Alpine grasslands,
grazing and climate change

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Author
Laia Jarque Bascuñana

Supervisors

Elena Albanell Trullas

Jordi Bartolomé Filella

Emmanuel Serrano Ferron

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El doctor **Emmanuel Serrano Ferron** investigador Ramón y Cajal del Departament de Medicina i Cirurgia Animal, i els doctors **Elena Albanell Trullas** i **Jordi Bartolomé Fiella**, professors del Departament de Ciència Animal i dels Aliments de la Facultat de Veterinària de la Universitat Autònoma de Barcelona

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Que la memòria titulada "**Alpine grasslands, garzing and climate change**", presentada per **Laia Jarque Bascuñana** per a l'obtenció del grau de Doctor en Biodiversitat per la Universitat Autònoma de Barcelona, s'ha realitzat sota la nostra direcció i, un cop considerada satisfactòriament finalitzada, autoritzem la seva presentació per tal que sigui avaluada per la comissió corresponent.

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director

Elena Albanell Trullas
directora

Jordi Bartolomé Fiella
director

Laia Jarque Bascuñana
doctorand

A l’àvia Rosa i a l’àvia Mercè

Agraïments

De petita el meu pare sempre em deia que per agafar el tren havies de ser a l'andana amb la maleta a la mà. Aquest consell m'ha acompanyat sempre i és que ja llavors tenia la intenció d'agafar-ne molts de trens i era conscient que alguns d'ells passarien molt poques vegades per la meva andana. Al llarg del meu camí he anat omplint la meva maleta de tot allò que he anat trobant interessant i m'ha sorprès com m'han fet servei un munt "d'andròmines" que en el seu moment hi vaig dipositar sense saber ben bé el perquè. És per això que quan l'Emmanuel em va llençar l'ham de col·laborar amb ell en algunes investigacions a la Facultat de Veterinària jo m'hi vaig enganxar, inconscient en aquell moment que el projecte aniria molt més enllà del que en un inici m'havia proposat.

I fins aquí hem arribat, la meva tesi ja és una realitat! Afegeixo una nova andròmina a la meva maleta. No ho he fet pas jo sola, no hi haguera arribat mai. Un munt de persones m'han ajudat. Els companys, estudiants i voluntaris de la Facultat de Veterinària que han anat a mostrejar al Pirineu totes les vegades que ha fet falta. La Míriam, que em va ajudar els primers temps de voltar jo pel laboratori i en Johan, amb qui compartírem part del nostre trajecte de doctorands. En Blas i la Carme, que van tenir paciència amb els meus horaris estranys al laboratori. Paciència infinita també la d'en Juancho, suport i contrapunt imprescindible, sempre tan didàctic i treballador. I, per descomptat, els tres directors d'aquesta tesi: l'Elena, en Jordi i l'Emmanuel. Van creure en el meu projecte i hi van posar totes les ganes per tal que arribés a bon port. Sense ells no ho haguera aconseguit. He après molt de vosaltres. Moltes gràcies!

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Isard dibuixat per Rafel Jarque Serena. Aquarel·la sobre cartolina.

Preface

Global warming affects physical and biological systems worldwide. The challenge is far from solved in the short-to-medium term, and scientists in particular must decrypt global warming and climate change processes to give humanity the tools it needs to address the consequences of these changes.

Dynamics in wild animal populations may be affected by global warming, but how climate change affects wild animal populations is an unsolved issue. Mountain ungulate populations for example rely on vegetation dynamics, which in turn are expected to be deeply affected by snow melt. The aim of this thesis is to improve our understanding of animal-plant interactions in the alpine ecosystem, in particular the Pyrenean chamois (*Rupicapra pyrenaica pyrenaica*)-alpine grass relationship in the Spanish Pyrenees.

This doctoral thesis has been accomplished with the collaboration and combined efforts of the Wildlife Ecology and Health Research Group (WE&H) and Group of Ruminant Research (G2R), both from the Facultat de Veterinària (Universitat Autònoma de Barcelona).

This work is based on three studies focused on improving knowledge about the relationship between animal grazing, plant production and quality and climate change in highly seasonal environments.

Firstly, in **study 1**, we assess the impact of grazing intensity on grassland biomass production and quality. In **study 2**, we compare two methodologies—fecal cuticle microhistology and Near Infrared Spectroscopy (NIRS) analysis—to assess diet composition of Pyrenean chamois. We furthermore highlight the limitations of both methodologies in the proper determination of diet composition. Finally, in **study 3**, we assess the short- and long-term impacts of local weather variations (precipitation, temperature and snow cover) on diet quality of a Pyrenean chamois population over ten years.

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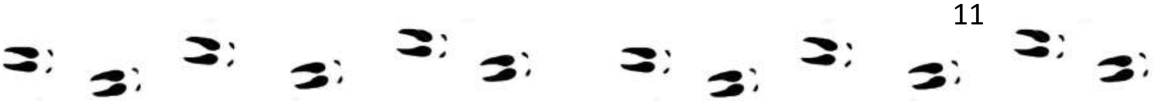


1. Abstract

Abstract

Climatic and land-use changes are affecting biological systems across the globe. Alpine ecosystems, as a fragile community, are especially threatened. Grasslands from these alpine ecosystems provide environmental services like biodiversity, greenhouse gas capture and food supply for domestic and wild herbivores. Assessing the impact of different grazing intensities and a warming climate on this ecosystem may provide important information to understand, or even to predict, the implications of climatic and land-use changes on alpine ecosystems.

Here, our main goal was to assess the impact of grazing, meteorological factors and climate change on the alpine grasslands of the Eastern Pyrenees, Spain. For this purpose, Near Infrared Spectroscopy (NIRS) was assessed as an emissions-free and low-cost predictive method.

We firstly combine two approaches at different spatial scales to assess grazing impact (biomass production and nutritional value) on grasslands (**study 1**). On the meso-scale, results showed that heavily grazed areas with livestock and wild ungulates were more productive than less grazed areas with only wild ungulates. Based on these results, three scenarios of contrasting grazing intensities (null, high and overgrazing) were recreated on the local scale approach, to assess the impact of overgrazing on both summer above-ground biomass production and the nutritional value of plants consumed by Pyrenean Chamois. Our results confirm that grazing intensity exerted significant changes on above-ground biomass production and the protein content of grasses, but had a negligible effect on woody vegetation such as *Calluna vulgaris*. On the other hand, overgrazing reduced biomass production over the

entire growing season but improved the nutritive values of some herbaceous plants such as *Festuca* spp. The worst scenario considered in production terms was under null grazing pressure, where primary production dropped.

We secondly evaluate the potential of NIRS analysis and highlighted the limitations of two methodological approaches (microhistological and NIRS analysis) used in the determination of the diet composition of herbivores (**study 2**). We found out that NIRS is a suitable technique only for wide taxa groups since it is accurate enough for rough diet composition studies and for long-term monitoring purposes of major plant groups. Nevertheless, NIRS technique would not be sufficient for determining the actual composition of the diet with high precision and/or at a species level.

Global climatic and land use changes impact the diet of alpine herbivores and could thus impact the performance of these species. Therefore, it is important to integrate dietary studies when assessing species' response to global changes. In the present thesis, we have combined information from Pyrenean chamois' diet quality and local climate to understand the effects of climate change on the diet of this herbivore (**study 3**). Our results indicated that local climate exerted high positive direct effect on primary production (EVI and LAI) and had indirect effect on Pyrenean chamois' diet (composition and protein content). In such ongoing climate change, temperature is predicted to rise and snow pack and snow melting date are predicted to reduce. Therefore, Pyrenean chamois' diet is predicted to improve. However, other factors than grassland production, such as the intensity of competition between animals, grassland composition and quality and

foraging strategies, may affect chamois diet quality. Further studies would be necessary to assess the effect of these factors on the chamois' diet quality.

Overall, chamois nutrient value and forage availability improve under certain grazing pressure, diet quality increases with warmer temperatures and the chamois diet can be monitored by using NIRS as a cheap, quick and reliable method. However, further studies should be carried out to describe how forage availability trend in a warming climate scenario could affect chamois population dynamics more broadly. These results and conclusions could be crucial to developing management guidelines to endorse profitable grazing activities and promote conservation of open landscapes in the alpine ecosystems under a global change scenario.

Keywords: alpine environment, climate change, diet composition, EVI, grassland management, NIRS, population dynamics, Pyrenean chamois

Resum

El canvi climàtic i en els usos del sòl afecten els sistemes biològics arreu del món. Els ecosistemes alpins, estan especialment amenaçats. Les pastures d’aquests ecosistemes proporcionen recursos com biodiversitat, captura de gasos d’efecte hivernacle i subministrament d’aliments pels herbívors domèstics i salvatges. Avaluar l’impacte del pasturatge i de l’escalfament global en aquest ecosistema pot proporcionar informació important per comprendre i, fins i tot, predir les implicacions del canvi climàtic i d’ús del sòl en els ecosistemes alpins.

El principal objectiu de la tesi és avaluar l’impacte del pasturatge, els factors meteorològics i el canvi climàtic en les pastures alpines del Pirineu Oriental, Espanya. Amb aquest propòsit, s’avalua l’espectroscòpia d’infrarojos propers (NIRS) com un mètode predictiu econòmic i lliure d’emissions.

En primer lloc, combinem dos enfocs a diferents escales espacials, per avaluar l’impacte del pasturatge a la vegatació alpina (**estudi 1**). A mesoescala, els resultats van mostrar que les zones intensament pasturades per bestiar i ungulats salvatges eren més productives que les zones menys pasturades (sols per ungulats salvatges). Basant-nos en aquests resultats, es van recrear tres escenaris d’intensitats de pasturatge contrastades (nul·la, alta i sobrepastura) des d’un enfoc a escala local, per avaluar l’impacte de la sobrepastura tant en la producció de biomassa com en el valor nutricional de les plantes consumides per l’isard. Els nostres resultats confirmen que la intensitat del pasturatge va exercir canvis significatius en les gramínies, però va tenir un efecte insignificant sobre les espècies llenyoses com *Calluna*

vulgaris. D'altra banda, la sobrepastura va reduir la producció de biomassa, però va millorar els valors nutritius d'algunes plantes herbàcies com *Festuca* spp. El pitjor escenari en termes de producció fou sota una pressió de pastura nul·la, on la producció primària es reduí. En segon lloc, avaluem el potencial de la tecnologia NIRS i analitzem les limitacions en front l'anàlisi microhistològica utilitzats en la determinació de la composició de la dieta dels herbívors (**estudi 2**). Hem comprovat que el NIRS és una tècnica adequada només per a grans grups taxonòmics, però útil per a estudis de composició aproximada de la dieta i pel control a llarg termini dels principals grups de plantes. Tot i això, la tècnica NIRS no seria suficient per a determinar la composició de la dieta a nivell d'espècie.

El canvi climàtic i d'ús del sòl afecten la dieta dels herbívors alpins i poden afectar el seu rendiment. És important, doncs, incorporar estudis dietètics a l'hora d'avaluar la resposta de les espècies als canvis globals. En la present tesi, hem combinat informació sobre la qualitat de la dieta de l'isard i el clima local per comprendre els efectes del canvi climàtic sobre la dieta d'aquest herbívor (**estudi 3**). Els nostres resultats indiquen que el clima local exerceix un elevat efecte directe sobre la producció primària i un efecte indirecte sobre la dieta de l'isard. En aquesta situació de canvi climàtic, es preveu l'augment de la temperatura i la reducció la quantitat de neu. Per tant, es preveu que la dieta dels isards millorarà. No obstant això, altres factors que no siguin la producció de pastures, com la competència intraespecífica, la composició i la qualitat de les pastures, poden afectar la qualitat de la dieta de l'isard.

Per a concloure, mitjançant l’ús de NIRS es podrien dur a terme estudis addicionals d’ampli espectre per descriure com l’augment de la disponibilitat de farratge en un escenari d’escalfament global podria afectar la dinàmica de la població d’isards. Aquestes conclusions podrien ser crucials per avalar activitats de pasturatge sostenibles i promoure la conservació dels ecosistemes alpins en un escenari de canvi global.

Paraules clau: canvi climàtic, composició de la dieta, dinàmica de poblacions, EVI, gestió de prats, isard, NIRS, prats alpins

2. General introduction

2.1. Alpine grasslands

Grasslands, defined as areas with very low abundance of trees and shrubs dominated by herbaceous, grass-like and wildflower species (Wilsey, 2018, see Fig. 2.1), represent around 40% of the terrestrial surface (Suttie et al., 2005). Grasslands exist under a wide precipitation range (250-1000 mm) and can easily become forested or desert depending on climate and human activities (Wilsey, 2018). Depending on temperature, precipitation and latitude, grasslands can be arctic, temperate or tropical (Olson et al., 2001; Wilsey, 2018). Grasslands around equator latitudes are called savanna, steppe grasslands are located along tropical, subtropical and temperate latitudes and tundra grasslands are located in the arctic latitudes. Montane or alpine grasslands are located on the summits of high mountains.

The alpine environment is defined as the upper mountain treeless zone (Nagy and Grabherr, 2009) found between 30° and 70° N and 30° and 70° S of the Earth’s equatorial plane. Alpine areas are highly seasonal with a short growing season between 3 and 6 months, mainly in summer. The harsh climatic conditions of this ecosystem (i.e., low temperatures, prolonged frost, heavy snow accumulation), limit the vegetative period to a few spring and summer months (Fig. 2.2) (García-González, 2008).

Figure 2.1. Alpine grassland at the Fontalba study site, located in the Freser-Setcases National Game Reserve (FSNGR), Catalan Pyrenees, northern Spain (Photo by Xavier Fernández Aguilar).

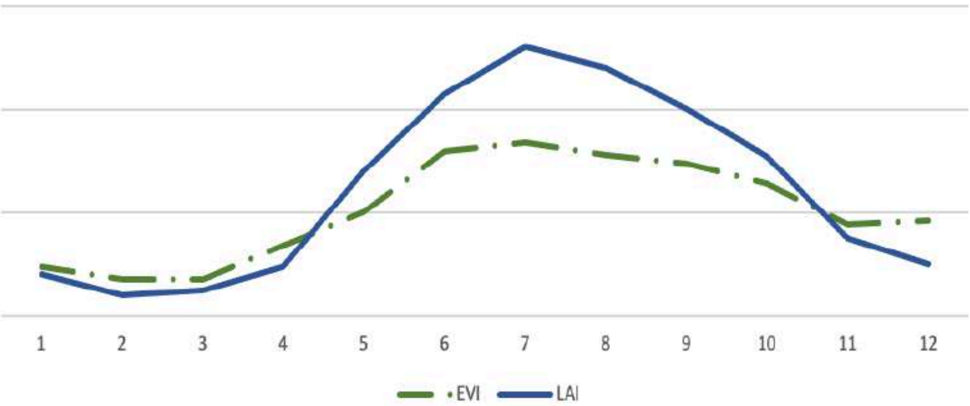


Figure 2.2. **Grassland phenology on the Leaf Area Index (LAI, solid line) and the Enhanced Vegetation Index (EVI, dashed and dotted lines).** The X-axis shows the months of the year whereas the Y-axes shows LAI and EVI values. Drawing adapted from Myneni et al. 2015.

In Spain, Pyrenean alpine grasslands are herbaceous plant communities above 1800 m. a. s. l., driven by low temperatures, snow and strong winds (Fillat et al., 2008; San Miguel Ayanz, 2001). Because of the altitude, precipitations is common and vegetation does not suffer from water stress (San Miguel Ayanz, 2001). Alpine grassland plants, however, develop thick cuticles, piles and whitish coloration to deal with high solar irradiation during summer. This adaptation reduces the palatability and nutritional value of many alpine plant species (San Miguel Ayanz, 2001), in particular of woody plants. Despite these limitations, the abundant water and the mild summer temperatures allow enough primary production to sustain transhumant livestock farming.

2.1.1. Global alpine grassland distribution

Alpine grasslands are located in all continents except Antarctica (Fig. 2.3). Asia is home to the largest surface area of grasslands, with the largest area on the Tibetan Plateau, followed by the Kamchatka Peninsula (in the east of the continent), the Urals range (in the west of the continent) and the western Asian mountains (between the Black Sea and the Caspian Sea). In South America, alpine grasslands are located all along the Andes range, including those located in northern Ecuador, Venezuela and Colombia. In North America, alpine grasslands are located in the Rocky Mountains range from California in the south to Alaska in the north. The South African grasslands is the largest alpine grasslands surface in Africa. Alpine grasslands are also located in some scattered and reduced areas in Africa (e.g., Grand Atlas, Mt. Kilimanjaro, Mt. Kenya, Rwenzori Mts.). In Oceania, alpine grassland areas are found in the Australian Alps (in the southeast of the continent), in the New Zealand Alps (located on New Zealand Island), as well as in the Central range of New Guinea (or the New Guinea Highlands), which crosses almost the entire land mass of New Guinea Island from east to west. European alpine grasslands are represented by the Alps and the Pyrenees range in the south, Scandinavian Alps in the north and the Carpathian Mountains at the eastern region.

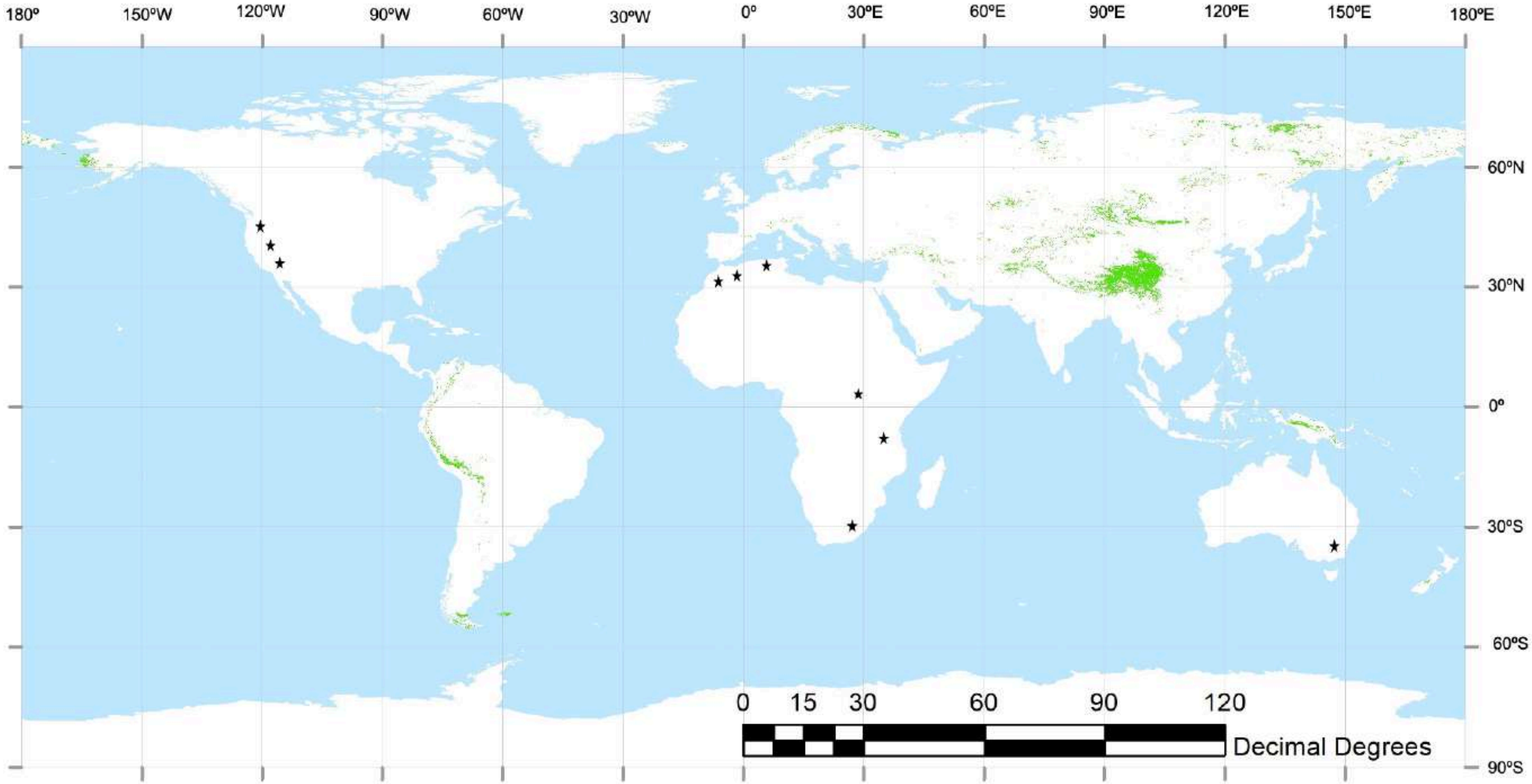
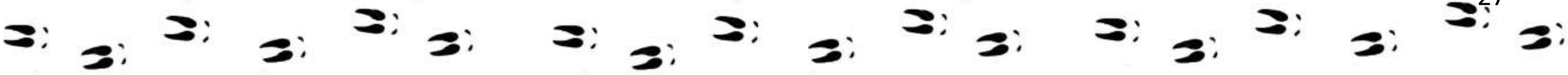


Figure 2.3. Approximated map of global alpine grasslands distribution Adapted from data courtesy of the U.S. Geological Survey, Dixon et al., 2014; Lechmere-Oertel, 2014; World Wild Life Fund, 2021; Walter and Breckle, 1986. Black stars indicate grassland alpine areas undetected by satellites.



2.1.2. Alpine grasslands provide ecosystem services

Alpine grasslands are valuable ecosystems in terms of three related perspectives: economics, biodiversity and livelihoods (Lechmere-Oertel, 2014):

I) The economic value of grasslands is associated with the livestock industry, mainly cattle production (Fig. 2.4). Taking advantage of their primary production, alpine grasslands are used to feed domestic livestock in summer (Carlier et al., 2009; Coventry, 2001; San Miguel Ayanz, 2001; Suttie et al., 2005), in addition to wild herbivores that graze in alpine grasslands year-round. Parallel to this, the tourist industry and outdoor recreation (both, winter and summer activities) in these alpine areas is experiencing an unprecedent boom. In some countries (e.g., Austria), former livestock villages have become tourist economies (Nagy and Grabherr, 2009).

II) From a biodiversity perspective, alpine grasslands are biodiversity hotspots (e.g., South Africa’s grasslands). In terms of plant species composition and growth forms they are very diverse, as well as in terms of all the major animals groups (mammals, birds, reptiles, amphibians and invertebrates) boasting great diversity in forms (Lechmere-Oertel, 2014; Wilsey, 2018).

III) Other multiple ecosystem services related to livelihoods perspectives are provided by alpine grasslands. Many people rely on this ecosystem for their daily living, either from direct benefits (livestock production or plants harvesting) or indirectly ones (cultural value, pollinators and butterfly habitat, water security, carbon-capture and global warming mitigation) (Lechmere-Oertel, 2014; McSherry and Ritchie, 2013; Minahi et al., 1993; Schmitz et al., 2018; Wilsey, 2018).

Figure 2.4. **Bruna del Pirineu calves** grazing in the Fontalba alpine grassland in Freser-Setcases National Game Reserve, Eastern Pyrenees. Photo by Xavier Fernández Aguilar.

2.1.3. Threats to alpine grasslands

Mountain environments are highly sensitive to human perturbations (Nagy and Grabherr, 2009). More than half of grasslands are affected by global changes such as land-use changes (Worbel, 2010), inappropriate grazing practices (Gordon, 2006; Worbel, 2010), or excessive nitrogen deposition (Nagy and Grabherr, 2009). Earlier green-up (Wolkovich et al., 2012) and flowering time (Fitter and Fitter, 2002), as well as changes in species composition (Wilsey et al., 2018) and timberline advance (Wilsey et al., 2018) have been attributed to global warming. Land abandonment, the main driver of woody plant encroachment (Espunyes, 2019), is provoking local ungulate overabundance probably affecting grassland communities.

2.1.4. Plant-animal interactions in alpine grasslands

Alpine grasslands are influenced by large herbivores (Améztegui et al., 2010; Bardgett et al., 2005; Billings, 1973; Körner, 2003; Krumins et al., 2015). Vegetation dynamics, biomass production and the chemical quality of plants (Austrheim et al., 2014; Cook et al., 1953), as well as the cycling of key nutrients (C-N-P) and the ability of pastures to sequester carbon (Cebrian, 1999; Hillebrand et al., 2008; Schmitz et al., 2018) rely on medium-sized and large herbivores. Grazing also increases species diversity reducing the dominance of colonizing plants (Casasús et al., 2003; Lu et al., 2017; Moen et al., 2006). The extent of these effects relies on the grazing intensity and the traits considered (Henkin et al., 2011; Komac et al., 2014; McNaughton, 1979; Thiel-Egenter et al., 2007). For example, biomass production of most pasture plants decreases with overgrazing (Lu et al., 2017; Matches, 1992; Parsons et al., 1983). Forage quality, however, does not follow such a direct pattern and depends on the plant species (Baron et al., 2002; Cook et al., 1953; Fanlo et al., 2015; Komac et al., 2014), and its adaptations to compensate for the effect of herbivores (see Billings, 1973 for a review). In other words, the entire functionality of these alpine ecosystems, as well as their ability to mitigate the effect of global warming is linked to the action of ungulates (Lu et al., 2017; Matches, 1992; McSherry and Ritchie, 2013; Minahi et al., 1993).

In turn, grasslands influence animal conditions, as well as animal population dynamics. As a capital breeders, large herbivores from alpine ecosystems, with a deep seasonality, store energy during spring and summer to meet the costs of reproduction (Jönsson, 1997) and thus rely on high-quality resources during this period. Population dynamics may be affected by global changes in grasslands such as land-use changes and global warming. Wilsey (2018) argues that phenological responses to global change in grasslands (i.e., green-up and flowering time) may drive plant-animal mismatches when animals use day-length as an activity cue. The same author pointed out that when animal activity is driven by other cues other than day-length (i.e., temperature, rainfall), plant and animal will shift in concert. Radchuk et al. (2019), however, concluded in their work that adaptive responses of animals to climate change were most likely insufficient.

