of the simpler model and the p-value for the chi-square test are shown.

In the Axial Pyrenees, the average group size was on average 5.7 individuals, while in the Pre-Pyrenees, it was higher, reaching 7.02 individuals. This implied that, according to the hypothesis accepted in Chapter 3, which stated that larger groups were formed during periods of lower food availability and smaller groups during periods of higher availability, there would be greater food availability and of more quality in the Axial Pyrenees. In Chapters 1 and 2, we already observed that graminoids dominated the diet composition in the Axial Pyrenees, which is a more nutritious group than woody plants while in the Pre-Pyrenees was mainly composed by woody plants. Therefore, in the Axial Pyrenees, it can be assumed that there was a higher food availability and better quality compared to the Pre-Pyrenees. The habitat use of the studied deer provided of transmitters also have shown, that, for the Axial Pyrenees they spent much more time in the richer habitats than in the poorest ones, while in the Pyrenees they spent much more time on the poorest habitats.

In the Axial Pyrenees, except winter and summer, the group sizes were significantly different between all seasons (Table 2 of Chapter 3). However, based on the results of Chapter 1, there were no significant differences in diet composition between seasons. Spring was the season with the largest groups, and although grass consumption stood out, the main reason for the formation of large groups, in this case, was the lower food availability due to the more extensive snow cover and the cold preventing the grass growth. In this season, individuals gathered in the valleys where they could find food, and the most abundant food were graminoids, which is why we found this food group dominant in the diet. Therefore, in this case, diet composition would not affect group size, and food availability would remain the main cause determining the aggregation behaviour. On the other hand, autumn was the season with smaller groups, a favourable season with ample food availability. The diet composition in this season was similar to that in summer, with a wide variety of food, which also does not imply a relationship between group size or composition and diet. However, we cannot forget that autumn is the rutting season, which, as described in Chapter 3, can be the most reasonable reason for the Autumn changes in group size.

In the Pre-Pyrenees, winter is the most unfavourable season, and there were significant differences in group size between this season and summer and autumn (Table 2 of Chapter 3). There are also significant differences in diet composition between seasons in this area, as shown in Chapter 1. The main difference between these seasons was a higher consumption of Quercus ilex in wintertime. In winter, there is a higher consumption of woody plants, and in summer and autumn, there is a higher consumption of graminoids, which coincides with larger groups in winter and smaller groups in these seasons. In the Pre-Pyrenees, there would be a relationship between group size and the availability and quality of food.

CLIMATOLOGY, A GREATER INFLUENCE ON STRESS THAN FOOD SHORTAGE

Finally, Chapter 4 showed a greater influence of climatology than food stress on cortisol levels in the Pre-Pyrenees population. As we have observed, the deer population in the Pre-Pyrenees had less food availability and lower quality compared to the Axial Pyrenees, and all this made the influence of climatology on cortisol levels greater. However, in the Axial Pyrenees, where climatic conditions were more severe, the individual condition, calculated through the KFI, was better. One of the reasons that would explain these conditions is that in the Pre-Pyrenees, there is sufficient food availability but of poor quality, so there would not be food stress. On the other hand, the climatic conditions in the Pre-Pyrenees, although not as severe as in the Axial Pyrenees, would also not be optimal for this species. It should be noted that the Iberian subspecies inhabits this area, which is not as well adapted to low temperatures as the population further north.

FUTURE STUDIES

One of the main uncertainties to be resolved in deer populations in the Pyrenees is demography. A demographic study is needed to determine population abundance, density, mortality, natality and sex-ratio. This study would require combining different techniques such as visual censuses or camera trapping. On the other hand, increasing the sample size in the GPS tracking study is necessary. The migration performed by some of our deer in the Axial Pyrenees is a very interesting novelty in our area, and it should be confirmed with other individualsor monitoring the whole population. Related to this topic, the strategy used during the rutting season in the Axial Pyrenees area should be studied, the “harems” were small, if they were harems, which leads us to think that they could be using another strategy during the rut. Related to the study stress, it would be interesting to know if ecotourism affects the intensity of male roar.

