Universitat Autònoma de Barcelona

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Informen:

Que la memòria titulada "**Effects of global change on the diet of a mountain ungulate: the Pyrenean chamois**", presentada per **Johan Espunyes Nozières** per a la obtenció del grau de Doctor en Veterinària per la facultat 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.

I perquè així consti als efectes que siguin oportuns, firmem el present informe a Bellaterra, 15 de febrer de 2019.

Emmanuel Serrano Ferron
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Mathieu Garel
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Doctorand

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I will never do a Phd, I am not crazy
Johan Espunyes. ***2014***

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Preface

Global change is having a colossal impact on physical and biological systems worldwide, with models predicting an intensification of these trends over the next century. Dietary studies are not only fundamental to understand the role of species in an ecosystem, but are also critical to predict their sensitivity, resilience and potential adaptations to these changes. However, when assessing the impact of anthropogenic global change, the dietary characteristics of the studied species are frequently ignored.

The main aim of this thesis is to improve the understanding of the effects of global change on the diet of a large alpine herbivore: the Pyrenean chamois (*Rupicapra pyrenaica pyrenaica*). We mainly focus on climate change and habitat change due to their strong impact in alpine ecosystems. This work could not have been accomplished without the collaboration and combined efforts of three actors from different countries with a common goal: the Wildlife Ecology and Health research group (WE&H) on the Spanish side, the Office National de la Chasse et de la Faune Sauvage (ONCFS) on the French side and the Government of Andorra through its predoctoral grant programme.

This doctoral thesis is based on four studies focussed on improving knowledge about the relationship between environmental conditions and resource-use in herbivores living in highly seasonal environments.

In **study 1**, we compare two methodologies—faecal cuticle microhistology and a refined and optimized novel DNA-based approach—to assess diet composition of wild herbivores. We furthermore highlight the limitations of both methodologies in the proper determination of diet composition. In **study 2**, we detail the intra-annual variation in the diet composition of Pyrenean chamois and its strong relationship to primary production trends. In **study 3**, we assess the mechanistic impact of climate change and intraspecific competition on the dietary composition and quality of Pyrenean chamois using a long-term (24 years) direct individual-based dietary monitoring scheme, coupled with multiple environmental databases. Finally, in **study 4**, we explore the effects of habitat change in alpine environments. Specifically, we address whether Pyrenean chamois and free-ranging livestock will be able, to maintain not only their feeding efficiency under varying conditions of resource availability, but also their role in preserving the structure and diversity of alpine ecosystems.

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1.1 Abstract

Herbivores play a fundamental role in maintaining the health and structure of ecosystems worldwide. However, recent evidence indicates that climatic and land-use changes are affecting biological systems across the globe at alarming rates, and more acutely in alpine ecosystems. Thus, predicting the impact of these changes on herbivores has become a key issue for the long-term conservation of ecosystems. Here, our main goal was to assess the impact of climate change and woody plant encroachment on the diet of a large alpine herbivore: the Pyrenean chamois (*Rupicapra p. pyrenaica*).

We firstly compared and highlighted the limitations of two methodological approaches used in the determination of diet composition of herbivores (study 1). We then combined information from two monitored populations of Pyrenean chamois with multiple databases on seasonal phenology, climatic conditions, population abundance and models of habitat evolution to understand understand the effects of climate change and woody plant encroachment on the diet of this herbivore. Our results indicate that Pyrenean chamois is well adapted to the variations in the seasonal phenology of plants in alpine habitats (study 2), but that these patterns can be affected by environmental conditions. For instance, the seasonal presence of livestock may affect the diet of chamois during the co-habitation period (study 2). Concurrently, the variations in the vegetation onset and intra-specific competition are regulating factors of diet quality and composition during spring (study 3). Finally, we observed that woody plant expansion in unmanaged alpine grasslands will also affect wild and domestic herbivores during summer and autumn, but that the magnitudes and direction of these effects will vary depending of their dietary preferences (study 4).

Overall, global changes are impacting the diet of alpine herbivores and could thus impact the performances of these species. We therefore reinforce the importance of integrating dietary studies when assessing species' response to global changes. However, further studies would be necessary to assess the effect of these dietary adaptations on the species' performance.

Keywords: Diet composition, Pyrenean chamois, alpine environment, climate change, woody plant encroachment, primary production, intra- and inter-specific interactions.

1.2 Resum

Els herbívors tenen un paper fonamental en el manteniment de la salut i l'estructura dels ecosistemes a nivell mundial. No obstant, evidències recents indiquen que el canvi climàtic i el canvi en l'ús del sòl afecten els sistemes biològics en tot el món a un ritme alarmant, i de manera més intensa en els ecosistemes alpins. Així doncs, predir l'impacte d'aquests canvis en els herbívors s'ha convertit en un tema clau per a la conservació a llarg termini dels ecosistemes. En aquest tesi, el nostre principal objectiu era avaluar l'impacte del canvi climàtic i l'expansió de les plantes llenyoses en la dieta d'un gran herbívor alpí: l'isard (*Rupicapra p. Pyrenaica*).

En primer lloc, hem comparat i ressaltat les limitacions de dos mètodes utilitzats en la determinació de la composició de la dieta dels herbívors (estudi 1). A continuació, s'ha combinat la informació de dues poblacions d'isards amb diverses bases de dades sobre la fenologia vegetal, les condicions climàtiques, l'abundància poblacional així com models d'evolució del hàbitat per entendre els efectes del canvi climàtic i l'expansió de les plantes llenyoses en la dieta d'aquest herbívor. Els nostres resultats indiquen que l'isard està ben adaptat a les variacions de la fitofenologia estacional en hàbitats alpins (estudi 2), però aquests patrons es poden veure afectats per les condicions ambientals. Per exemple, la presència temporal de bestiar domèstic pot afectar la dieta de l'isard durant el període de cohabitació (estudi 2). Al mateix temps, les variacions en la data d'inici del creixement vegetal i la competència intra-específica són factors reguladors de la qualitat i composició de la dieta durant la primavera (estudi 3). Finalment, hem observat que l'expansió de les plantes llenyoses en els prats alpins també afecta els herbívors salvatges i domèstics durant l'estiu i la tardor, però que la magnitud i direcció d'aquests efectes variaran en funció de les preferències dietètiques de cada espècie (estudi 4).

En general, els canvis globals tenen un impacte en la dieta dels herbívors alpins i, per tant, podrien afectar el rendiment d'aquestes espècies. Per tant, reforcem la importància d'integrar els estudis de dietes a l'hora d'avaluar la resposta de les espècies als canvis globals. Tanmateix, es necessiten estudis addicionals per avaluar l'efecte d'aquests canvis alimentaris sobre el rendiment d'aquestes espècies.

2. General introduction

2.1 About herbivores

> – It is true, isn't it, that sheep eat little bushes?
>
> – Yes, that is true.
>
> – Ah! I am glad!
>
> I did not understand why it was so important that sheep should eat little bushes. But the little prince added:
>
> – Then it follows that they also eat baobabs?
>
> I pointed out to the little prince that baobabs were not little bushes, but, on the contrary, trees as big as castles; and that even if he took a whole herd of elephants away with him, the herd would not eat up one single baobab. The idea of the herd of elephants made the little prince laugh:
>
> – We would have to put them one on top of the other, he said.
>
> **Antoine de Saint-Exupéry.** ***The Little Prince***

In most parts of the world, large herbivores are the most readily spotted and admired fauna in the landscape. They are often the flagship species of protected areas, and play a key role in attracting visitors to natural areas, providing economic contributions from ecotourism (Lindsey *et al.*, 2007; Maciejewski & Kerley, 2014). They are also important in cultural traditions and heritage, such as the reindeer for the Sami people in Scandinavia, the horses for Mongolian nomads and the elephants in Asia. They are even the national symbol of many countries (e.g. the vicuña in Peru or the moose in Sweden).

At the same time, they represent a notable economic resource through recreational hunting and game meat production. In Europe alone, 5.2 million large herbivores are harvested each year, representing more than 120.000 tonnes of meat and a potential revenue of several hundred millions of Euros (Apollonio *et al.*, 2010). In poor rural areas worldwide they also supply meat for subsitance (Milner-Gulland & Bennett, 2003). In the Congo Basin, for example, bushmeat consumption may exceed 1 million metric tons per year and is supplied primarily by ungulates (Wilkie & Carpenter, 1999).

The vast majority of large herbivores currently on earth are ungulates, a very diverse group of around 450 species, that have evolved to thrive in all of the main ecosystems (Fig 2.1; Groves and Grubb, 2011; Ripple et al., 2015). In this work we will use the terms “ungulate” and “large herbivores” interchangeably in referring to herbivorous ungulates.

Fig 2.1. A world full of herbivores. The evolution of large herbivores has produced a fascinating array of species able to survive in extreme habitats and under harsh climatic constraints form the ice and snow-covered high mountains to the torrid deserts, without surface water intake (adapted from Hofmann, 1989).

Ecological role of ungulates

From an ecological perspective, large herbivores are important agents of environmental change and the major drivers of the shape, function and diversity of terrestrial ecosystems (Danell *et al.*, 2006). By grazing, dunging, urinating and treading, they affect the physical and chemical properties of the environment and generate cascading trophic effects across the whole ecosystem (Daskin & Pringle, 2016; Harrison & Bardgett, 2008). While moving and resting, large herbivores tread and trample the vegetation, physically damaging plants and soil but also creating recruitment opportunities for other species (Hester *et al.*, 2006). Through dung and urine deposition, herbivores excrete nutrients in a form readily available for uptake by microbes and plants, accelerating the soil nutrient turnover (Hobbs, 1996). They also play an important role in seed dispersal, enhancing the colonisation of plants (Janzen, 1984). However, the greatest contribution of large herbivores to landscape heterogeneity and plant community dynamics lies in vegetation consumption *per se*. Indeed, vegetation consumption directly leads to important changes in the morphology of individual plants but also affects photosynthesis, respiration and growth rates as well as the resource allocation of plants (Gordon & Prins, 2008).

By creating different pressures on plant species, selective foraging also leads to changes in plant succession rates and therefore habitat composition (Pastor & Naiman, 1992). Plant species can react differently to grazing pressure and some preferred and defoliation-intolerant species can be eliminated from the community while browse-resistant or avoided species can expand (Tremblay *et al.*, 2006). Selectivity also depends on the size of the herbivore, as larger herbivores usually feed less selectively than smaller ones and create different impacts depending on the community composition of the herbivores (Bakker, 2003). As a consequence, these local differences contribute to landscape heterogeneity (Adler *et al.*, 2001; Huntly, 1991; Prins & van Langevelde, 2008). Together, these behaviours have the potential to modify, maintain and diversify terrestrial ecosystems and have led scientists to consider large herbivores as "ecosystem engineers" (Danell *et al.*, 2006; Smit & Putman, 2011).

These mammals even play a critical role in maintaining ecosystem health through strong cascading effects on numerous ecosystem communities and functions (Pringle *et al.*, 2007). The concept of ecosystem health implies a system able to maintain its structure and function over time in the face of external stress (Costanza, 2012). By maintaining natural processes, these animals provide a range of high-value ecological services (*i.e.* direct and indirect benefits that people obtain from the ecosystem), resulting in enormous direct and indirect economic revenues (Millennium Ecosystem Assessment, 2005). But above all, they indirectly contribute to maintaining ecological cycles that support human sustenance, development and health (Ripple *et al.*, 2015).

Altogether, these facts highlight the important ecological, economic and social role of large herbivores. Their study, management and conservation not only affect the ecosystem services they provide, but also have a cascade effect on the whole system by influencing all trophic levels. A scientific understanding of the ecology and dynamics of these populations, and the ecological systems they affect is fundamental to establishing conservation and management policies (Putman & Apollonio, 2014).

Drivers of large herbivores population dynamics

The populations of large herbivores are influenced by biotic and abiotic factors, including climate, pathogens, competitors, habitat composition, water availability, predators, and resource quality, among others. These forces determine population dynamics and community structure. At the same time, the performances and dynamics of large herbivore populations are shaped by a set of fitness components known as "life-history traits" (Stearns, 2000). These characteristics are directly related to the reproduction and survival of a population in a given environment. For example, the age of sexual maturity, the litter size or the fecundity rates are considered reproductive life-history traits while the survival of juveniles or the lifespan are considered life-history traits related to survival.

Understanding the sources of variation of these traits is key to apprehend the functional responses of herbivores and build analytical models that describe and, above all, predict the long-term dynamics of their populations. Using long-term data, several studies have addressed these pivotal questions by focussing on the impact of these traits on the fluctuations of large herbivore populations. For example, Gaillard *et al.* (2000) highlighted that the survival of immatures is a critical component of population growth.

Top-down regulation

The sources of variation in survival performances are age-dependent and there is a distinction between juvenile and adult. Juveniles are strongly affected by year-to-year variation in weather conditions through reduced maternal care or directly by death from starvation or freezing (Scornavacca *et al.*, 2016). Predation pressure, mainly by mesocarnivores, is an additional cause of juvenile mortality of herbivores (Linnell *et al.*, 1995). Indeed, through direct mortality, predation on juveniles, but also on adults, influences the population size of large herbivores and therefore plays an important role in their regulation (Messier, 1994). If we look at the trophic pyramid, with predators on the top and primary producers (vegetation) at the bottom, herbivores are in the central part of the food chain (Fig 2.2.). Therefore, the effect of predators on herbivores is known as a “top-down” effect (Gandiwa, 2013; Smith *et al.*, 2009a).

Historically, it has been assumed that natural carnivores were primarily responsible for the top-down regulation of herbivores. Nowadays, after the continuous alteration and fragmentation of habitats and the hunting, poisoning and trapping of large carnivores at a global scale (Woodroffe, 2000), humans are the species with the greatest impact on large herbivore populations through hunting (for meat, gaming or trophy purposes; through legal regulation or poaching). Indeed, one of the main threats to large (>100Kg) herbivores conservation is overhunting for meat and body parts, followed by livestock competition and habitat loss (Ripple *et al.*, 2015). However, this does not imply that carnivores no longer have an important top-down effect on herbivores. Despite the conservation concerns of large predator species, most herbivores continue to face top-down control by a wide range of carnivores, mainly mesocarnivores (Roemer *et al.*, 2009), but also large carnivores in some protected areas (Smith *et al.*, 2009b). Like predators, some diseases are also important drivers of wildlife population dynamics and performances that can strongly impact populations by causing temporary or permanent declines in abundance (Loison *et al.*, 1996; Smith *et al.*, 2009a). The 1890's rinderpest African panzootic that decimated more than 80% of several large herbivores, such as buffalos, giraffes, antelopes and wildebeest is a clear example of the substantial impact of diseases on herbivore populations. This epidemic even had cascading effects on carnivore populations, savannah ecosystems and the African economy (Mack, 1970).

Bottom-up regulation

While herbivore populations are subjected to this "top-down" regulation, they are also strongly limited by the availability of food resources (Gaillard *et al.*, 2000). Droughts, fires, floods, rainfall variability, and above all, changes in climatic conditions are affecting the resource productivity of the environment. In large herbivores, body weight has a critical impact on reproduction rates as well as juvenile and adult survival (Festa-Bianchet *et al.*, 1997; Gaillard *et al.*, 2000; Hempson *et al.*, 2015). Because body weight is directly linked to resource availability it is clear that nutrient availability has multiple impacts on the majority of herbivore life-history traits (Langvatn *et al.*, 1996). Although body weight can be influenced by other factors such as diseases, gastrointestinal parasitism or temperature (Irvine *et al.*, 2006; Pérez *et al.*, 2015; Saether, 1985), it is forage quality intake that primarily affects the body weight of individuals (White, 1983). Indeed, habitat quality usually correlates positively with body weight in numerous herbivore studies (Mysterud *et al.*, 2001; Pettorelli *et al.*, 2002; Simard *et al.*, 2008). This resource-related limitation is known as "bottom-up" regulation because the regulation originates in the resource flow from lower trophic levels (Turkington, 2009).

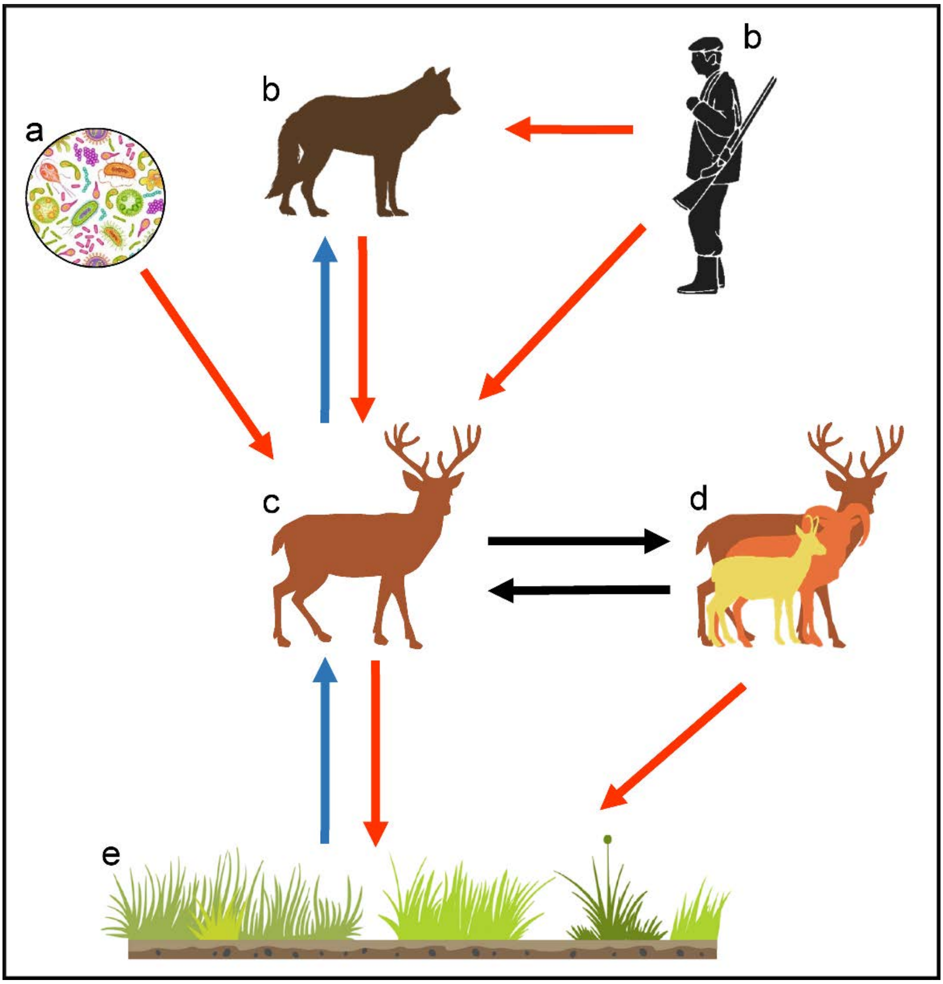


Figure 2.2: Trophic interactions in a classic carnivore-herbivore-plant food web. Top down effects are represented in red and bottom-up effects are represented in blue while transversal effects of inter- and intra-specific competition are represented in black. (a) diseases, (b) predators, (c) focal herbivore, (d) intra- and inter-specific competitors, (e) resource.

Because the resource availability is controlled by its quality but also by its quantity, this regulation is also negatively related to the density of herbivores. Individuals from the same species or from different species can compete for resources when they are in limited supply for both. Usually, increasing intraspecific competition for food when resources are limited results in a decrease in body mass affecting, in turn, individual performance by lowering survival and reproduction (Bonenfant *et al.*, 2009). Interspecific interactions, on the other hand, can cause lower densities or even the complete exclusion of one herbivore from its optimal habitat (Latham, 1999). This competition can be caused directly by the two species preventing each other from accessing the resource by physical displacement or grazing behaviour alterations. This phenomenon is called "interference competition" and is usually reported between free-ranging livestock and wild herbivores as, for example, between cattle and elk or between sheep and chamois (Chirichella *et al.*, 2013; Skovlin *et al.*, 1983). Another type of interaction, called "resource interaction", occurs indirectly when the amount of resource available for one species is reduced due to its use by another. When native wild ungulates have to coexist with livestock, it is usually the wild counterpart that ends up disadvantaged (Chirichella *et al.*, 2014).

"Why is the world green?"

For decades, ecologists have debated which of these two forces—bottom-up or top-down—is stronger in keeping herbivores in check so they do not damage their resources (Kay, 1998; Turkington, 2009). In 1960, Hairston *et al.* hypothesised that the world is "green" (*i.e.* dominated by plants) because herbivores are controlled by predators and pathogens. Without this top-down regulation, they would increase in numbers to such levels as to deplete all vegetation. However, this simple model has been strongly criticised (Murdoch, 1966). An opposing hypothesis has argued that the world is green because most plants provide inadequate nutritional requisites or defend themselves with indigestible components such as tannins and lignin or physical structures such as spines and prickles (Polis, 1999).

Despite the lack of consistent experimental approaches, most of the published literature now agrees with the premise that both processes act simultaneously on the dynamics of large herbivores (Hunter & Price, 1992; Turkington, 2009). The relative importance of these forces, however, is hard to assess because they are usually interrelated through trade-offs and feedbacks and above all, these processes are species- and environment-dependent. Studies in the Serengeti ecosystem have shown that the relative importance of these processes can be species-dependent because body size simultaneously affects the nutritional demand and the risk of predation. Larger herbivore species require greater resource biomass and are therefore more constrained by bottom-up processes of resource availability, whereas smaller species are more affected by predation and food quality (Hopcraft *et al.*, 2010). However, in high-latitude ecosystems, with typically few herbivores and even fewer predators (compared to tropical savannah and grasslands where as many as ten predators can coexist with tens of herbivore species), it is mostly bottom-up control that dominates the population dynamics of large herbivores (Sinclair, 2003). Food and nutrient availability influences most aspects of animal ecology and are ultimately the drivers of the reproductive cycle and survival of ungulates (Parker *et al.*, 2009).