2.1.5. Grazing and grasslands management

Grasslands have been heavily impacted by humans. Therefore, grassland conservation and restoration are important issues for natural resource conservation. This conservation usually involves plans to manage grazer stocking pressure and composition of grazer species, such that managed rangelands store more soil C contents and moisture than crop fields (Wilsey, 2018). Grazing management is, according to Vallentine (2001), the manipulation of animal grazing to achieve desired results to supply the quantity and quality of forage needed by the grazers, both domestic livestock and wild animals (including browsers). Grazing serves to not only harvest grazing resources, but also to improve the forage (amount and quality) resource (Vallentine, 2001). Nevertheless, optimum grazing management is required for these benefits. Sustainable exploitation is achieved by blending ecological, economic and social requirements. Properly managed grasslands have the potential to provide not only food for domestic and wild grazers, but also pollinators and butterfly habitat, repositories for biodiversity and greenhouse gas capture (Wilsey, 2018).

To develop proper management guidelines to reconcile exploitation and conservation of natural resources in alpine grasslands and probably in other northern grasslands, it is necessary to determine how grazing (exerted by both domestic livestock and wild animals) and climate conditions affect grassland primary production and quality.

2.2. Alpine grassland integrity through diet assessment

2.2.1. Herbivore feeding in alpine grasslands

Hofmann (1989) classified large herbivores into three flexible and overlapping feeding types based on their diet preferences and physiological evidence. Browsers (e.g., deer), which base their diet mainly on buds, stems, leaves and soft shoots of woody plants (e.g., shrubs). Grazers (e.g., cattle-type), which base their diet mainly on grass and low vegetation. Some herbivores may be classified as mixed, opportunistic or intermediate feeders (e.g., chamois), if they base their diet on a mixture of both groups of preferred plants mentioned above (Fig. 2.5). Clauss et al. (2008), however, underlined the limitations of this classification. In spite of this, the Hofmann classification system will be used here for its simplicity.

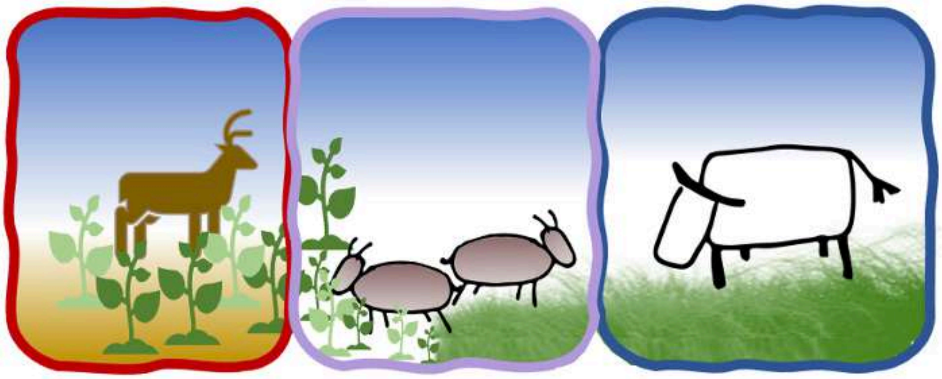


Figure 2.5. Schematic representation of dietary adaptations of herbivores. Browsers, in red rectangle (e.g., deer); mixed feeders, in purple rectangle (e.g., chamois) and grazers, in blue rectangle (e.g., cattle). Own elaboration figure.

According to the “optimal foraging theory” (Pyke, 1984), herbivores select plant species or parts with the highest energy content at the lowest energetic cost (e.g., time searching for and digesting food). Therefore, herbivores are able to adapt their feeding strategy to optimize their diet. In the alpine ecosystem, where the availability of nutritious vegetation is strongly affected by weather conditions, the life history of ungulates (e.g., mating and breeding, growth and migrations) is synchronised to grassland phenology (Fritz and Loison, 2006). Alpine grasslands show a very short vegetative period (60-120 days on average; Nagy & Grabherr, 2009), starting in late spring (May) and finishing in mid-summer (August) when plant senescence begins (Ernakovich et al., 2014). Survival of mountain ungulate populations and performance of livestock rely on the amount and quality of forage during this short period of time.

2.2.2. Global change and population dynamics of mountain ungulates

Warmer winters and wetter summers are expected to occur under a climate change scenario. Reduced snow cover and permafrost thawing trends have been detected, reporting longer growth periods and a thicker active layer (Folland et al., 2001; Moen et al., 2006). These changes favor the access to highly nutritious plants improving the quality of diets for ungulates living in temperate ecosystems (Lenart et al., 2002). It is unclear, however, whether this improvement affects the population dynamics of ungulates. Moen et al. (2006) suggested that offspring survival would increase during summer but that winter forages would be sufficient to support such high densities (Heggberget et al., 2002).

Significant land-use changes also affect European alpine grasslands. Since the mid-20th century human rural emigration has driven a pronounced decline in agropastoral activities in alpine grasslands (San Miguel Ayanz, 2001). Livestock pressure is thus declining and woody plants are colonizing the open spaces devoted to grasslands (Améztegui et al., 2016; Espunyes, 2019; Peñuelas et al., 2007). Woody plant expansion results not only in a loss of biodiversity (Koch et al., 2015; Tasser and Tappeiner, 2002), but also in lower availability of high quality forage (e.g., lower nitrogen/fibre ratio). This may affect grazers with a lower trophic plasticity (Espunyes et al., 2019b).

2.2.3. Diet assessment

Knowledge about herbivore diets is important for assessing ungulate body condition. Information about plant species composition and quality of animal diets is important to wildlife researchers monitoring animal nutritional condition and predicting population dynamics (Fig. 2.6). Body condition could be partially monitored and, in some cases, manipulated. That means that, firstly, monitoring wild animal nutrition performance could allow tracking body condition and indirectly predicting population dynamics (i.e., foreseeing high mortality by pathogen agents due to poor body condition). Secondly, proper management practices in grasslands may promote profitable grazing activities to optimize nutrient value and forage availability in the ecosystem and, in turn, improve animal nutrition condition.

Historically, microhistological analyses of herbivore feces have been the most commonly used method to determine diet composition (Holechek et al., 1982) since it is the cheapest and most non-invasive method to estimate herbivore diet. Other techniques have been used for wild herbivore diet studies but are too invasive and/or time-consuming, and inappropriate for continuous monitoring over time (Table 2.1). Despite its advantages, histological identification of plant fragments in feces may be both very laborious and time-consuming and has other disadvantages discussed by Holechek et al. (1982). For long-term period studies, a robust, quick and cheap non-invasive predictor method is needed.

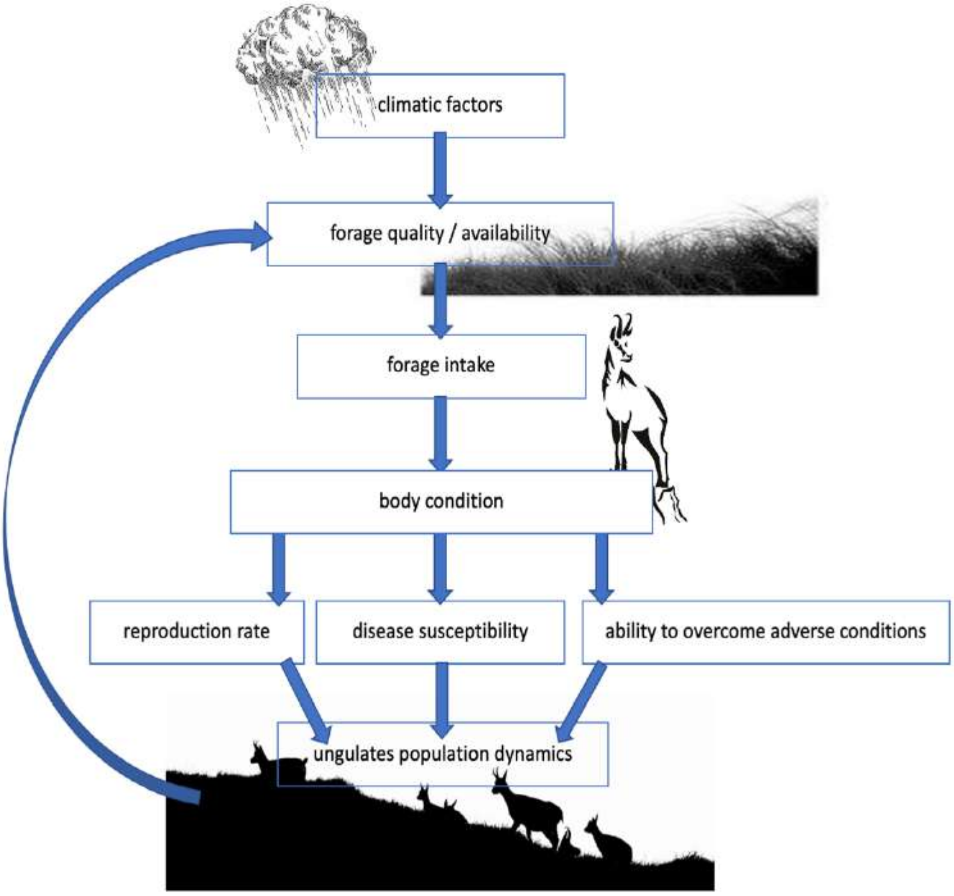
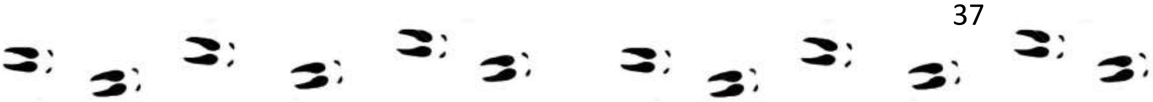


Figure 2.6. Flowchart representing the hypothetical links between climate and the viability of mountain ungulate populations through changes in the quality and availability of mountain grasses.

Table 2.1. Non-systematic review of invasive and non-invasive methods commonly used for diet composition assessment in wild and domestic ungulates. The pros and cons columns summarize some advantages and disadvantages of each technique. References have been included in the reference list of the introduction.

Type	Technique	Pros	Cons	Authors
Invasive methods	Rumen content	• Direct sample observation • Good estimation	• Inappropriate for continuous monitoring over time and/or protected species • Some species may be finely masticated and/or highly digested through the digestive tract	Andreoli et al., 2016
	Esophageal fistula			Vavra et al., 1978
Non-invasive methods	Direct grazing animal observation	• Simple • Small material investment • Can determine the species and plant parts that are consumed	• The accuracy strongly depends on the degree of training of the observer • Time consuming	Perle and Hamr, 1985; Takada and Minami, 2019
	Direct grazed plant observation			Valderrabano and Torrano, 2000
	Video recording			Newmaster et al., 2013
Fecal analysis	Cuticle microhistological analysis	• No animal stress • Small material investment	• No quantitative method. • Some species are highly digested through digestive tract • Time consuming • Long specialist trainging period • The accuracy strongly depends on the degree of training of the specialist	Bartolomé et al., 2011; Croker, 1959; Holechek et al., 1982; La Morgia and Bassano, 2009; Mohammad et al., 1995; Suter et al., 2004
	n-alkane markers (wax components)	• Useful on simple dietary mixtures of up to four components (livestock)	• Not effective enough on complexy diets (wild herbivores) • Expensive • Time consuming	Andriarimalala et al., 2020; Wright et al., 2019
	Isotopes			Codron et al., 2007
	DNA-barcoding	• The most powerful diet assessing method	• Variations in DNA content and different digestibility of several plants limit its accuracy • Less information than microhistological analysis • Long previous work period • Very expensive	Hebert et al., 2003; Pareja et al., 2021
	NIRS	• Quick, economic and non-destructive of samples	• No quantitative method • Some species are highly digested through the digestive tract • High starting investments	Albanell et al., 2011; Coates and Dixon, 2008; Keli et al., 2008; Núñez-Sánchez et al., 2016



2.3. Pyrenean chamois as the study case

Pyrenean chamois (*Rupicapra pyrenaica pyrenaica*) is a medium-sized mountain-dweller ungulate widely distributed in subalpine and alpine habitats of the Pyrenees and the Alps ranges in southern Europe (Corlatti et al., 2011, see Fig. 2.7). As a mixed feeder, the chamois adapts its diet to seasonal alpine ecosystem variations. During winter, its diet is mainly based on woody plants (e.g., *Calluna vulgaris* (L.) Hull and *Cytisus* spp.) and during summer herbaceous plants are more common (e.g., *Festuca* spp. and *Trifolium* spp., Espunyes et al., 2019a). As a capital breeder, the chamois stores energy during spring and summer to meet the high costs of reproduction (Jönsson, 1997) and thus relies on high-quality resources during this short growing period. Chamois hunting is allowed and carefully managed both in Spain and France, with important social and economic implications for local communities (Colom-Cadena, 2017).

Figure 2.7. Pyrenean chamois (*Rupicapra pyrenaica pyrenaica***) in Fontalba**, Freser-Setcases National Game Reserve (southern side of the eastern Pyrenees, Spain). Adult female (left) and males (top and bottom right photos). Photo by Jordi Bartolomé Filella.

2.3.1. Pyrenean chamois' s bad patch

The Pyrenean chamois is a success story of the Pyrenees. Even though many disease outbreaks (e.g., infectious keratoconjunctivitis, border disease virus) have affected chamois populations over the years, Pyrenean chamois populations have shown a high resilience to coping with the deleterious impact of pathogens.

The Infectious keratoconjunctivitis (IKC) cause caused by *Mycoplasma conjunctivae,* develops short duration outbreaks, with high morbidity and low mortality (Loison et al., 1996). Pneumonia mortality data caused by different Pasteurellaceae species are scarce and incomplete. In 2001 several outbreaks of a previously unreported disease associated with *border disease virus* (BDV) infection caused high mortality in Pyrenean chamois populations in the Pyrenees (Marco et al., 2008, 2009b). Marco's study (2008) indicated that the infection had become endemic and that it could have a significant impact on chamois population dynamics. The BDV was considered as an exceptional driver of Pyrenean chamois populations, given the high probability of host extinction (Serrano et al., 2015). Further studies (Fernández-Sirera et al., 2015), however, indicated at least two different epidemiological scenarios in the Pyrenees. On the one hand, in some areas, the disease had indeed become endemic and BDV circulated frequently in the chamois population, possibly having a negative impact on host population dynamics. In other areas, BDV did not seem to circulate and population dynamics were not affected. Several factors found to drive disease patterns including host factors (body condition, behavior and immunity at the population level and genetic variability), climate (especially the winter period) (Gonzalez and Crampe, 2001; Jonas et al., 2008; Willisch et al., 2013) and other ecological factors (anthropogenic, interspecific, food resources and other pathogens Colom-Cadena, 2017; Espunyes et al., 2019a; Jonas et al., 2008; Marco et al., 2009b, 2015, see Fig. 2.8). Pathogen virulence (through viral mutation) turned out to be the main driver of mortality patterns (Colom-Cadena et al., 2019).

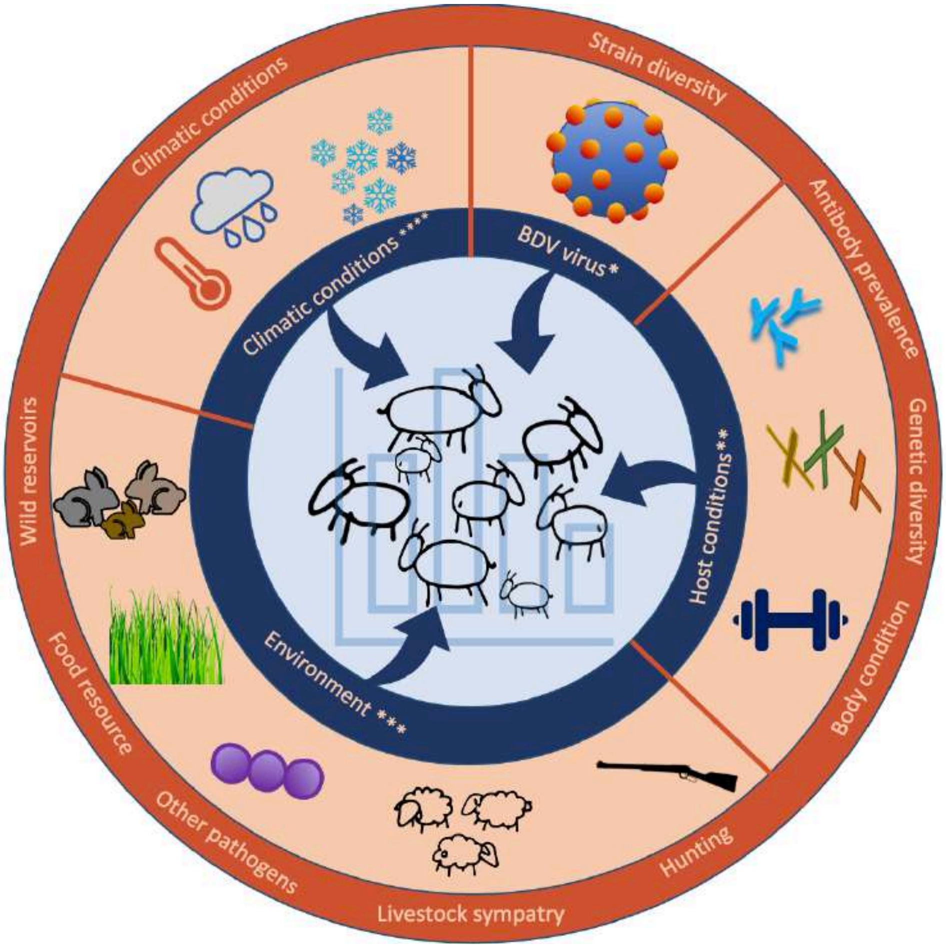


Figure 2.8. **Changes in the interplay of pathogens, host conditions, environmental factors and climatic conditions may drive different epidemiological scenarios during BDV virus outbreaks.** *Main driver, pathogen invasiveness through viral mutation (Strain diversity); **host factors (body condition, behavior, immunity at the population level and genetic variability); ***other environmental factors (wild reservoirs, food resource, other pathogens, livestock sympatry, hunting) and ****climatic conditions (temperature, precipitation, snow).

Today, BDV outbreaks in Pyrenean chamois populations do not lead to high mortality rates as previously seen. High seroprevalence detected in some regions indicates an endemic circulation of the virus (Jiménez-Ruiz et al., 2020). Populations may have acquired group immunity due to low virulence BDV strains. Even so, during those outbreaks periods with high mortality rates recorded in some areas, a need to forecast chamois population dynamics has arisen. Information about these factors that can drive chamois population dynamics (Table 2.2) is needed and knowledge about food resources as well as diet studies would also be useful. Chamois diet information allows, on the one hand, monitoring of wild animal nutrition (and indirectly, may predict population dynamics), and on the other hand, proper management practices of grasslands to optimize nutrient value and forage availability in this ecosystem.

Table 2.2. Factors affecting the viability of chamois populations.

Factor	Variables	References
Population and individual level	• Body condition • Reproductive rates • Juvenile survival	Hempson et al., 2015; Long et al., 2016; Pettorelli et al., 2002; Simard et al., 2008
Food resource*	• Amount • Quality	Gamelon et al., 2020; Jönsson, 1997
Climatic variables*	• Snow • Temperature	Gonzalez and Crampe, 2001; Jonas et al., 2008; Willisch et al., 2013
Anthropogenic	• Hunting • Livestock transhumance	Colom-Cadena, 2017
Inter-specific competition	• Livestock (mainly sheep)	Chirichella et al., 2015; Fankhauser, 2004
Diseases	• Pathogen type / virulence	Colom-Cadena, 2017; Marco et al., 2009b
	• Immune competence	Cavallero et al., 2012; Marco et al., 2009a; Pioz et al., 2007
	• Space-use patterns	Crampe et al., 2007; Marco et al., 2015
	• Wild reservoirs	Colom-Cadena, 2017

*Factors studied in this thesis.

3. Hypotheses and objectives

3.1. Hypotheses and predictions

Alpine grasslands provide value in terms of ecosystem services (economics, biodiversity and livelihoods), which are sensitive to excessive perturbation and are threatened mainly by global changes in climate and land use. To reconcile the exploitation and conservation of this ecosystem, knowledge about how alpine grasslands respond to global change must be broadened.

The survival of ungulates living in alpine grasslands relies on seasonal primary production. Monitoring short- and long-term variations in free-ranging ungulate diet quality and composition as well as proper grassland management practices will help to preserve these environments in the face of global change impacts.

To develop proper management practices that optimize nutrient value and forage availability, we firstly explore how alpine grasslands respond to domestic and wild grazing under different herbivores pressures (**study 1**). Due to the long history of herbivore-plant interactions in these ecosystems (Oksanen and Oksanen, 2000), the grazing optimization hypothesis implies that there is a correlation between grazing and biomass production (Austrheim et al., 2014; McNaughton, 1979). Therefore, we predict that biomass production will increase at high grazing levels but decrease under excessively high grazing and null grazing pressures. We also expect that excessively high grazing pressure will drive the nutritive value of plants depending on the plant type.

These changes in biomass availability due to the effects of grazing and/or other environmental determinants would affect the fitness of mountain ungulates. To monitor animal nutrition performance using a quick, cheap and reliable method, we assessed NIRS as a new technique for wild ungulate diet composition determination (**study 2**). The NIRS has been used to predict nutritional parameters and diet quality in fecal samples of both domestic (Li et al., 2007; Lyons and Stuth, 1992) and wild animals (Gálvez-Cerón et al., 2013; Kamler et al., 2004; Showers et al., 2006), as well as to predict diet composition just for domestic animals (Coates and Dixon, 2008; Keli et al., 2008; Núñez-Sánchez et al., 2016). Taking into account these previous works, we predicted that NIRS analysis would be a good proxy for rough diet composition and for long-term diet composition monitoring of wild ungulates such as Pyrenean chamois over the year. In particular, we expected that the predictive power of NIRS would be higher in periods when diet is more homogenous (e.g., summer, winter) than during transition periods with diverse diets.

Finally, to forecast the impact of global warming on feeding behavior of mountain ungulates, we explore the short- and long-term (10 years) effect of environmental variations on the diet quality and composition of Pyrenean chamois assessed by NIRS (**study 3**). Since alpine forage quality and availability is shaped mainly by climate conditions, we expected that an earlier onset of spring in a current global warming context would facilitate access to high-nutrition plants improving Pyrenean chamois diet quality.

3.2. Objectives

Grasslands have adapted to grazing through evolution. The growth of alpine grasses relies on climate conditions and herbivore activity, while the survival of ungulates living in these habitats depends on seasonal variations in primary production of the alpine meadows.

This thesis aims to improve our understanding of ungulate-grassland interactions in the alpine habitat providing ecological indicators for assessing the impact of global change on this emblematic ecosystem.

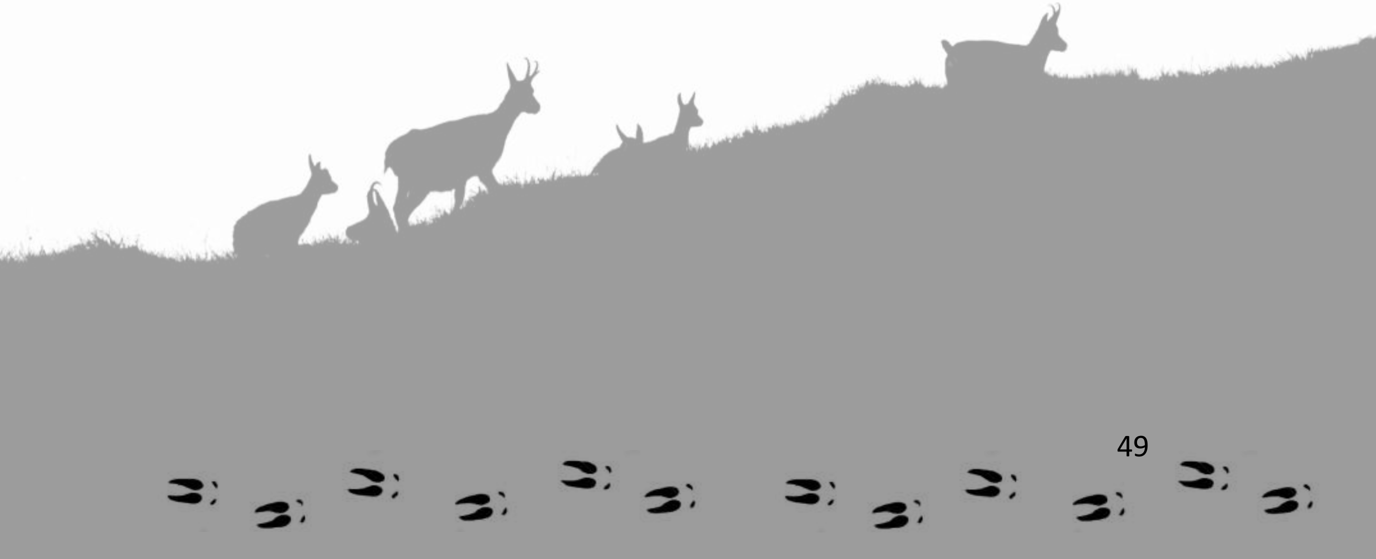
The specific objectives of this thesis are to:

1. Assess the impact of different grazing pressures on biomass production and nutritional value of plants consumed by herbivores.

2. Evaluate the potential of NIRS analysis as a new techinque to determine the diet composition of Pyrenean chamois.

3. Explore the long-term impact of local weather variation on the diet quality and composition of Pyrenean chamois assessed by NIRS.