SUPPLEMENTARY MATERIAL

Buxus scrubland	32aa and 31q
Crop fields	82c, 83a, 83f and 87a
Deciduous forest	31aa, 41h, 41n, 41r and 41s, 45d1
Genista scrubland	31t and 31u
Grassland	34a1, 34b, 34d, 34e, 34l, 34n, 35b, 35h, 36c, 36e, 36f2, 36g, 36n, 38b and 54a
Other habitats	31f, 31g, 31h, 31l, 32f, 32u, 32w, 43f, 44b, 37d, 45h, 61e, 61f, 62a, 62b and 62c
Pine forest	42ad, 42af, 42ah, 42f, 42g, 42h, 41i, 42j, 42k, 42m, 42p, 42r and 42t
Quercus forest	45f

Supplementary Table 1. Categories and habitats extracted from a CORINE map (Vigo et al., 2006).

Morphological variables

a. length (from upper lip to base of tail/cm),
b. height (from the end of the right leg before the withers/cm),
c. right tarsus length (cm)
d. maximum neck circumference (cm)
e. length of the right antler (cm)
f. length of the left antler (cm)
g. length of the right brow tine(cm)
h. length of the left brow tine (cm)
i. length of the right royal antler (cm)
j. length of the left royal antler (cm)
k. perish of the right pedicle (cm)
l. perish of the left pedicle (cm)
m. maximum perish between the right brow tine/royal antler (cm)
n. maximum perish between the left brow tine/royal antler (cm)
o. perish maximum between the right royal antler/crown tine (cm)
p. perish maximum between the left royal antler/crown tine (cm)
q. number of right tines
r. number of left tines
s. total number of tines
t. dist. maximum between each antler (cm)

CONCLUSIONS

The fast growth of ungulate populations in the northeaster Iberian Peninsula has led us to conduct a large number of studies in order to conserve and manage these populations and the habitats they use. The most relevant conclusion of this study is that red deer has a much greater capacity for adaptability to different ecosystems than we previously thought. In the introduction of this work, we have already seen that deer had a large distribution range, and therefore, it had to adapt to a wide variety of environments. However, in the case of our study, we have found new adaptations to very particular and closely related ecosystems within the Iberian Peninsula. The results from our study of diet and habitat use showed us that deer are not always browsers or mixed feeders, but in areas like the Axial Pyrenees, their diet positions them as grazers. We were also surprised to find that it is not always the males that are the most inclined towards browsing in all populations, but there are specific areas, such as the Pre-Pyrenees, where females can be more inclined towards browsing. We were also able to verify that climatology is an important determinant for habitat use, with changes in usage according to the harshness of the climate. On the other hand, we were able to confirm that diet is a key factor in understanding many aspects of deer biology, such as phenotypic plasticity or group ethology. In the study of the prevalence of supernumerary roots in molars, we were able to relate this trait to variations in the diet of each of the sampled populations. In the study of group size and composition, we attributed our results to changes in food availability. In contrast, in the case of the stress study in the Pre-Pyrenees population, we were found that changes in the concentration of stress hormones (a proxy for the stress response) are best explained by climate variables and the extremes of harsh weather conditions. In conclusion, diet and climate appear to be the main factors determining the adaptation of deer populations to the ecosystems under study. It is necessary to continue collecting data and expanding studies on these populations, as climate change will have a direct effect on them and the habitats they use.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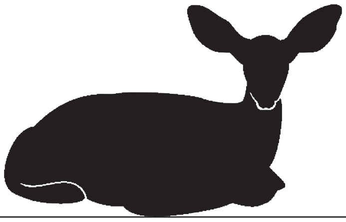

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This study focused on expanding our knowledge about the biology of red deer, analysing diet composition, habitat use, morphological plasticity in dental roots, group ethology and stress. It was determined that the population in the Axial Pyrenees consists mainly of grazers, while the population in the Pre-Pyrenees was more inclined towards browsing. The movements of the deer reflected the differences in diet and habitat between the areas. The habitat use was primarily for refuge in the Axial Pyrenees and for feeding in the Pre-Pyrenees due to the harshness of the climate in the northern area. The prevalence of supernumerary roots in molars and premolars was also analysed. The high prevalence in the M_1 was associated with the grazer condition in the Axial Pyrenees, while in the case of premolars in the Pre-Pyrenees was associated with a hard diet and dry climate. The size of deer groups varied with food availability, with large groups in unfavourable periods and small groups in favourable periods. During the rut, small mixed groups were formed, possibly due to the abundance of food. Furthermore, hormone stress levels were influenced by previous climatic conditions, and there were differences between adult males and other individuals. The study highlights the adaptability of red deer to different feeding and climatic conditions in the absence of natural predators.