Identifying the factors that explain changes in the performances and dynamics of a population is a crucial topic in ecology for understanding how communities are structured. This information is also becoming critically important as humans are drastically altering ecosystems around the globe. Human-induced global change is causing the loss of species diversity and is changing the intensity of top-down and bottom-up regulation in ecosystems worldwide (Ceballos *et al.*, 2015; La Pierre & Hanley, 2015). Understanding how ecosystems work is therefore a primary requisite to predicting the potential effects of these large-scale anthropogenic changes on ecosystem functioning.

2.2 About the importance of diet

> "For any frustrated field-mammalogists seeking observational data on their elusive study animals, dung may represent the most readily-available and easily-collected source of information upon which they may fall back in despair"
>
> **Rory Putman**, ***Facts form faeces***

Diet is a central concept in bottom-up regulation and herbivore species have adapted their physio-ecological characteristics to the forage available in their environment (Janis, 2008). The diversity of morphological, physiological and ecological adaptations of large herbivores contribute to the differentiation of their feeding niche, which explains their diversity and coexistence in a world of limited resources (Kartzinel *et al.*, 2015). Understanding species-specific feeding behaviours may allow us to explain dietary differentiation and environmental adaptations but also predict species' sensitivity to environmental change as well as their resilience and potential adaptations to these perturbations (Huey *et al.*, 2012).

Classification of herbivores

Large herbivores have been classified into three feeding categories based on their natural diet: browsers, which feed mainly on buds, stems, leaves and soft shoots of woody plants such as shrubs; grazers, which feed mainly on grass or other low vegetation; and mixed or intermediate feeders which feed on a mixture of both grasses and browses (Hofmann, 1989). This classification, based on the resources used, is useful and widely employed but also considered simplistic and limited (Clauss *et al.*, 2003). It does not include frugivors as an independent category (Bodmer, 1990) and the boundary of "mainly" is heterogeneous between studies as some researchers set the threshold at 75% (Mendoza *et al.*, 2002) and others at 90% (Hofmann & Stewart, 1972). Most grazers and mixed feeders have hypsodont teeth, a large mandibular size and long mandibular diastemata while browsers are usually brachydonts with small mandibles and short diastemata (Mendoza *et al.*, 2002).

However, aside from this resource-use classification, the world of large herbivores is much more complex and can be differently discriminated if we focus on the morpho-physiological adaptations of their digestive system. A first classification is based on their digestive process (Feldhamer *et al.*, 2015). Some herbivores are hindgut fermenters (e.g. equids) and others are foregut fermenters, also known as ruminants (e.g. cattle, sheep). Subsequently, hindgut fermenters are divided into colonic fermenters, which tend to be larger species such as horses or elephants, and caecal fermenters, which are smaller animals such as rabbits and rodents (Feldhamer *et al.*, 2015). Colon fermenters are able to retain and digest large particles over a long period of time, resulting in high fibre digestibility while caecum fermenters are able to selectively retain solutes and fine particles in the caecum while eliminating large, hard-to-digest particles (Barboza *et al.*, 2009a).

Ruminants can also be divided based on their forestomach morpho-physiology and are classified on a gradient from “cattle-type” to “moose-type”. The cattle-type species present a voluminous and muscular forestomach and a stratification of its contents allowing them to efficiently digest highly fibrous diets such as grass. The forestomach of the moose-type is less adapted than the cattle-type to digesting fibre (Clauss *et al.*, 2003). Their rumen content is much less stratified and they lack strong reticulorumen muscles. Such ruminants are typically browsers like the roe deer (*Capreolus capreolus*) or giraffids. However, among cattle-type ruminants, the degree of this morpho-physiologic adaptation is not clearly related to the percentage of grass in their natural diet (Codron & Clauss, 2010). In fact, several cattle type species such as European bison (*Bison bonasus*) or muskoxen (*Ovibos moschatus*) ingest significant amounts of woody plants (Clauss *et al.*, 2010).

Patterns of resource use

When feeding, herbivores face a landscape of highly variable nutritive quality known as a “foodscape” (Searle *et al.*, 2007). This foodscape is determined by species’ requirements, their foraging abilities, and the availability of resources. At the same time, herbivores must maximise their energy intake while minimising the costs related to searching and digesting food, following the “optimal foraging theory” (Pyke, 1984). They select plant species and parts with the highest energy return per time unit based on the availability across vegetation communities and their feeding adaptations. As forage quality and quantity is not spatially homogenous across habitats, animals should only select a more distant feeding patch when the travel costs are less than the energy gained, leading to a trade-off between maximising diet quality and patch departure (Fryxell, 1999; Searle *et al.*, 2005). Moreover, this decision is also affected by the temporal variability in forage quality and quantity (Albon & Langvatnt, 1992; Pettorelli *et al.*, 2005a), the inter- and intra-specific interactions and the risk of predation as previously explained. In addition, feeding behaviour and diet selection can be modified by individual determinants such as the reproductive status, sex or age (van Beest *et al.*, 2011; Froy *et al.*, 2018; Ingvartsen & Andersen, 2000). Habitat selection is therefore based on a range of scale-dependant decisions made at all levels in the foraging and ecological hierarchies, from the plant species consumed to the total landscape (Johnson, 1980; Senft *et al.*, 1987; Zweifel-Schielly *et al.*, 2009).

Why do dietary studies matter?

Even though studies on large-scale habitat selection have shown that seasonal space use is closely linked to resource quality (Bischof *et al.*, 2012; Pettorelli *et al.*, 2005a; Zweifel-Schielly *et al.*, 2009), it is crucial to study fine-scale dietary selection to understand the constraints related to food resources and habitat selection (Hebblewhite *et al.*, 2008).

A precise knowledge of the diet can, for example, identify key resources for a particular herbivore and therefore, aid in assessing the suitability of the habitat. This information is crucial when planning habitat management or endangered species policies. For example, studying the winter diet of woodland caribou has highlighted the importance of maintaining mature forest over managed forest due to their high consumption of lichen species, which are sensitive to herbicides used in forest management (Newmaster *et al.*, 2013a). Studying the variations in diet composition also allows an understanding of how herbivores cope with seasonal environmental constraints in food availability. The relative availability of resources along the seasons greatly influences the patterns of diet composition of herbivores and their seasonal habitat selectivity that will, in turn affect their movements, behaviour and performance (Mysterud *et al.*, 2011; Zweifel-Schielly *et al.*, 2012). Above all, understanding these resource utilisation patterns at the finest scale as well as the factors driving these patterns is not only fundamental to understanding plant-herbivore interactions, but also pivotal in understanding the mechanical links between variations in diet, environmental conditions and herbivores performance. In turn, the comprehension of these pathways is critical to studying current impacts of environmental change, predicting the vulnerability and response of organisms and modelling the distribution of biodiversity under future conditions (e.g. Thuiller et al., 2017).

2.3 About highly seasonal environments

> “In the lair under the uprooted old spruce, a heart beats faintly but steadily as the season marches on. All is quiet now, yet there is so much life in that still, dark hulk. Soon, barely waking, she will give birth to two or three cubs that will suckle in their mother’s warmth until they are as full of energy as the sunlight bouncing off the latewinter snow. It is just one of many landmark events passing unnoticed in a natural world that has paused from its usual business to carry out a few of its more important acts in relative privacy.”
>
> **Peter J. Marchand, *Life in the Cold: An Introduction to Winter Ecology*.**

Herbivores living in a highly seasonal environment such as the alpine region or the arctic tundra must cope with a wide range of environmental conditions that creates a seasonal cycle in vegetation growth. Winters are characterised by environmental constraints with important snow cover and reduced solar radiation resulting in cold temperatures and food limitations. Snow depth conditions and severe winter weather can have negative effects on recruitment and mortality (Garrott *et al.*, 2003; Gonzalez & Crampe, 2001; Mech *et al.*, 1987; Solberg *et al.*, 2008). In spring, the increase in incoming solar radiation induces snowmelt and the onset of the vegetation growing season (Billings, 1973) but due to low mean temperatures, plant growth and production are limited and this phenological period can be relatively short. After this brief green-up, a warm summer sets in, inducing a senescence period for plants until the first snow falls (Ernakovich *et al.*, 2014).

Special biological adaptations

Herbivores encounter special vegetation adaptations to life in these harsh environments. Plants present numerous processes and structures that minimise the impact of climatic stress, frost damage, snow cover and solar radiation. For example, deciduous plants avoid exposure to freezing winter temperatures through a short leaf life span coupled with the short spring (Kӧrner, 2011) and thus are only available for a short period of time. Evergreen plants, however, acquire frost resistance mechanisms such as cell wall impregnation with lignin or increasing phenol deposition (Neuner, 2014). As a result, these plants are hardly digestible for herbivores. Another large group of plants are perennial herbaceous plants that thrive as mats and cushion-like structures, an advantage in protecting their buds from environmental conditions but also in avoiding herbivory (Kӧrner, 2011). As a consequence, protein content, in terms of nitrogen/fibre ratios, peaks at early phenological growth stages (Mysterud *et al.*, 2011). Therefore, like climatic conditions, plant availability and digestibility also show a strong annual variability in these environments.

On the herbivore side, other types of strategies have been adopted in order to deal with these seasonal and spatial differences in forage availability that can strongly influence diet selection and energy intake (Goodson *et al.*, 1991). Some smaller species avoid energy expenditure during periods when sufficient food is unavailable by means of hibernation (e.g. alpine marmot *Marmota marmota*; Benedict and Lee, 1938). Others prefer to leave these particular environments during winter and move to other areas where resources are more available, performing long migrations as in the case of elks (*Cervus canadensis*, Houston, 1982). By migrating between summer ranges at high altitudes or latitudes and winter ranges at lower altitudes or latitudes, herbivores exploit the resources in one seasonal range while avoiding resource deficits in the other (Alerstam *et al.*, 2003). At the same time, during the migration to summer ranges as snow melts and new forage emerges, herbivores follow a wave of vegetation growth and therefore high-quality forage, a phenomenon known as "surfing the green wave" (Rivrud *et al.*, 2016).

Seasonality of life history traits

For those that do not rely on these survival strategies, extreme resource compensation tactics are necessary to survive over winter. During this season, food supply becomes severely restricted and body fat levels obtained during spring, summer and autumn serve as capital as energetic demands cannot be met by foraging alone (Mautz, 1978; Parker *et al.*, 1999). At the same time, harsh weather conditions and snow depth augments energetic costs of locomotion (Dailey & Hobbs, 1989) and thermoregulation (Signer *et al.*, 2011), and strongly influences body condition and energy balance (Douhard *et al.*, 2018; Garroway & Broders, 2005). As a consequence, the plant growing season (*i.e.* green-up period; Tveraa *et al.*, 2013) is the key season for food intake in these regions and consequent body growth, maintenance and reproduction for large herbivores (Fig. 2.3; Pettorelli et al., 2007; Hamel et al., 2009). The nutritional intake is used to restore the body weight lost during winter and to attain sufficient mass to enable the posterior winter survival. This is especially important in juveniles because they have limited body reserves and higher metabolic demands than adults. In fact, their survival rates during winter are lower than adults as they are more susceptible to harsh conditions (Loison & Langvatn, 1998).

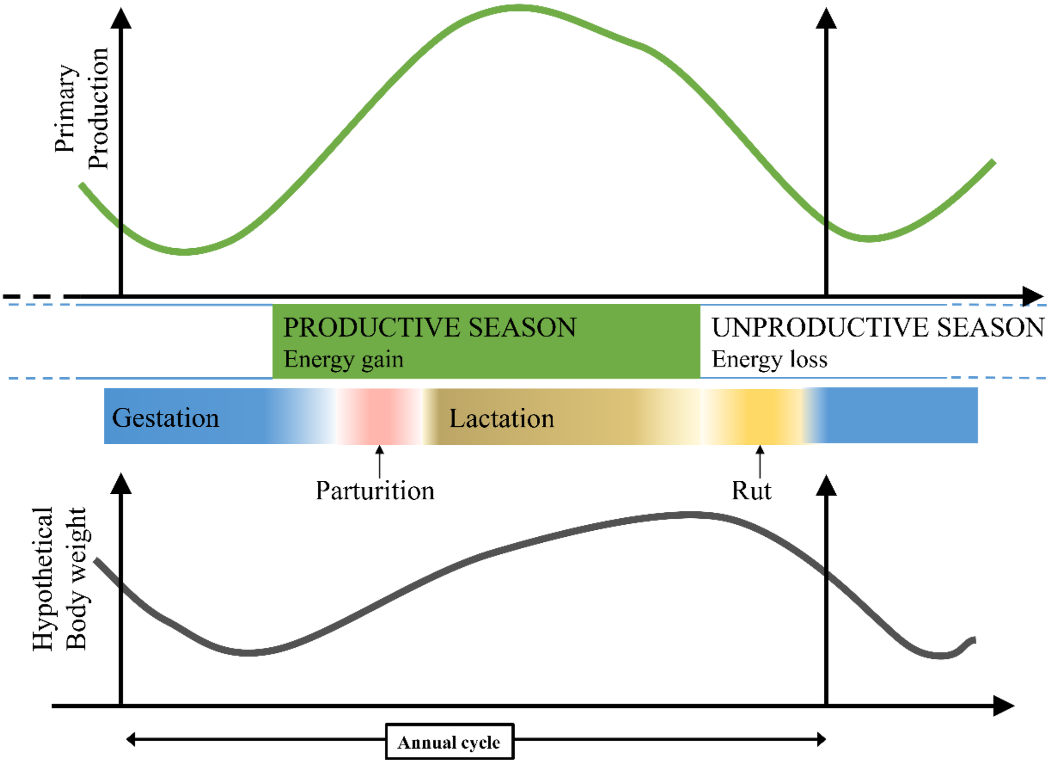


Figure 2.3. Life-history traits in seasonal environments. Schedules of growth, gestation, parturition, lactation and rut season of a capital breeder in a seasonal environment. All of these life-history traits are synchronised with the phenology of primary production (Adapted from Leader-Williams & Ricketts, 1982; Pettorelli, 2013; Varpe, 2017).

In addition to survival, maternal care and reproductive tactics are also greatly influenced by seasonality in these environments. In fact, seasonality is considered an important selective force that shapes the scheduling of annual activities and the developmental stages of organisms (Lindstedt & Boyce, 1985; Varpe, 2017). Herbivores usually time their parturition to coincide with patterns of plant phenology when resources are most abundant or of highest quality (Post, 2003). Furthermore, depending on their reproductive technique, ungulates can be classified on a capital-income breeder continuum. Capital breeders rely heavily on stored resources (*i.e.* body fat reserves) to produce and raise their offspring, decoupling feeding and reproduction. Income breeders, on the other hand, produce and raise offspring using food acquired during the same reproductive period, with the disadvantage of less temporal and spatial flexibility, but no costs of building and carrying body storage (Jönsson, 1997; Varpe, 2017). In highly seasonal environments, ungulates are considered capital breeders, such as the bighorn sheep (*Ovis canadensis*; Festa-Bianchet *et al.*, 1998), the Alpine ibex (*Capra ibex*; Toïgo *et al.*, 2002) and the reindeer (*Rangifer tarandus*; Reimers, 1983). As a consequence, reproduction is highly dependent on female body condition and thus on environmental conditions preceding birth (Toïgo *et al.*, 2002). When resources are scarce, females may even reduce resource allocation to foetus growth and maternal care to favour their own mass gain (Festa-Bianchet & Jorgenson, 1998). Consequently these thinner offspring will have a lower probability of survival because winter survival is correlated with summer mass (Douhard *et al.*, 2018; Lubow & Smith, 2004).

This thesis specifically focuses on alpine ecosystems, a scenery of high conservation priority in Europe (Council of the European communities, 1992). The alpine ecosystems are found in all the great mountain ranges worldwide. These ecosystems cover only 3% of the terrestrial land area but include many biodiversity hotspots and an important number of endemic species (Ko̎rner, 2011). Locally, as in Europe, they can represent one of the richest plant species communities as they comprise 20% of the native European flora, but cover only 3% of the entire continent (Väre *et al.*, 2003). In addition they are a key habitat for a large number of alpine-specialised species such as the Alpine ibex (*Capra ibex*), the rock ptarmigan (*Lagopus muta*) and the snow vole (*Microtus nivalis*). These species are especially vulnerable to variations in their environmental conditions due to their specific habitat requirements, seasonal adaptations, narrow distribution range, reduced population sizes and limited distribution capacity (Nagy & Grabherr, 2009). Ongoing global anthropogenic change is currently affecting plant phenology, species distribution and the geographical location of suitable habitats for animal species in alpine environments (Chen *et al.*, 2011; Morrison *et al.*, 2018). As a consequence, predicting the potential effects of global change on biodiversity has become a crucial field of research with high potential for conservation of alpine species (Guisan *et al.*, 2013; Thuiller *et al.*, 2005b, 2005a).

2.4 About global changes

> "The warnings about global warming have been extremely clear for a long time. We are facing a global climate crisis. It is deepening. We are entering a period of consequences"
>
> **Al Gore, *National Sierra Club Convention, 9 September 2005***

Climate change in mountainous ecosystems

The Intergovernmental Panel on Climate Change (IPCC) was established in 1988 to assess specific and global impacts of climate change. Through this panel, thousands of experts have unequivocally documented the widespread impact of anthropogenic climate change in physical and biological systems on all continent and across the oceans (IPCC, 2014). The alpine habitat, being on the coldest margins of life on Earth, is one of the habitats most vulnerable to rapid climate change (Ernakovich *et al.*, 2014).

Temperatures have increased 1.2°C in mountain ranges in the last century (Büntgen *et al.*, 2017a; Cannone *et al.*, 2007) and will continue to do so, as shown by projection scenarios (Loarie *et al.*, 2009; Nogués-Bravo *et al.*, 2007). This climate warming is having alarming consequences on biological systems worldwide and affecting a large range of species. Researchers have observed an advancement of spring events such as bird breeding (Tomotani *et al.*, 2018), migration (Charmantier & Gienapp, 2014), flowering (Franks *et al.*, 2007) and leaf unfolding (Menzel, 2000); species composition alterations (Walker *et al.*, 2006); and upward elevational range shifts of plants (Lenoir *et al.*, 2008) and animals (Büntgen *et al.*, 2017b; Wilson *et al.*, 2007). A recent meta-analysis estimated that during the last half-century, the distribution of species shifted to higher elevations at a median rate of 11m per decade, and to higher latitudes at a median rate of 17km per decade (Chen *et al.*, 2011). In mountain regions, however, it is likely that several mammals will be unable to undergo range shifts and colonise new areas to reduce climate change effects, highlighting a potential risk of biodiversity loss (Loarie *et al.*, 2009; Morrison *et al.*, 2018).

The warmer conditions at the end of winter are advancing the onset of plant growth and lengthening the growing season, therefore shortening the winter period of fasting (Post & Stenseth, 1999). In the Northern hemisphere, for example, the average beginning of the growing season has advanced by around 10 days in the last few decades with a variability between spatial and temporal projections (Chmielewski & Rötzer, 2002;

Linderholm, 2006; Park *et al.*, 2016). This earlier access to high-quality vegetation and longer forage availability positively influence the body mass of individuals before facing winter scarcity (Garel *et al.*, 2011; Herfindal *et al.*, 2006; Pettorelli *et al.*, 2005b). However, surprisingly, the dynamics of body size are not always positively affected by environmental variation. Studies on Soay sheep (*Ovis aries*) in Scotland have shown that lambs and adults are lighter and smaller today than 30 years ago. The warmer temperatures and milder conditions allow lambs to grow slower and survive winter without storing as much fat as before (Ozgul *et al.*, 2009). Similar declines in juvenile body mass have been detected in a population of Alpine chamois (*Rupicapra rupicapra*) but this was attributed, at least in part, to increased thermoregulatory demands and behavioural changes due to the higher temperatures in summer (Mason *et al.*, 2014).

These shifts in forage phenology may also impact species that synchronise parturition with the peak of vegetation flush. However, the degree of this impact will vary depending on whether the species relies on dietary income or stored capital to reproduce and if it is able to modify its gestation length (Clements *et al.*, 2011). As a consequence, reproductive success in income breeding roe deer, for example, has declined due to a mismatch between the two periods, triggering a decrease in early survival (Plard *et al.*, 2014). On the other hand, capital-breeding muskoxen are relatively unaffected by this mismatch as they use their own energetic reserves acquired prior to reproduction to invest in offspring production (Kerby & Post, 2013).

Human impact in mountainous ecosystems

In discussing global change, we refer not only to climate change and global warming, but also to effects of land transformation. In fact, global change involves several planetary-scale changes in the natural cycles due to human influences, all with important ecological consequences (Vitousek, 1994). Mountainous habitats are sensitive to climate change, but today land-use change appears as the major driving force of change in landscape patterns and in ecosystem functions and dynamics (Ameztegui *et al.*, 2016; Cernusca *et al.*, 1996; Prévosto *et al.*, 2011). For centuries, humans have inhabited the European mountains and have modified the environment to adapt it to their needs, using the natural resources of these areas (pastures, firewood, food, etc.). Although humans have been present in mountainous areas since the Palaeolithic (Before 9000AC; Bahn, 1995; Fedele, 1990), their presence in the alpine regions intensified after the last glaciation with the beginnings of agriculture (Chocarro *et al.*, 1990). Much later, during the Middle Ages and until recently, the use of mountains was exacerbated due to an increase in population size and the development of cultivation and stockbreeding. At that time, the economy of mountain regions was based on subsistence farming. Agricultural and pastoral lands were created using massive forest clearings (Chocarro *et al.*, 1990) and summer pastures at higher altitudes were developed. This long-term human impact contributed to the creation of rich, complex and contrasting semi-natural habitats of great ecological and cultural value, such as alpine meadows and grasslands (MacDonald *et al.*, 2000). At the same time, pastoralism was intensively developed in these regions (Chocarro *et al.*, 1990; Segard, 2009). This mobile system of livestock husbandry was characterised by the fact that livestock keepers in mountain regions moved their flocks, mainly composed of sheep and goats, between the lowland meadows in winter and the high-altitude pastures in summer, on a yearly basis (*i.e.* vertical transhumance; Dong 2016). Summer pastures are particularly sensitive to these dynamics since they depend on moderate grazing to maintain species diversity and succession patterns (Galop *et al.*, 2011; Loydi *et al.*, 2012; Wu *et al.*, 2011).