4. Studies



4.1 Study 1:

Grazing influences biomass production and protein content of alpine meadows

Science of Total Environment (under review)

Authors: Laia Jarque-Bascuñana, Juan Antonio Calleja, Miguel Ibañez, Jordi Bartolomé, Elena Albanell, Johan Espunyes, Arturo Gálvez-Cerón, Josep-María López Martín, Miriam Villamuelas, Diana Gassó, Xavier Fernández Aguilar, Andreu Colom-Cadena, Jennifer Adams Krumins, Emmanuel Serrano

Abstract

Alpine grasslands are essential for carbon sequestration and food supplying for domestic and wild herbivores inhabiting mountainous areas worldwide. These biomes, however, are alternatively threatened by the abandonment of agricultural and livestock practices leading to a fast-growing shrubification process while other mountain grasslands are suffering from the impacts of overgrazing. The functioning of alpine meadow ecosystems is primarily driven by climatic conditions, land-use legacies and grazing. However, although it is critically important, the role of large herbivores on the aboveground biomass and protein content of palatable plants is poorly understood for most of alpine meadows. In this work we explore the effects of grazing on grassland vegetation at two different spatial and temporal scales in the Eastern Pyrenees, Spain. Remote sensing was used to assess the effect of high and moderate grazing (HG and MG respectively) on grass biomass using the leaf area index (LAI) at meso-scale (patches between 2.3 and 38.7 ha), whereas eighteen 1x1 m^2 exclusion boxes were used to simulate null (NG), overgrazing (MO, mimicked overgrazing) and high (HG) grazing at local-scale. Historical satellite data showed that LAI values are higher in high than in low grazed areas (HG, mean = 0.66, LG, mean = 0.55). Along the same lines, high and moderate grazing pressures improved biomass production at local-scale (HG, mean = 590.3 g/m^2, MO, mean = 389.3 g/m^2 and NG, mean = 110.8 g/m^2). Crude protein content reached higher values under MO pressure than under HG pressure. Our results confirm that grazing intensity exerts significant changes on the above-ground biomass production and the protein content of plants consumed by domestic (cattle and horses) and wild ungulates (Southern Chamois, *Rupicapra pyrenaica pyrenaica*). We can conclude that ungulates sustain biomass and nutritive values of grass exerting a negligible effect on biomass and protein content of woody vegetation. Our results will inform management guidelines to support profitable grazing activities and promote conservation of the open landscapes in the alpine ecosystems under a global change scenario.

Key words. biomass production, forage quality, Grassland management, Mountain stock farming, Near-infrared spectroscopy, *Rupicapra pyrenaica pyrenaica*,

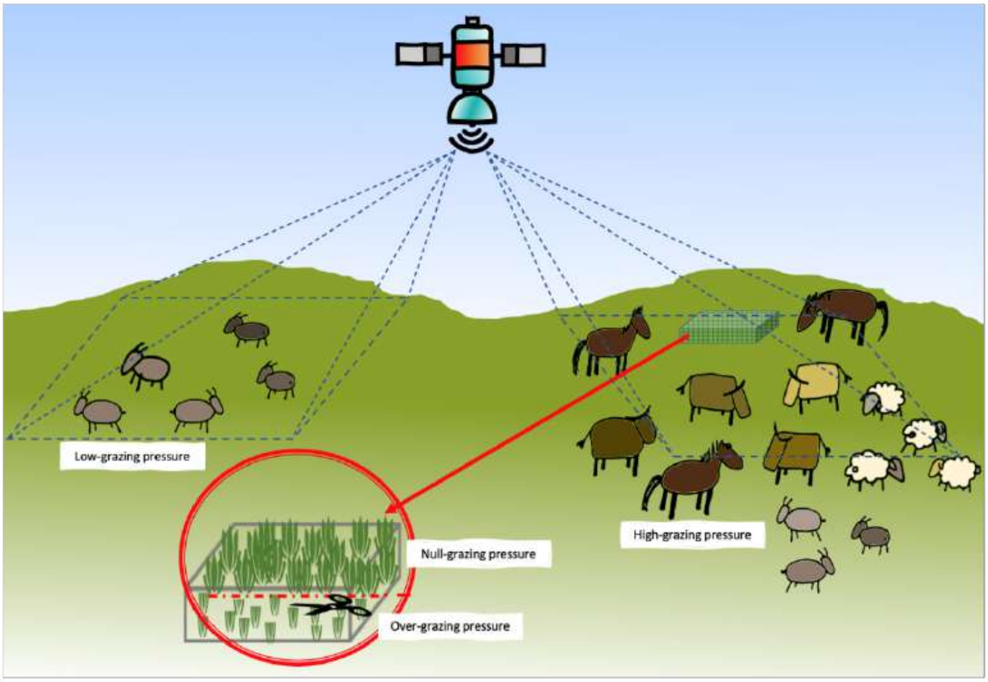


Figure 4.1.0 Graphical abstract from study 1. Grazing effects on grassland vegetation are studied at two different spatial and temporal scales.

Introduction

Grasslands represent around 40% of the terrestrial surface (Suttie et al., 2005), and provide multiple ecosystem services including economic and cultural value, food supply for domestic and wild ungulates (Carlier et al., 2009), carbon-capture and global warming mitigation (McSherry and Ritchie, 2013; Minahi et al., 1993; Schmitz et al., 2018). Unfortunately, more than half of the grasslands of the planet are being affected by land-use changes (Du Toit et al., 2010; Worbel, 2010) and inappropriate grazing practices (Du Toit et al., 2010). The impact of such alterations on the functionality of these ecosystems are not completely understood.

The integrity of grassland ecosystems is not only driven by climatic conditions or land-use legacies but also by the effect of large herbivores (Améztegui et al., 2010; Bardgett et al., 2005; Billings, 1973; Körner, 2003). Such large herbivores (i.e., mammals with an adult body weight of more than 5 kg, Fritz and Loison, 2006) influence vegetation dynamics, biomass production, chemical quality of plants (Austrheim et al., 2014; Cook et al., 1953), the cycling of key nutrients (C-N-P) and the ability of pastures to sequester carbon (Cebrian, 1999; Hillebrand et al., 2008; Schmitz et al., 2018). In other words, the entire functionality of these alpine ecosystems, as well as their ability to mitigate the effects of global warming, is in some way linked to the action of ungulates (Lu et al., 2017; Matches, 1992; McSherry and Ritchie, 2013; Minahi et al., 1993). But the extent of their effects rely on grazing intensity, the plant species and the trait considered (Henkin et al., 2011; Komac et al., 2014; Krumins et al., 2015; McNaughton, 1979; Thiel-Egenter et al., 2007). For example, though grassland biomass production increases after grazing

(López-Társico et al., 2015), overgrazing has clear deleterious effects on the growth rate of many grassland species (Lu et al., 2017; Matches, 1992; Parsons et al., 1983). Forage quality, however, does not follow such a direct pattern depending on the adaptations of plants to compensate for the effect of herbivores (Baron et al., 2002; Cook et al., 1953; Fanlo et al., 2015; Komac et al., 2014). In fact, few efforts have been oriented to understand its impact on the nutritional value and re-growth ability of plants with particular interest on domestic and wild ungulate nutrition (Fanlo et al., 2015; Leigh et al., 1991).

In the European alpine systems, the pressure of herbivory has diminished over time. After the peak of wild herbivore diversity inhabiting alpine areas in the late Pleistocene (Řičánková et al., 2018), wild species were progressively replaced by few domestic species (Chocarro and Reiné, 2008) that in turn are receding in the last half the 20th century (Flury et al., 2013; MacDonald et al., 2000; Nagy and Grabherr, 2009). The Pyrenees, in north Spain, are no exception to this trend (Espunyes, 2019; Muñoz-Ulecia et al., 2021).

Linked to this decrease in herbivore intensity and the land-use abandonment, European alpine grasslands are suffering from shrubification of meadows (Améztegui et al., 2010). Livestock is still moved up to alpine pastures in summer to take advantage of the scarce and nutritious meadows (Chocarro and Reiné, 2008; Mandaluniz et al., 2005), that in turn are also used by wild ungulates (La Morgia and Bassano, 2009; Mishra et al., 2004), resulting in local overgrazing (Du Toit et al., 2010; Gordon, 2006; Muñoz-Ulecia et al., 2021). Within this context, we seek answers regarding the consequences of this process at meso- and local-scales. Our meso-scale approach will help to understand how the seasonal coexistence of livestock and wild ungulates is affecting the productivity of grasslands over the years and to find out what happens if livestock are not present. The local-scale approach in grazed areas, however, will be focused to determine the effect of a degree of grazing pressure on biomass production and protein content of plant species preferentially consumed by a wild (Pyrenean Chamois, *Rupicapra pyrenaica pyrenaica*), and two domestic ungulates (cattle, *Bos taurus* “Bruna dels Pirineus” breed and horses, *Equus caballus* “Cavall dels Pirineus” breed) in the eastern Catalan Pyrenees, Spain. None fencing system was used.

Due to the long history of herbivore-plant interactions in these ecosystems (Oksanen and Oksanen, 2000), and the grazing optimization hypothesis (Austrheim et al., 2014; McNaughton, 1979), we predict that biomass production will increase at moderate grazing but decrease under overgrazing (Austrheim et al., 2014; Bardgett et al., 2005; Cook et al., 1953; Fanlo et al., 2015; Komac et al., 2014 but see Barthelemy et al., 2019). We also expect that overgrazing will drive the nutritive value of plants depending on the plant type (Cook et al., 1953; McNaughton, 1979). To test these hypotheses, we performed two methodological approaches embracing two spatial and temporal scales. We first analysed the trend in biomass production of different patches according to their herbivore pressure for the last 10 years. Then, for two growing seasons, we focused on the patches with high herbivore pressure and conducted a field experiment using eighteen $1m^2$ exclusion boxes set in six different zones considering three scenarios of contrasted grazing intensities: null and high grazing, and also mimicked overgrazing that is expected to happen in the remnant grasslands. Our objectives were three: 1) to evaluate the long-term trend in biomass production of subalpine grasslands with high and low livestock pressure; 2) to compare the accumulated above-ground biomass production among non-, highly and overgrazed, and 3) to evaluate the impact of grazing (high and overgrazing) on monthly above-biomass production and % crude protein over two (2013-14) growing seasons. This information is necessary to develop management guidelines to conciliate the exploitation and conservation of natural resources in alpine grasslands.

Material and methods

Study area

The study area is situated in Fontalba, in the Freser-Setcases National Game Reserve (FSNGR), Eastern Pyrenees, Spain (42º 22’ N, 2º 09’ E, Fig. 4.1.1A). The FSNGR is a mountainous area of 20.000 ha with an average altitude of 2.000 m. a. s. l. (from 1,220 to 2,910 m. a. s. l.) dominated by metamorphic rocks and oligotrophic soils (http://info.igme.es, Vigo, 2008). Vegetation belongs to the subalpine and alpine bioclimatic belts within the Alpine or Eurosiberian Region (Vigo, 2008). Vegetation above 2.000 meters is dominated by a mosaic of alpine meadows with perennial plants, mostly hemicryptophytes and rhizomatous herbs with scattered woody chamaephytes patches of *Calluna vulgaris*, *Juniperus communis* L. and *Cytisus* spp. Among herb species, tussock and stoloniferous graminoids taxa (e.g., *Festuca* and *Carex* genera) are dominant whereas other plants such as: *Gentiana* spp., *Hieracium pilosella* L., *Luzula* spp., *Trifolium alpinum* L., and *Plantago monosperma* Pourr, are abundant. Below 2,000 m, however, vegetation comprises a mosaic of subalpine meadows and forested patches of *Pinus uncinata*

Raymond ex A.DC., *Betula* spp., *Fraxinus excelsior* L., and *Cytisus spp., Juniperus communis*, *Rosa* spp. and *Rubus* spp., shrubs (Vigo, 2008). The annual mean temperature is 5.2ºC (min = -12ºC and max = 23.5ºC) and the mean yearly accumulated rainfall was 1206 mm (period 2013-2014, (www.meteocat.com, last access on 15 November 2016).

According to our monthly surveys in the study area (see Espunyes et al., 2019a for a description), a flock of about 90 – 100 chamois coexist with herds of approximately 97 – 160 cows (*Bos taurus* L. “Bruna dels Pirineus” cattle breed) and 25 – 27 horses (*Equus ferus caballus* L., “Cavall dels Pirineus” horse breed) from May to October. Based on that systematic count, we have defined meadows mainly grazed by chamois (LG areas) and by livestock and chamois (HG areas). According to these counts, herbivore density in HG areas is 5.5 LU/ha whereas 0.5 LU/ha in their LG counterparts. Livestock Units (LU) = 1 for horses older than 12 months and cows older than 2 years, and 0.15 for Pyrenean chamois. Note that there only were differences in the accessibility between the HG and LG areas, there were any difference else in the abiotic conditions among HG and LG areas.

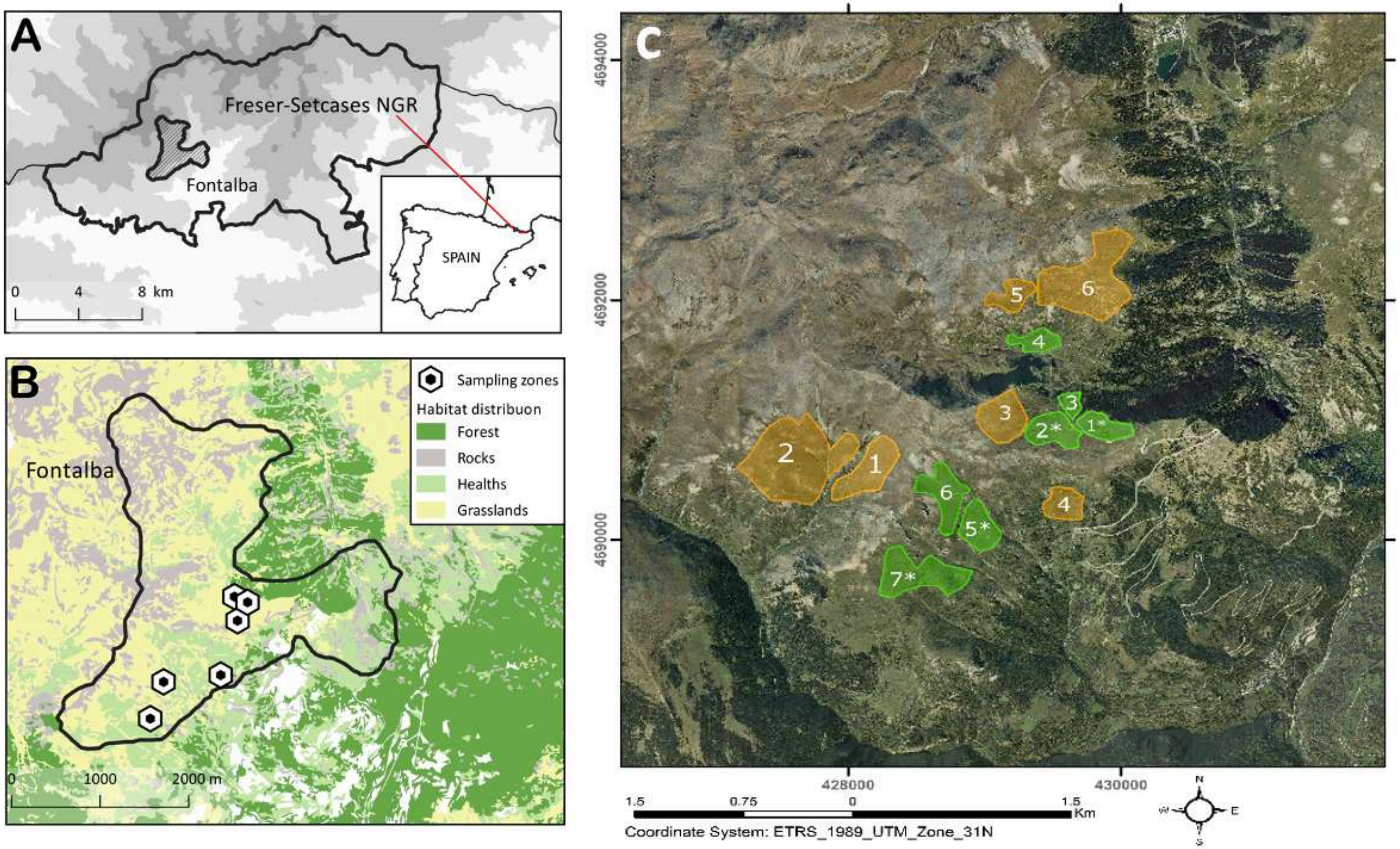


Figure 4.1.1. Our study area (Fontalba) is located in the Freser-Setcases National Game Reserve (FSNGR), Catalan Pyrenees, north Spain (**A**). A total of 18 exclusion boxes (1 m x 1 m) were placed in six sampling zones (**B**) were chamois, cattle and horses are commonly sight. The 13 selected meadows are shown in a satellite imagery (**C**) (in orange, meadows with wild herbivores and in green, meadows with both, wild and domestic herbivores).

LAI data for meso- scale comparisons

In the study area of Fontalba we selected 13 meadows ranging from 2.3 to 38.7 ha, with a contrasted history of herbivore pressure (Fig. 4.1.1C). These meadows are on average at 2,162.4 m. a. s. l. (1,995 to 2,335 m) and face a southeast orientation. Meadows have been categorised into a Low (0.5 LU) and High-grazing pressure (5.5 LU) according to the number cows, horses and chamois over the last ten years (2009-2019). Low-grazing pressure meadows are hardly visited by herds of cattle and horses. That LG areas are difficult for livestock to access due to cliffs and ravines whereas their high-grazing pressure counterpart areas are close to mountain tracks and pathways commonly visited by herds of cattle and horses (Fig. 4.1.1C).

We used the Application for Extracting and Exploring Analysis Ready Samples (AρρEEARS), to get a monthly Leaf Area Index (LAI, leaf area / ground area in m^2 / m^2) the previously mentioned HG and LG meadows. The period of study was comprised from February 2000 to April 2020. To get the LAI records, we created a shapefile containing the 13 study areas with ArcGIS v10.3.1 software (ESRI, 2011). Then, we requested the LAI values to the United States Geological Survey (USGS, see Neeley, 2018) through the AρρEEARS platform (https://lpdaacsvc.cr.usgs.gov/appeears/). To get the LAI data, we used the MOD15A2H geospatial product from the Moderate Resolution Imaging Spectroradiometer set in Terra spacecraft (MODIS, 2015). This approach based on the LAI has already been used to estimate biomass production in a large number of biomes variety (Asner et al., 2003; Myneni et al., 1997) and in alpine ecosystem in particular (Goswami et al., 2015; Wu and Fu, 2018).

Sampling procedure for local-scale comparisons

From July 2013 to October 2014, eighteen exclusion boxes (1x1 m^2, Fig. 4.1.2B) were set in six highly grazed areas of acid and oligotrophic soil at 2.000 m. a. s. l. grazed by livestock and chamois (Fig. 4.1.1B, Fig. 4.1.2A). Once a month, a total of 191 plant samples were collected inside and outside the boxes to allow grass regrowth. In winter (November to April), sampling was precluded by the snow accumulation. We recreated three degrees of grazing namely: high grazing (HG) which represents the areas grazed in summer by both livestock and wild ungulates with 5.5 LU; mimicked overgrazing (MO) exerted by clipping shoots and leaves; null grazing (NG) in which plants remained untouched (Fig. 4.1.2A). MO and NG treatments were applied inside the exclusion boxes splitting the covered surface into two different halves of 0.5 m^2 each (1 m x 0.5 m). Note that due to the exclusion boxes, MO and NG treatments did not have any direct trampling or fertilization from herbivore dung. However, due to the box size, both treatments might have been fertilized by superficial runoff after rainy events. To mimic overgrazing, the MO half was cut once a month with secateurs from May to October (108 samples in total). In contrast, the NG complementary half remained uncut until the last sampling session to get the accumulated biomass production (18 samples in total). HG, however, was recreated in a randomly selected 0.5 m^2 plot 10 meters away from the exclusion boxes (6 samples each sampling session, 36 in total). To avoid resampling of HG sites, we labelled the cut areas carefully.

Plant samples were collected in individually labeled plastic bags, transported to the laboratory and stored frozen at -20ºC until analysis. Then, samples were naturally unfrozen at laboratory conditions. A quarter of the sample was used to classify our vegetal sample into four plant groups: *Festuca* spp.; Other graminoids (Poaceae family plus *Carex* spp., *Juncus* spp., *Luzula* spp. and *Kobresia myosuroides* (Vill.) Fiori, Forbs (e.g., *Gentiana* spp., *Hieracium pilosella, Plantago monosperma, Trifolium alpinum*), and *Calluna vulgaris*. Once plant classification was performed, we got the fresh and dry weight (60º stove for 24 - 48h) for each group of plants to extrapolate to the whole sample.

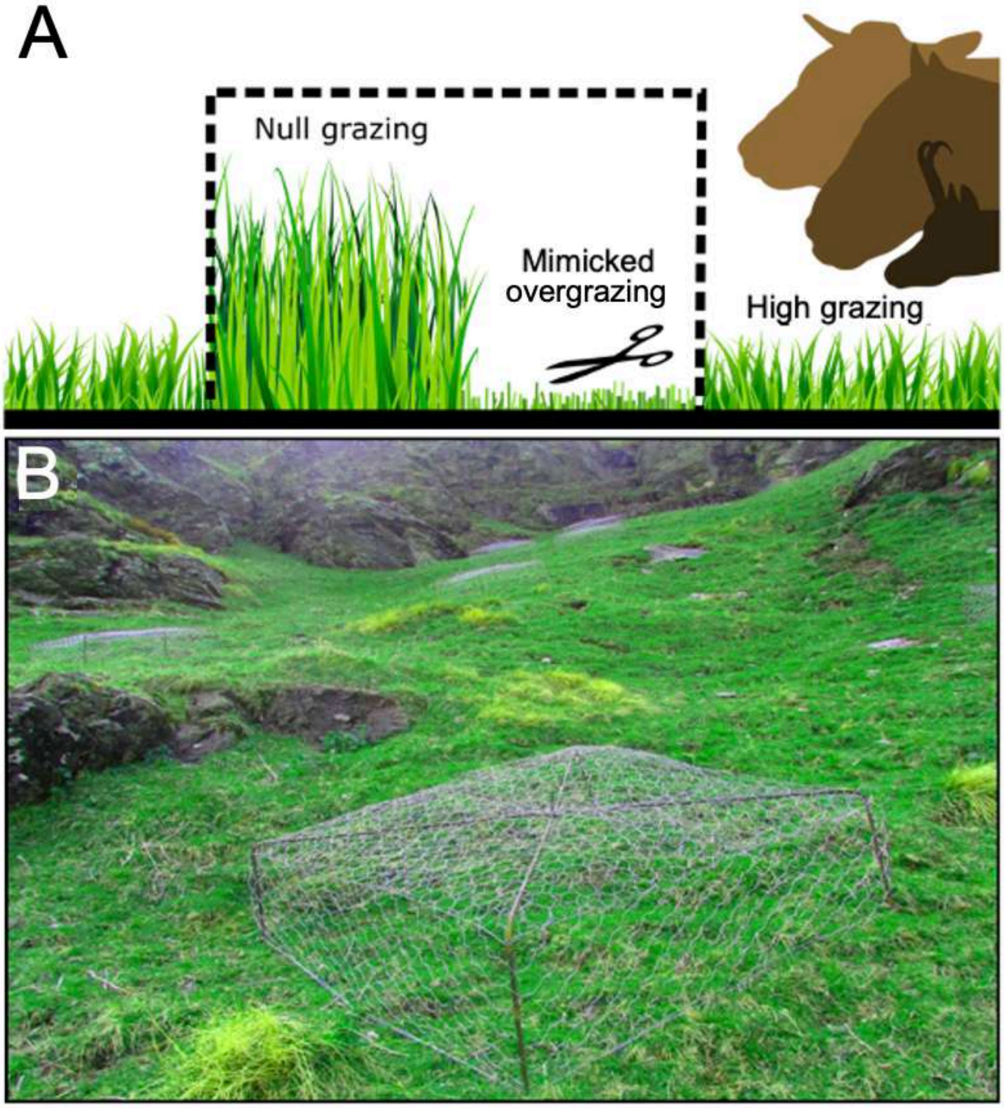


Figure 4.1.2 Three grazing treatments (e.g., Null grazing, High grazing and Mimicked Overgrazing) were performed (**A**) from May to October (2013 – 2014) in each exclusion box (**B**),

Then, plant subsamples were milled in a Cyclotec mill of 0.5 mm screen (FOSS Tecator, Höganäs, Sweden), packed in a ring-cup sample cell, and then scanned from 1000 to 2000 nm using a NIRSystems 5000 scanning monochromator (FOSS, Hillerød, Denmark). The analysis was performed twice by the same person (LJB). A WinISI III (v1.6) software program was employed for spectra data analysis and development of chemometric models. Crude protein concentration in each subsample was assessed NIRS analysis based on calibration made with 26 samples (13.61% of the total sample size) collected from three exclusion boxes under high grazing (HG). Ten of those samples were mono-species and 16 were multi-species samples containing: *Festuca* spp. (33.84%), other graminoids such as *Carex caryophyllea* (30.66%), forbs such as *Trifolium alpinum* (27.65%) and *Calluna vulgaris* (7.84%). Laboratory nitrogen determination was carried out by the Dumas dry combustion method using a LECO analyzer (LECO Corporation, St. Joseph, Michigan, USA).

Data analyses

To compare LAI values in meadows with high and low herbivore pressure (Fig. 4.1.3C), we used generalized additive mixed models (GAMM, Zuur et al., 2009). This additive modelling allows the fit of non-linear response variables using smoothed and non-smoothed predictor variables (Wood, 2016). LAI values were used as response variable and the interaction between the month of the years (smoothed term), and herbivore pressure (low- and high-grazing pressure) as fixed explanatory variables (Fig. 4.1.3A, 4.1.3C). Meadow identity was used as random term in the GAMM analysis. We also followed the same

GAMM approach for comparing monthly-accumulated LAI values between areas (Fig. 4.1.3B).

On the other hand, we fit a set of Generalized Linear Mixed Models (GLMM with a normal error distribution and identity link function), to explore the effects of grazing intensity (NG, HG, MO) on the accumulated biomass production (in g/cm^2) recorded in our box enclosures. For monthly biomass production and crude protein content (CP, in %), we fit another set of GLMM including the month of sampling, grazing intensity (only HG and LG in that case) and their interaction as fixed terms. In all cases, the exclusion box nested to sampling zone was included as a random intercept term in our GLMM (Zuur et al., 2009). Because of our moderate sample size ($n < 40$), model selection was based on the Second-order Akaike Information Criterion (AICc, Burnham and Anderson, 2002). The Akaike weight (ωi) for each competing model was also calculated. For GAMM we used the library mgcv 1.8-31 version (Wood, 2011), for GLMM the library lme4 1.1-15 version (Bates et al., 2015). The library MuMin 1.43.6 version (Barton, 2019) was used to assess the marginal and conditional contribution of the fixed and random terms in the GLMM (Nakagawa and Schielzeth, 2013). All statistical analyses have been performed in the R Statistical Software 4.0.5 version (Lüdecke et al., 2021).