Land-use changes in Europe

However, during the mid-20th century, socio-economic changes in Europe led to rural outmigration, depopulation and a rapid decline in human activities in mountainous regions (Flury *et al.*, 2013; MacDonald *et al.*, 2000). Mountain production changed from self-sufficiency towards market production and farming became less economically viable and disadvantaged in a globalised market (MacDonald *et al.*, 2000). Therefore the portion of the population employed in agriculture dropped drastically. Mountain agriculture underwent considerable changes that led to farm and land abandonment and a general decrease in livestock densities. Transhumance slowly disappeared and shifted to a system in which animals are kept in stalls during winter, with the subsequent need to reduce flock numbers. Sheep were also gradually replaced by cattle due to their less demanding management requirements and a better suitability to the actual socio-economic circumstances (a review of these causes can be read in: Garcia-Ruiz and Lasanta-Martinez 1990). Mountainous areas were released from agriculture and deforestation pressure and became available for trees and shrubs re-growth, modifying the land cover and the mountain ecosystems (Gellrich *et al.*, 2007). Even though global warming is having a dramatic effect on landscape patterns, land-use changes are considered to be the major driving forces of these alterations (Ameztegui *et al.*, 2016; Cernusca *et al.*, 1996). The structure and diversity of the vegetation have been affected by successional processes involving shrub encroachment—also known as shrubification (Mod & Luoto, 2016)—of open areas as well as forest expansion and densification (Améztegui *et al.*, 2010; Prévosto *et al.*, 2011). Broadly, in a first stage, herbaceous areas are progressively colonised by shrubs, which are then slowly substituted by trees in a second successional stage, with the exception of the areas above the tree line ecotone, which are only affected by the first stage (Wallentin *et al.*, 2008). However, because the natural location of the tree line is closely related to climatic factors (Körner & Paulsen, 2004), the current increasing temperatures favour the upward shift of the potential tree line range (Case & Duncan, 2014). Therefore, land use abandonment and climate change are leading to a transformation of the landscape in European mountains, characterised by the domination of homogeneous shrub and forest landscapes (Kulakowski *et al.*, 2011).

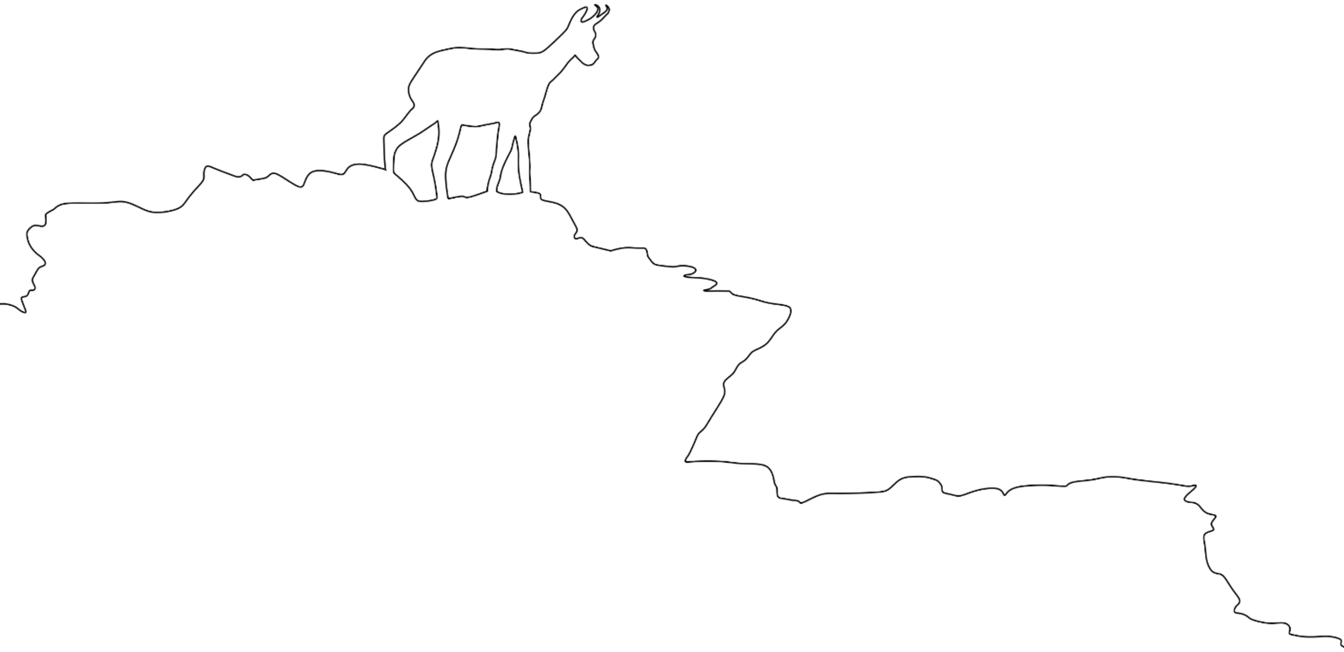
This phenomenon of woody-plant expansion is common over several other of the world's grassland ecosystems, such as the African savannahs (Roques *et al.*, 2001), the North American grasslands (Van Auken, 2000), the Himalayan meadows (Brandt *et al.*, 2013) and the Arctic tundra (McLaren *et al.*, 2017). The causes, however, vary depending on the environment, and overgrazing by domestic livestock, changes in fire frequency or invasions by alien plant species are additional triggers of shrub establishment worldwide (Van Auken, 2009; Lesoli *et al.*, 2013; Roques *et al.*, 2001). This phenomenon generally induces a decrease in plant species diversity (Koch *et al.*, 2015; Ratajczak *et al.*, 2012), an alteration of the community structure and composition (Costello *et al.*, 2000) and a reduction in the productivity of the environment (Lett & Knapp, 2003). Grasslands are among the most plant species-rich habitats in the world (Wilson *et al.*, 2012) and they support a large variety of mammals, birds and insects. However, several studies showed that mammalian diversity and total abundance are negatively correlated with woody-plant encroachment (Stanton *et al.*, 2018). Regrettably, these encroachment trends will manifestly continue as models predict a continuous increase in temperatures (López-Moreno *et al.*, 2008) and a decline in rural agro-pastoral activities (Mann, 2013; Verburg *et al.*, 2010). Nonetheless, the established link between shrub encroachment and ecosystem degradation is not universal and can exhibit variable and mixed responses depending on the examined variable or the specific ecosystem (Eldridge *et al.*, 2011).

Because resource quality and availability affects the mass gain and survival of ungulates (Garel *et al.*, 2004; Parker *et al.*, 2009), we may expect that habitat change also impacts these phenotypic traits. However few studies have documented these effects. For example, Garel *et al.* (2007) reported a decline in lamb body mass of mouflons linked with habitat closure by shrubification. Some studies also showed a significant reorganisation of the ungulate assemblage composition with densities of grazers being negatively affected by shrub encroachment (Ben-Shahar, 1992; Smit & Prins, 2015; Soto-Shoender *et al.*, 2018). However, these same results are context-dependent because shrub cover can also be used by grazers to avoid predation risk effects (Schwarz *et al.*, 2018).

Therefore, food and the herbivore-plant interactions are the main links between global changes and animal performance. Studies focussing on the impact of those changes on feeding behaviour are thus crucial to understanding how large herbivores are responding to these global changes in climatic conditions and vegetation structure.

3. About our study model: the Pyrenees and the Pyrenean chamois

> “Qu'il fait bon s'endormir
> Au refuge le soir
> Près du feu qui s'éteint
> Au pays des isards”
> **Edmond Duplan – *Le refuge***



The Pyrenees: a paradigmatic example of global change

The Pyrenees are a mountain range in southwest Europe, stretching along 430Km from the Atlantic Ocean on the west to the Mediterranean Sea on the east, forming a natural border between France and Spain. Despite an average altitude of 1,067 m.a.s.l., they include 212 peaks over 3,000 m.a.s.l. with the highest point being Aneto Peak at 3,404 m.a.s.l., in the Central Pyrenees. The north-west side, with an oceanic influence, is characterised by greater precipitation, humid conditions and a smaller temperature range than the Mediterranean south-eastern area which is mainly characterised by warm summer droughts. The central sector, with a continental influence, is affected by colder and drier weather.

The Pyrenees represent a natural laboratory for ecological research on mountain fauna as a result of the large variety of habitats and the profound impact of glacial and interglacial periods (Valbuena-Ureña *et al.*, 2018). They host a large number of emblematic mountain species and subspecies—many endemic to the Pyrenees—such as the Pyrenean newt (*Calotriton asper*), the Bearded vulture (*Gypaetus barbatus*) and the Pyrenean desman (*Galemys p. pyrenaicus*). However, in the last few decades, the Pyrenees has undergone rapid changes in environmental conditions. Indeed, the average yearly temperature has experienced a clear and unprecedented increase of 1.2ºC in the last six decades (OPCC-CTP, 2018). Regarding seasonal temperatures, the more drastic increase has been detected in summer. At the same time, there is an overall decrease in winter and summer precipitation despite some regional and seasonal variability (Vicente-Serrano *et al.*, 2007). As a consequence, snow accumulation has also decreased in the last several decades, but with a variable signal in the last two decades due to an important interannual variability of the Atlantic North Oscillation (OPCC-CTP, 2018). Alarmingly, climate change projections in the Pyrenean Mountains reveal that minimum and maximum temperatures will continue to increase through the rest of the century (OPCC-CTP, 2018).

Additionally, this region is one of the clearest examples of rural depopulation and land abandonment in mountainous areas. In the last century, rural populations have declined sharply in this mountain range, reaching even 94% depopulation in some valleys (Lasanta-Martínez *et al.*, 2005; Metailié & Paegelow, 2005). This rural exodus, coupled with a decline in traditional practices is leading to a drastic reduction in livestock densities and the abandonment of cultivated farmlands and traditional land-use practices such as logging. Lasanta *et al.* (2005) estimated that in the Borau Valley (Spanish Central Pyrenees), sheep flock size was reduced by 85% and farmlands were reduced by 82%, simultaneous to a population decline of 84%. Gartzia *et al.* (2016) detected a similar trend in sheep flock reduction (-79%) and a substitution by cattle herds (+120%) in the Pyrenean municipalities of Alto Gállego and Sobrarbe Counties (Spanish Central Pyrenees).

Both of these major changes (climate and land use) are contributing to a shift in the composition of the vegetation in mountainous areas. Indeed, Gartzia et al. (2014) indicated that more than 20% of summer grasslands between 1,980 and 2,000 m.a.s.l. are covered by woody plants in the Central Pyrenees. Simultaneously, 35% of previous shrubby areas are now occupied by trees. The Pyrenean tree line has moved upwards an average of 35 m (Ameztegui *et al.*, 2016) and the recruitment and density of trees has increased in forests near this tree line (Batllori & Gutiérrez, 2008). Similar patterns of shrubification of grasslands and forest densification and expansion have been noted along the entire Pyrenean range, with local variations depending on previous human-induced disturbances and spatial organisation of vegetation (Fig 3.1, Galop *et al.*, 2011; Camarero *et al.*, 2015; Sanjuán *et al.*, 2016).

For all of these reasons, the Pyrenees is a paradigmatic example of mountains undergoing rapid changes in environmental conditions. They represent an exceptional ecology field lab to study the complex dynamics of natural systems and the effects of global change on natural communities (Dover *et al.*, 2017; Zamora *et al.*, 2017).

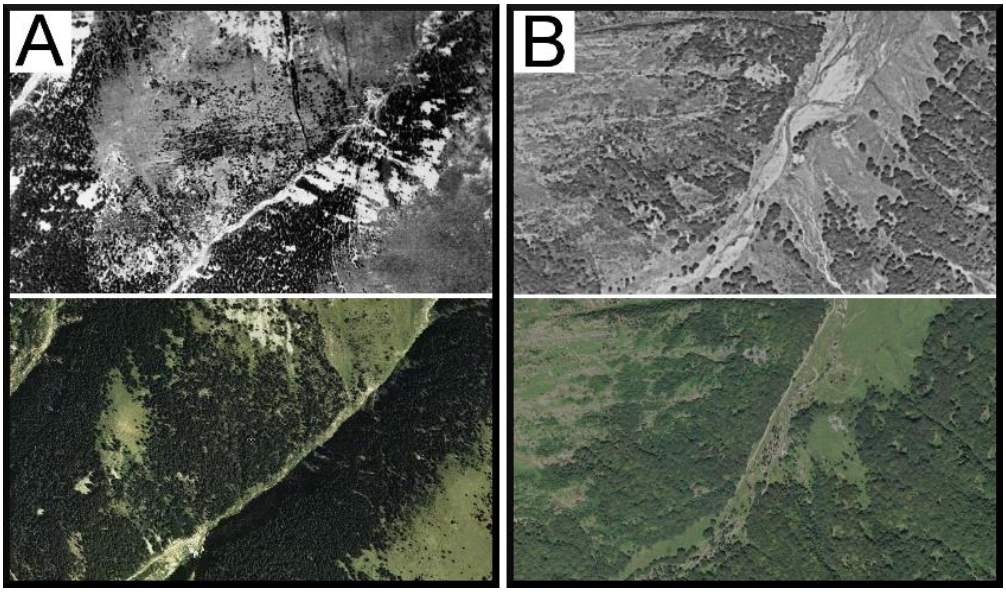


Figure 3.1: **Woody plant expansion and densification in the Pyrenees**. Aerial photographs of two eastern Pyrenean landscapes: Costabona (A) and Jasse d'en Gaudu (B) in 1956 (up) and 2016 (down). A clear expansion and densification of the forested areas can be observed. Aerial photographs were obtained from the French National Institute of Geographic and Forest Information and Spanish National Geographic Institute.

The Pyrenean chamois: a model of adaptation

In particular, this thesis will focus on one mountain-dwelling ungulate inhabiting the Pyrenees: the Pyrenean chamois (*Rupicapra pyrenaica pyrenaica*). This is a particularly interesting species because, as we will see, the Pyrenean chamois is well adapted to environmental and nutritional contrasts and is therefore an excellent model to study the seasonal aspects of foraging behaviour in alpine environments, as well as the effects of global change on this behaviour.

Chamois usually inhabit mountainous environments of Europe and Western Asia, up to around 3100 m.a.s.l. (Aulagnier *et al.*, 2008). They must endure the environmental constrains of these highly seasonal habitats and cope with the limited period of plant growth and winter scarcity. Their lower altitudinal limit is, however, not related to biological factors but rather to anthropogenic pressure, as chamois can also sporadically colonise low mountains down to 400 m.a.s.l., with the extreme situation of a small group in the Trieste Karst that inhabits an area just near the sea (Carnevali *et al.*, 2009).

Figure 3.2. Pyrenean chamois, male (top) and female with a kid and two second-years (bottom).

Box 3.1. The Pyrenean chamois: an overview

Chamois can be found in several mountain ranges in southern Europe from the Cantabrian Mountains in Spain to the Greater Caucasus Mountains in Azerbaijan (Fig 3.3). The genus Rupicapra is divided into two species: the Northern chamois (*Rupicapra rupicapra*) with seven subspecies, and the Southern chamois (*Rupicapra pyrenaica*) with three subspecies (Corlatti *et al.*, 2011). In fact, they are the most common ungulates in European mountains (Corlatti *et al.*, 2011). Small introduced populations also inhabit the Southern Alps of New Zealand (Forsyth, 2005) and the Neuquén province of Argentina (Corlatti *et al.*, 2011). In the early 2000's, the total population of Pyrenean chamois was estimated at 53.000 individuals (Herrero *et al.*, 2004a). However these numbers have not been updated and since, several populations have declined due to different outbreaks of a disease caused by a pestivirus (Marco *et al.*, 2007).

This medium-sized mountain ungulate has a shoulder height of around 70cm and a total length between 100 and 120cm when adult (Pérez-barbería *et al.*, 2010). Both sexes display thin, black horns that rise straight from the head and end in a backward hook (Fig 3.2). Males have longer and thicker horns than females and their hook is more acute (Pepin *et al.*, 1991). Horn growth is continuous throughout the animal's life but interrupted during winter, producing sharp marks along the horn between consecutive annual segments (i.e. annuli). These annuli are used to date the age of chamois (Corlatti *et al.*, 2015) and in long-unhunted populations they can live up to 20 years (Gonzalez & Crampe, 2001). However, senescence—decreasing survival and reproductive capacity with increasing age (Gaillard *et al.*, 2003)—onsets at around 8 years old, with a lower survival in males and an increased gender difference with age (Loison *et al.*, 1999a).

Chamois are highly adapted to life in alpine environments. At high altitudes, the low atmospheric pressure reduces the exchange of gas in the lungs. To overcome this obstacle, chamois have large lungs and a high number of red blood cells (12-13 million/ml) but also thick heart walls that can tolerate over 200 beats/minute for a relatively long time, more than most mammals of the same size (Lovari, 1987). To cope with harsh rocky mountain terrain topography, they also present several anatomic adaptations of the hoof, such as an interdigital membrane to favour mobility on snow, sharp hoof edges to facilitate rock climbing or the ability to spread apart their hoof-halves by over 90° to ease climbing or running down steep slopes (Lovari, 1987). Finally, to endure the wide temperature variations of mountain environments, chamois have two seasonal coats. A summer coat with short ruddy brown hair and a dense dark brown winter coat with long hairs.

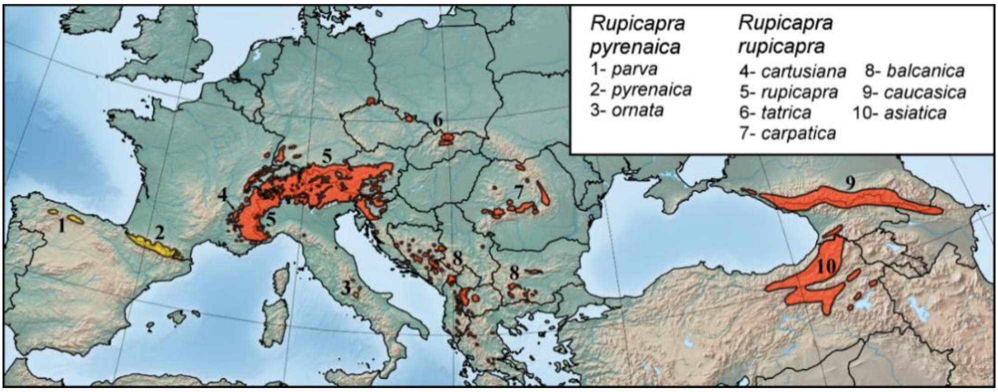


Figure 3.3. Map of the current distribution of *Rupicapra* spp. in Europe and Western Asia. The distribution of the three Southern chamois subspecies (*Rupicapra pyrenaica*) is represented in yellow while the seven Northern chamois subspecies (*Rupicapra rupicapra*) are represented in red. Data from IUCN.

They are considered intermediate feeders, capable of adapting their digestive system to woody plants or grasses depending on their availability (García-gonzález & Cuartas, 1996; Hofmann, 1984; Redjadj *et al.*, 2014). During the warm months, chamois feed in the alpine meadows in search of the most nutritious and digestible plants. They show a preference for herbaceous plants such as forbs and grasses (Ferrari *et al.*, 1988; La Morgia & Bassano, 2009), presumably adapting their feeding behaviour to the phenological plant phases (Duparc, 2016; Ferrari & Rossi, 1985). In fact, most of the chamois' life history traits rely on the amount of energy stored during summer. Maternal care and, therefore, the winter survival of kids, for example, are directly linked to high-quality food resources during nursing and weaning (Scornavacca *et al.*, 2016). As capital breeders, the energy acquisition before winter is also capital in the individual growth and capability to cope with the costs of reproduction (Richard *et al.*, 2017) and winter fasting (Garel *et al.*, 2011).

Births occur in May after 160-170 days of gestation with a birth rate of around 70-90% (Pérez-barbería *et al.*, 2010; Richard *et al.*, 2017). However, juvenile survival is relatively low during the first year. A first peak of mortality is usually detected during mid-summer, related to a decrease in maternal immunity, and a second, more important peak, during winter, related to harsh conditions, delayed parturition and deficient nutrition (Crampe, 1997). Altogether, mortality rates of juveniles can reach up to 50% (Crampe, 1997; Levet *et al.*, 1995). The recruitment rate increases with the age of females but, even if highly variable among years and areas, is relatively low and reaches only around 10 to 50% (Crampe *et al.*, 2006; Pérez-barbería *et al.*, 2010; Richard *et al.*, 2017).