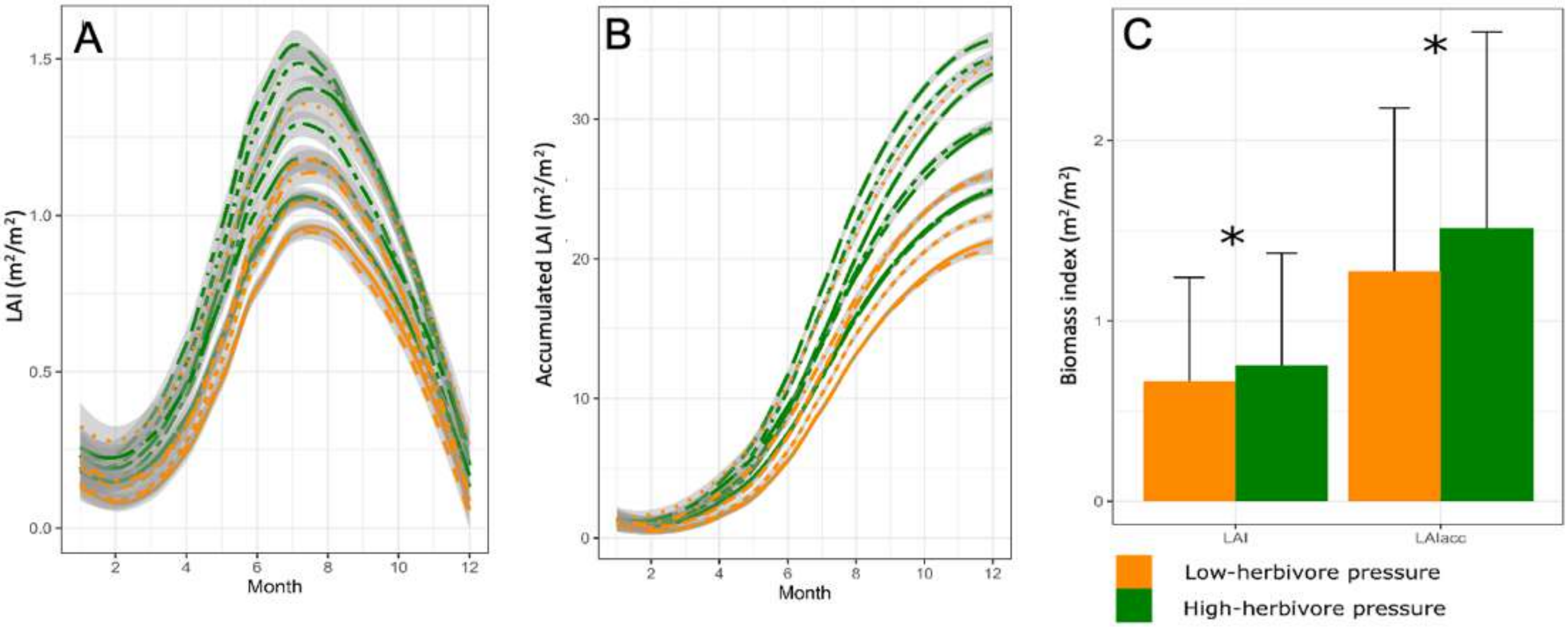


Figure 4.1.3 Monthly mean Leaf Area Index (LAI) (A) and accumulated LAI (B) values from the in the 13 selected meadows from February 2000 to April 2020. Biomass index comparative (**C**) between low-herbivore pressure meadows (in orange) and high-herbivore pressure meadows (in green). Bars represent the standard deviation of the mean. * Denotes statistically significant differences.

Results

Biomass production at meso-scale

Descriptive statistics (mean± SE, minimum and maximum) of LAI and accumulated LAI are summarised in Table 4.1.1. LAI followed the typical seasonal pattern of alpine meadows with a progressive increase in biomass production from February to July followed by a slowdown (Fig. 4.1.3A, Table 4.1.1). According to our GAMM this seasonal pattern differed between HG and LG areas ($F_{Month*LG}$ = 6665, edf = 8.35, p= 2e-16; $F_{Month*HG}$ = 10857, edf = 8.52, p= 2e-16, Fig. 4.1.3A). This GAMM including the interaction between grazing intensity and the month of the year explained 60% of the observed LAI variability. Along the same lines accumulated LAI also differed between grazing areas ($F_{Month*LG}$ = 918.4, edf = 8.78, p= 2e-16; $F_{Month*HG}$ = 1348.1, edf = 8.82, p= 2e-16, Fig. 4.1.3B). In fact, the additive model including the interaction between grazing intensity and the month of the year explained 88.1% of the observed accumulated LAI variability (Fig. 4.1.3B). These patterns were also observed comparing yearly mean LAI (t = 11.9, df = 11607, p = 2.2e-16), and accumulated LAI (t = 12.24, df = 11607, p = 2.2e-16) values between Low- and High-herbivore pressure areas (Fig. 4.1.3C).

Accumulated biomass production at local-scale

In highly grazed areas in which exclusion boxes were set, the greatest values of grass accumulated biomass occurred under high grazing intensity of summer (HG, mean = 590.3 g/m^2), whereas the lowest in places under mimicked overgrazing (MO, mean = 389.3 g/m^2), or null grazing (NG, mean = 110.8 g/m^2, Fig. 4.1.4A). However, the woody plant *Calluna vulgaris* show no differences in biomass production (Fig. 4.1.4B).

Table 4.1.1. Mean, SE, min and max values of LAI (leaf area / ground area in m^2 / m^2), and accumulated LAI recorded monthly from 2000 to 2019 in 13 Alpine meadows ranging from 2.3 to 38.7 ha with a long history of low and high herbivore pressure in the Catalan Pyrenees, northeast Spain (Fig. 4.1.1C).

	LAI $_{m2/m2}$		**Accumulated LAI** $_{m2/m2}$	
Month	**Low-Herbivore pressure**	**High-Herbivore pressure LAI**	**Low-Herbivore pressure**	**High-Herbivore pressure LAI**
January	0.20±0.01 (0-0.9)	0.25±0.08 (0-1.5)	0.65±0.02 (0-2.7)	0.8±0.02 (0-2.85)
February	0.13±0.006 (0-0.7)	0.16±0.005 (0-0.7)	1.15±0.03 (0-4.5)	1.4±0.03 (0.1-4.30)
March	0.16±0.008 (0-1)	0.21±0.08 (0-1)	1.78±0.05 (0.1-6.9)	2.17±0.05 (0.4-6.75)
April	0.22±0.01 (0-1.2)	0.36±0.01 (0-5.5)	2.55±0.08 (0.2-8.85)	3.11±0.07 (0.6-11.1)
May	0.49±0.01 (0-1.7)	0.66±0.01 (0-2)	3.92±0.11 (0.45-12.40)	5.03±0.1 (0.8-14.60)
June	1±0.01 (0.1-3.1)	1.22±0.02 (0.1-3-10)	7.07±0.14 (1.2-17.69)	9.00±0.13 (1.65-26.95)
July	1.21±0.01 (0.1-2.5)	1.38±0.02 (0.1-3.95)	11.63±0.17 (4.6-24.70)	14.32±0.16 (6.15-26.95)
August	1.08±0.15 (0.1-20.4)	1.26±0.01 (0.1-3.4)	16.22±0.18 (7.9-30.5)	19.57±0.18 (10.20-33-7)
September	0.87±0.1 (0.1-1.9)	1.01±0.01 (0.1-2.05)	19.95±0.2 (11.76-35.31)	23.97±0.19 (14.22-38.15)
October	0.64±0.1 (0-5.8)	0.71±0.01 (0-20.5)	22.59±0.24 (13.93-38.20)	27.05±0.22 (16.72-40.81)
November	0.36±0.01 (0-2.4)	0.43±0.01 (0-1.40)	24.22±0.24 (15.5-38.90)	28.93±0.21 (18.45-42.40)
December	0.2±0.008 (0-1.2)	0.31±0.01 (0-7)	25.10±0.24 (16.06-41.00)	30.24±0.22 (19.12-44.00)

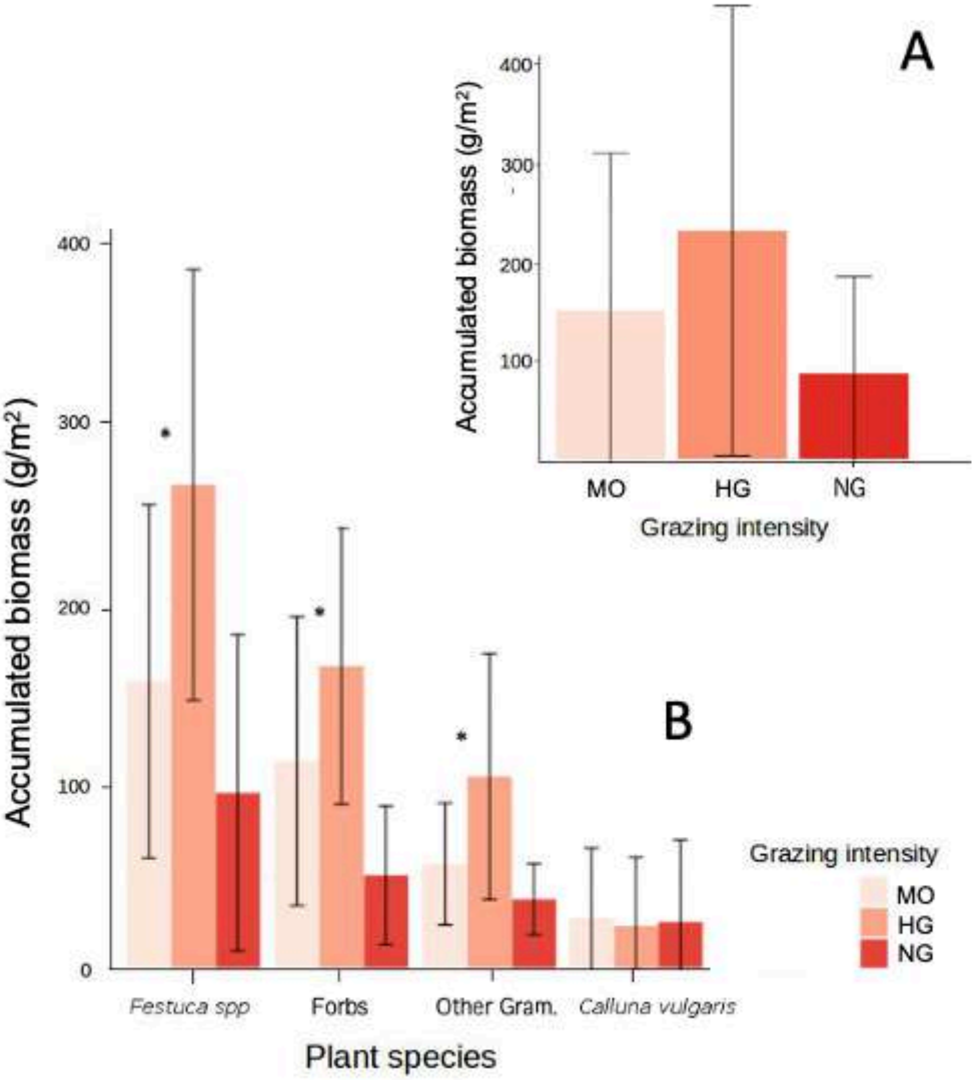


Figure 4.1.4. Accumulated biomass (g/m^2) of all groups plant (A) and different alpine plants (*Festuca* spp., Other graminoids, Forbs, and *Calluna vulgaris*) (B) harvested in 24 squares of 0.5 m^2 placed in six sampling zones located around 2000 m.a.s.l. in the Freser-Setcases National Hunting Reserve, Catalan Pyrenees, northeast Spain, from May to October (2013 – 2014). Sampling areas were exposed to high grazing intensity (HG) (n = 6 for each sampling session), to mimicked overgrazing intensity (MO) (n = 18 for each sampling session), and to a null grazing intensity (NG) (n = 18 at the end of fieldwork). Bars represent the standard deviation of the mean. * Denotes statistically significant differences.

Grazing intensity clearly drove mean accumulated biomass of *Festuca* spp plants ($F_{2, 33}$= 11, p = 0.03, Fig. 4.1.4B). In HG areas, for example, accumulated *Festuca* spp biomass (267.9 g/m^2 ± 282.8, min = 98.6, max = 410.4) was greater than in NG (97.1 g/m^2 ± 65.61, min = 15.7, max = 304.8; $\beta_{LG\text{-}NG}$ = 1.2, t = 4.25, p = 0.005), or MO areas (158.8 g/m^2 ± 23.1, min = 37.6, max = 403.9; $\beta_{HG\text{-}NG}$ = 0.6, t = 3.44, p = 0.005). Along the same lines, the accumulated biomass of other graminoids under HG intensities (106.1 g/m^2 ± 27.82, min = 41.64, max = 216.9 g/m^2) was higher than in areas under NG ($\beta_{LG\text{-}NG}$ = 1.69, t = 7.1, $p < 0.001$) or MO intensities ($\beta_{HG\text{-}NG}$ = 1.1, t = 6.2, $p < 0.001$). Similarly, the highest forb accumulated biomass was observed in areas of HG intensity (167.2 g/m^2 ± 133.4, min = 93.8, max = 276.3, $\beta_{LG\text{-}NG}$ = 2.31, t = 6.3, $p < 0.001$) followed by areas under MO (114.5 g/m^2 ± 18.8, min = 18.5, max = 351.4, $\beta_{HG\text{-}NG}$ = 1.76, t = 7.2, $p < 0.001$). In contrast, the accumulated biomass of *Calluna vulgaris* did not respond to changes in the grazing intensity (Fig. 4.1.4B).

The HG areas show a considerable environmental heterogeneity. In fact, 28.2% of the observed *Festuca* spp accumulated biomass variability was due to changes in the grazing intensity whereas 41.8% due to differences among boxes and sampling areas (Table 4.1.2). In contrast, grazing intensity explained 55.57% of changes in the accumulated biomass of other graminoids, 52.7% of Forbs and 3.3% of the woody *Calluna vulgaris*. Differences among boxes and sampling sites explained 16.2% of the biomass variability in other graminoids, 23.9% in forbs and 70.9% in *Calluna* plants.

Table 4.1.2. Proportion of explained variability (%) in a set of GLMM to explore the effects of grazing intensity and the month of sampling on the accumulated and monthly biomass (g/m²) and the crude protein contents (%) in four alpine plant types (i.e., *Festuca* spp., Other graminoids, Forbs and *Calluna vulgaris*) consumed by wild (*Rupicapra pyrenaica pyrenaica*) and domestic (cows and horses) ungulates in the Catalan Pyrenees, northeast Spain. Null and high grazing plus mimicked overgrazing intensities were considered in the GLMM for biomass production, whereas high and mimicked overgrazing in the GLMM for crude protein contents. The exclusion box nested in the sampling was the random intercept term in all mixed models whereas the fixed term (a combination of grazing intensity and month of sampling), varied according to our model selection (see Table 4.1.3).

	Factor	***Festuca* spp.**	**Other graminoids**	**Forbs**	***Calluna vulgaris***
Accumulated Biomass	Fixed term	28.2	55.57	52.7	3.3
	Random term	41.8	*16.2*	23.9	70.9
Biomass	Fixed term	37.7	41.7	26.7	2
	Random term	11.9	13.2	16.9	51.1
Crude protein	Fixed term	45.9	26.3	18.9	5.6
	Random term	10	24.4	16.3	17.2

Monthly biomass production at local-scale

Regarding the highly grazed areas in which the exclusion boxes were set, the best mix model shows that 64.9 % of the observed monthly biomass variability of our four plant groups depended on the month of the year and grazing intensities ($F_{16,\ 621\ \text{Month * Plant type + Grazing intensity}} = 4.49$, p = 0.001). Accordingly, the effects of grazing intensity on *Festuca* spp biomass production depended on the month of sampling ($\text{AICc}_{\text{Grazing intensity * Month}} = 234.3$, Wi = 0.68, Table 4.1.3, Fig. 4.1.5A), in other words, HG resulted in a higher *Festuca* spp productivity in May, July and October, but not in August or September (β = 3.8, SE = 0.23, t = 16.4, p > 0.001). Most of the observed *Festuca* spp. biomass variability (37.7%) was due to monthly differences in grazing intensities whereas 11.9 % due to differences among boxes and sampling sites (Table 4.1.2).

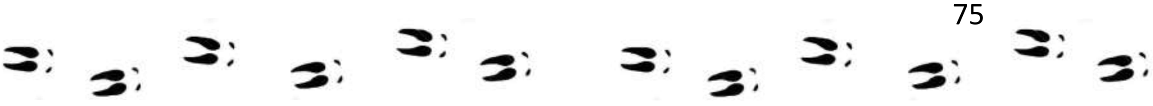
Regarding other graminoid plants, the retained GLMM supported that the effects of grazing relied on the month of sampling ($\text{AICc}_{\text{Grazing intensity * Month}} = 131.3$, Wi = 0.84, Table 4.1.3, Fig. 4.1.5B). However, statistically significant differences between grazing treatments were not observed in any of the months of sampling, presumably because of the great variability in the biomass harvested in each month of sampling (Fig. 4.1.5B). In fact, 41.7% of the observed biomass variability was due to grazing intensities, whereas 13.2% due to differences among exclusion boxes and sampling sites.

Forb biomass production, however, was higher under HG intensity (Mean = 32.88, SE = 4.9, min = 0.49, max = 124.18) than under its MO counterpart (Mean = 23.95, SE = 2.98, min = 0, max = 188.91, $F_{HG} = -0.25$, SE = 0.1, t = -2.51, p = 0.02) all over the period of sampling ($\text{AICc}_{\text{Grazing intensity + Month}} = 1124.4$, Wi = 0.76, Table 4.1.3, Fig. 4.1.5C). On the other hand, forb biomass production strongly relied on the month of sampling ($F_{4,\ 103}$ = 13.14, p-value = 0.001), peaking in July (Mean = 53.99, SE = 8.7, min = 0, Max = 188.9, Fig. 4.1.5 C). For this grass type, 26.7% of the observed biomass variability depended on the month of sampling whereas 16.9% on the random term.

Biomass production of *Calluna* plants was neither explained by the effects of grazing nor by the month of sampling ($AICc_{Mo}$ = 127.4, Wi = 0.46, Table 4.1.3, Fig. 4.1.5D, Table 4.2.2). The second candidate model (< 2 AIC units), however, included the month of sampling to explain the variability in biomass production of *Calluna* plants ($AICc_{Month}$ = 128.8, Wi = 0.23, Table 4.1.3). In fact, biomass production of this woody species in October was below the average value recorded over the other months. (β = -0.2, SE= 0.09, t = -2.2, p = 0.02, Fig. 4.1.5D). For this woody plant, most of the observed biomass variability relied on differences among exclusion boxes and sampling sites (51%, Table 4.1.2).

Table 4.1.3. Summary of the best generalized linear mixed models (GLMM) analyzing the effects of grazing intensity (high and mimicked overgrazing) on monthly biomass production of four types of alpine grasses consumed by wild (*Rupicapra* pyrenaica *pyrenaica*) and domestic (cows and horses) ungulates. Grasses were kept away from herbivores for 14 months using 18 exclusion boxes of 1 m x 1 each placed in 6 sampling stations located around 2000 m.a.s.l in the Freser Setcases National Hunting Reserve, Catalan Pyrenees, northeast Spain. K = number of parameters, AICc = Second-order corrected Akaike Information Criterion for small sample sizes, Δi = difference of AICc with respect to the best model, ωi = Akaike weight. The best model is shown in bold. Only models with Δi lower than 10 have been presented.

Plant	**Biological model**	**K**	**AIC_C**	***Δi***	**$ω_i$**
Festuca spp.	**Grazing intensity * Month**	**13**	**234.3**	**0**	**0.68**
	Grazing intensity + Month	9	236.3	2.1	0.25
	Month	8	238.8	4.5	0.07
Other graminoids	**Grazing intensity * Month**	**13**	**131.3**	**0**	**0.84**
	Grazing intensity + Month	9	134.4	3.4	0.15
	Month	8	140.1	9.1	0.01
Forbs	**Grazing intensity + Month**	**9**	**221.1**	**0**	**0.79**
	Month	8	224.7	3.7	0.13
	Month * Grazing intensity	13	225.7	4.7	0.08
Calluna vulgaris	**Mo**	**4**	**127.4**	**0**	**0.46**
	Month	8	128.8	1.4	0.23
	Grazing intensity	5	129.42	2.0	0.17
	Grazing intensity + Month	9	130.9	3.5	0.08
	Grazing intensity * Month	13	131.7	4.3	0.05



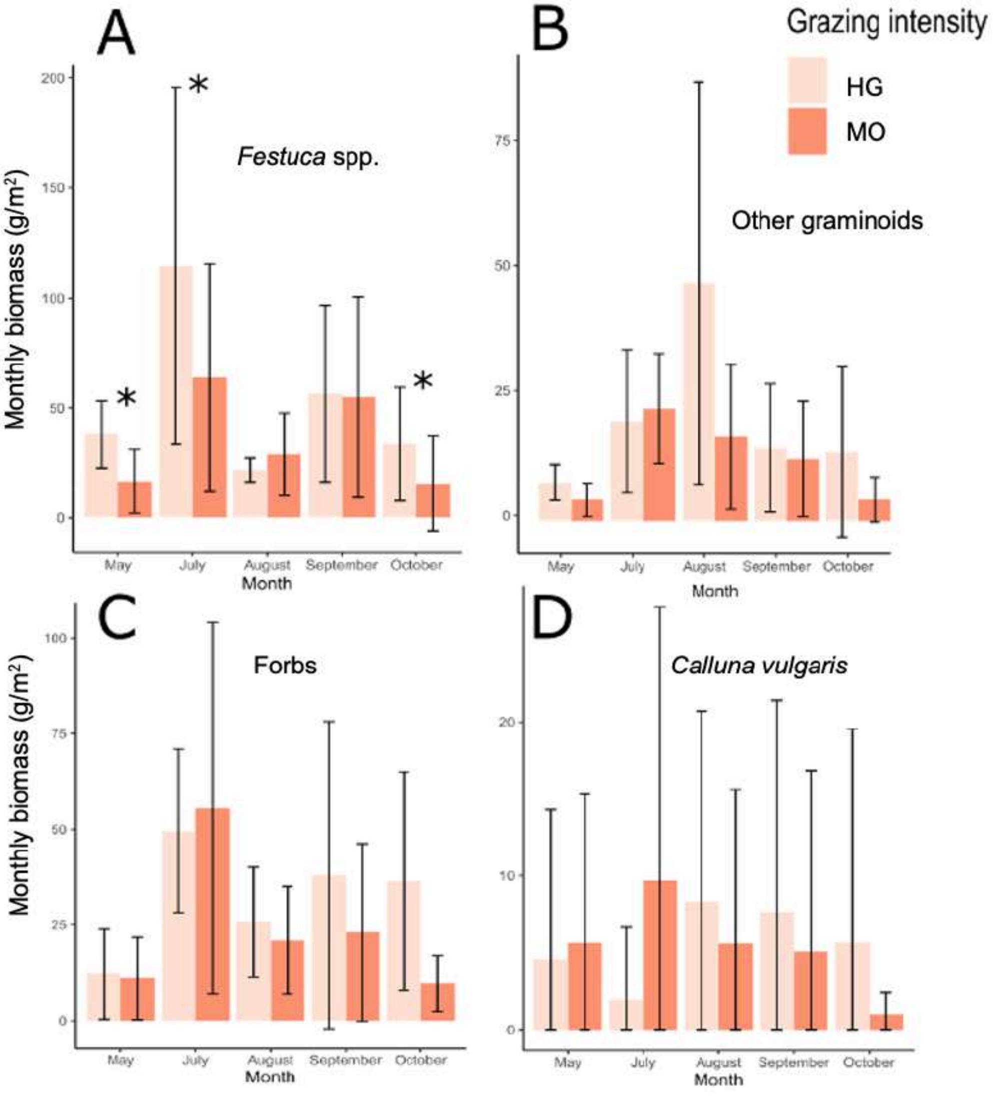


Figure 4.1.5. Monthly biomass production (g/m^2) of *Festuca* spp. (A), Other graminoids (B), Forbs (C), and *Calluna vulgaris* (D) harvested in 24 squares of 0.5 m^2 placed in 6 sampling zones located around 2000 m.a.s.l. in the Freser-Setcases National Hunting Reserve, Catalan Pyrenees, northeast Spain, from May to October (2013 – 2014). Sampling areas were exposed to high grazing intensity (HG) (n = 6 for each sampling session) and to mimicked overgrazing intensity (MO) (n = 18 for each sampling session). Bars represent the standard deviation of the mean. * Denotes statistically significant differences.

Nutritional quality at local-scale

In highly grazed areas in which exclusion boxes were set, the effects of grazing intensity on the crude protein contents of our groups of alpine plants depended on plant type and the month of sampling ($F_{12, 936} = 6.8$, $p < 0.01$, Fig. 4.1.4). Accordingly, grazing effects on the protein content of *Festuca* spp plants relied on the month of sampling ($AICc_{\text{Grazing intensity * Month}} = 1090.9$, $Wi = 0.68$, Table 4.1.4, Fig. 4.1.6A). In fact, the MO grazing treatment improved the protein contents of *Festuca* spp in July ($\beta = 1.5$, $SE= 0.73$, $t = 2.1$, $p = 0.04$, Fig. 4.1.6A), August ($\beta = 1.6$, $SE= 0.72$, $t = 2.3$, $p = 0.02$, Fig. 4.1.6A) and September ($\beta = 2.1$, $SE= 0.64$, $t = 3.2$, $p = 0.001$, Fig. 4.1.6A), but not during other months.

Along the same lines, the protein content of other graminoid plants was affected by both grazing intensities and the effect of months ($AICc_{\text{Grazing intensity + Month}} = 1126.7$, $Wi = 0.59$, Table 4.1.4, Fig. 4.1.6B). In general, protein contents under the MO effect was on average 1.1 times higher (Mean = 13.49, SE = 0.19, min = 3.84, max = 19.35) than under their HG counterpart (Mean = 12.05, SE = 0.29, min = 6.30, max = 18.32, $\beta = 1.4$, $SE= 0.54$, $t = 2.6$, $p = 0.01$, Fig. 4.1.5B). Regarding the monthly effects ($F_{4, 230} = 25.55$, $p < 0.01$), protein contents peaked in July (Mean = 14.19, SE = 0.28, min = 9.17, max = 19.07) and August (Mean = 14.04, SE = 0.26, min = 10.33, max = 18.17) and achieved the minimum in October (Mean = 10.64, SE = 0.35, min = 3.8, max = 15.21). In this case, grazing explained 26.3% of the protein content whereas 24.4% was due to differences among boxes and sampling sites.