Both sexes have a relatively similar growth rate during the first two summers of life. At least in Alpine chamois, females reach their adult body mass during their third year but males grow for a longer period than females and reach their adult body mass almost three years later (Garel *et al.*, 2009). Adult Pyrenean chamois females weight between 18 and 32Kg and males between 22 and 33Kg depending on the area (Pérez-barbería *et al.*, 2010). In Northern chamois, males can weigh 30 to 40% more than females due to greater summer accumulation of fat and muscle mass (Rughetti & Festa-Bianchet, 2011), but this dimorphism is not as acute in the Pyrenean species and sometimes even non-existent (Pepin *et al.*, 1996). This body weight difference is important because in November, when the rut period begins, males reduce their food intake and adopt an energy saving strategy (Willisch & Ingold, 2007). Nonetheless, they can lose up to 20% of their body weight during this period (Herrero *et al.*, 2003) and must face winter scarcity with the remaining fat reserves. Females, however, only begin to lose weight in December, when winter begins (Pepin *et al.*, 1996). Unfortunately, information on the feeding behaviours of Pyrenean chamois in winter and spring is practically non-existent due to the difficulties in obtaining samples in mountainous environments during these seasons. However, the scarce published information has highlighted a high consumption of woody-plants during these seasons because it is usually the only available resource (Berducou, 1974). In fact, during winter, most of the individuals move to the nearest forest habitats to avoid the challenges of snowy conditions at high altitude (Michallet *et al.*, 1999). Some can even undergo short seasonal altitudinal migrations of no more than 10km in response to winter harshness (Crampe *et al.*, 2007). However, they are usually sedentary and over-wintering species (Crampe *et al.*, 2007; Gálvez-Cerón *et al.*, 2013).

The Pyrenean chamois is the most representative and famous ungulate of the Pyrenean range. It is a charismatic species of cultural and social importance. Above all, its impact stems from its hunting interest, different depending on the area. In France and Andorra, hunting quotas are decided by the regional administration (with the collaboration of hunters in France) and mainly based on a non-profit hunting scheme (Berducou *et al.*, 2004; Naudí & Riba, 2004). The hunt in these countries is usually an important social and cultural event. In Andorra, for example, an important event is

"Chamois hunting week" when hunting is allowed in the "common use zones" for only one week. During this event, more than 500 hunters, gathered in groups of 4 to 6 people, are allowed to hunt most of the hunting quota. In contrast, on the Spanish side, chamois hunting also provides huge economic benefits to the local administration and indirectly to the rural communities by attracting hunters from around the world. Hunting is performed in the company of a ranger who controls and selects the animals in regional units known as Hunting Reserves (Herrero *et al.*, 2004b; Padrós Selma *et al.*, 2004). The most prized specimens, based on their horns and known as trophies, are sold at auction with prices starting at 2.000€ and reaching up to more than 8.000€.

Due to its social, cultural, economic and ecological importance, hunters, conservationists, governments, wildlife biologists and the local population are concerned about the conservation and careful management of this species. In the past, overhunting was the main threat to Pyrenean chamois, coupled with some outbreaks of infectious keratoconjuntivitis (Fernandez-Aguilar, 2017), but with the current strict hunting regulations of national administrations, the problem of overhunting has been resolved. Today, the major threat to Pyrenean chamois is a novel border disease virus (BDV). This first epidemic was detected in 2001 and since then the virus has caused dramatic decreases, and in some cases collapses, of Pyrenean chamois populations (Marco *et al.*, 2015). The sudden deaths of this herbivore caused major concern amongst the public and hunting was suspended in the affected areas. Despite the numerous studies regarding the consequences of BDV (Serrano *et al.*, 2015), its ecological consequences are still unclear despite the key role of Pyrenean chamois in the alpine ecosystem. Today, 15 years after the first epidemics, the effects remain in the affected populations and the virus still circulates and causes fatalities in the Pyrenees, mainly on the southern side (Colom Cadena, 2017)

In the present thesis, we will use the monitoring of two Pyrenean chamois populations as a model to assess the effects of environmental variation on the diet of a large herbivore inhabiting highly seasonal environments (Fig 3.4). We will not only address the link between plant phenology and changes in the diet composition of this mountain species but will also study the effects of climatic mismatch and habitat change on its foraging strategies.

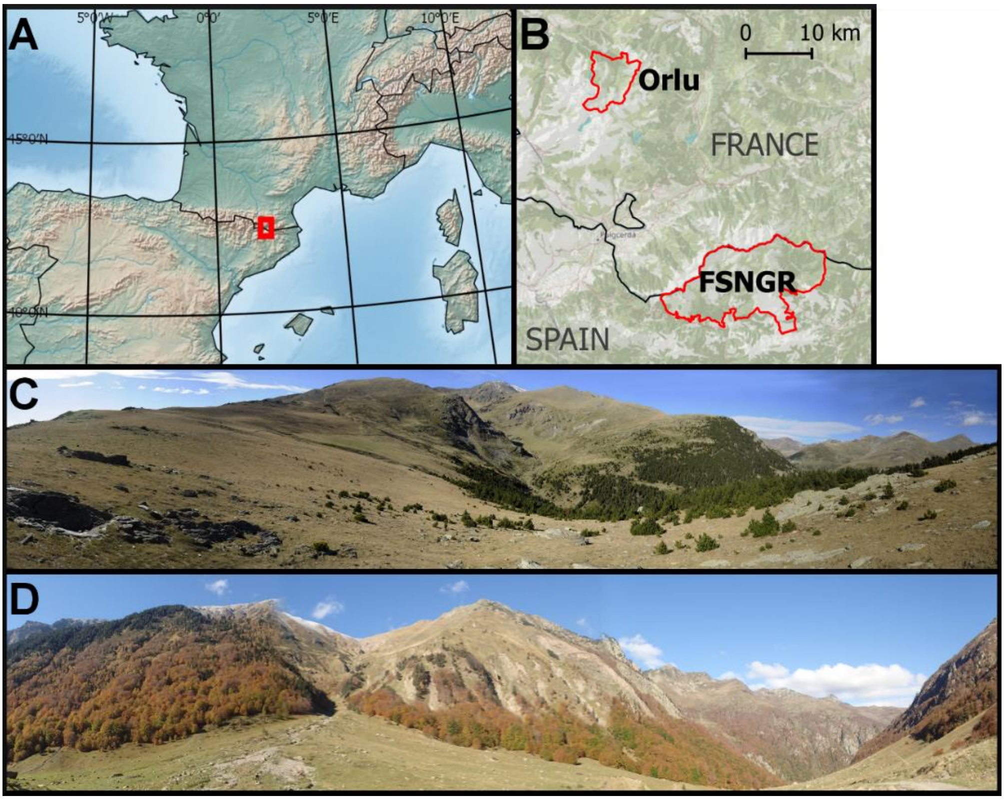


Figure 3.4. The two study areas. (A) Map of south-western Europe with the location of our study areas noted with a red rectangle. (B) Zoom in on the previous red rectangle with the location of the Orlu National Game and Wildlife Reserve (Orlu) in France and the Freser-Setcases National Game Reserve (FSNGR) in Spain. (C) Photograph of the Fontalba Valley in the FSNGR. (D) Photograph of the Jasse d'en Gaudu in Orlu. More detailed information about our study areas can be found in the Material and Methods section of our studies

4. Hypotheses and objectives

4.1. Hypotheses

In large herbivores, animal performance is primarily affected by resource availability and quality, which in turn are influenced by environmental conditions. Global change is having a colossal impact on biological systems worldwide but, despite the fundamental role of resource quality on animal performances, research rarely assesses the mechanistic relationship between environmental changes and dietary choices. Research effort on the dietary habits of herbivores can contribute to understanding the pathways by which environmental variation can impact these species.

The aim of this thesis is to improve our understanding of the effects of climate and habitat change on the diet of large alpine herbivores such as the Pyrenean chamois.

Assessing the use and selection of forage by herbivores is an essential step toward understanding plant-herbivore interactions (Bison, 2015; Duparc, 2016). In that sense, non-invasive techniques for the analysis of herbivore diet are widely established (See Annex) but their effectiveness and practicality have stimulated debate (Barboza *et al.*, 2009b). In a first methodological approach, we aimed to improve our basic understanding of the reliability of two non-invasive techniques to assess the diet composition of an herbivore. We refined and optimised a DNA-based methodology for higher resolution in the determination of diet composition and compared its effectiveness to the well-established cuticle microhistological analysis (study 1).

In alpine habitats, climatic conditions generate seasonal variability in forage availability for herbivores such as the Pyrenean chamois (Billings, 1973). In this scenario, herbivores should adapt their feeding behaviour to the dynamics of the plants to optimise their energy intake (Parker *et al.*, 2009). By studying the dietary patterns of a large herbivore that is highly selective in terms of high-quality forage (Bison *et al.*, 2015; Redjadj *et al.*, 2014), we aimed to understand how seasonality affects the feeding behaviour of alpine herbivores. We studied and described the monthly changes in diet composition of Pyrenean chamois and assessed their adaptation to the seasonal phenology of plants (study 2). Understanding the functional adaptation of the species to seasonal environmental variations is a crucial step toward assessing the impact of changes on these mechanisms (Williams *et al.*, 2017). At the same time, inter-specific interactions may also shape the feeding behaviour of herbivores (Ferretti *et al.*, 2015). The spatial overlap between chamois and livestock in our study areas may lead to changes in the feeding behaviour independent of plant phenology. We therefore studied two populations of Pyrenean chamois with contrasting inter-specific interactions to explore the effects of livestock on such seasonal feeding behaviour (study 2). We focussed on the impact of sheep due to their similar morphologies with chamois and because sheep are able to spatially segregate and modify the feeding habits of chamois (La Morgia & Bassano, 2009; Rüttimann *et al.*, 2008).

Forage availability shapes herbivore populations through density-dependent and density independent processes (see chapter 2.1). However, very few studies have explicitly assessed the mechanistic impact of environmental factors on the dietary composition and quality of herbivores despite the fundamental role of resource-use strategies in life-history traits (Parker *et al.*, 2009; White, 1983). The ongoing increases in spring temperatures are advancing the spring green-up and therefore advancing access to high-quality vegetation during a strategic period for herbivores (Cleland et al., 2007). Conversely, population abundance would impact resource availability due to intra-specific competition (Kausrud *et al.*, 2006; Nicholson *et al.*, 2006). Using a 24-year monitoring scheme of a Pyrenean chamois population with highly contrasting demographic periods, we studied the mechanistic impact of environmental change on the resource-use strategies of herbivores. Specifically, we assessed the effects of spring temperatures and animal densities on the dietary quality and composition of this species. We expected that an earlier onset of spring would advance access to higher quality vegetation (Klein, 1990) and, conversely, that population abundance would impair access to this high-quality vegetation due to intra-specific competition (Stewart *et al.*, 2011) (study 3).

Environmental changes are affecting biological and ecological systems across the globe at alarming rates (IPCC, 2014). As part of this scenario of change, woody plant expansion is threatening the extent of alpine grasslands worldwide. This phenomenon potentially affects herbivore assemblages by conditioning their resource availability and increasing their intra and inter-specific interactions—mainly through resource competition (Putman, 1996). Herbivores will need to adapt their diet, adjust their behaviour or move to another habitat to survive to these changes (Visser, 2008). The extent to which species-specific phenotypic plasticity and biotic interactions mediate organismal adaptation to changing environments, however, remains poorly understood (Charmantier *et al.*, 2008). Given the diverse dietary requirements of herbivore species, we hypothesise that changes in land cover would affect species differently depending on their feeding preferences, plasticity and specialisation (Somero, 2010). We therefore explored the potential impact of the expansion of woody plant coverage on the feeding efficiency of a contrasting group of herbivores. We aimed to predict if these species would efficiently use their food landscapes with their current dietary specificities in different scenarios where shrubland and woodland proliferate at the expense of grassland (study 4).

4.2. Objectives

The objectives of the present thesis are:

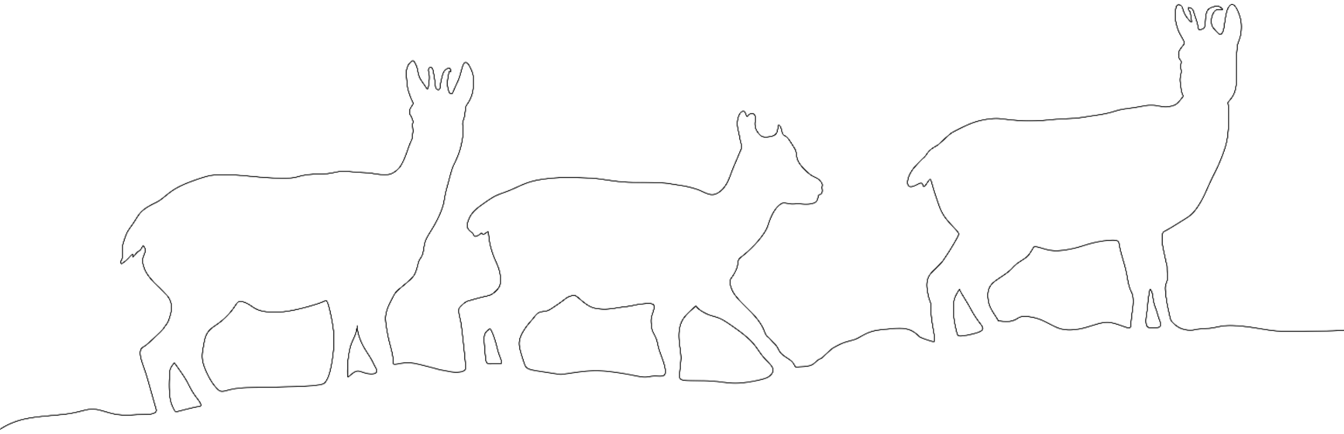
1. Compare the reliability of cuticle microhistological analysis and PCR-CE to assess diet composition of wild herbivores.

2. Describe the seasonal feeding patterns of Pyrenean chamois and assess their relationship with primary production trends.

3. Assess the impact of climate change and population densities on the dietary quality and composition of Pyrenean chamois and estimate the relationship between forage consumption strategies and diet quality.

4. Explore the effects of shrub expansion in alpine environments by addressing whether Pyrenean chamois and free-ranging livestock under varying conditions of resource availability are able to efficiently utilise their food landscape.

5. Studies



5.1 Study 1:

Comparing the accuracy of PCR - capillary electrophoresis and cuticle microhistological analysis for assessing diet composition in ungulates: A case study with Pyrenean chamois.

PlosOne (Under review)

Authors: Johan Espunyes, M. Carme Espunya, Sara Chaves, Juan Antonio Calleja, Jordi Bartolomé, Emmanuel Serrano

Abstract

The study of diet composition is required to understand the interactions between animal and plant ecosystems. Different non-invasive techniques applied on faecal samples have commonly been used for such purposes, with cuticle microhistological analysis (CMA) and emerging DNA-based methods being the most relevant. In this work, we refined and optimized a novel DNA-based approach combining PCR amplification of long trnL(UAA) and ITS2 fragments and capillary electrophoresis (PCR-CE), instead of short trnL(UAA) fragments and massive sequencing technologies commonly reported. To do so, we developed a controlled diet assay using a stabled Pyrenean chamois specimen (*Rupicapra pyrenaica pyrenaica*), which included representative herbaceous and shrubby plant species. We also assessed the impact of sample freshness on the diet determination of this mountain caprinae by exposing faecal samples to the outdoor environment for three weeks. Faecal samples from both experiments were analysed by PCR-CE and CMA in order to compare the pros and cons of both approaches. Our results show that all of the offered plant species were detected by both methodologies although CMA over-detected shrubs compared to herbaceous species. At the same time, sample degradation due to sustained climate exposure is a limiting factor for molecular analysis, but not for CMA. Taken together, our results suggest that the qualitative information obtained by CMA and PCR-CE can be interchangeable when faecal samples are fresh (less than one week after deposition) but, afterwards, molecular analysis underestimates diet composition probably due to DNA degradation. CMA, however, can accurately be used at least three weeks after defecation. Moreover, the use of this novel PCR-CE methodology provides a reliable, feasible and more affordable DNA-based alternative for multiple and routine analyses of complex samples.

Introduction

Individual food selection is a central concept in wildlife nutrition studies (Barboza *et al.*, 2009a). Although food intake evaluation is the starting point for any study of animal nutrition, field ecologists struggle with the challenging task of assessing diet composition. Feeding strategies of free-ranging species are not only influenced by foraging behaviour, food quality and availability but also by the individuals' requirements for reproduction and maintenance (Illius, 2006). Large herbivores play a major role in forming the shapes and functions of terrestrial ecosystems as they exert an impact on landscape dynamics and biodiversity (Danell *et al.*, 2006). Comprehensive data on their feeding habits are crucial to understand their influence on the ecology of other organisms as they can drive the viability of plant communities (Virtanen *et al.*, 2002), modify nutrient cycles (Pastor *et al.*, 2006) and regulate carnivore populations (Pierce *et al.*, 2000). On the other hand, this information is necessary to evaluate their impact on threatened flora (Velamazán *et al.*, 2017) and hence to elaborate plans for plant protection (Brookshire *et al.*, 2002) or population control (Tanentzap *et al.*, 2009). Therefore, knowing the diet composition of herbivores is an essential step towards building an ecological model of plant-herbivore interactions and is basic to many aspects of wildlife and plant research and conservation (Kowalczyk *et al.*, 2011; Mishra *et al.*, 2004).

Along these lines, non-invasive techniques for the analysis of herbivore diets (e.g. faeces collection or animal observation) are broadly established since they do not imply animal culling. The simplest procedures are the *in situ* observation of herbivore ingests of available vegetation, and the time-lapse visualization of chewed plants (Gordon, 1995). However, these approaches are both highly time-consuming because extensive and representative geographical areas need to be monitored in order to obtain conclusive information. Alternatively, information on diet composition can be inferred from the corresponding herbivore faeces through the analysis of plant material remaining after digestion. Two effective faeces-related methodologies should be noted. The first, cuticle microhistological analysis (CMA), is a traditional and well-established method that can provide reliable semi-quantitative data through the identification of plant cell structures, mainly the epidermis and trichomes, visualised under an optical microscope (Anthony & Smith, 1974; Holechek *et al.*, 1982; Stewart, 1967). CMA has been extensively applied to different types of complex samples, including faeces, rumen contents and even coprolites

(Rivera *et al.*, 2014), due to the strong resistance of plant epidermal cuticles to climate and environmental degradation processes. However, CMA presents important drawbacks that limit its use. Significant expertise in microscopic skills is required (Alipayo *et al.*, 1992). In addition, identification of plant structures is frequently impaired by the strong similarity of anatomic structures between taxonomically related genera to the extent that in some cases only the family level can be determined (Holechek *et al.*, 1982).

The second category of faeces-related methodologies involves the analysis of remaining plant genomic DNA isolated from faeces, also known as DNA barcoding methods (Angers-Loustau *et al.*, 2016; Coissac *et al.*, 2016; Hajibabaei *et al.*, 2007). DNA barcoding is considered a powerful and accurate alternative to morphological methods and has been applied in many biological studies including the authentication of herbal medicinal products (Newmaster *et al.*, 2013b; Parveen *et al.*, 2016), ancient permafrost sediment analysis (Bellemain *et al.*, 2013; Haile *et al.*, 2007; Hofreiter *et al.*, 2000), and the assessment of herbivore diet composition (Burkepile & Parker, 2017; Czernik *et al.*, 2013; Hibert *et al.*, 2013; Rayé *et al.*, 2011; Soininen *et al.*, 2009; Valentini *et al.*, 2009a). Next-generation sequencing (NGS) technology, is currently the most used DNA barcoding method and consists of the identification of different taxa using marker gene sequences, also called barcodes, through amplification by PCR and subsequent sequencing by massive NGS (Angers-Loustau *et al.*, 2016; Hollingsworth *et al.*, 2011; Pompanon *et al.*, 2012; Shokralla *et al.*, 2012; Staats *et al.*, 2016). In plants, different chloroplastic genes have been extensively used - e.g. *matK*, *trnL(UAA)*, *rbcL* and *trnH-psbA* - either alone or combined with nuclear genes, such as the Internal Transcribed Spacer regions of ribosomal DNA, *ITS1* and *ITS2* (Chen *et al.*, 2010; Hollingsworth *et al.*, 2011, 2009; Yao *et al.*, 2010). However, NGS-DNA barcoding implementation has been hampered by the economic cost of sequencing technologies (Pompanon *et al.*, 2012; Staats *et al.*, 2016) whereas an alternative method involving capillary electrophoresis has emerged as a more affordable and suitable option for multiple and routine analysis of complex samples (Czernik *et al.*, 2013; Pegard *et al.*, 2009). This alternative technique, hereafter called PCR-capillary electrophoresis (PCR-CE), applies capillary electrophoresis to determine the amplicons lengths, instead of DNA-sequencing as in NGS-DNA barcoding. We obtain a migration pattern that allows the detection of several peaks characteristic of multi-species samples. The subsequent botanical adscription of each peak is realized through matching experimental data with those available in the GenBank database (NCBI, 2016). PCR-CE is not commonly used in studies of diet composition despite being fast, inexpensive and frequently applied in the food industry ((Bazakos *et al.*, 2012; Spaniolas *et al.*, 2006), but see (Czernik *et al.*, 2013)). Usually, the resolution of this method is considered low for complex samples because plant species with similar fragment sizes can account for the same peak due to a visual overlap (Czernik *et al.*, 2013). However, this drawback can be overcome by combining simultaneous PCR amplification of two complementary genes (Pegard *et al.*, 2009).