Table 4.1.4. Summary of the best generalized linear mixed models (GLMM) analyzing the effects of grazing intensity (high and mimicked overgrazing) on the protein contents of alpine grasses consumed by wild (*Rupicapra* pyrenaica *pyrenaica*) and domestic (cows and horses) ungulates. Grasses were kept away from herbivores for 14 months using 18 exclusion boxes of 1 m x 1 each placed in 6 sampling stations located around 2000 m.a.s.l in the Freser Setcases National Hunting Reserve, Catalan Pyrenees, northeast Spain. K = number of parameters, AICc = Second-order corrected Akaike Information Criterion for small sample sizes, Δi = difference of AICc with respect to the best model, ωi = Akaike weight. The best model is shown in bold. Only models with Δi lower than 10 have been presented.

Response variable	**Selected model**	**K**	**AIC_C**	***Δi***	**$ω_i$**
Festuca spp.	**Grazing intensity * Month**	**13**	**1090.9**	**0.0**	**0.68**
	Grazing intensity + Month	9	1093.4	2.5	0.20
	Month	8	1094.9	4.0	0.09
	Grazing intensity	5	1097.7	6.8	0.02
	Mo	4	1099.3	8.3	0.01
Other graminoids	**Grazing intensity + Month**	**9**	**1126.7**	**0**	**0.59**
	Grazing intensity * Month	13	1127.91	1.2	0.32
	Month	8	1130.41	3.7	0.09
Forbs	**Month**	**8**	**1767.91**	**0**	**0.67**
	Grazing intensity + Month	9	1769.62	1.7	0.28
	Grazing intensity * Month	13	1773.14	5.2	0.07
Calluna vulgaris	**Grazing intensity**	**5**	**318.18**	**0**	**0.45**
	Mo	4	318.67	0.5	0.35
	Grazing intensity + Month	9	321.06	2.9	0.11

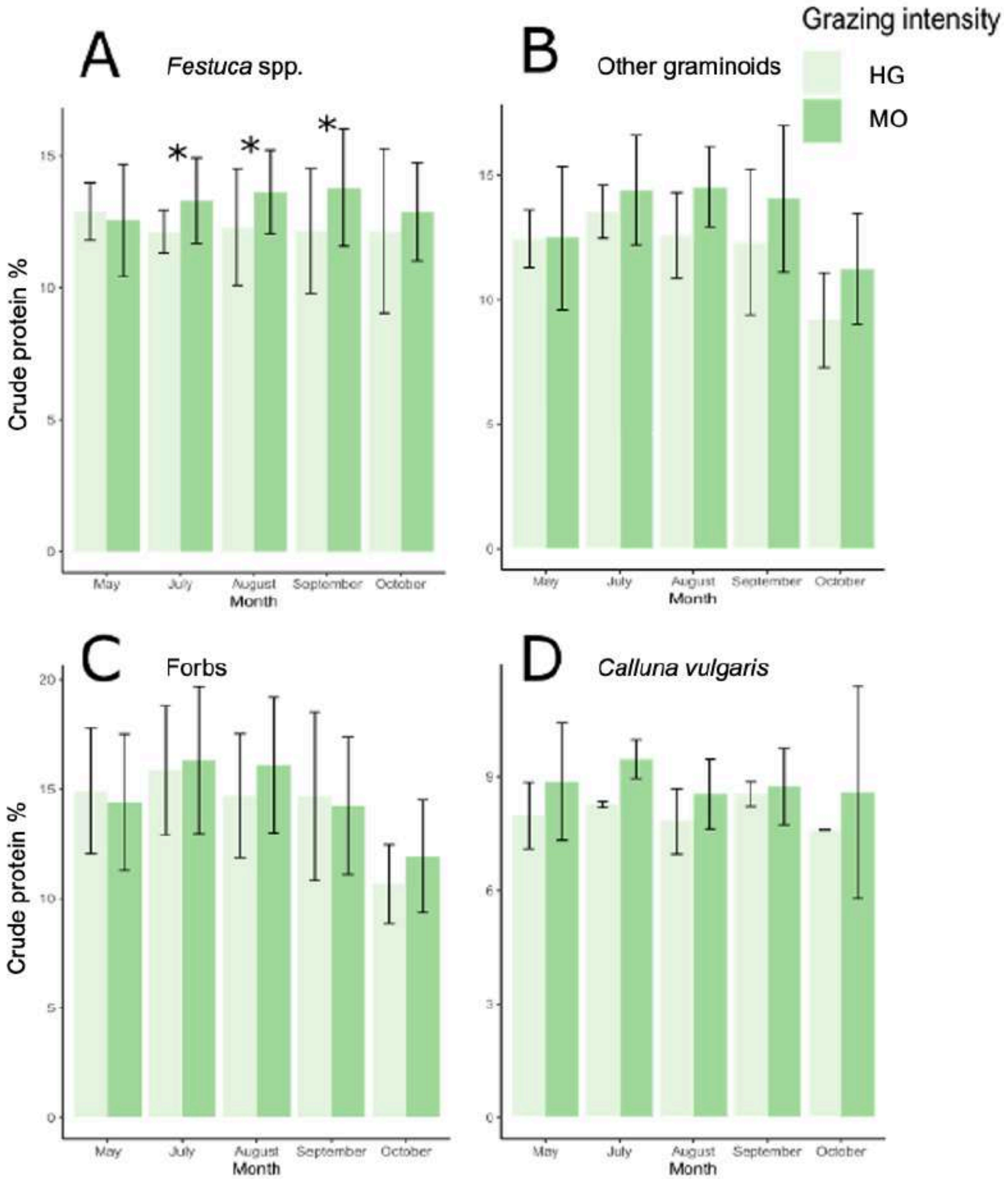


Figure 4.1.6. Monthly protein contents (%) of *Festuca* spp. (A), Other graminoids (B), Forbs (C), and *Calluna vulgaris* (D) harvested in 24 squares of 0.5 m^2 placed in 6 sampling zones located around 2000 m.a.s.l. in the Freser-Setcases National Hunting Reserve, Catalan Pyrenees, northeast Spain, from May to October (2013 – 2014). Sampling areas were exposed to high grazing intensity (HG, n = 6 for each sampling session) and to mimicked overgrazing intensity (MO, n = 18 for each sampling session). Bars represent the standard deviation of the mean. * Denotes statistically significant differences.

The protein content of forbs, however, were mainly affected by the effects of months ($AICc_{Month} = 1767.91$, Wi = 0.67, Table 4.1.2, Fig. 4.1.6C). In line with the phenology of other graminoids, protein content of forbs peaked in July (Mean = 16.19, SE = 0.36, min = 10.85, max = 24.39, $\beta = 1.85$, SE= 0.51, t = 3.6, $p < 0.01$, Fig. 4.1.6C) and August (Mean = 15.73, SE = 0.38, min = 10.54, max = 23.37, $\beta = 1.4$, SE= 0.53, t = 2.6, $p < 0.01$), achieving the minimum concentrations in October (Mean = 11.61, SE = 0.33, min = 4.64, max = 16.43, $\beta = -2.7$, SE= 0.55, t = -4.8, $p < 0.01$). For this plant type, 18.9% of the variability of protein content was due to monthly differences and 16.3 % due to the random term.

Finally, the protein content of *Calluna* was slightly affected by the effects of grazing and not by the effect of months ($AICc_{Grazing\ intensity} = 318.18$, Wi = 0.45, Table 4.1.4, Fig. 4.1.6D). In fact, protein content under MO was only 1.08 times higher than under a HG pressure ($\beta = 0.77$, SE = 0.46, t = 1.65, p = 1.13). In this woody plant, protein content was most affected by differences among sampling sites (17.2% of observed protein content variability was due to the random term) than due to changes in the grazing intensity (5.6%, Table 4.1.2).

Discussion

As we have seen, grazing intensity played a considerable but plant-specific influence on biomass production and the nutritional quality of alpine pastures at different spatial and temporal scales. More specifically, the month of sampling had a greater influence on biomass production whereas grazing intensities influenced the protein content of plants.

Effects of grazing intensity on biomass production

A long history of grazing has driven monthly and accumulated biomass (LAI values) in our alpine system (Fig. 4.1.3C). Our observations are in line with other reports that support the positive effect of grazing on biomass production in other mountain ecosystems (Duan et al., 2012; Gong et al., 2015). This positive effect of grazing is corroborated by our local-scale survey where in non-grazed areas alpine herbaceous plants (*Festuca* spp., Forbs and other graminoids) accumulated less above-ground biomass than under high grazing, or even overgrazing (Fig. 4.1.4). Despite the tissue damage or removal exerted by natural grazing (HG) and mimicked overgrazing (MO), the observed increment in above-ground biomass can be understood as a compensatory response induced by herbivory (McNaughton, 1979) in line with the exploitation ecosystems (Oksanen and Oksanen, 2000) and grazing optimization hypotheses (Austrheim et al., 2014). Grazing triggers a cascade of processes that increase biomass production in plants: reduction of old (and less) efficient vegetative structures and transpiration surface with a subsequent increase in light availability, water-use efficiency, hormonal redistributions promoting cell division, translocation of stored nutrients, and photosynthesis activity (Billings, 1973; McNaughton, 1979 and references therein; Bardgett et al., 2005; Austrheim et al., 2014). Moreover, soil fertilization due to animal feces support plants growth as nutrients return to the soil (Aldezábal et al., 2002; Barthelemy et al., 2019; Hobbs, 1996; Krumins et al., 2015; Vallentine, 2001). Likewise, the above-ground biomass increase is fuelled by vegetative resprouting displayed by these alpine perennial herbaceous plants and their relatively large underground structures (roots or rhizomes) which store organic resources to cope with hazardous climatic conditions and grazing (Billings, 1973; McNaughton, 1979; Skarpe and Hester, 2008). However, our alpine grasses showed a lower accumulated biomass under overgrazing probably because we overpassed the ability to compensate for loss of biomass in absence of fertilization (McNaughton, 1979; Skarpe and Hester, 2008). In places with a long-history of overgrazing, however, grazing exclusion increases forage production and soil carbon sequestration (Xiong et al., 2014).

Our results also indirectly support the relevant role of grazing and the impact of overgrazing on greenhouse gases balance (McSherry and Ritchie, 2013; Schmitz et al., 2018) and the consequent effect on the ongoing climate crisis (Jones and Donnelly, 2004; O'Mara, 2012; Soussana et al., 2010). The current grazing of wild and domestic herbivores in our studied alpine system contributes to the sequestration of CO_2 by significantly promoting biomass production that is also mediated by regional climate and nutrients availability (Conant and Paustian, 2002; Dlamini et al., 2016; Welker et al., 2004). On the contrary, the expected overgrazing in alpine grasslands will limit CO_2 uptake by plants and soils (Conant and Paustian, 2002; Jiang et al.,

2020) and therefore enhancing the detrimental effects of such gas in our climate (Jones and Donnelly, 2004; McSherry and Ritchie, 2013; O’Mara, 2012; Schmitz et al., 2018). This fact is influenced by the marked seasonal growing pattern of our alpine plants, and monthly biomass dynamics differed among plant groups (Fig. 4.1.5). In line with other mountain ecosystems (Dhaulakhandi et al., 2010; Henkin et al., 2011), the onset of biomass production starts in May, peaks between July to August and drops in late October (Fig. 4.1.5). As we have seen, this seasonal growing pattern was affected by the grazing intensity. In fact, overgrazing reduces growth rates, the final size and hence the viability of the affected plants (Lu et al., 2017), and alpine grasslands are not the exception (Austrheim et al., 2014).

In contrast to herbaceous plants (*Festuca* spp., other graminoids, and forbs), *Calluna vulgaris* biomass production was equally affected by our contrasted grazing treatments (Fig. 4.1.4). The resprouting and survival capacities of *Calluna vulgaris* are significantly reduced by cutting (Calvo et al., 2002) and hard climatic conditions (Henning et al., 2017). Its performance is in line with other slow-growing plants that show a limited physiological ability to cope with the recurrent impact of herbivores, a negative annual carbon balance, extreme temperatures, and water and/or nutrient shortage (Ammer, 1996; Bellingham and Sparrow, 2000; Skarpe and Hester, 2008; Smith et al., 2003). Therefore, herbivory appears to be an important driver for fast-growing and palatable herbaceous plants but not for their woody counterparts (Cebrian and Duarte, 1994).

Effects of grazing intensity on the forage quality

Herbivores exert a crucial effect on growth strategies and chemical contents of plants (Gordon, 2006). In our study system, nutritional value (protein content) of most of the studied plants, *Festuca* spp, other graminoids, and the woody *Calluna vulgaris*, improved under mimicked overgrazing compared to high grazing (Fig. 4.1.6). Moreover, found out that grazing exclusion had no impact on total C, N, and P storage, (in both, total biomass and soil fractions) in the alpine grassland ecosystem. In our study, the grazing intensity effect on forage quality was relevant but mainly in the optimum period for growing (i.e., summer), being especially significant in *Festuca* spp. Then, all the surveyed herbaceous alpine plants (*Festuca* spp., other graminoids and Forbs) show decay at the end of the growing season as reported in other studies (Bovolenta et al., 2008; Henkin et al., 2011). However, we do not find a general pattern. The grazing intensity and the season showed negligible effects on forbs and *Calluna vulgaris* respectively (Fig. 4.1.6). Thus, both grazing intensity and time drive the quality of alpine plants as happens in temperate grasslands (Mysterud et al., 2011) but not necessarily in a combined way, maybe due to inter-species differences in leaves/stem ratio and other physiological and anatomical traits (Bovolenta et al., 2008; De Santis et al., 2004; Fanlo et al., 2015; Komac et al., 2014; McNaughton, 1979).

Overgrazed plants are forced to an immature stage (Hebblewhite et al., 2008; Mysterud et al., 2017). That entails an active regrow with poorly developed stems that increases photosynthesis activity and protein contents (high protein/fibres index, Bovolenta et al., 2008; McNaughton, 1979). This fact is exacerbated in *Festuca* spp. and other graminoids, the preferred taxa consumed by Pyrenean chamois (Espunyes, 2019), in mimicked overgrazing areas compared to high grazed areas. The grazed dwarfs of *Festuca* spp and graminoids have almost null stems (when overgrazed) and show an intense vegetative regrowth (Henkin et al., 2011; Komac et al., 2014). In addition, protein content in overgrazed plants must be enhanced by the mineralization of nitrogen from herbivores faeces (Austrheim et al., 2014; Hillebrand et al., 2008; McNaughton et al., 1997).

The registered highest values in protein content of the surveyed herbaceous alpine plants (*Festuca* spp., other graminoids, and forbs) agree with the highest population densities of Pyrenean Chamois and its maximum fecal nitrogen (Gálvez-Cerón et al., 2013) in the eastern Pyrenees. Moreover, it is in summer when the quality of some herbaceous plant groups (*Festuca* spp., other graminoids) significantly differs with respect to high grazing. It seems that the long-term interaction between plants including alpine ones and herbivores would have selected grazing-tolerant taxa that are able to maintain high nutritive values (Henkin et al., 2011; Mysterud et al., 2017). Thus, paradoxically, despite the overlapping diets of Pyrenean chamois and domestic herbivores in summer (Espunyes, 2019), overgrazing in diminishing alpine areas might benefit the diet quality of mountain wild ungulates (e.g., Pyrenean Chamois) rather than impair it (Mishra et al., 2004). In fact, highly aggregated patterns in herbivore population enhance forage quality (Fryxell, 1991), and livestock (cattle) has been known to improve forage quality and thus improve wild ungulate population fitness (Anderson and Scherzinger, 1975).

Random effects may be driving much of the results we report here. There is not a clear influence of grazing intensity and time on protein content and biomass production of the plants studied, and the random term of our analyses also influences the observed variability (Table 4.1.2). The micro-scale environmental heterogeneity might be the explanation. Subtle variations in lithological, edaphic and topographic characteristics drive the availability of water and soil nutrients which, in turn, shape the species composition of the below and above-ground microbiological, floristic and fauna communities with inherent mutualistic or antagonistic interactions (Burke et al., 1998; Hodkinson, 1999; Michalet et al., 2002). The interplay of such abiotic and biotic factors and processes finally determines a proportion of the ability of plants to grow (accumulated biomass) and regrow after grazing (e.g., Guitian and Bardgett, 2000). In addition, intrinsic factors at the species or individual level must influence since some taxa, especially *Festuca* spp. and *Calluna vulgaris*, show remarkable variations. Taxon, development stage, leaves/stem ratio, size or age of plants and their physiological and anatomical traits render inter and intra-species differences in biomass production and protein content under the same grazing intensity (Bovolenta et al., 2008; Briske and Richards, 1994; Fanlo et al., 2015; Pyke, 1987).

Prospects on alpine grasslands management and environmental implications

At a global scale, traditional seasonal pasturing in alpine ecosystems is dropping since the mid-20th century (Körner, 2003). This fact, linked with forest expansion and climate change impacts leads to grasslands reduction (Améztegui et al., 2016) and local overgrazing (Jackson et al., 2002; Rogiers et al., 2005). Grasses show strategies to survive grazing conditions, but they disappear under the current forest expansion (Espunyes *et al.*, 2019a). This ongoing trend, acting as a negative feedback process, might exacerbate the reduction in biomass and forage quality affecting wild herbivores and farming practices (Montero et al., 2009). Mountain wild Caprinae species (wild sheep, goats and relatives) are an extremely valuable group of mammals that rely on grasslands to survive. Over 70% of Caprinae taxa are threatened and thrive in environments with relatively low productivity affected by habitat loss and overgrazing (Shackleton, 1997).

Our results have implications for economic and environmental management in alpine grasslands since grazing is the only component that can be easily manipulated, and therefore is now a key tool in conservation management (Adler et al., 2001; Pardo et al., 2015). Additionally, grazing management entails environmental effects beyond the alpine ecosystems due to its role in carbon soil sequestration (Rogiers *et al.*, 2005). Thus, promoting suitable grazing conditions in this ecosystem increases biomass production and the quality of preferred plants for wild grazers and livestock. This in turn favours the conservation of our current alpine ecosystems and profitable farmland activities (Espunyes *et al.*, 2019a) and the carbon balance at the global scale (McSherry and Ritchie, 2013).

Conclusion

In summary, livestock grazing in alpine ecosystems increases biomass production and quality of preferred plants for wild grazers and livestock. This is positive, not only for herbivore condition and their offspring survival, but also for reducing climate change impacts as long as grasslands biomass production contributes to the sequestration of CO_2. Proper management practices are required to optimize nutrient value and forage availability in this fragile ecosystem.

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4.2 Study 2:

Near infrared reflectance spectroscopy analysis to predict diet composition of a mountain ungulate species.

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Authors: Laia Jarque-Bascuñana, Jordi Bartolomé, Emmanuel Serrano, Johan Espunyes, Mathieu Garel, Juan Antonio Calleja Alarcón, Jorge Ramón López-Olvera, Elena Albanell

Abstract

The diet composition of ungulates is important to understand not only their impact on vegetation, but also to understand the consequences of natural and human-driven environmental changes on the foraging behavior of these mammals. In this work we evaluated the use of near infrared reflectance spectroscopy analysis (NIRS), a quick, economic and non-destructive method, to assess diet composition of Pyrenean chamois *Rupicapra pyrenaica pyrenaica*. Fecal samples (n = 192) were collected from two chamois populations in the French and Spanish Pyrenees. Diet composition was initially assessed by the fecal cuticle microhistological analysis (CMA) and categorized into four functional groups, namely: woody, herbaceous, graminoid and Fabaceae plants. Regressions of modified partial least squares and several combinations of scattering correction and derivative treatments were tested. The results showed that models based on the second derivative processing got the higher determination coefficient for woody, herbaceous and graminoid plants (R^2_{CAL}, coefficient of determination in calibration, ranged from 0.86 to 0.91). The Fabaceae group, however, was predicted with lower accuracy ($R^2_{CAL} = 0.71$). Even though an agreement between NIRS and CMA methods was confirmed by a Bland-Altman analysis, confidence limits of agreement differed up to 25%. Our results support the viability of fecal NIRS analysis to study spatial and temporal variations of the chamois' diets in summer and winter when differences in the consumption of woody and annual plants are the greatest. This new use for the NIRS technique would be useful to assess the consequences of global change on the feeding behavior of this mountain ungulate and also in other ungulate counterparts.

Keywords: diet composition; fecal NIRS; foraging ecology; global change; *Rupicapra pyrenaica pyrenaica*.

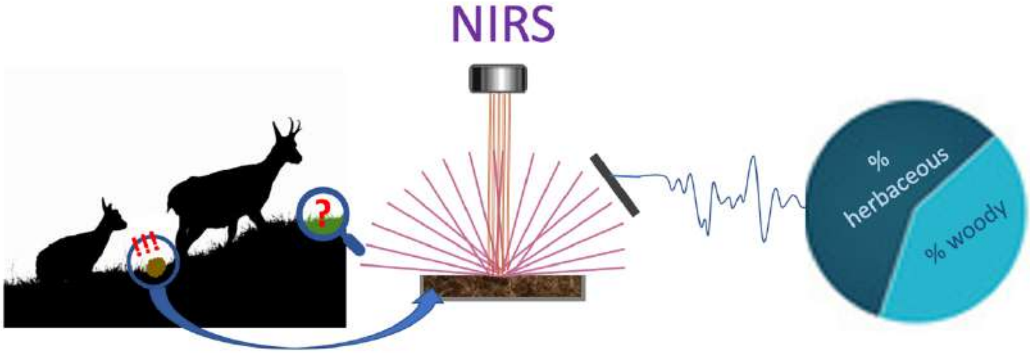


Figure 4.2.0. Graphical abstract from study 2. NIRS analysis on fecal samples is tested as a method to determinate diet composition of Pyrenean chamois.

Introduction

Information about plant species composition and quality of animal diets is important for wildlife researchers in order to monitor the animal nutritional condition and to predict population dynamics. Body condition (Pettorelli et al., 2002; Simard et al., 2008), reproductive rates (Hempson et al., 2015) and juvenile survival (Long et al., 2016) rely on the availability of highly nutritious plants. Likewise, knowledge of the range of herbivore diet quality is essential for a sustainable management of natural resources. Vegetal landscapes are fast-changing due to global warming and land-use transformation (Elmendorf et al., 2012; Nogués-Bravo et al., 2007; Shi et al., 2021) which affect the quality and availability of forages and probably the foraging behavior of ungulates (Bhattacharyya et al., 2019; Espunyes et al., 2019c). Therefore, detecting diet change trends may be useful for uncovering clues about how climate, landscape and vegetation changes affect the diet of free-ranging herbivores and, in turn, their health condition and their chances of overcoming the current warming climate and landscape transformation.

A wide variety of invasive and non-invasive methods have been used to assess the diet composition of wild ungulates. Invasive methods (e.g., esophageal fistula application (Vavra et al., 1978) or rumen content inspection (Andreoli et al., 2016) are inappropriate for continuous monitoring over time, especially for protected populations or out of the hunting season, since animal capture (López-Olvera et al., 2006) or certainly shooting (Serrano et al., 2011, 2008) is required. Non-invasive methods, however, allow an assessment of diet composition without animal behavior interference. The most common are direct monitoring of grazing animals (through direct observation (Perle and Hamr, 1985; Takada and Minami, 2019), video recording (Newmaster et al., 2013)), analyzing animal feces through fecal cuticle microhistological analysis (CMA, Croker, 1959), chemical analysis (Andriarimalala et al., 2020; Wright et al., 2019), radioactive isotopes (Codron et al., 2007) or DNA-barcoding (Hebert et al., 2003). Traditionally, researchers have relied on fecal CMA of herbivore feces to determine diet composition through the study of plant cuticle (Croker, 1959; Holechek et al., 1982) since it is the cheapest diet estimation method compared to the other non-invasive methods mentioned above. This indirect assessment has been by far the most common technique for assessing diet selection in both domestic (Bartolomé et al., 2011) and wild ruminants (La Morgia and Bassano, 2009; Mohammad et al., 1995; Suter et al., 2004). In spite of its advantages, the histological identification of plant fragments in feces is a very laborious and time consuming task, and presents other disadvantages which were discussed by Holechek et al. (1982). For these reasons, a reliable, quick and cheap non-invasive predictor method is needed for diet composition studies.

Near infrared reflectance spectroscopy (NIRS) is a non-destructive, emission-free and low-cost predictive technique which needs low amounts of samples and reduces the use of conventional time-consuming and laborious methods. The NIRS technique has already been used to predict nutritional parameters and diet quality in fecal samples (fecal NIRS) of domestic (e.g., sheep Li et al., 2007 and cattle Lyons and Stuth, 1992) and wild animals (e.g., Pyrenean chamois, *Rupicapra pyrenaica pyrenaica* (Gálvez-Cerón et al., 2013); red deer, *Cervus elaphus* and roe deer, *Capreolus capreolus* (Kamler et al., 2004); white-tailed deer, *Odocoileus virginianus* (Showers et al., 2006)) and other herbivores species (Alonso et al., 2014; Dixon and Coates, 2009; Li et al., 2007; Liu et al., 2017; Tolleson et al., 2004; Villamuelas et al., 2017). Later on, some studies also used fecal NIRS to determine the plant species consumed by domestic animals (cattle, Coates and Dixon, 2008; sheep, Keli et al., 2008; Núñez-Sánchez et al., 2016 cattle and sheep, Volesky and Coleman, 1996 and pigs Schiborra et al., 2015). However, few works have been carried out to achieve a NIRS prediction model for the diet composition of wild ungulates, surely because the broad dietary niche of these animals is a challenge for NIRS calibration. Nevertheless, in a previous NIRS study we applied it with success using a multispecies calibration model obtained from fecal samples of four wild herbivores (Albanell et al., 2011).