Here, we wanted to refine and optimize a PCR-CE analysis for a higher resolution in the determination of diet composition. For this purpose, we subjected a stabled chamois specimen to a controlled diet, which included representative herbaceous plants and woody shrubs of the eastern Pyrenees ecosystem. We developed a novel method combining simultaneous PCR amplification of two genes, *trnL(UAA)* and *ITS2*, and capillary electrophoresis to determine the corresponding fragments length. We expected this PCR-CE alternative method to led to a higher fragment resolution and more precise plant species identification compared to conventional amplifications methods previously described (Nichols *et al.*, 2016; Taberlet *et al.*, 2007) avoiding, at the same time, the high cost and the need for specific expertise on NGS. Simultaneously, cuticle microhistological analysis was also carried on these samples in order to compare both technical approaches.

On the other hand, one of the major concerns in the determination of diet composition is knowing to what extent the freshness of faecal samples may compromise the obtaining of reliable results. In that way, we also aimed at determining if the time-lapse between animal deposition and faecal sampling impacts on CMA and PCR-CE achievements. As a pilot assessment, we exposed a pool of faeces obtained from hunter-harvested Pyrenean chamois (*Rupicapra pyrenaica pyrenaica*) to outdoor environmental conditions for several weeks. We then determined the number of plant species detected by both methodologies every week along the study. Being used in archeobotany (Rivera *et al.*, 2014), we expected CMA results not to be influenced by environmental exposure. On the other hand, due to the high degradability of DNA (Deagle *et al.*, 2006), we expected an effect of the time of exposure on the number of plants detected by the DNA-based method. At the same time, this assessment allowed us to test the efficiency of PCR-CE on a more species-diverse sample.

Materials and Methods

Study area and sampling procedure

Fresh plant samples for the controlled diet tests were collected in June 2015 in the Freser-Setcases National Game Reserve (FSNGR), Eastern Pyrenees, Spain (42º 22' N, 2º 08' E). The FSNGR is a typical sub-humid subalpine and alpine bioclimatic region with a noticeable Mediterranean climatic influence. It covers an area of 20,200 ha, ranging from 1,800 to 2,910 m.a.s.l. and hosts a population of about 3,000 Pyrenean chamois (Villamuelas *et al.*, 2016). The fresh faecal samples used for the time-course degradation study were collected from 12 hunter-harvested Pyrenean chamois during the hunting season of 2014-2015 in the same area. Faecal and plant samples were refrigerated at 4°C during transport to our facilities and stored frozen at -20ºC until processing.

Controlled diet test

A twelve-year-old female Pyrenean chamois was fed on several diets that overall included 13 vascular plant species, comprising herbaceous plants and woody shrubs representative of the subalpine and alpine grassland ecosystem of the FSNGR. Three different plant mixtures were consecutively offered and intercalated with a four-day exclusive *Medicago sativa* diet, as shown in Table 5.1.1. Fresh faeces were collected before beginning the controlled diet (day 7) and the day after the corresponding diet intake, and were immediately stored at -20ºC until processing. The animal used in this study was maintained in our facilities for two months. The animal was being translocated and was opportunistically used for this study. Animal care and procedures were in accordance with the guidelines of the Good Experimental Practices.

Table 5.1.1. Schedule of the controlled diet offered to a specimen of Pyrenean chamois. Composition of the plant mixtures is indicated and the weight of each plant species in the mixture is given in grams (g) and in percentage (%). Plant species initials are listed between brackets.

Days	Diet components	Weight (g)	Percentage (%)
1 to 7*	*Medicago sativa* (Ms)	*ad libitum*	
8	Diet 1:		
	Calluna vulgaris (Cv)	28.5	39.8
	Carex caryophyllea (Cc)	13.5	18.8
	Festuca spp.[1]	29.7	41.4
9* to 12	*Medicago sativa*	*ad libitum*	
13	Diet 2:		
	Helianthemum nummularium (Hn)	17.3	18.5
	Juncus trifidus (Jt)	6.7	7.2
	Nardus stricta (Ns)	47.0	50.3
	Trifolium alpinum (Ta)	22.5	24.1
14* to 17	*Medicago sativa*	*ad libitum*	
18	Diet 3:		
	Arrenatherum elatius (Ae)	76.4	32.3
	Hypochoeris radicata (Hr)	68.7	29.2
	Vaccinium uliginosum (Vu)	89.4	38.5
19*	*Medicago sativa*	*ad libitum*	

[1] Includes a mixture of *F. eskia* (Fe), *F. gautieri* (Fg), *F. glauca* (Fgl), and *F. violacea* (Fv)

* Days of faeces collection

Time-course degradation

Fresh faeces from the hunter-harvested Pyrenean chamois were randomly mixed in 4 pools and left outdoor at the Autonomous University of Barcelona campus (Bellaterra, Spain) for three weeks starting in January 2016. This experiment was set up as a pilot assessment to evaluate the connection among the time-lapse between chamois deposition and faecal sampling - a potential degree of sample degradation - and the performance of both CMA and PCR-CE methods. In that period, temperatures ranged from 2.5ºC to 21.7ºC (mean= 11.1ºC) with short periods of rain between weeks 2 and 3 (See S5.1.1 Fig). Pellets from the four different mixtures were collected every week and kept at -20ºC until processing. After three weeks of harvesting, they were subsequently tested as described below and the number of identified plant species was monitored using the two methods.

Genomic DNA extraction and multiplex-PCR DNA amplification

Genomic DNA from plants and chamois faeces was extracted from 200 mg of raw material with the DNeasy Plant Mini Kit and the QIAamp DNA Stool Kit (Qiagen, Germany), respectively, following manufacturer's instructions. Genomic DNA was eluted in 100 µl of milli-Q water and kept at -20ºC. DNA concentration was measured with a NanoDrop 1000 Spectrophotometer (ThermoScientific, USA) and DNA quality was analysed on a 0.8% agarose gel electrophoresis in 1 x TBE buffer.

Multiplex-PCR reactions for specific amplification of chloroplastic *trnL(UAA)* and nuclear *ITS2* sequences in plants and faeces were carried out in a total volume of 20 µL containing 1x MyTaq Reaction Buffer (Bioline Reagents Ltd, United Kingdom), 0.4 µM each oligonucleotide (Stab Vida, Portugal; see S5.1.1 Table for detailed oligonucleotide sequences), 1 U MyTaq HS DNA polymerase and 20 ng of genomic DNA template. Oligonucleotides trnL-G and trnL-D were used to amplify the *trnL(UAA)* region (Taberlet *et al.*, 2007), while oligonucleotides S2F and S3R amplified the *ITS2* region (Chen *et al.*, 2010). Oligonucleotides trnL-D and S2F were fluorescence-labelled with 6-FAM (carboxyfluorescein) and HEX (hexachloro-6-carboxyfluorescein) fluorochromes, respectively, to allow simultaneous fluorescent detection by capillary electrophoresis.

Cycling conditions were optimized for multiplex DNA amplification and comprised an initial denaturation for 1 min at 95ºC, followed by 35 cycles of denaturation for 15 s at 95ºC, annealing for 15 s at 56ºC and extension for 45 s at 72ºC, and a final extension step for 3 min at 72ºC. PCR reactions were run on a MJ Research PTC-100 Thermal Cycler (MJ Research Inc., Canada) and amplicons were visualised on a 1.5% agarose gel electrophoresis in 1 x TBE buffer.

DNA sizing

Amplicon lengths were determined by automated capillary electrophoresis with a Genetic Analyzer 3130xl system (Applied Biosystems, USA) using LizS600 as a size standard. Electropherograms were *in silico* analysed with provided Peak Scanner Software version 2.0 (Applied Biosystems, USA). Plant genomic sequences of the *trnL(UAA)* and *ITS2* regions were retrieved from the GenBank database using the nucleotide BLASTN tool (e-value < e-04) (Altschul *et al.*, 1990). Sequence lengths were calculated by flanking corresponding primers to each plant sequence.

Slide preparation for CMA analysis

Fresh faeces were lightly ground by hand in a mortar to separate out the epidermal fragments (Holechek, 1982). From each sample, 0.5 g of ground faeces were introduced into a test tube with 3 ml of concentrated $HN0_3$ to allow non-epidermal tissue digestion. The test tubes were placed in a water bath at 80ºC for 2 minutes and the samples were then diluted with 200 ml of distilled water. This suspension was successively sieved through 1 mm and 0.25 mm filters. The 0.25 to 1 mm fraction was dispersed in a 50% (v/v) aqueous solution of glycerin. Samples of the suspensions were spread on glass microscope slides at a density that prevented any significant overlapping of fragments. Cover slips (24 x 60 mm) were then fixed to the slides with DPX varnish (Herter Instruments, Spain). Three slides were prepared from each sub-sample. A reference collection of the different plant species included in the study was set up in parallel using two different methods. First, fresh plant material was coarsely ground by hand and then processed in the same way as the faeces samples in order to simulate the fragmentation of the epidermis during ingestion and digestion. Second, fresh abaxial and adaxial leaf surfaces were obtained with a scalpel and mounted on a glass microscope slide allowing a more accurate identification of the main morphological features of the different species.

Fragment Identification

Each slide was examined under a Motic BA210 optical microscope (MoticEurope SLU, Spain) at 400x magnification. Three traverses were scanned, each one 2 mm wide and 60 mm long with 3 mm between traverses. Up to 200 epidermis fragments were identified per sample using the reference collection of plant species, based on the shape of epidermal cells, trichomes and stomata surrounding cells. The data were then pooled and converted to percentages. Reference collection images were captured with a Moticam 2300 camera using provided Motic Images Plus 2.0 software.

Statistical analysis

We used a linear model (LM) to explore the effects of the elapsed time between faecal deposition and sampling on the number of plant species identified (response variable) by CMA and PCR-CE. The LM included the sampling period (fixed categorical factor with four categories: Initial and 1st, 2nd, and 3rd week) and the identification technique (CMA and PCR-CE) as fixed explanatory factors, and the number of identified plant species as a response variable. Due to our moderate sample size (n= 32, two techniques x 4 determinations per week), model parameter uncertainty was estimated by residual-based bootstrap methods using the Boot package 1.3-20 version (Canty & Ripley, 2017). LM assumptions were evaluated by exploring the residual pattern (homoscedasticity, linearity and normality) (Zuur *et al.*, 2010). All statistical analyses were performed in R version 3.4.3 (R Core Team, 2018).

Results

DNA fragments length and CMA reference databases

We established a database of different plants, 13 of them representative of the eastern Pyrenees ecosystem and *Medicago sativa*, based on the size of multiplex-PCR amplified *trnL(UAA)* and *ITS2* genomic sequences with primers trnL-G/D and S2F/S3R, respectively. Both genes were successfully amplified in all the plants tested, confirming the universality of the oligonucleotides previously designed for plant DNA barcoding studies (S5.1.2 Fig) (Chen *et al.*, 2010; Taberlet *et al.*, 2007).

The size of the fluorescently labelled PCR products determined after capillary electrophoresis ranged from 303 to 593 bp for *trnL(UAA)* amplicons and from 463 to 500 bp for *ITS2* amplicons, which strongly match the lengths calculated from retrieved GenBank sequences (S5.1.3 Fig and Table 5.1.2). Despite the fact that *trnL(UAA)* exhibits higher resolution than *ITS2*, since the corresponding PCR products show a wider range of sizes, the data obtained from the two genomic sequence amplifications is complementary. Thus, *Calluna vulgaris* (L.) Hull, *Carex caryophyllea* Latourr. and *Vaccinium uliginosum* L. showed similar *ITS2* fragment sizes (488, 490 and 492 bp, respectively), but they could be clearly discriminated through their *trnL(UAA)* fragments (492, 593 and 464 bp, respectively). The species of the genus *Festuca* were almost indistinguishable by their *ITS2* fragments (472-475 bp) but exhibited distinct *trnL(UAA)* fragment sizes (504-517 bp), with the exception of *F. eskia* Ramond ex DC. and *F. gautieri* (Hack.) K.Richt., which were identical. Furthermore, *Nardus stricta* L. and *Trifolium alpinum* L. could be clearly differentiated by their *ITS2* fragment (471 and 480 bp, respectively), better than by the corresponding *trnL(UAA)* fragment (518 and 516 bp). Three species, *Arrhenatherum elatius* (L.) P. Beauv. ex J. Presl & C. Presl, *M. sativa* and *Juncus trifidus* L., exhibited a shorter *trnL(UAA)* fragment, which sharply differed from the rest of the plant species.

Concerning CMA, all plant epidermis fragments were easily distinguishable at the genus level based on their anatomical structures (Johnson *et al.*, 1983). In summary, graminoids presented rectangular epidermic cells but differences between the species in terms of cell size, cell wall shape and the presence and shape of trichomes and stomata (Fig 5.1.1, A). *C. cariophyllea* fragments presented square to rectangular epidermic cells with wavy cell walls, prickle-shape trichomes and diacytic stomata. *J. trifidus* also presented rectangular elongated epidermal cells but the wall was smoother. *A. elatius* displayed rectangular epidermic cells with wavy walls intercalated with short cells. *N. stricta* also had rectangular epidermis cells with wavy walls but were longer than *A. elatius* and some prickle-shaped trichomes could also be observed. *Festuca* spp. showed long epidermic cells with wavy walls separated by C-shaped short cells, paracytic stomata and spindle-shaped trichomes. Depending on the *Festuca* species, cell walls displayed variable waviness, yet fragments could not be characterised at the species level due to the extraordinary morphological similarity exhibited by this genus.

Table 5.1.2. DNA fragment lengths reference database for the plant species included in the controlled diet test. Amplicon sizes were determined by capillary electrophoresis in this study. Fragment sequence lengths were calculated from retrieved GeneBank sequences. (bp), base pairs. NA, not available.

	***trn*L(UAA)**			**ITS2**		
Plant species	amplicon size (bp)	fragment sequence length (bp)	Gene Bank accession	amplicon size (bp)	fragment sequence length (bp)	Gene Bank accession
Arrhenatherum elatius	303	310	EU434100	471	NA	-
Calluna vulgaris	492	495	KP737377	488	497	KP737514
Carex caryophyllea	593	593	EU288467	490	499	HG915830
Festuca eskia	517	519	AF478508	473	484	KF917344
Festuca gautieri	517	519	KP699267	472	482	AF303414
Festuca glauca	504	506	JX871940	475	485	AY327792
Festuca violacea	508	510	EF593012	472	484	EF584979
Helianthemum nummularium	491	NA	-	463	479	GU327669
Hypochoeris radicata	530	532	AY504774	483	485	AF528461
Juncus trifidus	343	345	AY437971	500	495	AY973508
Medicago sativa	326	327	GQ488614	484	486	AF053142
Nardus stricta	518	520	EU434097	471	479	KJ477049
Trifolium alpinum	516	522	DQ311725	480	484	DQ311995
Vaccinum uliginosum	464	466	DQ860640	492	498	DQ217769

Dicots were also clearly distinguishable by their specific micro-anatomical features, especially trichomes and stomata (Fig 5.1.1., B). For Ericaceae, *C. vulgaris* presented lobed epidermal cells and anisocytic stomata protected by medium-sized, wavy trichomes while *V. uliginosum* displayed angular to lobed epidermal cells, paracytic stomata and no trichomes. Additionally, veins were easily observable in the *V. uliginosum* fragments. *Helianthemum nummularium* showed angular polygonal epidermal cells, cyclocytic stomata and star-shaped trichomes. *Hypochoeris radicata* presented irregular rectangular cells with angles often different than 90 degrees, anamocytic stomata and large multicellular hairs. Finally, Fabaceae *T. alpinum* fragments showed angular polygonal epidermal cells, anomocytic stomata and medium-sized trichomes with pointed tips, and *M. sativa* displayed polygonal epidermal cells with undulated cell walls and long single-cell trichomes with a reflective dual-cell base.

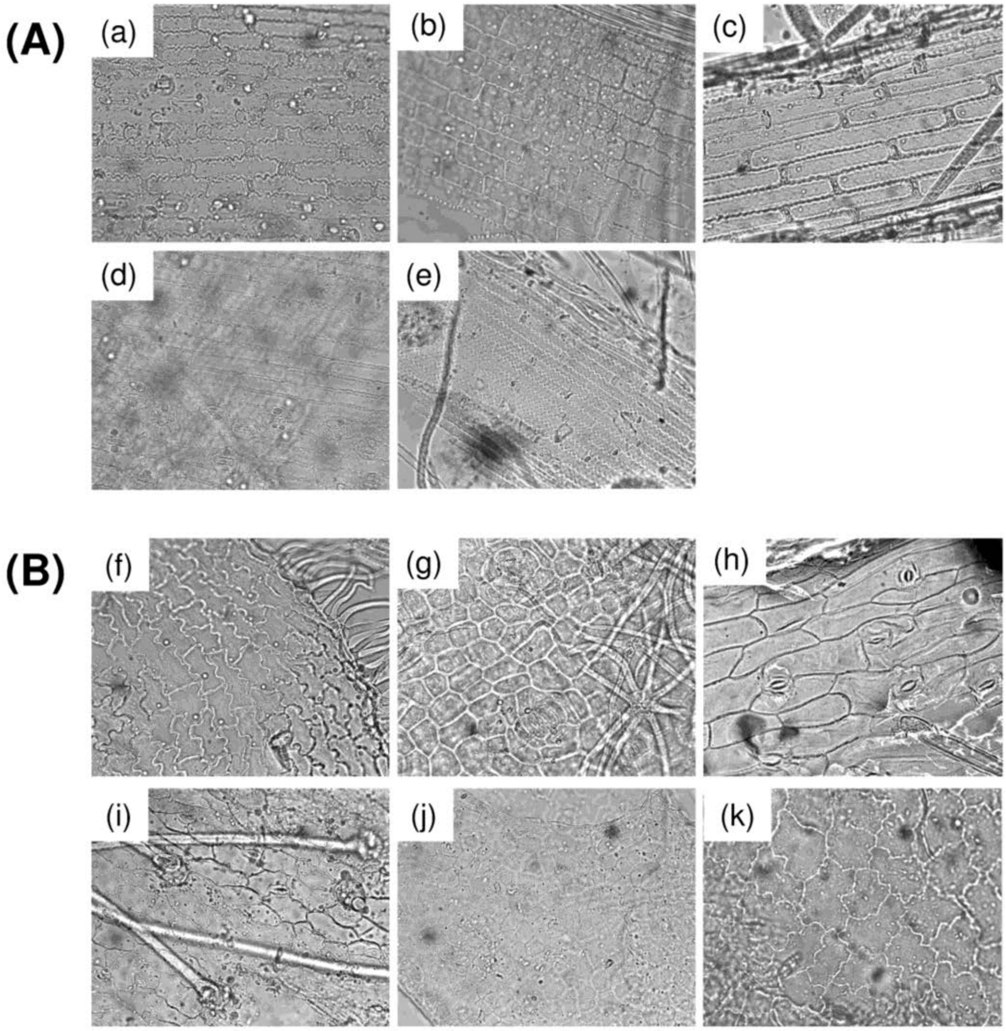


Fig 5.1.1. Microscopical images showing the main epidermal anatomical features of the plant species used in this study. (A) Graminoid species: (a) *Arrhenatherum elatius*, (b) *Carex caryophyllea*, (c) *Festuca* spp., (d) *Juncus trifidus*, (e) *Nardus stricta*; (B) Dicotyledonous species: (f) *Calluna vulgaris*, (g) *Helianthemum nummularium*, (h) *Hypochaeris radicata*, (i) *Medicago sativa*, (j) *Trifolium alpinum*, (k) *Vaccinium uliginosum*. Images were obtained under 400 x magnification.

Analysis of the botanical composition of chamois faeces subjected to a controlled diet

The 13 plant species that were distributed in three different mixture diets and offered to a Pyrenean chamois were completely ingested within the timeframe they were offered. Multiplex PCR amplification of *trnL(UAA)* and *ITS 2* sequences from genomic DNA extracted from chamois faeces yielded products of expected size in all the samples tested (S5.1.4 Fig). Analysis of fluorescently labelled PCR products by capillary electrophoresis showed precise and independent peaks of different lengths that could be roughly ascribed to a particular plant species (Fig 5.1.2). We were able to identify all the plants included in the controlled diets. For a better interpretation of the electropherograms, the two fluorescence channels were independently preselected. Two common peaks among the three controlled diets of 326 and 484 bp for *trnL(UAA)* and *ITS2* amplicons, respectively, were observed and would correlate with *M. sativa* background intake.

In diet 1 electropherograms, 6 peaks for *trnL(UAA)* and 5 peaks for *ITS2* amplicons were distinguished, which may correspond to the 7 plant species included in the diet (Fig 5.1.2, A). In contrast, two species of the genus *Festuca*, *F. eskia* and *F. gautieri* remained undetermined. A wider peak of 474-476 bp in the *ITS2* amplification was assigned to the *Festuca* genus. Diet 2 electropherograms exhibited 7 peaks for *trnL(UAA)* and 6 peaks for *ITS2* amplicons that could be assigned to the 4 diet species (Fig 5.1.2, B). One *trnL(UAA)* peak, adjacent to *H. nummularium*, did not match any plant species but may match a particular amplification profile for this species (S5.1.3 Fig). Furthermore, one of the peaks for each gene correlated with *C. vulgaris* size amplicons suggesting that residual genomic DNA of this species belonging to diet 1 was present in the analysed faeces. Moreover, diet 3 *trnL(UAA)* electropherograms displayed a total number of 6 peaks: 3 of them corresponded to the diet species, the other 2 to residual *H. nummularium and* N. *stricta* from diet 2, and the common *M. sativa* peak. For the *ITS2* channel, results were less resolved as two of the peaks (472 and 483 bp) matched more than one species (Fig 5.1.2, C).

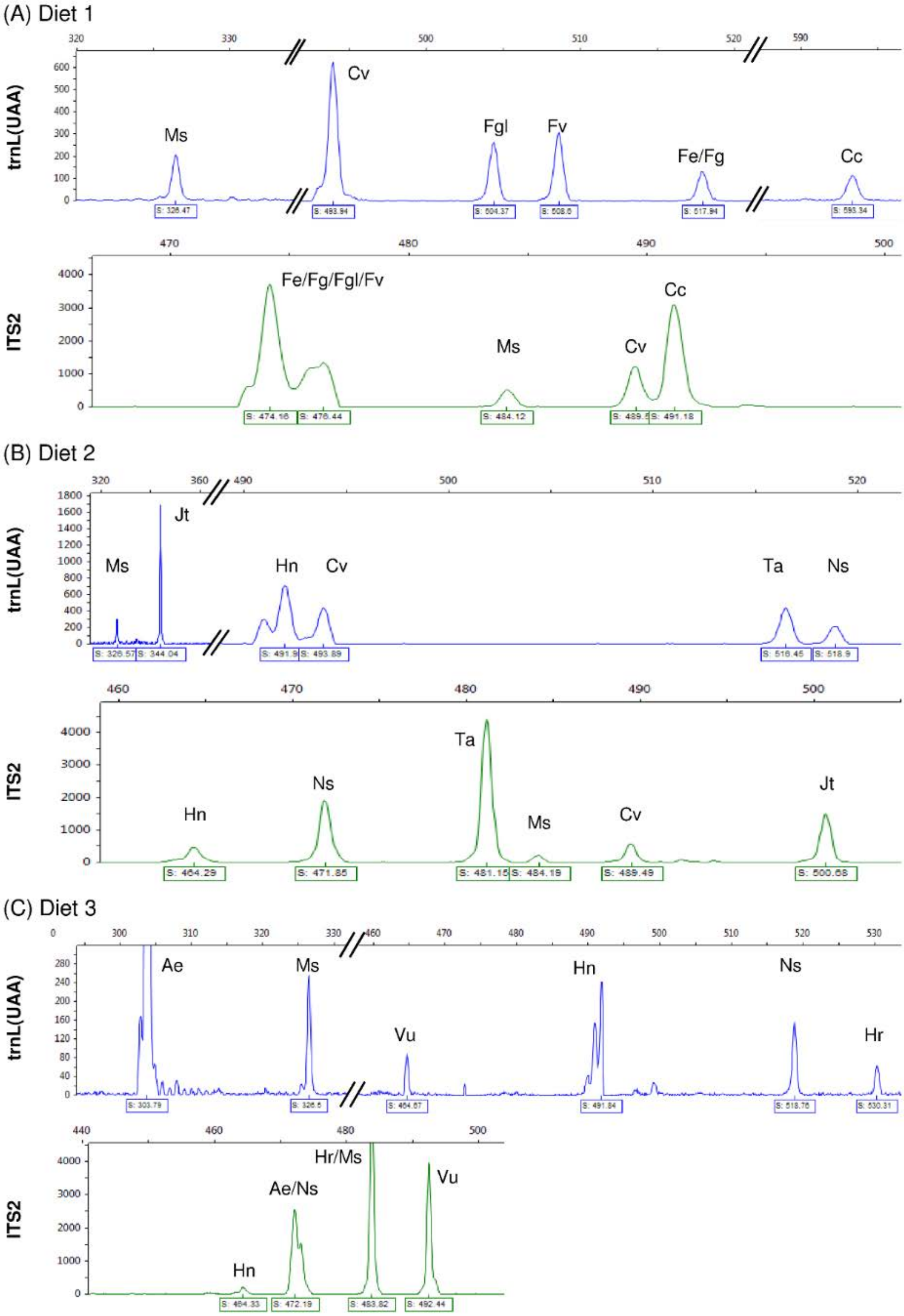


Fig 5.1.2. Electropherograms peaks of the three controlled diets obtained by capillary electrophoresis of the fluorescence labelled PCR products corresponding to *trnL(UAA)* (blue) and *ITS2* (green) multiplex amplification. For a better interpretation, the two fluorescence channels are shown separately. Plant species initials are as in Table 5.1.1.

Regarding CMA analysis, all the species included in the study were identified and quantified in the faeces, with the exception of the species of the genus *Festuca*, which could not be discriminated due to their almost identical epidermal structures. Results are given as percentages of epidermal fragments quantified in faecal samples (Table 5.1.3). Quantification of *M. sativa* was discarded for better determination of experimental plants. Our results show that epidermal fragments from the different species were preserved in the successive faecal samples throughout the experiment to the extent that species included in diets 1 and 2 were also detected in diet 3.

Table 5.1.3. Percentages of epidermal plant fragments quantified in faecal samples (EPF) determined by cuticle microhistological analysis. (SE), standard error.

Diet 1	EPF(%)	± SE
Calluna vulgaris	74.8	4.4
Festuca spp.	16.1	3.3
Carex caryophyllea	9.1	0.7

Diet 2	EPF %)	± SE
Helianthenum num.	28.3	2.9
Trifolium alpinum	27.8	6.5
Calluna vulgaris	16.6	3.6
Nardus stricta	9.7	2.3
Festuca spp.	9.4	1.6
Juncus trifidus	7.9	1.2
Carex caryophyllea	0.3	0.3

Diet 3	EPF(%)	± SE
Arrenatherum elatius	18.7	4.2
Nardus stricta	17.6	5.6
Festuca spp.	14.9	3.5
Hipochoeris radicata	10.9	3.1
Vaccinum uliginosum	9.4	1.9
Helianthemum num.	8.8	1.5
Calluna vulgaris	7.5	3.3
Juncus trifidus	4.9	0.7
Carex caryophyllea	4.5	1.0
Trifolium alpinum	2.9	1.7

Furthermore, the CMA approach enables the possibility of linking percentages of epidermal fragments quantified in faeces (% EPF) with those of plant ingested weights (% I). Thus, a EPF/I ratio can be defined for each species in the corresponding diet, providing information about a putative over-detection of a particular species in faeces when EPF/I > 1 (Table 5.1.4). For a simplification of the model, only original diet species have been included in ratio calculations.

Table 5.1.4. Ratios of epidermal fragments quantified in faeces by CMA (EPF) *versus* ingested plant weights (I) for each species in the corresponding diet. Lignin content values were taken from the indicated references. ND, data not reported in the bibliography.[1]Bartolome *et al.*, 1998; [2] Marinas & García-González, 2006, [3] Hayes *et al.*, 2016; [4] Heggberget *et al.*, 2002; [5] Asshoff & Hättenschwiler, 2005; [6] Hendriks *et al.*, 1999.

	Plant species	Herbaceous /shrub	% ingest (I)	% EPF (EPF)	EPF/I	Lignin (% of dry matter)	Ref.
Diet 1	*Calluna vulgaris*	Shrub	39.8	74.8	1.9	17.8	[1]
	Carex caryophyllea	Herbaceous	18.8	9.1	0.5	3.3	[2]
	Festuca spp	Herbaceous	41.4	16.1	0.4	5.4	[1]
Diet 2	*Helianthemum num.*	Shrub	18.5	28.3	1.5	7.4	[3]
	Juncus trifidus	Herbaceous	7.2	7.9	1.1	2.8	[4]
	Nardus stricta	Herbaceous	50.3	9.7	0.2	5.4	[2]
	Trifolium alpinum	Herbaceous	24.1	27.8	1.1	6.9	[2]
Diet 3	*Vaccinum uliginosum*	Shrub	38.5	9.4	0.2	7.4	[5]
	Arrhenaterum elatius	Herbaceous	32.3	18.7	0.6	ND	-
	Hypochoeris radicata	Herbaceous	29.2	10.9	0.4	3.1	[6]

For example, in diet 1, ingested quantities of *C. vulgaris* and *Festuca* spp were equivalent and double that of *C. caryophyllea* ingests (39.7%, 41.4% and 18.8%, respectively) but the first was the predominant species detected in faeces (74.8%, 16.1% and 9.1% resp.). EPF/I ratios suggest an over-detection of *C. vulgaris* (EPF/I=1.9) and a significant equivalent infra-detection of *Festuca* spp (EPF/I=0.4) and *C. caryophyllea* (EPF/I=0.5). A similar behaviour is observed in diet 2. Despite the fact that *H. nummularium* was not the most ingested species, it was over-detected in the corresponding faeces (EPF/I=1.5), while *N. stricta*, which was the main species in the diet, was poorly detected, showing a EPF/I ratio of 0.2, whereas for *J. trifidus* and *T. alpinum* the EPF/I was 1.1. The three plant species included in diet 3 were infra-detected in faeces showing EPF/I ratios of 0.2-0.6.

Time-course degradation analysis

Descriptive statistics for the mean number of plant species found in our pool of faecal samples exposed to outdoor climatic conditions is shown in Table 5.1.5. According to our LM, 94.4% of the observed variability in the number of plant species detected was explained by the interaction between the time elapsed since samples were exposed to the environment and the detection technique ($F_{7, 14} = 40.5$, $p < 0.001$). The mean number of plant species detected by PCR-CE was notably higher than the mean number detected by CMA at the initial starting point of outdoor exposure. However, while the number of species identified by CMA was fairly stable during the trial, the species detected by the molecular method dropped by more than half since the first week. In the faecal material exposed to the environment for three weeks, no species were detected by this technique.

Table 5.1.5. Mean ± standard deviation, minimum and maximum number of plant species detected in a pool of Pyrenean chamois faeces using CMA and PCR-CE.

Sampling period	CMA	PCR-CE
Initial	8.75 ± 0.5 (8-9)	11 ± 1.83 (9-13)
1st Week	8.75 ± 1.5 (7-10)	4.25 ± 0.71 (3-5)
2nd Week	8.75 ± 0.96 (8-10)	1 ± 0.82 (0-2)
3rd Week	8 ± 0.82 (8-10)	0 ± 0 (--)

Discussion

In this article, we describe and compare two technical approaches for the study of the diet of herbivores using faecal samples as a source for analysis: a modified PCR-based DNA amplification of marker genes (PCR-CE approach) and a microhistology-based identification of the remaining plant epidermis (CMA approach). In both methodologies, a previous description of the plant species that constitute the available resources for herbivores is an essential prerequisite. The identification of the putative ingested plant species is supported by the collation of the information obtained from the remaining plant epidermis and/or genomic DNA in the faeces with those available in plant reference databases.

Concerning the molecular approach, we have developed a novel method for multiplex-PCR DNA amplification of two genomic sequences including chloroplastic *trnL(UAA)* and nuclear *ITS2* plant genes using total genomic DNA extracted from herbivore faeces as a template. The use of fluorescently labelled oligonucleotides enables the subsequent determination of the amplicon sizes by capillary electrophoresis, which in turn is the basis for plant species determination in diet composition. It has been previously described that DNA barcoding of *trnL(UAA)* using conserved oligonucleotides G and H broadly amplifies a fragment of 10-143 bp covering the P6 loop of the plant *trnL(UAA)* intron, and plant species adscription relies on the application of massive sequencing technologies (Taberlet *et al.*, 2007; Valentini *et al.*, 2009a). We show in this work that the amplification of larger *trnL(UAA)* fragments (310-593 bp), including the conserved oligonucleotides G and D and comprising the intron and the exon 2 gene regions, greatly improves the discriminating capacity of the technique due to the hypervariable length of the region and enables a more accurate identification of plant species. Moreover, the Internal Transcribed Spacer 2 (*ITS2*) region, situated between the 5.8S and 26S genes of the nuclear ribosomal cistron, has also been considered an optimal candidate gene marker, due to its appreciable interspecific divergences even in closely related species (Chen *et al.*, 2010; Yao *et al.*, 2010). Since the unique plant structures that can be distinguished in faeces samples are the epidermal cells, which lack chloroplastic organelles, the concomitant amplification of a nuclear genomic sequence, such as *ITS2*, serves as a control for DNA template quality. In addition, *ITS2* analysis supplements the data obtained from *trnL(UAA)* concerning plant species identification belonging to a particular genus (i.e., *Festuca* genus), suggesting that the information obtained from both genes may be complementary. Furthermore, genomic sequences of *trnL(UAA)* and *ITS2* genes from many plant species are freely accessible through public databases, such as GenBank (NCBI, 2016), enabling the *in silico* prediction of gene lengths. It is noteworthy that retrieved GenBank sequences corresponding to both genes are a few base pairs longer compared to the fragment amplicon sizes determined experimentally, probably due to the intrinsic sensitivity of the capillary electrophoresis procedure, as previously described (Czernik *et al.*, 2013). However, these slight variations do not affect the identification of the plant species since fragment sizes determined by capillary electrophoresis are equivalent in fresh plant tissues and in faeces samples analysis. Although we tested the efficacy of PCR-CE combining two complementary genes on faecal samples from free-ranging chamois, the reliability and resolution of the method should be tested on even more species-diverse diets (Newmaster *et al.*, 2013a).

CMA provides valuable information about herbivore diet choices through microscopic recognition of digested plant epidermises (Holechek, 1982; Stewart, 1967). This method has been widely used for decades due to its versatility and low economic cost, although a high degree of observer expertise is required for accurate microscope interpretation and the level of discrimination between some related plants is relatively low (Carrière, 2002). Microhistological analysis of faeces is more precise than macroscopic identifications of rumen contents (Mohammad *et al.*, 1995; Nichols *et al.*, 2016), and may serve to assess diet composition not only in game ungulates but also in species with some degree of protection (e.g., Balkan chamois among others), thus offering the opportunity to avoid invasive hunting praxis. One of the strong features of CMA is that it offers the possibility to perform semi-quantitative determinations of herbivory diet composition, through the direct extrapolation of the plant epidermal fragment quantities present in the faecal samples (Alipayo *et al.*, 1992). However, differing degrees of digestibility of plant species that may determine the remaining plant material in the faeces must be considered (Bartolome *et al.*, 1998; Holechek *et al.*, 1982; Leslie *et al.*, 1983; Vavra & Holechek, 1980). To explain this apparent inconsistency**,** we propose an index of preservation for each plant species, called an EPF/I ratio, that would link the relative quantity of one species detected in a faecal sample (EPF) to the corresponding ingested quantity (I). The index would reflect a putative over-detection of a plant species in the faeces when EPF/I > 1, due to a slow or a reduced digestion process of the ingested plant tissues. Considering the plant species included in the study, the shrubby or herbaceous constitution of the plant is most likely the main factor determining the digestibility of a particular plant species. The plant cell wall is composed of polysaccharides and secondary metabolites that assemble to generate complex structures. Shrubs are persistent woody plants with reinforced and thicker cell walls compared to herbaceous plants, partially due to their high lignin content, and therefore are prone to a relatively low digestibility (Anthony & Smith, 1974; Heggberget *et al.*, 2002; Marinas & García-González, 2006). Intriguingly, the lignin content values reported in the bibliography for the species included in this study, describe an overall correlation with their structural constitution, where the highest lignin contents are consistent with the shrubby species - *C. vulgaris* and *H. nummularium* - and, concomitantly, with their average over-detection in faeces (Table 4). This observation suggests that lignin may exert a protective effect over plant structures throughout the digestion process, which would lead to their preservation, thus triggering the over-detection of shrubby compared to herbaceous species in the faeces. However, lignin content and plant constitution may not be the only explanations for the discrepancies between plant ingests and plant fragments detected in faeces (EPF/I ≠ 1), since in our experimental conditions two herbaceous species, *J. trifidus* and *T. alpinum,* showed equivalent EPF/I ratios, but different lignin contents. It is also notable that the concomitant detection of many plant species hinders the observation under the microscope and debases the relative species quantification, thus hampering the conclusive establishment of EPF/I correlations. This fact occurred in the corresponding faecal samples from diet 3 and especially in the case of the shrubby *V. uliginosum*. We propose that further determinations of individual EPF/I ratios for preferred plant species would provide valuable and more reliable information in CMA-based herbivore diet studies.

The greater resistance to digestion of some shrubby plant species observed by CMA was also perceived at the molecular level since *C. vulgaris* (diet 1) and *H. nummularium* (diet 2) were PCR-amplified in faecal samples belonging to diets 2 and 3, respectively, while none of the herbaceous plants showed this behaviour. Furthermore, PCR amplification is roughly independent of the structural constitution of a plant species but strongly hinges on sample freshness. Thus, while the number of species detected by CMA remained stable in samples kept outdoors for three weeks, the number of species amplified by PCR markedly decayed within the first week. The length of the PCR amplicons, which were 450 bp on average, provides high resolution to discriminate between plant species, but the progressive degradation of the genomic DNA due to deficient preservation conditions is a drawback for amplification success (Czernik *et al.*, 2013; Deagle *et al.*, 2006; King *et al.*, 2008; Parveen *et al.*, 2016). Although PCR amplification of shorter genomic fragments (70-100 bp in length) from ancient samples containing partially degraded genomic DNA has been previously described in DNA barcoding studies, plant species adscription was performed through massive DNA sequencing instead of amplicon length determination (Bellemain *et al.*, 2013; Haile *et al.*, 2007; Hofreiter *et al.*, 2000). Massive sequencing technologies are powerful tools that provide accurate information but require expensive technology and specific bioinformatic expertise that can serve as a barrier to standard implementation. We propose that determination of amplicon sizes by capillary electrophoresis is a precise, less time-consuming, standardisable and affordable method and that it can replace complex massive sequencing technologies in routine studies of diet composition from fresh faecal samples, as has been previously pointed out (Czernik *et al.*, 2013; Pegard *et al.*, 2009).

Conclusions

Taken together, our results suggest that PCR-CE combining the simultaneous amplification of two genes – long *trnL(UAA)* and *ITS2* - is a reliable technique for the determination of diet composition in herbivores. However, while this DNA-based method is qualitative in our experimental conditions, CMA can provide semi-quantitative data, since individual percentages of ingested plant species can be calculated, although a differential index of plant preservation should be taken into account. Additionally, the experimental conditions of outdoor exposure acted concomitantly with proper digestion processes to trigger genomic DNA degradation. Consequently and as expected, faeces freshness and preservation conditions are critical and determinant factors that compromise downstream DNA amplification efficiency but are not limiting in the case of CMA. We conclude that CMA and PCR-CE have both strong and weak features that make the two approaches complementary.

Acknowledgements

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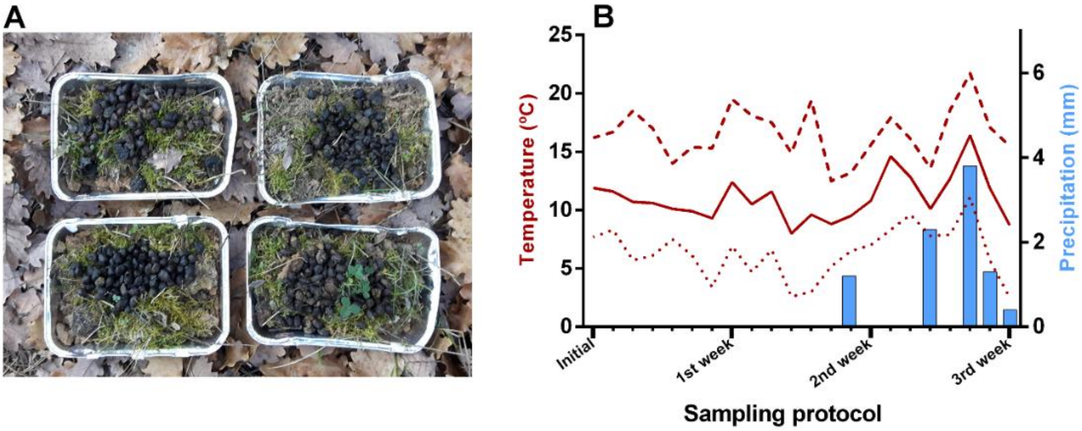
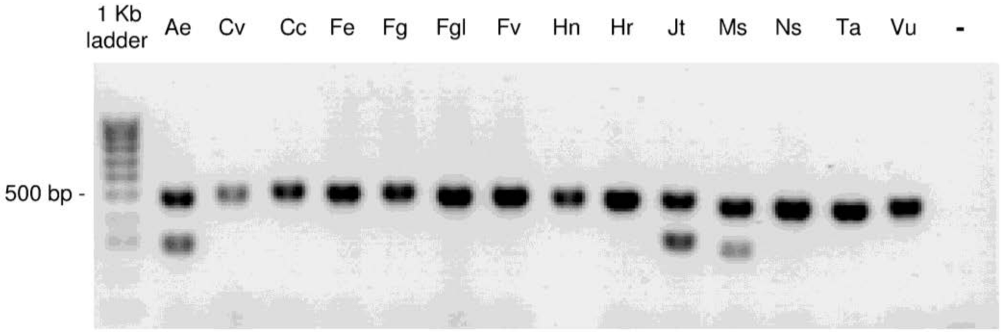
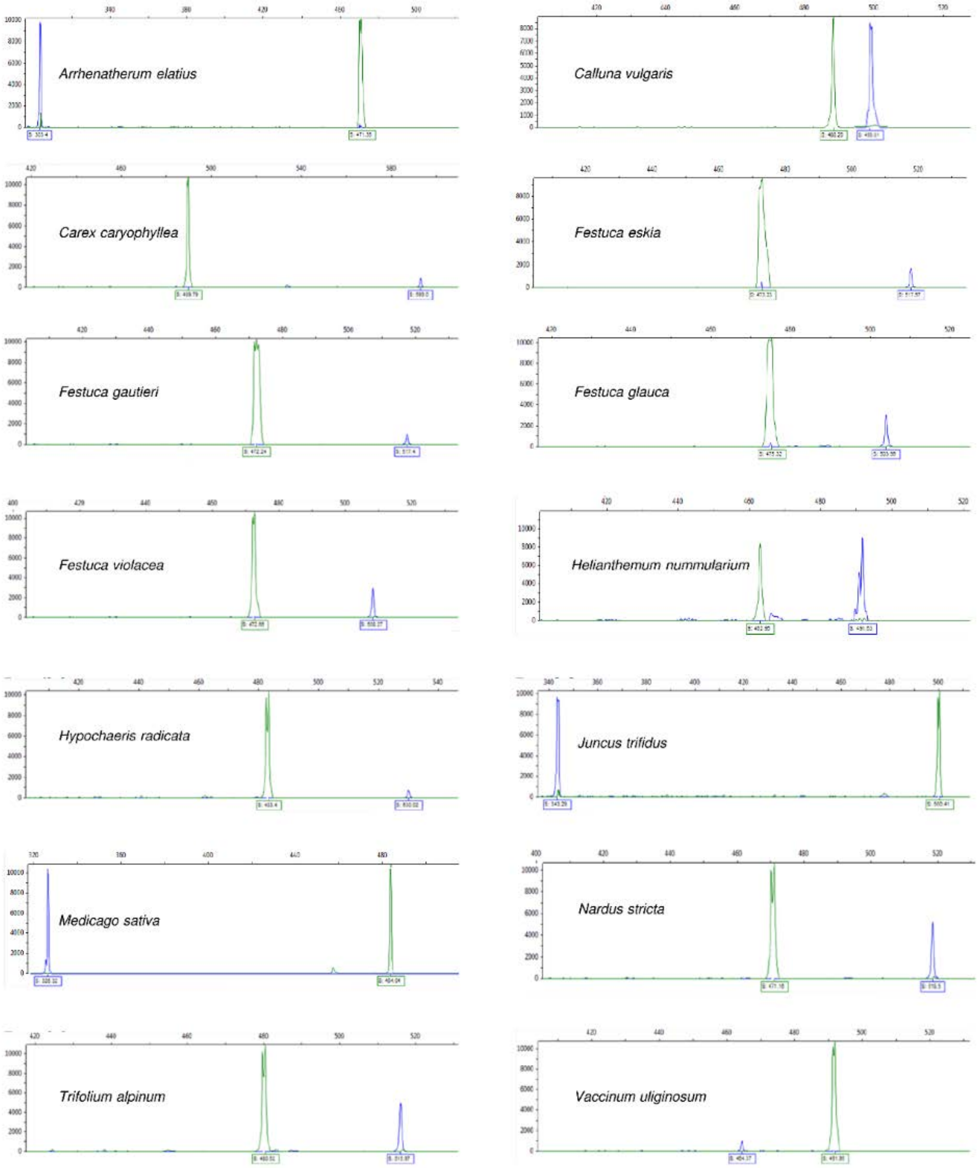
Supporting information