The European alpine grasslands are threatened due to a process of shrubification that is linked to land abandonment and climate change (Améztegui et al., 2015; Lasanta et al., 2019; Prévosto et al., 2011). Thus, grazing pressure may be exacerbating the threat in the remaining open areas and disrupting the herbivore-plant relationships at different spatial and temporal scales. A cheap, quick and reliable diet composition assessment technique would provide valuable information to monitor the nutritional condition of wild mountain herbivores, to find out how climate warming and land use shifts are affecting these animals and to predict their population dynamics in this current scenario.

The aim of this study was to explore the pros and cons of fecal NIRS to predict diet composition of mountain ungulates, using the fecal CMA as the reference method, since, as mentioned above, this is the most common technique for diet composition assessment. We focused on Pyrenean chamois, a medium- sized mountain-dwelling ungulate widely distributed in subalpine and alpine habitats of the Pyrenees (Corlatti et al., 2011).

Materials and Methods

Sampling area

Chamois fecal samples were collected in the Freser-Setcases National Game Reserve (FSNGR) and the National Game and Wildlife Reserve of Orlu (NGWRO, Fig. 4.2.1). The FSNGR is located on the southern side of the eastern Pyrenees, Spain (42°22'N, 2°09'E, see Fig. 4.2.1). This is a mountainous area of 20,200 ha with an altitude average of 2000 meters above sea level (m.a.s.l.) ranging from 1200 to 2910 m.a.s.l. Annual mean temperature is 6.0 °C and mean yearly accumulated rainfall is 963.4 mm (data from Núria meteorological station located at 1971 m.a.s.l. in the core FSNGR, Servei Meteorològic de Catalunya (www.meteocat.com, last access on 29 October 2014). The FSNGR vegetation is represented by sub-Mediterranean, Medio-European and Boreo-Alpine vegetation communities linked to acidic and calcareous soils of the eastern Pyrenees (Ninot et al., 2007; Vigo, 2008). At lower altitudes, the vegetation is dominated by oak (*Quercus* spp.) and Scots pine (*Pinus sylvestris)* forests with anthropic grasslands. At higher altitudes, the landscape is a mosaic of patches of mountain pine (*Pinus* *uncinata*), shrublands (*Cytisus oromediterraneus* and *Juniperus communis* subsp. *alpina*) and subalpine grasslands (e.g., *Festuca* spp., *Nardus stricta*, *Trifolium alpinum*).

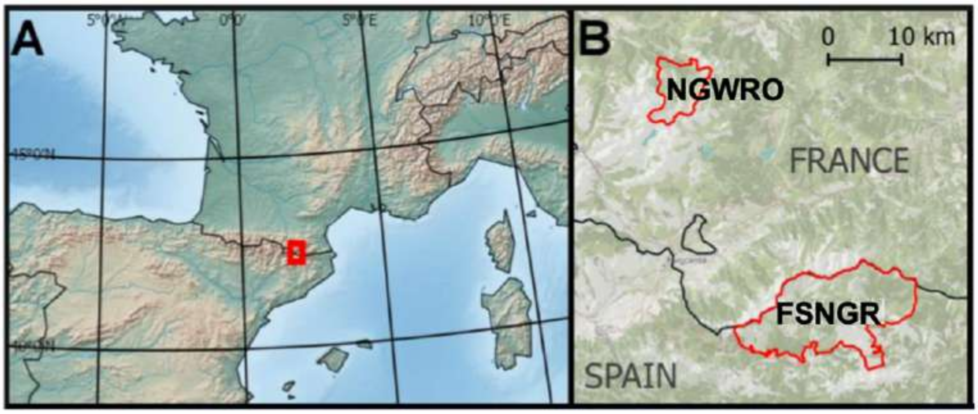


Figure 4.2.1. The two study areas. (**A**) Map of south-western Europe with the location of our study areas noted with a red rectangle. (**B**) Zoom on the previous red rectangle with the location of the National Game and Wildlife Reserve of Orlu (NGWRO) in France and the Freser-Setcases National Game Reserve (FSNGR) in Spain (both delimitated by a red line). Thick black line represents France-Spain border.

The NGWRO is also located in the eastern Pyrenees, but on the northern side, in France (42.66°N, 1.97°E, see Fig. 4.2.1). It is a protected area of 4250 ha ranging from 920 to 2765 m.a.s.l. The area presents a mountainous oceanic climate with annual mean temperatures of 8 °C and mean yearly accumulated rainfall of 1200 mm (Meteorological data collected by Meteo France weather station of Ascou-Pailhères, 1120 m.a.s.l., 42.72°N, 1.89°E). The vegetation is mainly composed of beech forests (*Fagus sylvatica*) with Atlantic chamaephytes and hemicriptophytes in the montane stage. In contrast, the subalpine stage is dominated by mosaics of shrublands of *Rhododendrum ferrugineum* and *Vaccinium* spp., grasslands of *Festuca* spp. and *Nardus stricta* and mountain pine (*Pinus uncinata*) patches. In the higher slopes, grasslands, rocks and cliffs are the prevailing habitats (Espunyes, 2019).

Fecal sampling procedure

In the FSNGR, 449 fresh chamois fecal samples were collected from May 2009 to May 2012. Each month, the study area was surveyed by two observers following defined transects of about 5 km each. Covering the whole altitude range and the main vegetation communities of the study area, the observers located chamois groups using 10 x 42 binoculars and 20 - 60 x 65 spotting scopes. Once group size, composition and precise location of chamois were recorded, observers collected fresh droppings at the exact place where animals were sighted and their surroundings. In the NGWRO, 277 fecal samples were collected from April 1992 to May 2015 in a long-term capture-mark-recapture monitoring project. The number of animals trapped each year varied from 2 to 66, using different methods as corral nets, drive nets, snares, or tele-anesthesia. In both study areas, fecal samples were placed in individually labelled plastic bags and transported to the laboratory where they were frozen at -20 °C for further laboratory analysis.

Fecal cuticle microhistological analysis

From all the collected samples, 192 randomly chosen were microhistologically analyzed for this study (95 from FSNGR and 97 from NGWRO). The procedure employed was developed based on work by Stewart (1967). Once samples were thawed, part of each sample was water-washed to remove extraneous material and then ground in a mortar to separate the epidermal fragments. After that, 10 g of sample were placed in a test tube with 5 ml of 65% concentrated HNO_3. The test tubes were then boiled in a water bath for 1 min. After digestion in

HNO_3, the samples were diluted with 200 ml of water. This suspension was then passed through 1.00 and 0.25 mm filters. The 0.25–1.00 mm fraction was spread on glass microscope slides in a 50% aqueous glycerin solution and cover-slips were fixed with DPX microhistological varnish. Two slides were prepared from each sample. Later, the slides were examined by the same operator under a microscope at ×100 and ×400 magnifications and plant fragments were recorded and counted until 200 leaf epidermis units identified. An epidermis collection of 55 main plant taxa of the study area was made and used as a reference method for fragment identification (Bartolomé and López-Garrido, 2020). Plant taxa were pooled into two main functional groups namely: woody species and herbaceous species. The group of woody species was composed by the genera *Calluna*, *Cystius*, *Genista*, *Hedera*, *Juniperus*, *Pinus*, *Quercus*, *Rosa*, *Rhododendron*, *Rosmarinus*, *Rubus, Teucrium, Thymus* and *Vaccinium*. The group of herbaceous species was composed by *Anthylis, Arrhenatherum, Astragalus, Avenula, Carex, Festuca, Juncus, Lotus, Nardus, Poa, Trifollium*, and 14 more genera. Moreover, regardless of this dichotomous classification, the Fabaceae group was also considered as well as graminoid species, which includes Poaceae, Juncaceae and Cyperaceae families. This CMA was considered as the gold standard method for our NIRS calibration.

NIRS Analysis and Spectral Data Analysis

To perform the NIRS analysis, the material not used in the CMA was dried at 60 °C in a stove during 48 hours. After, it was milled in a Cyclotec mill of 0.5 mm screen (FOSS Tecator, Höganäs, Sweden) and packed in a ring-cup sample cell. The samples were then scanned from 1100 to

2500 nm using a NIRSystems 5000 scanning monochromator (FOSS, Hillerød, Denmark) using a PbS detector. The analysis was carried out in duplicate using a closed ring cup cell (35 mm diameter) with quartz glass windows containing 2-3 g of the sample and absorbance was recorded as log (1/reflectance) (log 1/R) at 2 nm intervals, resulting in 692 data points for each sample. All measures were performed by the same operator.

One hundred and fifty samples were used for calibration (approximately 78% of the total samples) and 42 random samples (around 22% of the total samples) were separated previously and used as an external source of samples for validation. A WinISI III (v1.6; Infrasoft International, Port Matilda, PA, USA) software program was employed for spectra data analysis to develop chemometric models. The spectra were corrected emphasizing peaks and valleys and reducing noise for the effects of light scattering and particle size, using the standard normal variate, detrend (DT) or multiplicative scatter correction (MSC). Several prediction models were tested, taking into account different independent factors in addition to major group plants (woody/herbaceous, graminoids and Fabaceae), such as the study area (NGWRO/FSNGR) and the season (winter/summer). The prediction models were performed by modified partial least square regression using first and second derivatives of the spectra. In short, the results of the calibration were checked by observing (t) and global (GH) outliers, with the extreme values of $t > 2.5$ and GH > 10 not being considered for calibration.

The optimum prediction model was selected on the basis of minimum standard error of calibration (SEC) and greatest coefficient of determination (R^2_{CAL}). Performance of prediction model was evaluated using: i) the coefficient of determination of the external validation (R^2_{VAL}); ii) the ratio of performance to deviation (RPD), defined as the ratio of the range in the reference data from the validation set to the standard error of prediction (SEP); and iii), the range error ratio (RER), described as the ratio of the range in the reference data (validation set) to the SEP. Additionally, we also considered other statistics, such as bias to measure the accuracy between predicted (by NIRS) and reference values (MCA) in an external validation. For a detailed description, see (Williams, 2014; Williams and Sobering, 1996).

Relationships between fecal CMA and NIRS methods

We explored the relationships between fecal CMA and NIRS by two approaches: model selection and Bland-Altman regressions in order to test agreement between methods. The model selection approach was used to explore whether the relationships between diet composition predicted by NIRS and diet composition estimated by the CMA varied between plant groups. Model selection procedure based on the Akaike Information Criterion (AIC) was performed (Burnham and Anderson, 2002). The model with the lowest AIC was retained, and the remaining competing models were ordered according to their Akaike differences (Δi) with respect to the best model (lowest AIC). The Akaike weight (Wi) for each competing model was also calculated. Later, we used the Bland–Altman analysis to explore the agreement between the proportion of each functional group of plants assessed by the CMA and the predictions made by our NIRS analysis (Bland and Altman, 1986). This graphical method is the most popular to compare two measurement techniques by means of the representation of the mean differences (Y-axis) and magnitude of such measurements (X-axis, for a review of agreement approaches see Zaki et al. (2012)). The limits of agreement (LoA) at 95% defined as the mean differences ± 1.96 x SD (standard deviation), are also estimated and represented. Bland-Altman analysis was performed in the “blandr” package version 0.5.1 (Datta, 2017), the R software 4.0.2 version (Team R, 2019).

Results and Discussion

Spectral characteristics of samples

The Fig. 4.2.2A shows the average raw NIR spectrum of fecal samples used. Regardless of their plant species composition, all fecal samples showed local peaks at 1450, 1724, 1762, 1930, 2100, 2310 and 2350 nm. These absorption peaks were similar to those reported previously by Villamuelas et al. (2017) in Pyrenean chamois fecal samples, where NIRS was able to predict fecal nitrogen using a multispecies calibration. A derivative transformation was performed on the raw spectra to narrow the bandwidths and also remove some of the baseline variations. This transformation facilitated the analysis of the results. It made the absorption bands much more evident since overlapping absorbances become separated and the peak resolution was improved. The Fig. 4.2.2B shows the average second derivative NIR spectrum of fecal samples analyzed. The same trend of absorption bands of major components as in the raw spectra were observed in this derivative transformation. However, the absorption bands at 1914 and 2270 nm became clearer than in the raw spectra. Shenk et al. (2001) reported that the peak at 1914 nm is associated with the absorption of the OH stretch while the 2270 nm wavelength is due to overtone and combination CH stretches from the various CH group absorption bands, probably related to water and lignin content respectively. Cozzolino et al. (2002) found similar derivative spectra in beef fecal samples.

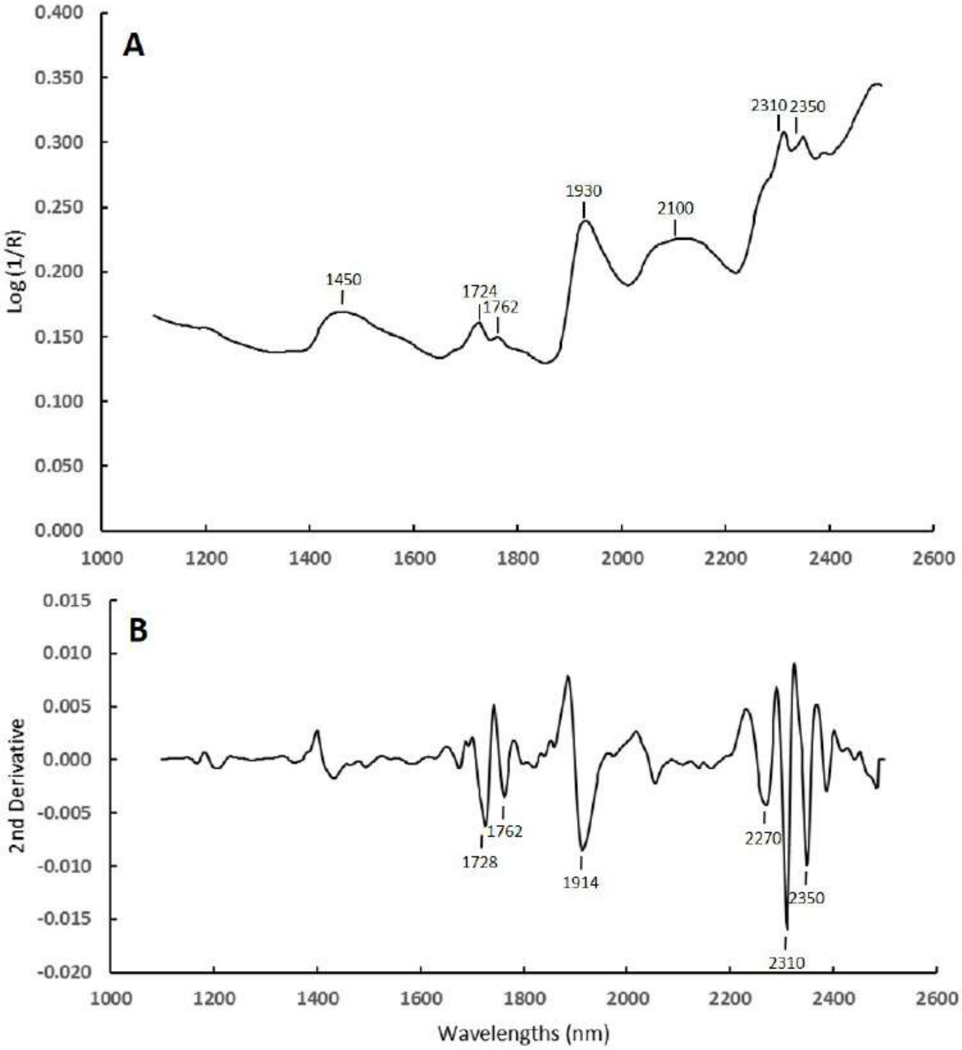


Figure 4.2.2. Near-infrared reflectance spectra used to build a prediction model for diet composition of Pyrenean chamois. It shows the bands of the main absorption. (**A**) Raw average spectrum of fecal samples, (**B**) the same spectrum after a second derivative and detrend treatment (R = reflectance).

Development of prediction models

Descriptive statistics of both, calibration and validation sets, are shown in Table 4.2.1. The broad ranges observed in the values were probably due to the sampling method, which covered large sampling zones, located in two different areas, over several years through all seasons, and showed the wide composition variability of the chamois diet. The Pyrenean chamois has already been described as an intermediate feeder, capable of adapting its diet composition to woody or herbaceous plants depending on their availability (Espunyes et al., 2019a; Garcia-Gonzalez and Cuartas, 1996). A high range in the reference values, allows a better establishment of prediction through NIRS. Furthermore, the calibration and validation matrices covered a similar broad range, thus ensuring the robustness of the calibration models (Næs et al., 2002).

Table 4.2.1. Plant species composition (%) of Pyrenean chamois fecal samples used in the calibration and validation sets.

	Calibration set				**Validation set**			
	N	**Range**	**Mean**	**SD**	**n**	**Range**	**Mean**	**SD**
Woody	150	0.5-95.0	50.42	28.17	42	3.5-87.5	46.26	26.83
Herbaceous	150	5.0-99.5	48.90	27.68	42	12.5-96.5	53.11	26.55
Graminoids	150	5.0-91.5	32.04	21.47	42	10.0-75.0	33.39	19.71
Fabaceae	150	0.0-70.0	22.69	15.16	42	1.5-55.0	22.69	13.49

Number of samples for calibration (N), number of samples for validation (n), interval between the maximum and the minimum value of data set (Range), standard deviation (SD)

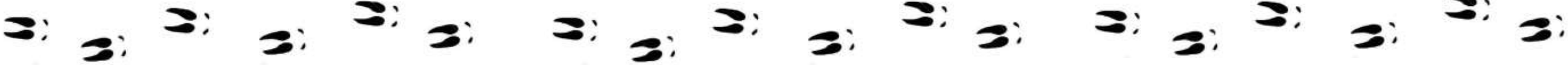
The Table 4.2.2 shows the statistical results from NIRS calibration equations and their corresponding validations (both cross and external validations). The best math treatment for each category was selected from those with lower SECV and/or SEC values. The best prediction model results were obtained from second derivative combined with MSC, DT or without scatter correction. Other prediction models were tested taking into account other independent factors than the major plant groups such as study area (NGWRO/FSNGR) and season (winter/summer), but neither of them showed better prediction models.

We achieved acceptable predictive models for woody, herbaceous and graminoids composition (Table 4.2.2), since NIRS equations owning coefficient of determination in calibration (R^2_{CAL}) ≥ 0.80 are considered acceptable (Williams, 2001; Williams and Sobering, 1996). The SEC and SECV values were also acceptable for woody and herbaceous, since for a good predictive power SECV value must be close to SEC (Williams, 2001; Williams and Sobering, 1996). Likewise, RPD values for woody and herbaceous suggested prediction models appropriate for screening purposes (between 2.0 and 2.4) according to some studies (Magwaza et al., 2012; Williams, 2014; Williams and Sobering, 1996). However, lower RPD values were found for graminoids and Fabaceae plants (1.79 and 1.47, respectively), which indicated a poor prediction model (RPD $\leqslant$ 1.9) (Magwaza et al., 2012; Williams, 2014; Williams and Sobering, 1996). Those low RPD values in graminoids and Fabaceae plants might be partially explained due to the fact that these two groups are more taxa specific and their predictions are not so accurate as generalist ones (woody and herbaceous). In addition, woody plants are easier to detect in fecal analysis because they are less digestible than the other groups (Anthony and Smith, 1974; Heggberget et al., 2002; Marinas and García-González, 2006) and lignin is easily detectable in NIR analysis (Cozzolino et al., 2002).

Table 4.2.2. Calibration and validation statistics of prediction models used to determine the diet composition (% presence) in Pyrenean chamois fecal samples by near infrared reflectance spectroscopy analysis.

	Calibration				Cross validation		External validation					
	Math treatment[a]	Scatter[b] correction	R^2_{CAL}	SEC	R^2_{cv}	SECV	R^2_{VAL}	SEP	Bias	Slope	RPD	RER
Woody	2,5,5,1	MSC	0.90	9.39	0.85	11.10	0.83	11.29	-0.94	0.88	2.38	7.44
Herbaceous	2,4,4,1	none	0.91	8.49	0.82	11.48	0.81	11.88	2.82	0.90	2.24	7.07
Graminoids	2,4,4,1	DT	0.86	7.70	0.71	11.24	0.70	11.03	0.74	0.92	1.79	5.89
Fabaceae	1,4,4,1	none	0.71	7.81	0.52	9.79	0.55	9.20	1.39	0.80	1.47	5.82

[a]Math treatment: derivative order, subtraction gap, first smoothing, second smoothing. [b]MSC multiple scatter correction, DT detrend. R^2_{CAL} coefficient of determination for calibration, SEC standard error of calibration, R^2_{cv} coefficient of determination for cross validation, SECV standard error of cross validation, R^2_{VAL} coefficient of determination for external validation, SEP standard error of prediction, RPD ratio of performance to deviation (SD/SEP), RER range error ratio (Range/SEP).



Other works provided similar or better results developing fecal NIRS prediction models to estimate plant species diet composition in livestock: cattle (R^2_{CAL} = 0.94 just for monocots and dicots proportions (Coates and Dixon, 2008; Dixon and Coates, 2009)); goat (R^2_{CAL} = 0.95 to 0.99 depending on the plant taxa hay, *Pistacia lentiscus, Phillyrea latifolia* and *Pinus brutia* (Landau et al., 2004); R^2 = 0.85 for herbaceous vegetation as one category; R^2 = 0.89 for *Phillyrea latifolia*; R^2 = 0.77 for tannin-rich *Pistacia lentiscus* (Glasser et al., 2008)); and sheep (R^2_{CAL} = 0.96 for *Artemisia tridentata* (Walker et al., 2002); R^2_{CAL} = 0.86 to 0.97 depending on the plant taxa species: alfalfa, cereal straw, maize and forage (Núñez-Sánchez et al., 2016)). The better NIRS prediction power in plant taxa diet composition in the livestock studies may be due to the fact that the reference method was the actual diet intake since diets were already known and very few species were represented in those diets. However, in these studies, researchers used direct observation of bites (Glasser et al., 2008) or controlled formulation diets (Coates and Dixon, 2008; Dixon and Coates, 2009; Keli et al., 2008; Núñez-Sánchez et al., 2016; Volesky and Coleman, 1996) as reference methods, which are not viable approaches for wild herbivore studies as in the case of the CMA.

To the best of our knowledge, only two studies have used microhistological identification as a reference method in wild ruminants. Our previous work (Albanell et al., 2011) for a multispecies calibration obtained from fecal samples of four wild herbivores (red deer, Barbary sheep, *Ammotragus lervia,* mouflon, *Ovis orientalis musimon* and rabbit, *Oryctolagus cuniculus*) reached values of R^2_{CAL} = 0.98 and R^2_{CAL} = 0.97 for woody and herbaceous components, respectively. Jean et al. (Jean et al., 2014) were also able to predict the amount of coniferous fragments in the diet of wild ruminants (white-tailed deer) with a similar precision to our results (R^2_{CAL} = 0.89).

In Fig. 4.2.3, the best regressions obtained between NIRS prediction and reference data for woody (A), herbaceous (B), graminoids (C), and Fabaceae (D) are represented. Good models should result in a slope very close to 1.0, and this was the case for woody, herbaceous and graminoids. The number of PLS factors or terms (Fig. 4.2.3) is a measure of the potential effectiveness of the calibration, which, according to Williams et al. (2017), should range from six to eight factors.

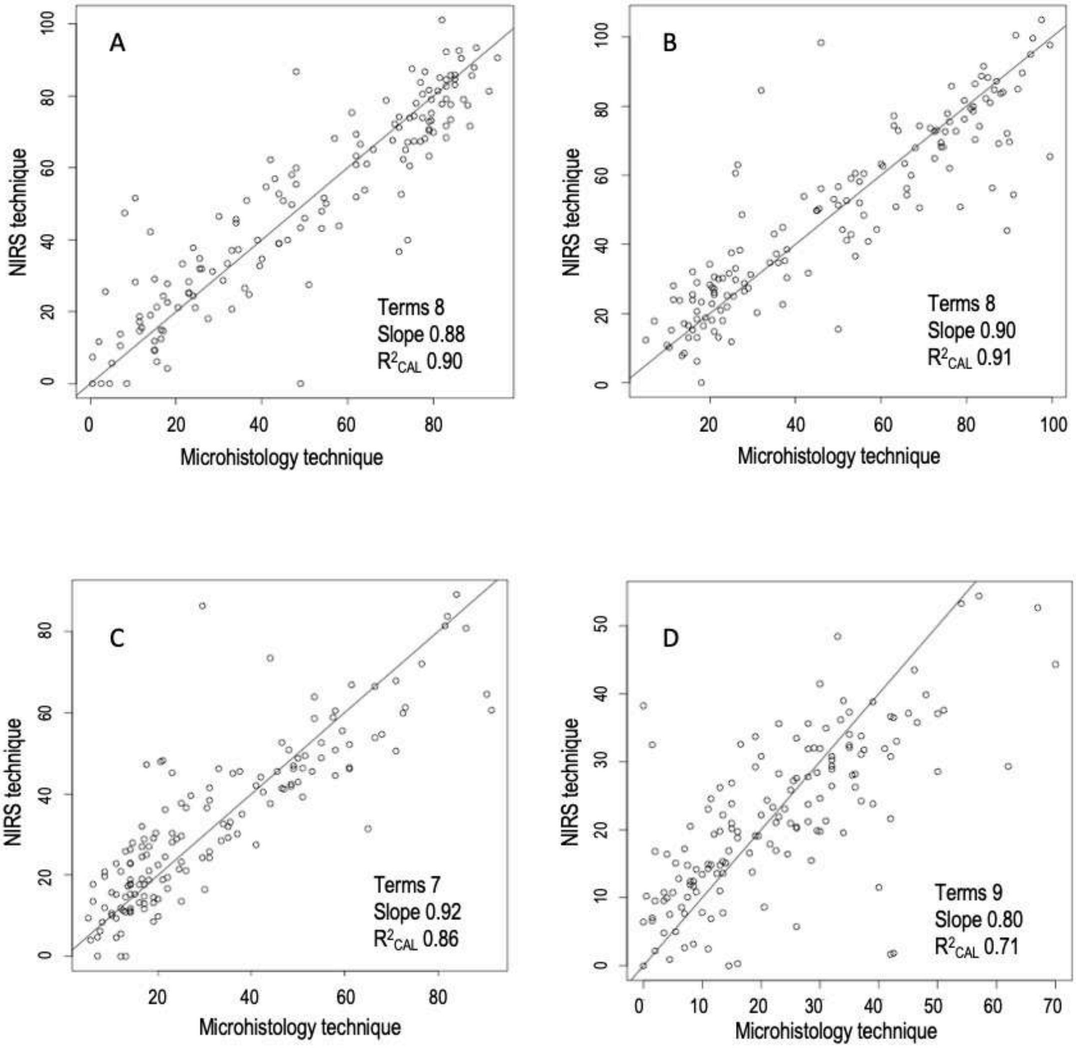


Figure 4.2.3. Linear regression between fecal cuticle microhistological analysis and near infrared reflectance spectroscopy predictions for four functional groups of plants: woody (**A**), herbaceous (**B**), graminoids (**C**) and Fabaceae (**D**) found in 150 fecal samples of Pyrenean chamois. The coefficient of determination for calibration (R^2_{CAL}), the slope and the numbers of terms are also shown.