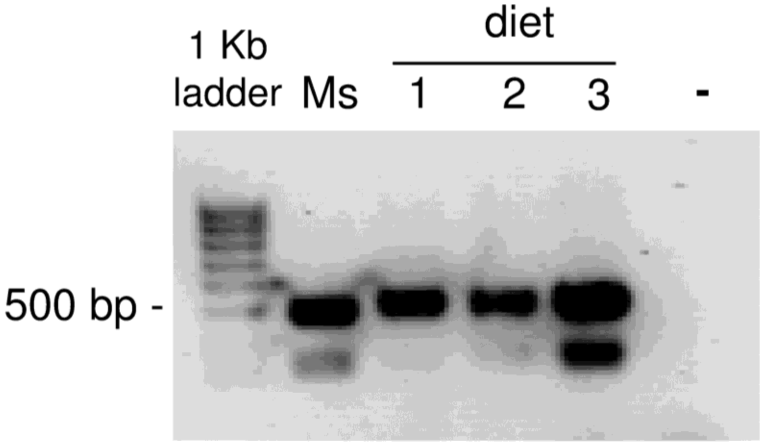
S5.1.1 Fig. (A) Picture of the four pools of faecal samples from the time-course degradation experiment. (B) Environmental conditions while faecal samples where left outdoor (red line: mean daily temperature; red dotted line: minimum daily temperature; red dashed line: maximum daily temperature; blue bars: daily cumulative rainfall)



S5.1.2 Fig. Migration patterns on a 1,5 % agarose electrophoresis of *trnL(UAA)* and *ITS2* PCR products corresponding to the reference plant database amplifications. Ae: *Arrhenatherum elatius,* Cv: *Calluna vulgaris*, Cc: *Carex caryophyllea*, Fe: *Festuca eskia,* Fg: *Festuca gautieri,* Fgl: *Festuca glauca*, Fv: *Festuca violacea*, Hn: *Helianthemum nummularium,* Hr: *Hypochoeris radicata*, Jt: *Juncus trifidus*, Ms: *Medicago sativa,* Ns: *Nardus stricta*, Ta: *Trifolium alpinum*, Vu: *Vaccinum uliginosum.* (-): non template negative PCR control. 1Kb ladder is a reference ladder marker.



S5.1.3 Fig. Electropherogram peaks of the 14 plant species included in this study, obtained after capillary electrophoresis of the fluorescense labelled PCR products corresponding to *trnL(UAA)* (blue) and *ITS2* (green) multiplex amplification.



S5.1.4 Fig. Migration patterns on a 1,5 % agarose electrophoresis of *trnL(UAA)* and *ITS2* PCR products amplified from previous controlled diet faeces genomic DNA (*Medicago sativa* intake, Ms) and from faeces genomic DNA corresponding to diets 1 to 3. Composition of diets is detailed in Table 5.1.1. (-): non template negative PCR control. 1Kb ladder is a reference ladder marker.

S5.1.1 Table. Universal oligonucleotide sequences used for specific PCR amplification of chloroplastic *trnL(UAA)* and nuclear *ITS2* regions. Fluorescence-labelling of oligonucleotides TRNL_D and S2F is indicated. Expected amplicon size is given in base pairs (bp). TGS= Target genomic sequence. [1] Taberlet *et al.*, 2007; GenBank; [2] Chen *et al.*, 2010.

	Sequence	Position	Target genomic sequence	Fluorochrome dye	Amplicon size (bp)	Ref.
TRNL_G	$^{5'}$gggcaatcctgagccaaatc$^{3'}$	Forward	*trnL(UAA)*	-	310-593	[1]
TRNL_D	$^{5'}$ggggatagagggacttgaac$^{3'}$	Reverse		6-FAM		
S2F	$^{5'}$atgcgatacttggtgtgaat$^{3'}$	Forward	*ITS2*	HEX	195-510	[2]
S3R	$^{5'}$gacgcttctccagactacaat$^{3'}$	Reverse		-		

5.2 Study 2:

Seasonal diet composition of Pyrenean chamois is mainly shaped by primary production waves

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Abstract

In alpine habitats, the seasonally marked climatic conditions generate seasonal and spatial differences in forage availability for herbivores. Vegetation availability and quality during the growing season are known to drive life history traits of mountain ungulates. However, little effort has been made to understand the association between plant phenology and changes in the foraging strategies of these mountain dwellers. Furthermore, this link can be affected by the seasonal presence of livestock in the same meadows. The objective of this work was to study the seasonal changes in diet composition of Pyrenean chamois (*Rupicapra p. pyrenaica*) and its relationship to primary production trends in a Mediterranean alpine environment. Moreover, diet composition in two populations with contrasting livestock pressure was compared in order to study the effect of sheep flocks on the feeding behaviour of chamois. From 2009 to 2012, monthly diet composition was estimated by cuticle microhistological analysis of chamois faeces collected in the eastern Pyrenees. The primary production cycle was assessed by remote sensing, using the Normalized Difference Vegetation Index. Additionally, the diet of sheep sharing seasonally the subalpine and alpine meadows with chamois was analysed. Diet selection of chamois and sheep and their overlap was also assessed. Our results show an intra-annual variation in the diet composition of Pyrenean chamois and demonstrate a strong relationship between plant consumption dynamics and phenology in alpine areas. In addition, *Calluna vulgaris*, *Cytisus* spp. and *Festuca* spp., as well as forbs in the summer, are found to be key forage species for Pyrenean chamois. Furthermore, this study couldn't detect differences between both chamois populations despite the presence of sheep flocks in only one area. However, the detection of a shift in the diet of chamois in both areas after the arrival of high densities of multi-specific livestock suggest a general livestock effect. In conclusion, Pyrenean chamois are well adapted to the variations in the seasonal availability of plants in alpine habitats but could be disturbed by the seasonal presence of livestock. Due to the key plants in their diet, we suggest that population management programmes should focus on the preservation of mixed grasslands composed of patches of shrubs and herbs. The effects of climate change and shrub expansion should be studied as they may potentially affect chamois population dynamics through changes in habitat composition and temporal shifts in forage availability.

Introduction

The alpine ecosystems are some of the most seasonal biomes in the world, covering around 3% of the earth's surface area (Nagy & Grabherr, 2009). Alpine landscapes remain free of snow for a short period of time (60-120 days on average (Ernakovich *et al.*, 2014; Nagy & Grabherr, 2009)), limiting not only opportunities for vegetation growth but also the availability of food resources for primary consumers (Christianson & Creel, 2009; Jonas *et al.*, 2008). Outside this short green period, annual plants become dry and nutrient-poor (Körner, 2011) and the evergreens develop frost-resistance mechanisms, which in turn reduce their palatability (Neuner, 2014). In such extreme environments, herbivores must decide which plants should be consumed, and when and to what degree, in order to maximise their reproductive potential (Morano *et al.*, 2013; Tollefson *et al.*, 2010), health (Treanor *et al.*, 2015) and ultimately survival rates (Cook *et al.*, 2004).

Diet selection, therefore, plays a central role in herbivore-plant interactions, with consequences for plant community composition and, in turn, the herbivore's population viability (Bergman *et al.*, 2014). Surprisingly, little effort has been devoted to addressing this link between plant phenology and changes in the foraging strategies of mountain species whereas plant phenology and resource availability have been repeatedly shown to affect multiple fitness components in large herbivores (Hamel *et al.*, 2009; Pettorelli *et al.*, 2007; Treanor *et al.*, 2015). Diet selection studies can also contribute to identifying key forage species used to maximize body condition gains during the growing season (Rominger *et al.*, 1988), or to increase the chance of winter survival (Takahashi & Kaji, 2001). Research effort in that sense provides important clues for developing population management programmes aimed at preserving areas of special interest (Lopez *et al.*, 2017).

The Pyrenean chamois (*Rupicapra pyrenaica pyrenaica*) is a medium-sized mountain ungulate widely distributed in alpine and subalpine habitats of the Pyrenees (Corlatti *et al.*, 2011). Chamois are considered capital breeders (they store energy as body reserves to meet the high energetic costs of reproduction (Jönsson, 1997)) and intermediate feeders, capable of adapting their digestive system to woody plants or grasses depending on availability (García-gonzález & Cuartas, 1996). Most of the chamois' life history traits such as nursing, weaning or body mass rely on the amount of energy stored during summer (Corlatti *et al.*, 2013; Scornavacca *et al.*, 2016). Although chamois can undergo short seasonal altitudinal migrations of no more than 10km in response to winter hardness and variations in food quality, they are usually a sedentary and over-wintering species (Crampe *et al.*, 2007; Gálvez-Cerón *et al.*, 2013). Winter diets, on the other hand, compensate for the high locomotion and thermoregulation costs in snowy landscapes (Bocci *et al.*, 2010; Rughetti *et al.*, 2011). Considerable research effort has been directed towards summer (Ferrari *et al.*, 1988; Ferretti *et al.*, 2015; García-González, 1984) and autumn diets (Andreoli *et al.*, 2016; Rayé *et al.*, 2011), whereas information on the spring and winter feeding behaviours of chamois is practically non-existent.

In most mountainous regions, humans have traditionally moved livestock up to alpine pastures during summer to take advantage of the nutritious growing plants (Montserrat & Fillat, 1990). This transitory increase in the number of ungulates can be huge (e.g., more than 300.000 livestock units in Switzerland (Mack *et al.*, 2013)) and may result in an overgrazing of the most palatable plant species (Evju *et al.*, 2006). As a result, livestock can become a potential competitor for native mountain ungulates by equally using the same finite resources (scramble competition (de Boer & Prins, 1990)), depleting the availability of a resource for native species (exploitation competition) or directly disturbing the behaviour of these species (Chirichella *et al.*, 2014). The consequences of such interactions between ecologically close ungulate species constitute a major challenge for community and foraging ecologists (Loison *et al.*, 2003). In European mountains, for example, chamois shares habitat with livestock during summer, mainly horses, cattle and sheep (Chirichella *et al.*, 2014). Because of their morphological and functional similarities, resource competition between chamois and domestic sheep (*Ovis aries*) has been the most studied by far (Berducou, 1986; La Morgia & Bassano, 2009; Rebollo *et al.*, 1993). In fact, flocks of sheep seem to force chamois to graze in suboptimal areas (Chirichella *et al.*, 2013; Mason *et al.*, 2014; Rebollo *et al.*, 1993), decreasing the proportion of highly digestive forbs in their diets and probably affecting body mass gains during summer.

In this work, we describe the seasonal changes in diet composition of Pyrenean chamois and their relationship to primary production trends, using three years of monthly faecal sampling in two populations from the Catalan Pyrenees (north-eastern Spain). In particular, we describe the seasonal feeding behaviours of chamois through faecal cuticle microhistological analysis and assess the cycles of primary productivity through the NDVI (Normalized Difference Vegetation Index). As foraging behaviour of northern ungulates is presumed to be mainly determined by primary production cycle (Parker *et al.*, 2009; Villamuelas *et al.*, 2016), we expected that chamois adapt their feeding strategy to plant phenology. We will also explore the effects of livestock on such seasonal feeding behaviour by studying the diet composition of chamois in two areas with different livestock communities (i.e., with and without flocks of sheep). Due to the impact of sheep flocks on chamois behaviour (La Morgia & Bassano, 2009), we expected to observe a use of suboptimal resources by chamois during the cohabitation period in the area where sheep was present. Diet selection of chamois and sheep in July (i.e., taking into account plant availability), and their overlap is also assessed and discussed in order to understand the potential interactions between these species.

Materials and Methods

Study area

The study was conducted in the Freser-Setcases National Game Reserve (FSNGR), eastern Pyrenees, Catalonia, Spain (42° 22' N, 2° 09' E, Fig 1). The FSNGR is a mountainous area of 20.200 ha where subalpine and alpine ecosystems predominate with an average altitude of 2000 m. a. s. l. (1200 - 2910 m.a.s.l. at Puigmal peak).

Sampling was carried out in two areas separated by 20km of rough terrain called Costabona and Fontalba (Fig 5.2.1). The former is around 410ha and located in the north-eastern part of the FSNGR and the latter is 717ha and located in the western part of the game reserve. The two areas range from 1.700 to 2.500 m.a.s.l. and are characterised by similar features in terms of vegetation composition and structure, typical of the sub-humid subalpine and alpine bioclimatic belts of the southern slopes of the Pyrenees with a noticeable climatic Mediterranean influence (Vigo, 2008). During the study period, annual mean temperature was 5.7 ºC (min = 4.68, max = 6.21) and mean yearly accumulated rainfall was 1042.4 mm (min = 762.6, max = 1282.8). From July to September, mean temperature was 12.7ºC (min= -12.6, max=12.8) and mean accumulated rainfall was 214.5 mm (min=169.2, max=283.0). From November to April, mean accumulated snowfall was 1124mm (min=877, max=1354) and mean snow depth was 128mm (min=41.2, max= 336.7, period 2009-2012, data from Nuria meteorological station located at 1971 m.a.s.l. in the core FSNGR, Servei Meteorològic de Catalunya < www.meteocat.com >).

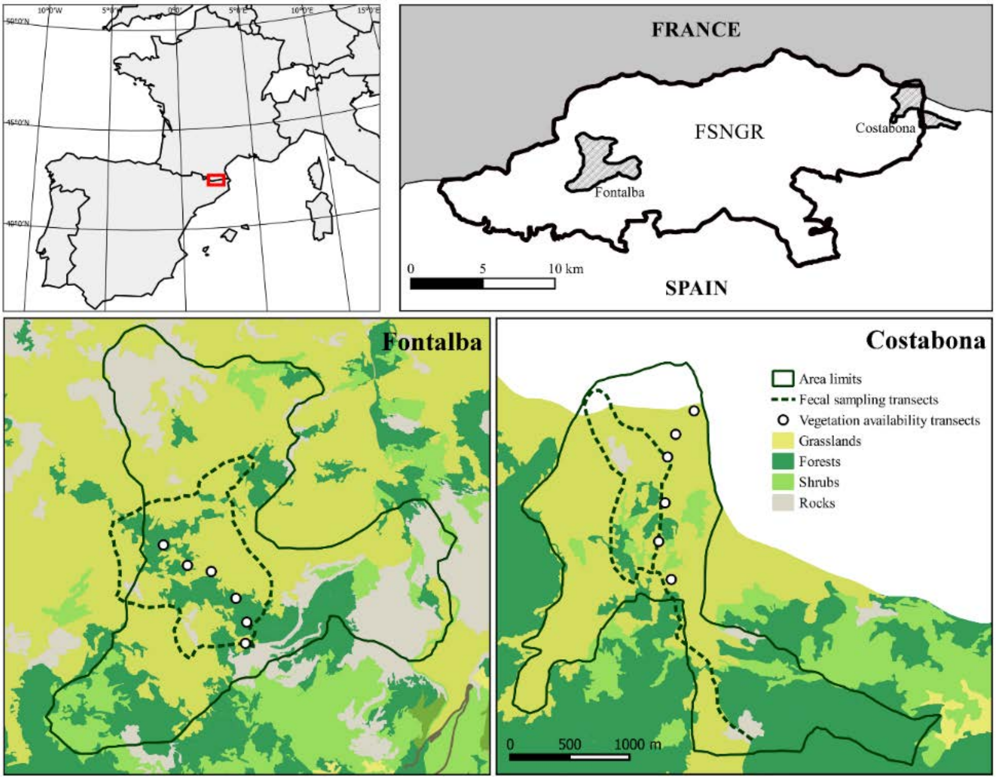

Fig 5.2.1. Location and map of the two study areas (Fontalba and Costabona) in the Freser-Setcases National Game Reserve. Transects for faecal sampling (dotted lines) and for vegetation availability assessment (black circles) are shown.

In Fontalba, from mid-May to late October, at least 220 chamois share habitat with cattle (n = 309) and horse (n=47) herds. In Costabona, a group of approximately 100 chamois coexists with herds of at least 647 cattle, 71 horses and 352 sheep. Stocking rates are around 0.57 livestock units (LU)/ha in Fontalba and 2LU/ha in Costabona (S5.2.1 Table). Sporadically, other herbivores such as roe deer and mouflon were observed in the study areas but they were not taken into account in this study due to their low densities (less than 300 for the whole FSNGR in the case of mouflon) and encounter rate.

Vegetation availability

The vegetation of the study areas mainly consists of subalpine and alpine grasslands dominated by graminoid taxa (e.g., *Festuca* and *Carex* genera) and patches of *Trifolium alpinum* and *Calluna vulgaris*. Scattered *Pinus uncinata* forest patches also grow with an understory of small woody groundcover shrubs (e.g., *Juniperus communis, Rhododendron ferrugineum*) (Vigo, 2008). The vegetation availability during June 2011 was assessed following the point-intercept method proposed by Daget & Poissonet (1971). In brief, we defined 6 transects of 10m (see Fig 5.2.1) at different altitudes in each study area (from 1900 to 2500 m.a.s.l.). In each transect, at every 10 cm interval, a 50 cm steel needle was vertically planted in the ground and all plant species touching the needle were identified and recorded (i.e. 100 points of vegetation measurement per transect). The frequency of occurrence for each plant species was then calculated by transect as: specific number of occurrences / total number of occurrences.

Faecal sampling procedure

From the 22nd of May 2009 to the 30th of November 2012, fresh chamois faecal samples were collected monthly by at least two observers following defined transects of about 5 km each within the two study areas (see Fig 1), between 1900 and 2400 m.a.s.l. Observers located chamois groups using 10 x 42 binoculars and 20 - 60 x 65 spotting scopes. These transects encompassed the main vegetation communities and the altitudinal movement of chamois throughout the year within each study area. Once group size, composition and precise location of chamois were recorded, observers collected fresh droppings at the exact location where animals were seen and in their surroundings. Based on the colour, texture and the presence of mucus, we estimated a maximum of five hours between defecating and collection (Hibert *et al.*, 2011). Six faecal samples per transect were collected in separated labelled plastic bags and transported to the laboratory where they were frozen at -20°C. Groups of animals that were observed twice were only sampled once in order to avoid double-sampling the same individual. For further cuticle microhistological analyses, all six samples collected along the transect were gathered into a monthly sample (86 monthly samples in total: 44 for Fontalba and 42 for Costabona).

The extent of our study areas was based on the monitoring of these populations of chamois through nine years of a capture-mark-observation study (unpublished data).

In the Costabona area, sheep faecal samples were also collected monthly from June to October 2011 and 2012 following the same sampling protocol. Similarly, six faecal samples were collected along the same transect were pooled into a monthly sample (9 monthly samples in total, as in September 2012, sheep were not observed in the area and faecal samples could not be collected).