Comparison between NIRS predictions and microhistologic method

The best model to explain the observed variability of CMA and NIRS methods for predicting diet composition is independent of the plant group analyzed (ω_i = 0.85, Table 4.2.3). Therefore, NIRS method predictions and MCA estimates did not vary across plant groups. In the same line, the Bland-Altman analysis showed a good agreement between NIRS and MCA for all functional group of plants (Table 4.2.4). In this analysis, the bias indicates the average differences of each functional group recorded in our fecal samples, proximity to zero indicating a good agreement in the detection of plants by both methods. Both bias and the number of observations outside the upper and lower limits of agreement were lower for woody, herbaceous and graminoid plants, with Fabaceae species showed higher values for both indicators. Altogether, this agrees with MCA and NIRS showing a better correspondence for the first three groups of plants than for Fabaceae. However, in spite of these differences, the lower and higher confidence limits of agreement were overall similar for each plant group (Table 4.2.4 and Fig. 4.2.4). Overall, this analysis confirmed that both MCA and NIRS methods are interchangeable to predict the fecal diet composition in Pyrenean chamois for the groups of plants studied.

Table 4.2.3. Model selection to explore whether the relationships between diet composition of Pyrenean chamois (% presence) assessed by the cuticle microhistological method (reference method) and predicted by near infrared reflectance spectroscopy varies among woody, herbaceous, graminoids and Fabaceae groups.

Selected model	K	AIC	*Δi*	ω_i
NIRS method	**3**	**4616.2**	**0**	**0.852**
NIRS method + Group plant	6	4619.7	3.49	0.148
NIRS method * Group plant	9	4625.0	10.08	0.005
Group plant	5	5420.73	804.93	0.000

K = number of parameters, Δi = difference of AIC with respect to the best model, Wi = Akaike weight. The best model is indicated in bold.

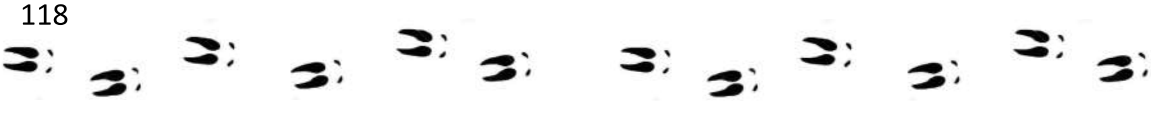


Table 4.2.4. Descriptive statistics for the Bland-Altman analysis of agreement between the fecal cuticle microhistological analysis and near infrared reflectance spectroscopy predictions for four functional groups of plants found in fecal samples of Pyrenean chamois, collected in the French and Spanish Pyrenees.

Functional group	**Parameter**	**Unit**	**CI at 95%**	
			minimum	**maximum**
Woody	Mean differences (bias)	-0.05	-2.1	2.04
	SD	1.06	--	--
	ULoA	25.46	21.87	29.07
	LLoA	-25.57	-29.17	-21.97
Herbaceous	Mean differences (bias)	0.14	-1.91	2.21
	SD	12.77	--	--
	ULoA	25.18	21.65	28.71
	LLoA	-25.01	-28.42	-21.36
Graminoids	Mean differences (bias)	- 0.05	-1.69	1.58
	SD	0.83	--	--
	ULoA	19.87	17.17	22.79
	LLoA	-19.98	-17.17	-22.79
Fabaceae	Mean differences (bias)	1.28	-0.37	2.94
	SD	10.29	--	--
	ULoA	21.46	18.61	24.31
	LLoA	-18.88	-21.721	-16.04

Confidence interval (CI), standard deviation (SD), upper and lower limits of agreement (ULoA and LLoA respectively).

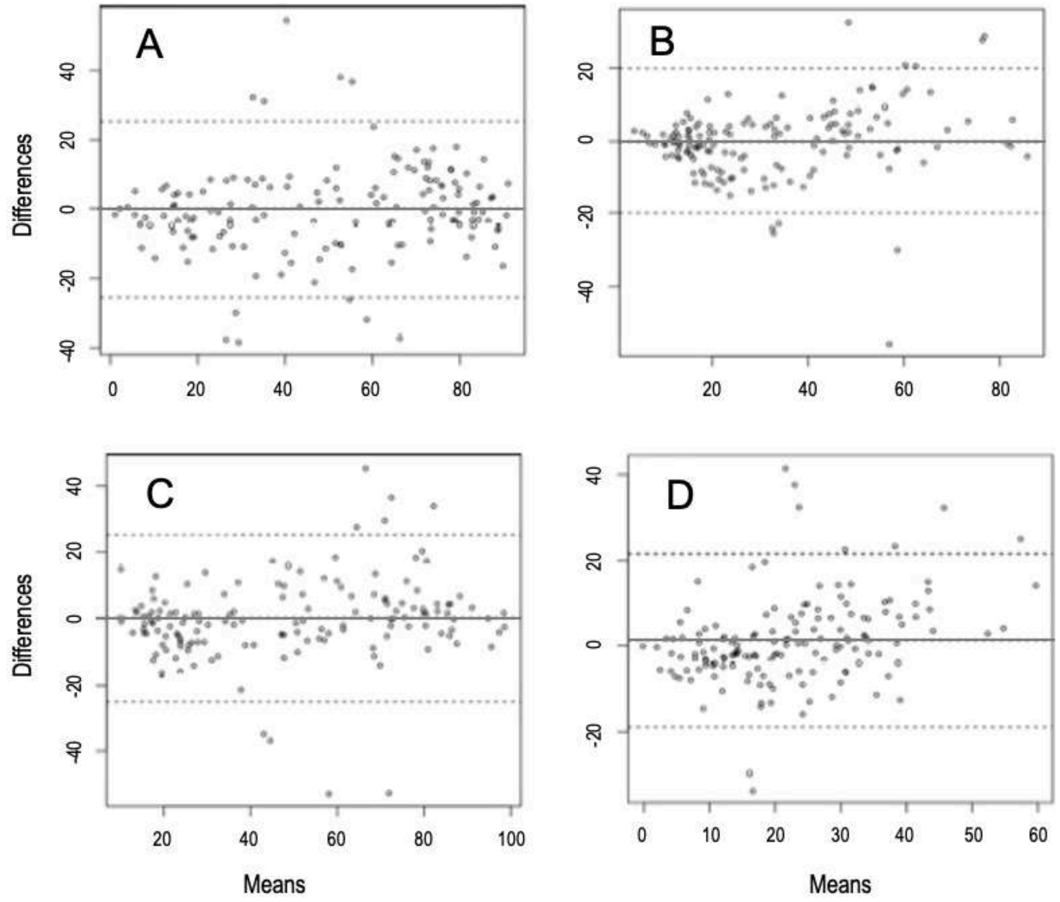


Figure 4.2.4. **Bland-Altman plots showing the agreement between fecal cuticle microhistological analysis (CMA) and near infrared reflectance spectroscopy (NIRS) predictions** for four functional groups of plants: woody (**A**), herbaceous (**B**), graminoids (**C**) and Fabaceae (**D**) found in fecal samples of Pyrenean chamois. Y axis represents the difference between the proportion of each functional group of plants observed by the CMA and the NIRS prediction. X axis represents the mean of the observed and the predicted proportion for each functional group of plants. The limits of agreement (dotted line), from-1.96SD (standard deviation), to +1.96SD have also been represented.

Our study showed that NIR spectra of feces can be used to predict major groups of plants (namely woody and herbaceous, and even for graminoids) as a diet composition proxy for Pyrenean chamois. Regarding the better results in livestock studies with known diet components as a reference method, it could be suggested that the CMA may not be the ideal reference method. Still, it is currently the best available noninvasive approach to study the diet of wild and protected species. The main disadvantage of the CMA is that highly digestive plant species could be underrepresented in the estimated diets, whereas less digestible plants could be overrepresented (Bartolomé et al., 1995). Shaffer et al. (1990) and Volesky and Coleman (1996) achieved better results estimating plant species composition using NIRS (R^2_{CAL} = 0.96 for both) with samples collected directly from the field or from domestic animals esophageal extrusa, respectively. Those better results could be adjudged to the fact that field and esophageal extrusa samples were not digested or not totally digested. Several authors (Bartolomé et al., 1998; Holechek et al., 1982; Leslie et al., 1983; Vavra and Holechek, 1980) pointed out that the differing degrees of digestibility of plant species must be taken into account, as long as it may determine fragment detection in fecal samples. Espunyes et al. (2019b) proposed an index of preservation for each plant species to link the relative quantity of a specie detected in fecal sample to the corresponding ingested quantity. Plant digestive pattern is not the only problem with this method; CMA requires a good knowledge of the micromorphology of different plant tissues; and human error, if untrained, could be quite important when identifying some plant groups.

Our current prediction models show some limitations to depict accurately the diet composition of Pyrenean chamois. However, we show that our models can be used as a screening technique for woody and herbaceous taxa, particularly when or where chamois diet woody/herbaceous ratio is highly contrasted (e.g., summer/winter) (Espunyes et al., 2019a). In the context of alpine ecosystem, Pyrenean chamois diet shows a relative abundance of woody species, improving, with that feature, the screening monitoring. The reason that NIRS could be used as a rough diet composition proxy is probably due to high woody species percentage (more than 70%) and that subalpine and alpine ecosystems are relatively simple, with only a few winter forage types available to intermediate feeders.

This is a relatively new application for NIR spectra, since previous research on wild ungulates primarily focused on estimates of the nutritional quality of diet rather than its plant species/groups composition (Kamler et al., 2004; Showers et al., 2006). Although we expected to predict the four forage groups, being able to predict just woody species and herbaceous content is already quite useful for diet composition and ecological studies. Alpine ecosystems are changing in a very fast way due to changes in land uses and global warming. Therefore, better tools to evaluate the impact of such environmental changes on animal-plant interaction are needed. Taking into account that feeding habits are crucial to population dynamics, NIRS method to predict plant taxa in chamois diet composition may provide important information about diet quality and possible changes in vegetation availability.

Conclusions

Our results demonstrated the potential of fecal NIRS to estimate major plant groups composition for Pyrenean chamois as a quick, cheap and practical option, with good accuracy but limited precision power. It is adequate for rough analysis, for monitoring purposes (e.g., to compare independent variables effects on diet preference) focused on major plants groups, but would not be enough if it is necessary to know the actual composition of the diet with high precision and/or at species level.

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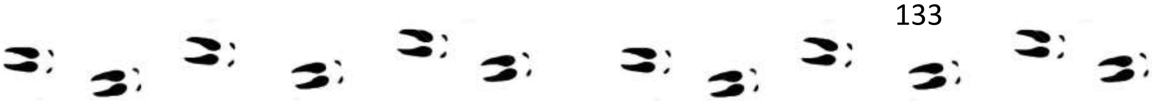
4.3 Study 3:

Local climatic conditions drive diet quality of Pyrenean chamois. A ten years study.

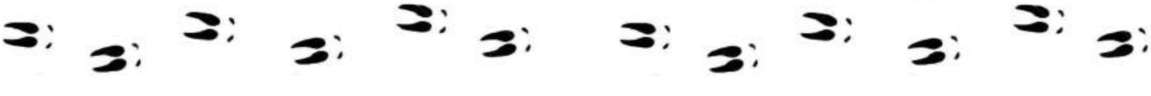
Unpublished manuscript



Abstract

Vegetation availability and quality drive life history of mountain ungulates living in seasonal environments. In the alpine ecosystems, for example, the access of herbivores to highly nutritious vegetation relies on snow cover, summer rainfall and spring temperatures. The link between changes in environmental variation and diet composition and quality of herbivores living in seasonal ecosystems is poorly understood. Here, we explore direct and indirect relationships among local weather, primary productivity and diet quality of a Pyrenean chamois (*Rupicapra pyrenaica pyrenaica*) population in the National Game Reserve of Freser-Setcases, Catalonia, Northeast Spain. The percentage of fecal nitrogen, neutral detergent fibre, acid detergent fibre, lignin and diet composition were assessed by Near Infrared Spectroscopy (NIRS) analysis of 802 fecal samples collected on a monthly basis from May 2009 to December 2019. Local weather information was recorded in a local meteorological station located at 1971 m.a.s.l. Primary productivity was estimated by the Enhanced Vegetation Index (EVI) and the Leaf Area Index (LAI) recorded from Terra and Aqua satellites. Our results indicated that local weather exerted a positive direct effect on primary productivity and on the Pyrenean chamois diet. Snowfalls, however, had a direct negative effects on the primary productivity and indirect effect on the nutritional value of the Pyrenean chamois diet. We can conclude that less snow cover duration and higher mean temperatures will positively affect the diet quality of chamois in the short- medium-term. Understanding the link between local weather, primary productivity and diet of herbivore is crucial to develop management plans oriented to preserving this fragile ecosystem.

Key words. Diet quality, fecal nitrogen, *Rupicapra pyrenaica pyrenaica*, climate change, population dynamics, EVI, LAI.



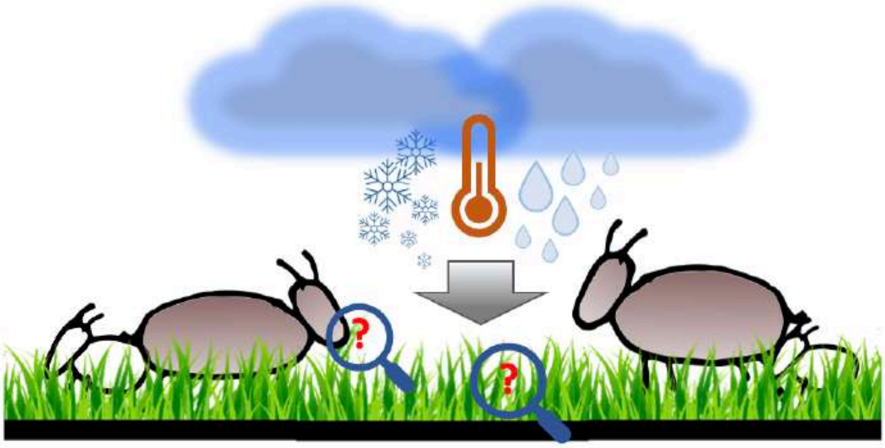


Figure 4.3.0. Graphical abstract from study 3. The effects of local weather on grasslands primary productivity and Pyrenean chamois diet are studied.

Introduction

Alpine regions are highly seasonal ecosystems. Primary productivity in these landscapes is relatively high during short period of time (60-120 days in average, Nagy and Grabherr, 2009). The growing season starts in late spring and finishes in mid-summer when plant senescence begins (Ernakovich et al., 2014). Herbivores inhabiting in these extreme environments are adapted to alternate periods of high food availability with food shortages.

Diet quality drives life history of many ungulate species (Gamelon et al., 2020; Jönsson, 1997 for Pyrenean chamois, DeGabriel et al., 2014 for mammalian browsers in general). The access to highly nutritious vegetation influences body weight (White, 1983), population dynamics (Flajšman et al., 2018; Hewison and Gaillard, 2001; Zini et al., 2019 for roe deer, *Capreolus capreolus* and Miyashita et al., 2008 for sika deer, *Cervus nippon*), diseases susceptibility (Beldomenico and Begon, 2010; Fordyce et al., 1990; Hudson et al., 2002; Treanor et al., 2015), and the ability to cope with environmental changes (Korslund and Steen, 2006; Loison & Langvatn, 1998; Clutton-Brock et al., 1983). Thus, variations in the availability of nutritious plants due to changes in environmental conditions play an important role on ungulate population dynamics (Cook et al., 2004).

Wild mountain Caprinae species (wild sheep, Pyrenean chamois and relatives) adapt their energetic demands to the cycles of primary production characterized by short periods of plant growth in late spring and summer alternating with long periods of plant senescence (Hebblewhite et al., 2008; Mysterud et al., 2017; Parker et al., 2009). The body reserves of mountain ungulates rely on the access to alpine grasslands during summer (Parker et al., 2009). Changes in vegetation growth patterns due to global warming (Ernakovich et al., 2014; Peterson et al., 2014) have strong repercussions on the growth rates and population recruitment of-mountain goats (*Oreamnos americanus*), bighorn sheep (*Ovis canadensis*) and Alpine ibexes (*Capra ibex,* see Pettorelli et al., 2007). The mechanism behind this process is that an early vegetation onset result in higher plant productivity for shorter periods of time reducing the opportunity for ungulates to exploit high-quality forage (Pettorelli et al., 2007). This may cause a mismatch between plant phenology and ungulate population cycles (Bhattacharyya et al., 2019; Espunyes et al., 2019a). How mountain ungulates overtake climate warming is a hot topic in conservation biology.

This work focuses on the causal relationship between environmental variation and diet quality of Pyrenean chamois (*Rupicapra p. pyrenaica*). Pyrenean chamois is a medium-sized mountain-dwelling ungulate widely distributed in subalpine and alpine habitats of the Pyrenees and other mountain ranges in southern Europe (Corlatti et al., 2011). Chamois show an intra-annual variation in their diet composition, and depend on woody plants (e.g., *Calluna vulgaris* (L.) Hull and *Cytisus* spp.), during winter and herbaceous (e.g., *Festuca* spp. and *Trifolium* spp) during summer (Espunyes et al., 2019a). As a capital breeder, they store energy during spring and summer to meet the high costs of reproduction (Jönsson, 1997) and thus rely on high-quality resources during this period. European alpine grasslands occupied by Pyrenean are threatened due to a shrubification process linked (Améztegui et al., 2010) and climate change (Ernakovich et al., 2014). Grazing pressure, on the other hand, may exacerbate in the remaining open areas (Espunyes et al., 2019b) and disrupt herbivore-plant relationships at different spatial and temporal scales (Lu et al., 2017).

In this study, we explore the causal relationships between local climatic conditions and diet composition and quality of chamois using path models. Taking advantage of 802 fecal samples collected on a monthly basis for ten years, we investigate direct and indirect relationships among local weather conditions (e.g., Tmax, Tmin, accumulated precipitation, mean precipitation), snow cover (mean and total accumulated snow), primary production (LAI, EVI), diet composition (herbaceous, graminoids and woody vegetation), and diet quality (fibre and protein), in a population of Pyrenean chamois.

Materials and methods

Study area

The study area is located in Fontalba, in the Freser-Setcases National Game Reserve (FSNGR) core. The FSNGR is a mountainous area of 20.000 ha with an average altitude of 2.000 m.a.s.l. (from 1.220 to 2.910 m.a.s.l.) in the Eastern Pyrenees, Spain (42° 22’N, 2° 09’ E, Fig. 4.3.1). The reserve is dominated by metamorphic rocks and oligotrophic soils (http://info.igme.es, Vigo, 2008). Vegetation composition and structure belong to the subalpine and alpine bioclimatic belts within the Alpine or Eurosiberian Region (Vigo, 2008). On the one hand, vegetation above 2.000 m.a.s.l. is mainly represented by a mosaic of alpine grasslands dominated by perennial plants. Mostly of them are hemicryptophytes and rhizomatous herb species, were tussock and stoloniferous graminoid taxa (e.g., *Festuca* and *Carex* genera) are dominant and *Gentiana* spp., *Hieracium pilosella* L., *Luzula* spp., *Trifolium alpinum* L., and *Plantago monosperma* Pourr. patches are abundant. Woody chamaephyte are found with scattered patches (*Calluna vulgaris*, *Juniperus communis* L. and *Cytisus* spp.). On the other hand, below 2.000 m.a.s.l. vegetation comprises a mosaic of subalpine grasslands and forested patches dominated by *Pinus uncinata* Raymond ex A.DC., *Betula* spp., and *Fraxinus excelsior* L. with shrubs (*Cytisus spp., Juniperus communis*, *Rosa* spp. and *Rubus* spp., Vigo, 2008). In the study area the mean annual temperature was 5.6 °C, with a maximum of 22.0 °C and a minimum of -13.7 °C. The mean yearly accumulated rainfall was 1101 mm (period 2009-2019, www.meteo.cat accessed on 9 May 2020). The maximum snow cover thickness recorded was 103.8 cm. According to the monthly population counts, throughout the study period (from 2009 to 2019), a sedentary Pyrenean chamois flock of about 90 – 100 individuals coexisted (from May to October) with herds of approximately 97 – 160 cows (*Bos taurus* L.) and 25 – 27 horses (*Equus ferus caballus* L.). The livestock density was 1.9 – 2.2 LSU.

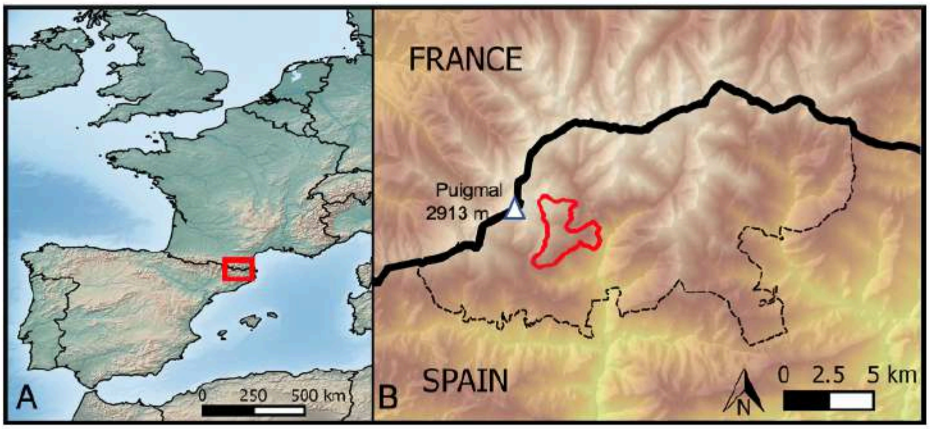


Figure 4.3.1. Study area. (A) Map of south-western Europe with the study area represented by a red rectangle. (B) Detail of the Freser-Setcases National Game Reserve (dashed line), and the study area (red line). Average altitude is 2.000 m.a.s.l. peaking in the Puigmal, marked by a white triangle. The thick black line represents the France-Spain border.

Fecal sampling procedure and processing

From May 2009 to December 2019, 802 fresh Pyrenean chamois fecal samples were collected for this study. Following defined transects of about 5 km each, the study area was surveyed monthly by two observers. These observers located Pyrenean chamois groups using 10 x 42 binoculars and 20 – 60 x 65 spotting scopes. The transects covered the whole altitudinal range and the main vegetation communities of the study areas. Fresh droppings were collected at the exact place where animals were sighted, once group size, composition and the precise location of Pyrenean chamois were recorded. Fecal samples were collected in individual labelled plastic bags and transported to the laboratory where they were frozen at -20 °C for further laboratory analyses.

Samples were dried in a forced-air oven (60 °C for 24-48h). After that period, samples were milled in a Cyclotec mill of 0.5 mm screen (FOSS Tecator, Höganäs, Sweden) and stored frozen at -20 °C until analysis.

Fecal nitrogen and diet composition assessment

Fecal nitrogen was determined using a Near Infrared Spectroscopy (NIRS) analyzer (NIRSystems 5000, FOSS, Hillerød, Denmark), as described by Villamuelas et al. (2017). Calibration was checked using the Dumas dry combustion method using a LECO analyzer (LECO Corporation, St. Joseph, MI, USA), according to AOAC (2012) protocols. A correlation between fecal nitrogen values and diet quality is assumed in ungulates in general (Kamler et al., 2004; Verheyden et al., 2011) and for Pyrenean chamois in particular (Villamuelas et al., 2017). Likewise, fecal nitrogen (FN) values can also be used for forage intake and digestibility evaluation in grazing ruminants (Peripolli et al., 2011). Neutral detergent fibre (NDF), acid detergent fibre (ADF) and especially lignin contents (ADL), are indicators of low digestibility in herbivores (Barboza et al., 2009). The NIRS was also used to assess fibre contents of our fecal samples.

Along the same lines, diet composition estimation using NIRS was calibrated and validated using 192 fecal samples microhistologically analyzed following the procedure of Stewart (1967), modified by Bartolomé et al. (1998). A correlation between diet composition estimation using NIRS and diet composition estimation using microhistological analysis discriminating major plant groups (woody and herbaceous) was demonstrated by Albanell et al. (2011). Predictive models used were published by Jarque-Bascuñana et al. (2021).

Local climate data

Meteorological data (temperature, precipitation and snow cover) were collected from the Núria meteorological station located at 1971 m.a.s.l. (Servei Meteorològic de Catalunya) in the study area core (Fig. 4.3.2).

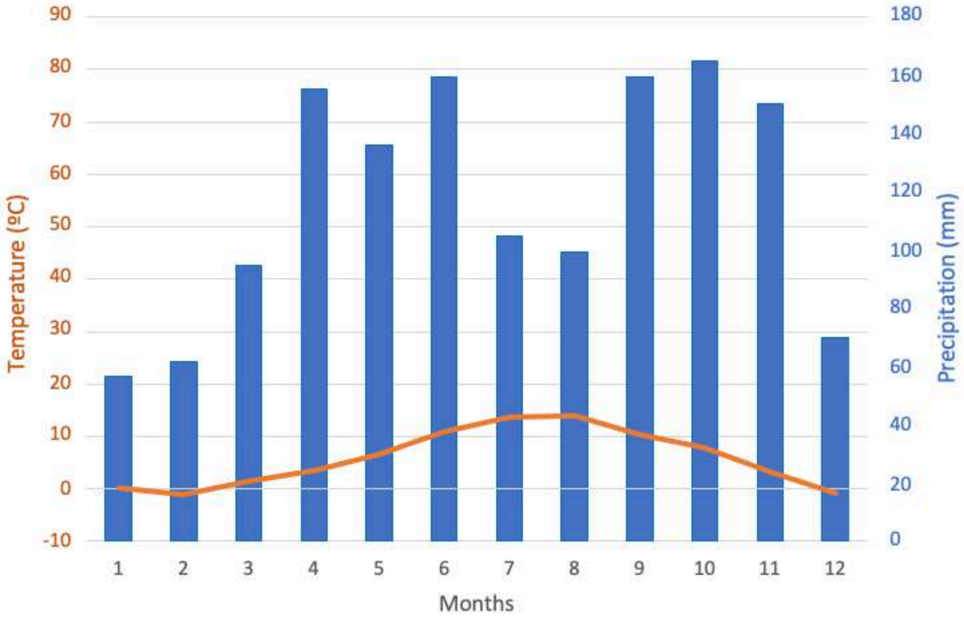


Figure 4.3.2. Climograph of the study area. Meteorological data were collected from Núria meteorological station located at 1971 m.a.s.l. (Servei Meteorològic de Catalunya) from 2009 to 2019. Bars represent the mean monthly accumulated precipitation and the solid line the mean monthly temperature.

Mean monthly temperature of maximum and minimum values, as well as mean monthly rainfall and cumulative monthly rainfall were considered as local weather variables (Table 4.3.1). Snow cover has been recorded daily using two calibrated stakes in meters and centimeters. The readings are taken by sighting over the undisturbed snow surface to the stake.

Table 4.3.1. Variables used in this study. Variables initially included in the PLS-PM outer model describing causal relationships among local weather conditions, snow, primary production, and diet quality (fibre and protein) and composition (herbaceous, graminoids, and woody plants) in a southern Pyrenean chamois population from the Freser-Setcases National Game Reserve, Northeast Spain. Meteorological variables (temperature, precipitation and snow) were recorded from the Núria meteorological station, located at 1971 m.a.s.l. (Servei Meteorològic de Catalunya). Primary production variables were collected by the Moderate Resolution Imaging Spectroradiometer set in Terra spacecraft (MODIS), and Pyrenean chamois diet composition and quality was assessed by NIRS on fecal samples. Abbreviations appear in parentheses.

Block	Variable	Description
Time	Years	Years (2009-2019)
Local weather	Tmax	Average of monthly maximum temperatures (°C)
	Tmin	Average of monthly minimum temperatures in °C
	AcPp	Monthly accumulate precipitation (mm)
	MeanPp	Mean monthly precipitation (mm)
Snow	Mean snow accumulation	Ratio between accumulated snow and number of days between the first and last snow falls from November to April (cm/day)
	Accumulated snow	Accumulated snow from November to April (cm)
Primary production	Enhanced Vegetation Index (EVI)	EVI is a proxy for vegetation greenness
	Leaf Area Index (LAI)	The ratio between the foliar area and the unit of soil surface. LAI is a proxy for plant biomass production
Diet composition	Herbaceous	Proportion of herbaceous, excluding graminoids, in chamois diet (%)
	Graminoids	Proportion of graminoid plants in chamois diet (%)
	Woody plants	Proportion of woody plants in chamois diet (%)
Fibre content	Neutral detergent fibre (NDF)	NDF (%) are structural components of the plant, specifically cell wall including hemicellulose, cellulose and lignin
	Acid detergent fibre (ADF)	ADF (%) are the least digestible plant components, including cellulose and lignin
	Acid detergent lignin (ADL)	ADL (%) are indigestible part of the plant cell
Protein content	Fecal nitrogen (FN)	Metabolic and food residual N per gram of dry fecal matter (%)

Primary production

Plant phenology, obtained from the enhanced vegetation index (EVI) data, has been used to assess grazers diet quality and availability of mountain ungulates (Garel et al., 2011). The EVI data were collected from MODIS (Moderate Resolution Imaging Spectroradiometer), aboard the Terra (originally known as EOS AM-1) and Aqua (originally known as EOS PM-1) satellites. This index is calculated from the red-infrared reflectance ratio taking into account the reflectance in the blue band, which makes it less sensitive to the influence of atmospheric aerosols and improves sensitivity to vegetation signals as compared to the NDVI (Huete et al., 2002).

The leaf area index (LAI), defined by Stewart (1967) as the ratio between the sum of the foliar area and the unit of soil surface, is a key variable for characterizing different plant canopies because it is related to light and energy capture. Is an indirect measure of ecosystem productivity. The LAI data were collected also from MODIS, aboard Terra and Aqua satellites. This index is calculated taking into account the heterogeneity of the plant canopy that can be described by the three-dimensional leaf area distribution function. The LAI was calculated by the Myneni et al. (1999) equation.

Data analyses

Partial Least Square Path Modelling (PLS-PM) was used to explore causal relationships among local weather conditions (temperature and precipitation), snow cover, primary production and diet composition and quality in chamois. Briefly, this approach quantifies the network relationship between a set of unobservable latent variables (LV) and a set of manifest variables (MV, i.e., parameters directly measured in the field or in the laboratory). The LVs are conceptual variables defined by one or several MVs and organized in a network of relationships where the connections among LVs are assumed to represent a cause-effect process. This network of relationships among LVs forms the inner model (also called structural model) while each group of MVs linked to a LV represents the outer model. The links among LVs are quantified through path coefficients while the links between LV and MV are quantified through weights (Hair et al., 2013). Our analysis included fifteen MVs organized in seven LVs namely (Table 4.3.1): time in years (I); local weather, defined by the maximum and minimum temperatures and the accumulated and mean precipitation in the study area (II); snow cover, defined as the mean and the accumulated snow in the study area (III); primary production assessed by the Enhanced Vegetation Index (EVI) and the Leaf Area Index (LAI, IV); diet composition in terms of forbs, graminoids and woody plants (V); and fibre (NDF, ADF and ADL, VI), and protein contents (FN, VII) assessed in chamois fecal samples. After fitting the first model including all the variables, a model simplification was performed by removing those MVs uncorrelated with their own LVs. The PLS-PM approach does not depend on any distributional assumptions, therefore, a resampling procedure or bootstrap validation was used to get confidence intervals for evaluating the precision of the PLS parameter estimates (e.g., path coefficients, total effects and fit indices such as R^2). Statistical analyses have been performed in the R Statistical Software 3.3.0 version (Team R, 2019).

Results and Discussion

Average of monthly primary production values (EVI and LAI) as well as Pyrenean chamois diet quality values (% FN and % herbaceous) for the whole study period in the study area are represented in Fig. 4.3.3. A seasonal pattern is detected for these variables. Primary production rises in summer, reaching its peak in June-July, and it is low in winter, with February being its minimum. Fecal nitrogen content and portion of herbaceous plants in the Pyrenean chamois diet reach their peak in June and their minimum in January-February.

Descriptive statistics for the variables used in the full PLS-PM to investigate environmental drivers of diet composition and quality in chamois are in Table 4.3.1. The final outer model including the set of retained variables with a significant contribution to their LVs is in Table 4.3.2. The mean monthly precipitation, accumulated snow, graminoids and woody plant consumption, and ADL showed no significant contribution to their LVs and thus were excluded from our final PLS-PM (Table 4.3.2). The goodness of fit for the final PLS-PM was 0.70 explaining between 55% (R^2) to 66% of the observed variability in fibre and protein contents of the chamois diet (Fig. 4.3.4).

From the results of this preliminary work, it is worth highlighting the high positive effect of local weather (temperature and precipitation) ($\lambda = 0.77$, Table 4.3.3) on primary production (EVI and LAI) and positive indirect effects on the nutritional value of the Pyrenean chamois diet through the gain in herbaceous consumption ($\lambda = 0.45$), and in turn their protein contents ($\lambda = 0.47$, Table 4.3.3). As expected, local weather exerted indirect negative effects on fibre content in the Pyrenean

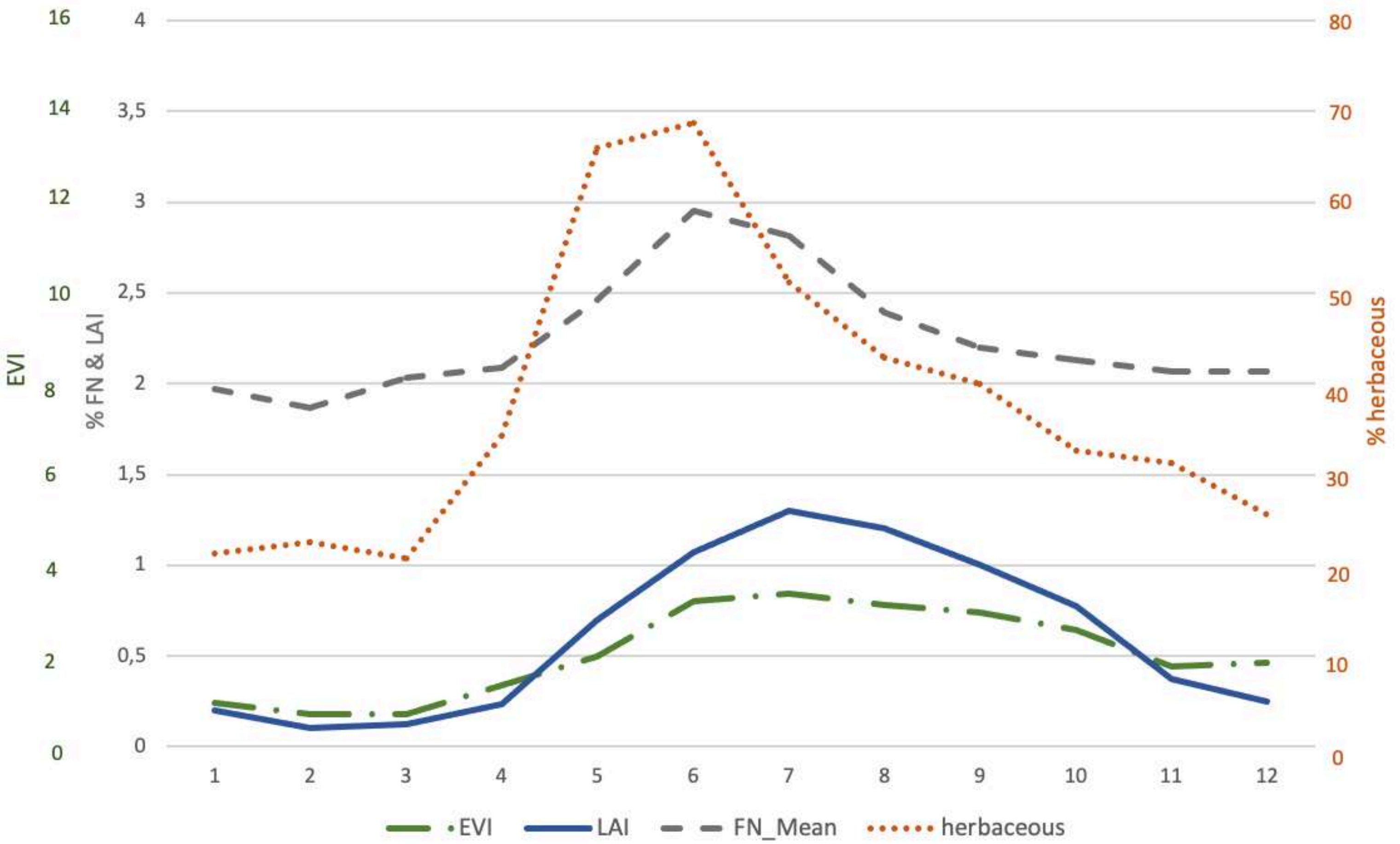


Figure 4.3.3. Primary production (LAI and EVI), diet composition (herbaceous plants %), and quality (fecal nitrogen, FN %) of Pyrenean chamois in the FSNGR. Primary production has been assessed by the LAI (solid line) and the EVI (dashed and dotted line). Diet quality of chamois is represented by the proportion of FN (dashed line), and herbaceous plants in (dotted line). The X-axis shows the months of the year whereas the Y-axis on the left EVI and LAI values. Fecal nitrogen (%) and the proportion of herbaceous plants in the diet have been represented by secondary Y-axes.

chamois diet (λ = - 0.51, Table 4.3.3). Other studies found high correlation values between temperature variables and plant production (R^2= 0.7 to 0.8). (Jonas et al., 2008; Qun et al., 2015 for grasslands and Bertin, 2008 for ecosystems in general). High correlation values between climatic variables and herbivores diet were also found in several works (Langvatn et al., 1996; Mysterud et al., 2003).

Table 4.3.2. Outer model describing the set of manifest variables included in the PLS-PM model for exploring causal relationships among local weather, snow, primary production and chamois' diets. Some manifest variables initially included in the Outer model (e.g., Mean**Pp**) are excluded here due to their low contribution to their respective blocks. Weights are the relative importance of each manifest variable inside a block (e.g., Primary production). Loadings are correlations. A loading of 0.99, that more than 0.99^2 = 98% of the variability in an indicator (e.g., Tmax) is captured by its block. Communality is the proportion of variance in common between the block and a given indicator.

Outer Model	**Weight**	**Loading**	**Communality**
Time			
Years	1	1	1
Local weather			
Tmax	0.5	0.99	0.97
Tmin	0.47	0.99	0.97
AcPp	0.03	0.19	0.04
Snow			
Mean snow accumulation	1	1	1
Primary production			
EVI	0.46	0.97	0.95
LAI	0.55	0.98	0.96
Diet composition			
Forbs	1	1	10.89
Fibre content			
ADF	0.89	0.99	0.99
NDF	0.12	0.89	0.79
Protein content			
FN	1	1	1

The effect of time had a total positive effect on the diet quality of Pyrenean chamois (λ= 0.23 for FN and λ= 0.13 for fibre, Table 4.3.3). These effects were direct (λ= 0.18 for both, FN and fibre, Table 4.3.3, Fig. 4.3.4), and indirect through a slight but positive influence on primary production (λ= 0.08).

Snowfall, however, had a clear indirect negative effect on the nutritional value of chamois diet (λ = - 0.13 for FN and λ= 0.15 for fibre, Table 4.3.3), through its direct influence on primary production (λ = - 0.23) and indirect on the amount of forbs in the diet of this ungulate (λ = - 0.13). Snow typically reduces primary production and plant coverage because of the low temperatures and the prevention of capturing solar radiation energy due to the snow pack. Accumulated snow is expected to decrease (Steger et al., 2013; Xie et al., 2020), increasing the length of snow-free periods. Other studies (Xie et al., 2020), however, point out that variations in melt-out dates do not change plant growth patterns. Jonas et al. (2008) suggest that changes in snow cover would affect plant communities rather than plant productivity, at least in the short or mid-term. A shift in the vegetation onset due to warmer temperatures after an early melt-out may favor fast-growing plants increasing primary productivity of mountain grasslands. Choler (2015) found out that longer snow-free periods increase primary productivity of mountain grasslands and, as we have seen, forage availability for chamois. This increase of primary production may not result in a long-term improvement of diet quality of Pyrenean chamois due to the expected encroachment of the Pyrenean environment (Espunyes et al., 2019b).

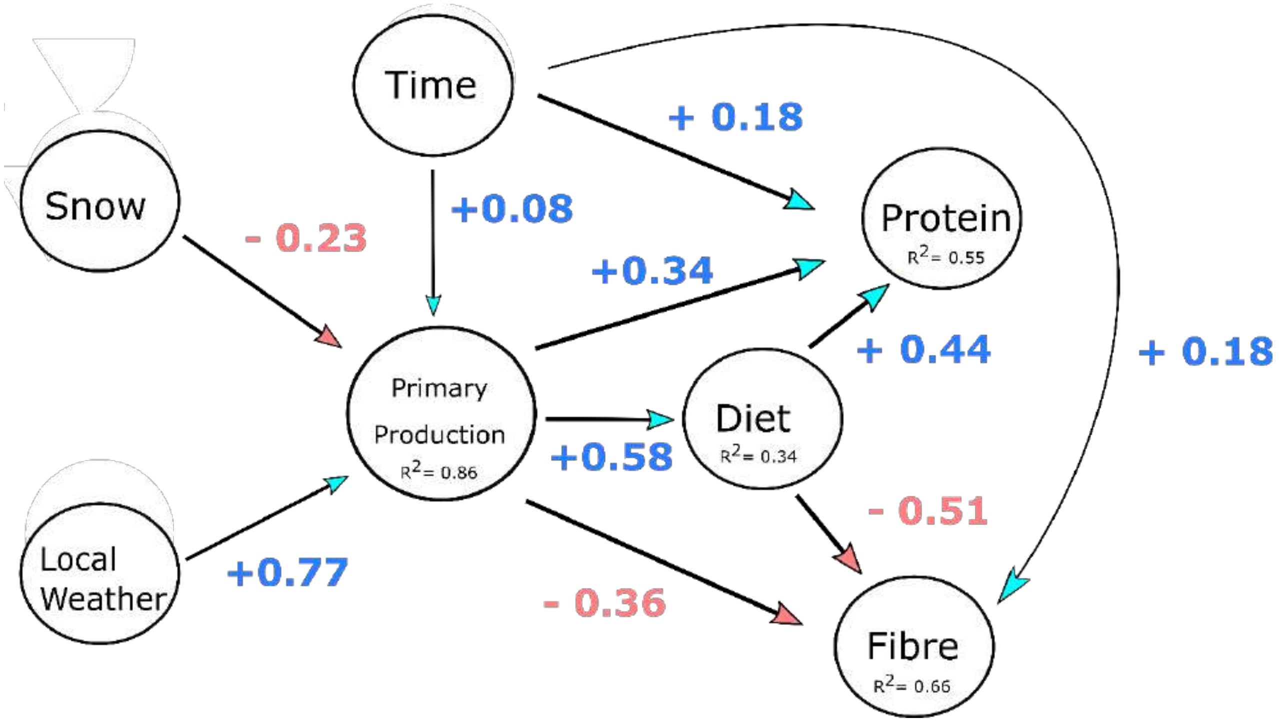
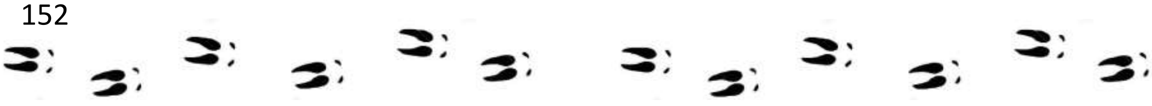


Figure 4.3.4. **Final path model describing** the effects of time, snow and local weather conditions on primary production, diet composition and quality (fibre and protein contents) of Pyrenean chamois. Blue and red arrows and coefficients represent positive and negative associations between latent variables (LV). The R^2 are coefficients of determination for each latent variable and indicate the amount of variance explained by their independent latent variables.

Table 4.3.3. **Direct, indirect and total effects of the PLS-PM** exploring the causal relationships between environmental variation and diet composition and quality of southern chamois. The ns symbol indicates non-significant path coeficients

Relationships	**Direct**	**Indirect**	**Total**	**Inner Model**
Time > Primary production	0.08	0	0.08	t-value = 2.4 p-value = 1.6e-2
Time > Diet composition	0	0.04	0.04	ns
Time > Fibre content	0.18	-0.05	0.13	t-value = - 3.5 p-value = 6.4e-4
Time > Protein content	0.18	0.05	0.23	t-value = 3.1 p-value = 2.5e-3
Snow > Primary production	-0.23	0	-0.23	t-value = - 5.6 p-value = 8.45e-8
Snow > Diet composition	0	-0.13	-0.13	ns
Snow > Fibre content	0	0.15	0.15	ns
Snow > Protein content	0	-0.14	-0.14	ns
Local weather> Primary production	0.77	0	0.77	t-value = - 1.9 p-value = 1.5e-39
Local weather> Diet composition	0	0.45	0.45	ns
Local weather> Fibre content	0	-0.51	-0.51	ns
Local weather> Protein content	0	0.47	0.47	ns
Primary production> Diet composition	0.58	0	0.58	t-value = 8.34 p-value = 8.34-14
Primary production> Fibre content	-0.36	- 0.30	0.66	t-value = - 5.5 p-value = 2.2e-7
Primary production> Protein content	0.34	0.25	0.60	t-value = 4.7 p-value = 5.6e-6
Diet > Fibre content	-0.51	0	-0.51	t-value = -7.8 p-value = 1.74e-12
Diet > Protein content	0.44	0	0.44	t-value = 6.5 p-value = 1.4e-8



Conclusions

In summary, this preliminary work shows how local weather exerted high positive direct effects on primary production (EVI and LAI) and an indirect (and lower) effect on the Pyrenean chamois diet. In the ongoing global warming scenario, temperature increasing while snow cover decreasing. This may lead to higher primary production and higher nutritional value of chamois diets, probably favoring Pyrenean chamois populations. However, these changes would also bring a good opportunity for other ungulate species such as the red deer (*Cervus elaphus*), increasing interspecific competition (Corlatti et al., 2019), in detrimental of chamois populations (Donini et al., 2021). Shrub encroachment, on the other hand, is increasing due to the rise of temperatures and land abandonment (Alados et al., 2018). This process will also result in a negative impact on chamois diets due to the increase of woody plants with a lower nutritional value (Espunyes et al., 2019b). Further studies should be carried to investigate how landscape changes and the interspecific competition with other ungulates will influence the potential advantages of global warming on the diet quality of this mountain dweller.

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5. General discussion

In this thesis, we have explored the relationship between Pyrenean chamois (selected as a model of an alpine grassland ungulate), grasslands and local weather, and the impact they have on alpine ecosystems in a global change scenario, and we have presented an alternative methodology for monitoring the diet composition of ungulates. In this penultimate chapter, we discuss the results obtained in our three articles in a more integrated way.

Global change affects biological and ecological systems worldwide and alpine grasslands are no exception. Global change directly affects grassland vegetation and thus indirectly affects wild animals living in these grassland ecosystems. Warmer temperatures are giving Pyrenean chamois earlier access to high-quality forage (Chirichella et al., 2021; Espunyes, 2019) due to earlier vegetation onsets and to higher forage availability due to primary production increase (**study 3**). In this ongoing scenario of climate change, snow pack decline and earlier snowmelt is predicted. These trends in snow dynamics may also improve primary production and the quality of Pyrenean chamois diet (**study 3**). Even with these increasing trends in amount and quality of forage for Pyrenean chamois, these results must be interpreted with caution when looking for repercussions on Pyrenean chamois population dynamics. Considering a more global context, these changes in grassland ecosystems could drive intraspecific competition. As a mixed feeder, the Pyrenean chamois is well adapted to variations in the seasonal phenology of plants in alpine habitats, but animal densities are important drivers of diet composition and quality. Intraspecific competition deteriorates the quality of the diet of Pyrenean chamois by driving it toward a diet richer in woody plants and poorer in forbs and graminoids (Espunyes et al., 2019a). Consequences of global change other than this intraspecific competition may affect the diet composition and quality of Pyrenean chamois. Plant-animal mismatches (Wilsey, 2018) or shorter periods of availability of high-quality forage (Pettorelli et al., 2007) may affect the diet of Pyrenean chamois.

Beyond global climate change, alpine grasslands are also affected by land-use changes. In the European alpine systems, during the last half of the 20th century, the pressure of herbivory has been receding (Flury et al., 2013; MacDonald et al., 2000; Nagy and Grabherr, 2009). The Pyrenees, in north Spain, are no exception to this trend (Espunyes, 2019; Muñoz-Ulecia et al., 2021). Alpine meadow abandonment drives an increase in shrubification, thus reducing grassland areas (Espunyes et al., 2019b). This decrease in farming activity will result in a lower nutritional value of the alpine meadows probably affecting the diet quality of Pyrenean chamois (**study 1**). Grazing action, even under high pressure, increases biomass production and the quality of plants in alpine ecosystems. The worst-case scenario would be herbivore disappearance and null grazing pressure leading to declines in grassland primary biomass (**study 1**). Grazing is necessary to keep biomass production and nutritional value of alpine meadows.

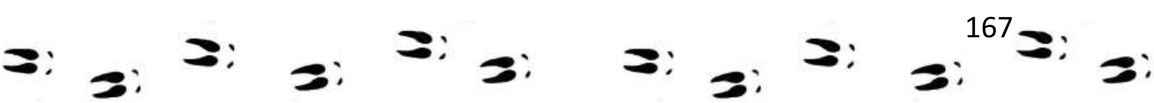
How these climate and land-use changes might affect Pyrenean chamois population dynamics in the long-term remains unknown. Therefore, taking into account that global climate and land use changes affect grasslands ecosystems and that the only factor we really can modify is land use, we suggest that habitat management programs for grassland protection should focus on the preservation of a certain amount of grazing pressure to optimize nutrient value and forage availability. In view of current land abandonment processes and woody plant encroachment, proper management practices are required to maintain this fragile ecosystem. The increase in grasslands' primary production is positive, not only for herbivore conditions and their offspring survival, but also for reducing climate change impacts as long as grassland biomass production contributes to the sequestration of CO_2. To design habitat management programs for grassland protection, further studies should be carried out describing these issues in a warming climate scenario and possible effects on Pyrenean chamois population dynamics more broadly. Pursuing this goal, the long-term assessment of diet composition of Pyrenean chamois is recommended. Fecal NIRS analysis would be suitable to conduct an early discrimination of diet composition in Pyrenean chamois living in contrasted environments (**study 2**). Fecal samples could be used as a forage composition proxy to monitor long-term changes in nutritional condition through changes in the environmental conditions of a given population (Blanchard et al., 2003; Gálvez-Cerón et al., 2013). Managers, with routine sample collection and NIRS analysis, could use dietary composition as a practical, quick and low-cost option to monitor population conditions as a complement to other indicators of ecological change (Morellet et al., 2007; Santos et al., 2014).

6. Conclusions

1. Grazing is necessary to keep biomass production and nutritional value of alpine meadows. The decrease in farming activities will result in a lower nutritional value of the alpine meadows probably affecting the diet quality of Pyrenean chamois.

2. NIRS analysis on fecal samples would be suitable to conduct an early discrimitation of diet composition in Pyrenean chamois living in contrasted environments. The long-term assessemt of diet composition of Pyrenean chamois using NIRS is also recommended.

3. Warmer temperatures have a positve effect on primary production of alpine meadows and on proportion of hebaceous plants in the diet of Pyrenean chamois. In a global warming scenario the long access to high nutritious forrages would have a positive effect of Pyrenean chamois performance at least at short and medium term.



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