Diet composition assessment

A cuticle microhistological analysis of chamois and sheep faecal samples was used for our diet composition assessment (Croker, 1959). This non-invasive method has been widely used for studying diet composition of both wild (La Morgia & Bassano, 2009; Suter *et al.*, 2004) and domestic animals (Bartolomé *et al.*, 2011) without interfering with their feeding behaviour (Bartolomé *et al.*, 1998). Following Stewart's protocol (Stewart, 1967), samples were thawed, washed with distilled water and ground in a mortar to separate the epidermal fragments. Ten grams of sample were then placed in a test tube with 5 ml of 65% concentrated HNO3. The test tubes were then boiled in a water bath for 1 min. After digestion in HNO3, the samples were diluted with 200 ml of water. This suspension was then passed through 1.00 and 0.25 mm filters. The 0.25–1.00mm fraction was spread on glass microscope slides in a 50% aqueous glycerine solution and cover-slips were fixed with DPX microhistological varnish. Two slides were prepared from each sample. Later, slides were examined by the same operator under a microscope at ×100 and ×400 magnifications and plant fragments were recorded and counted up to 200 units of leaf epidermis. An epidermis collection of 55 main plant taxa from the study area was made and used as a reference for identification of fragments in faecal samples. Since 28 plant taxa were present at levels less than 1% of the fragments, all recorded plants were pooled into five functional groups, namely: leguminous woody plants (hereafter LW), non-leguminous woody plants (NLW), graminoid plants (GR), leguminous forb plants (LF) and non-leguminous forb plants (NLF).

Primary production assessment

We used the Normalised Difference Vegetation Index (NDVI) as a proxy for vegetation productivity and phenology in Fontalba and Costabona (for review, see (Pettorelli, 2013)). We worked with MOD13Q1 NDVI data extracted from the MODIS repository (Moderate Resolution Imaging Spectroradiometer) provided by NASA (Didan, 2015). NDVI time series were calculated for Costabona and Fontalba for the period from January 2009 to December 2012 with 16 day composites at a spatial resolution of 250 m. NDVI pixels (146 pixels for Fontalba and 86 for Costabona) that fell within the boundaries of the study area shape files were extracted and an average of these pixel values was calculated.

Statistical analysis

Firstly, differences in vegetation availability between the two study areas were checked using a permutational multivariate analysis of variance (PERMANOVA). This resemblance-based permutation method allows a geometric partitioning of variation across a distribution-free multivariate dataset using distance matrices (Anderson, 2017). PERMANOVA is widely used in ecology to compare communities across ecological gradients (Anderson & Walsh, 2013). In our case, the multivariate response variable was the percentage of the total count of the five plant groups (NLW, LW, Gr, NLF and LF), whereas the study area (Fontalba vs Costabona) was our fixed categorical factor. Manhattan dissimilarity index was used, as it had the highest rank-order similarity with gradient separation in our community matrix.

Seasonal changes in the diet composition of chamois, in terms of plant use, were described performing the same PERMANOVA approach using the five plant groups as response variables. Three phenological periods (called Green-up from 1 March to 30 June, Plateau greenness from 1 July to 31 August , and Senescence periods from 1 September to 28/29 February; according to Villamuelas *et al.*, (2016), and two contrasting livestock periods (presence of livestock from June to October and absence of livestock from November to May) and their two-way interaction with the study areas were used as explanatory variables. In this case, the Bray-Curtis dissimilarity index was used as it had the highest rank-order similarity with gradient separation in this community matrix.

The relationships between diet composition and NDVI were explored using generalised additive models (GAM), based on the cubic regression splines method. We fitted a separate GAM for each plant group (LW, NLW, Gr, NLF and LF), using NDVI values as fixed explanatory factors and the percentages of each plant group as response variable. The assumptions of normality, homoscedasticity and independence were previously checked by the residual analysis. As NDVI values correspond to 16 day composites, we selected the value with the closest date to each sampling day. This GAM analysis is commonly used to explore non-linear relationships in ecology due to its robustness and flexibility (Fewster *et al.*, 2000). Furthermore, the phenological dates for the start of the growing season (SOS, day of the year identified as having a consistent upward trend in the NDVI time series) and the peak of production (POP, corresponding with the day of the year when the NDVI reaches its maximum value in an annual time series) were also calculated. A further description of these metrics can be found in Forkel *et al.* (2015).

Additionally, diet preferences of both chamois and sheep were explored through a plant selectivity analysis (Manly *et al.*, 1993). This technique compares the resources used by the animals (e.g., faecal microhistological analyses) with the resources available in the area (e.g., plant availability). In particular, a Type II selectivity analysis was performed, as information about the utilised resources is available at the individual level but available resources were assessed at the population level. Values under one indicate avoidance, values around one indicate an opportunistic consumption and values above one indicate preference.

Finally, diet overlap between Pyrenean chamois and sheep in the Costabona area was calculated using Horn's index of overlap (Horn, 1966), as it is considered the method least biased by sample size when resource use is expressed as proportions (Smith & Zaret, 1982):

$$R_o = \frac{\sum(P_{ij} + P_{ik})\log\left(P_{ij} + P_{ik}\right) - \sum P_{ij} log P_{ij} - \sum P_{ik} log P_{ik}}{2 log 2}$$

where P_{ij} and P_{ik} are the proportions of resource i used by species j (Pyrenean chamois) and species k (sheep). Index values range from zero (no resources used in common) to one (complete overlap).

All the statistical analyses were performed using the statistical software R version 3.4.2 (R Core Team, 2018) and the significance threshold was set at 0.05. The PERMANOVA approaches were conducted with the R-package "Vegan" (version 2.4-5, Oksanen *et al.*, 2017). GAMs were implemented using the R-package "mgcv" (Version 1.6-1, Wood, 2011) and the phenological dates were calculated with "Greenbrown (version 2.4.3; Forkel *et al.*, 2013) while "AdehabitatHS" (version 0.3.13, Calenge, 2006) was used in the plant selectivity analysis.

Ethics Statement

All necessary permits were obtained for the described field studies. Permission to conduct research in the Freser-Setcases National Game Reserve was obtained from the reserve director. Because only faecal material was collected for the present study, no Institutional Animal Care and Use Committee (IACUC) approval was required.

Results

Vegetation availability

We recorded 70 plant taxa (65 different taxa in Fontalba and 65 in Costabona) with 60 of these detected in both areas. Only 13 of these plants were present at levels greater than 1% of the fragments (representing 83.2% of the total availability, See Table 5.2.1). A mean of 0.25% of the observed fragments could not be identified (min=0%, max=6.5%). The PERMANOVA test confirmed that plant availability was similar between our studied areas (Pseudo $F_{1,10}$ = 0.37, p-value = 0.792).

Graminoids were the most abundant functional group followed by forbs and woody species in decreasing order of importance. Graminoids represented half of the vegetation cover, with *Festuca* spp. the dominant species followed by *Carex cariophyllea*. Forbs, covering almost one third of the area, were equally represented by legumes and non-legume plants. Leguminous forbs were dominated by *Trifolium alpinum* and no non-leguminous forb species could be considered as dominant. The rest were woody plants, where the most common were some dwarf shrubs (*Calluna vulgaris* and *Juniperus communis* ssp. *alpina*) and legumes *Cytisus* spp.

Table 5.2.1. Plant availability (i.e., proportion of a given plant taxa with respect to the total of recorded plants in %) assessed by the line-intercept method in 6 transects of 10m length conducted at altitudes ranging from 1900 to 2500 m.a.s.l. in two areas (Costabona and Fontalba) in the Freser-Setcases National Game Reserve, Catalonia, north-eastern Spain. Only plants present at levels greater than 1% are represented.

Plant	Fontalba	Costabona
Non-leguminous woody		
Calluna vulgaris	7.36	7.92
Juniperus communis	5.12	4.62
Pinus uncinata	1.26	1.73
Other NLW	0.85	0.51
Leguminous woody		
Cytisus spp.	4.88	4.32
Other LW	0.69	1.12
Graminoids		
Avenula pratensis	1.26	1.88
Carex caryophyllea	9.63	12.44
Festuca spp.	33.86	32.30
Nardus stricta	3.58	2.49
Other Gr.	0.61	2.54
Non-leguminous forbs		
Cruciata glabra	1.42	0.66
Hieracium pilosella	2.68	1.12
Plantago monosperma	1.99	2.13
Other NLF	10.20	11.27
Leguminous forbs		
Lotus corniculatus	1.34	1.02
Trifolium alpinum	11.99	7.52
Other LF	1.26	4.37

Diet composition of Pyrenean chamois

Our PERMANOVA analysis also revealed that the diet composition of Pyrenean chamois was similar between the two study areas (Pseudo $F_{1,85}$ =0.63, ns) and differed significantly along the three Phenological periods (Pseudo $F_{1,85}$ = 11.83, R^2 = 0.222, p-value = 0.001) and the two Livestock periods (Pseudo $F_{1,85}$ = 18.26, R^2 = 0.179, p-value = 0.001), with no significant Area x Phenological period (Pseudo $F_{1,85}$ = 0.79, ns) or Area x Livestock period interaction (Pseudo $F_{1,85}$ = 0.76, ns). A total of 39 plant taxa were microscopically identified in faecal samples from chamois during the three years of study and only 13 appeared in an amount greater than 1% of the total number of fragments. These 13 taxa represented 91.9% of the diet composition (Fig 5.2.2).

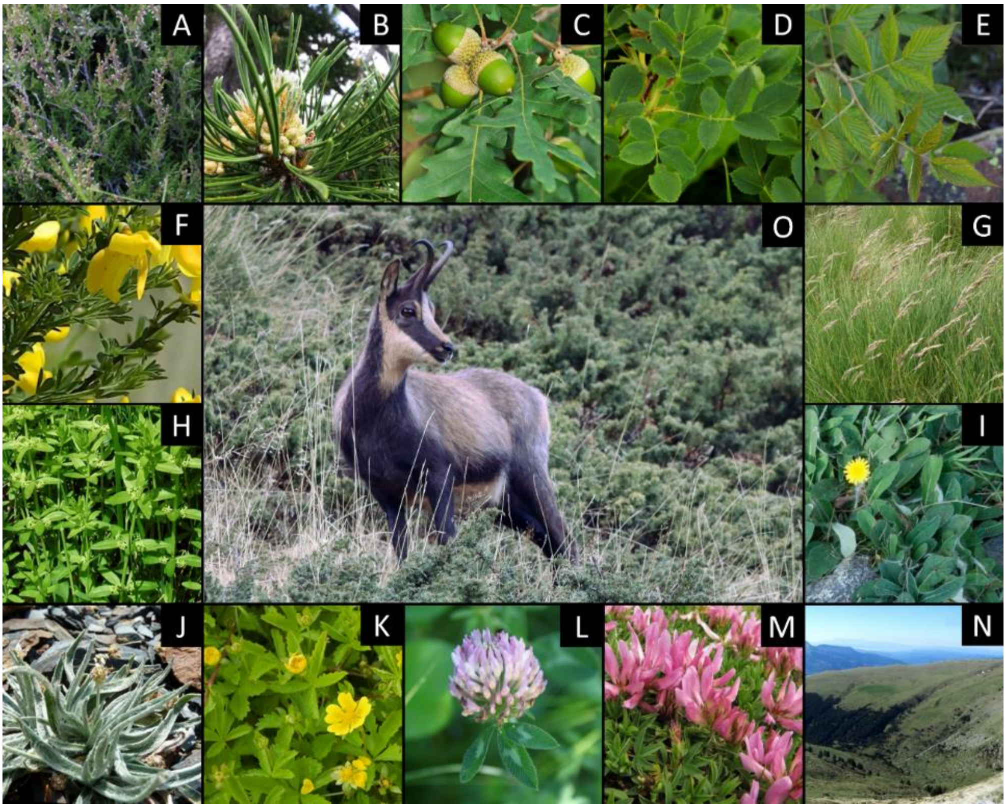

Fig 5.2.2. Pictures of the 13 plants most consumed by Pyrenean chamois in the Freser-Setcases National Game Reserve (FSNGR), Catalonia, north-eastern Spain. A-*Calluna vul*garis; B-*Pinus uncinata*; C-*Quercus* sp.; D-*Rosa* sp.; E-*Rubus* sp.; F-*Cytisus* spp.; G-*Festuca* spp.; H-*Cruciata glabra*; I-*Hieracium pilosella*; J-Plantago monosperma; K-Potentilla sp.; L-*Trifolium alpinum*; M-*Trifolium pratense*. Additionally, a picture of the FSNGR (N) and a Pyrenean chamois (O).

As we can observe in tables 5.2.2 and 5.2.3, during the green-up period (i.e., from March to June) more than half of the diet was composed of woody species (51.6%), with *Calluna vulgaris* (21.3% of the total of fragments) and *Cytisus* spp. (17.0%) the most consumed plants. Graminoids were also an important component of the diet (32.1%) with a predominance of *Festuca* spp. (27.0%). The rest of the diet was composed of forbs (16.4%) and their consumption intensified along the months from 5.6% in March to

35.5% in June at the expanse of woody plants. *Trifolium alpinum* (3.1%) was the most consumed plant among LF, while *Plantago monosperma* (2.1%) dominated NLF.

The plateau greenness period (i.e., from July to August) was still characterised by a major consumption of woody plants (52.0%), mostly *Cytisus* sp. (25.1%) and *Calluna vulgaris* (19.0%), but unlike the green-up period, forbs (32.6%) were more present than graminoids (15.6%).

Finally, during the senescence period, the consumption of woody plants reached its peak (70.3%) and the consumption of forbs reached its lowest point (11.9%). Graminoids, however, were still consumed at nearly the same rate as during the plateau greenness period (17.8%). *Calluna vulgaris* (34.3%) and *Cytisus* spp. (21.1%) were still the most consumed plants during this period, followed by *Festuca* spp. (15.2%) and *Quercus* sp. (6.1%).

Table 5.2.2. Non-leguminous woody (NLW), Leguminous woody (LW), and Graminoids (GR) plants in the annual diet composition of Pyrenean chamois from the Freser-Setcases National Game Reserve (Catalonia, north-eastern Spain), estimated by cuticle microhistological analysis of faecal samples. Values represent mean percentage of fragment frequency (min-max). Forbs content are presented in Table 5.2.3. Only plants with a consumption of more than 1% are represented.

	Green-up period				**Plateau greenness**		**Senescence Period**					
	March	**April**	**May**	**June**	**July**	**August**	**September**	**October**	**November**	**December**	**January**	**February**
NLW												
Calluna vulgaris	32.9 (11.5-53.0)	39.5 (20.0-53.0)	10.6 (0.0-29.5)	2.1 (0.0-6.0)	14.1 (2.5-25.0)	23.8 (8.5-44.5)	27.0 (9.5-44.0)	23.6 (10.0-33.5)	39.0 (30.5-47.0)	40.2 (35.5-45.5)	41.3 (29.0-64.5)	34.6 (22.0-46.0)
Pinus uncinata	11.9 (2.0-31.5)	4.5 (1.0-15.5)	2.2 (0.0-8.5)	0.9 (0.0-3.0)	0.5 (0.0-2.0)	0.4 (0.0-1.0)	0.1 (0.0-0.5)	0.2 (0.0-1.5)	1.6 (0.0-8.5)	0.4 (0.0-1.5)	0.4 (0.0-1.0)	3.0 (0.0-10.0)
Quercus sp.	3.3 (0.0-10.5)	2.6 (0.0-13.0)	0.0 (0.0-0.0)	0.1 (0.0-0.5)	0.0 (0.0-0.0)	0.4 (0.0-1.0)	0.9 (0.0-3.5)	3.0 (0.0-7.5)	5.1 (0.0-12.5)	9.4 (3.0-13.0)	11.1 (5.5-18.0)	7.0 (0.0-13.5)
Rosa sp.	0.6 (0.0-2.5)	0.2 (0.0-1.0)	1.0 (0.0-2.5)	1.9 (0.0-5.5)	1.9 (0.0-4.5)	1.4 (0.0-10.0)	2.7 (0.0-4.5)	4.1 (0.0-10.0)	0.4 (0.0-3.0)	0.0 (0.0-0.0)	0.0 (0.0-0.0)	0.2 (0.0-1.5)
Rubus sp.	6.1 (0.0-18.5)	7.8 (0.0-28.0)	3.1 (1.5-6.0)	5.8 (2.0-14.0)	4.1 (0.5-8.0)	6.9 (0.0-18.5)	8.3 (0.0-17.0)	13.8 (5.0-27.0)	3.3 (1.0-8.0)	3.3 (0.0-6.5)	2.6 (0.5-6.5)	3.5 (0.0-15.0)
Other NLW	0.4 (0.0-1.5)	0.4 (0.0-2.0)	0.3 (0.0-1.5)	0.3 (0.0-1.0)	0.3 (0.0-1.0)	0.0 (0.0-0.0)	0.5 (0.0-1.5)	2.3 (0.0-8.0)	0.6 (0.0-1.5)	0.2 (0.0-0.5)	0.4 (0.0-1.0)	1.4 (0.0-3.5)
Total	**55.1**	**54.9**	**17.1**	**11.1**	**20.9**	**32.9**	**39.5**	**47.0**	**50.0**	**53.5**	**55.8**	**49.7**
LW												
Cytisus spp.	24.5 (2.0-41.5)	19.6 (12.5-35.5)	9.3 (4.5-17.0)	14.8 (3.5-31.5)	24.6 (9.5-42.5)	25.6 (8.0-54.5)	25.1 (4.0-51.5)	14.8 (8.0-22.5)	15.6 (5.5-24.0)	22.0 (10.0-37.5)	24.9 (16.5-32.5)	24.1 (7.5-32.5)
Total	**24.5**	**19.6**	**9.3**	**14.8**	**24.6**	**25.6**	**25.1**	**14.8**	**15.6**	**22.0**	**24.9**	**24.1**
GR												
Festuca spp.	12.8 (4.5-21.0)	16.9 (14.5-24.5)	47.6 (34.0-60.5)	30.9 (17.5-50.0)	13.9 (6.0-18.0)	13.8 (6.0-24.0)	15.9 (7.5-27.5)	17.0 (4.5-32.5)	15.8 (8.0-25.0)	12.2 (7.0-18.0)	14.5 (11.0-22.0)	15.6 (7.5-23.5)
Other Gr.	2.0 (0.0-4.0)	6.0 (0.5-10.0)	5.9 (0.0-19.5)	6.3 (1.0-10.5)	2.1 (1.0-4.5)	1.5 (0.0-3.0)	2.6 (0.5-6.5)	1.6 (0.0-6.5)	4.1 (0.0-10.0)	3.0 (0.0-7.0)	2.0 (0.0-6.0)	2.5 (0.5-8.0)
Total	**14.8**	**22.9**	**53.5**	**37.2**	**16.0**	**15.3**	**18.5**	**18.6**	**19.9**	**15.2**	**16.5**	**18.1**

Table 5.2.3. Non-leguminous forbs (NLF) and leguminous forbs (LF) in the annual diet composition of Pyrenean chamois from the Freser-Setcases National Game Reserve (Catalonia, north-eastern Spain), estimated by cuticle microhistological analysis of faecal samples. Values represent mean percentage of fragment frequency (min-max). Graminoid and woody plants content are presented in Table 5.2.2. Only plants with a consumption of more than 1% are represented.

	Green-up period				**Plateau greenness**		**Senescence Period**					
	March	**April**	**May**	**June**	**July**	**August**	**September**	**October**	**November**	**December**	**January**	**February**
NLF												
Cruciata glabra	0.9 (0.0-5.5)	0.0 (0.0-0.0)	4.6 (1.0-15.0)	1.4 (0.0-5.5)	1.8 (0.5-4.0)	2.1 (1.0-4.0)	2.0 (0.5-4.0)	4.7 (0.0-10.5)	0.4 (0.0-2.0)	0.3 (0.0-1.0)	0.3 (0.0-2.0)	1.4 (0.0-9.5)
Hieracium pilosella	0.3 (0.0-1.0)	0.7 (0.0-3.0)	1.6 (0.0-3.5)	5.2 (0.0-10.5)	4.3 (0.5-17.0)	2.8 (0.0-9.0)	1.2 (0.0-4.0)	1.8 (0.0-4.0)	2.2 (0.0-11.0)	2.1 (0.0-4.5)	0.6 (0.0-1.0)	1.1 (0.0-5.5)
Plantago monosperma	0.1 (0.0-1.0)	0.3 (0.0-1.0)	2.4 (0.5-7.5)	5.4 (1.5-9.0)	3.1 (1.5-5.0)	2.5 (0.0-6.0)	2.1 (0.0-5.0)	0.3 (0.0-2.5)	0.9 (0.0-5.0)	0.3 (0.0-0.5)	0.1 (0.0-0.5)	0.1 (0.0-0.5)
Potentilla spp.	0.0 (0.0-0.0)	0.1 (0.0-0.5)	3.1 (0.5-6.5)	3.3 (1.5-6.0)	4.9 (3.0-8.5)	2.5 (0.0-6.5)	0.9 (0.0-2.5)	0.1 (0.0-1.0)	0.5 (0.0-2.5)	0.0 (0.0-0.0)	0.0 (0.0-0.0)	0.4 (0.0-3.0)
Other NLF	0.9 (0.0-3.0)	0.5 (0.0-1.0)	2.2 (0.0-6.5)	5.8 (2.5-10.0)	6.1 (2.5-14.5)	5.1 (0.0-22.5)	2.7 (0.0-7.5)	3.5 (0.0-13.0)	0.9 (0.0-4.5)	0.5 (0.0-1.5)	0.1 (0.0-0.5)	0.2 (0.0-1.0)
Total	**2.1**	**1.6**	**13.9**	**21.1**	**20.2**	**15.0**	**8.9**	**10.4**	**4.9**	**3.1**	**1.1**	**3.2**
LF												
Trifolium alpinum	2.6 (0.0-16.0)	0.0 (0.0-0.0)	2.3 (0.0-6.0)	7.5 (3.0-13.0)	7.9 (5.5-11.0)	5.9 (1.5-16.5)	5.8 (0.5-24.5)	7.4 (2.5-10.0)	7.8 (4.5-11.5)	4.8 (0.0-7.5)	1.6 (0.0-4.0)	4.2 (0.0-12.0)
Trifolium pratense	0.0 (0.0-0.0)	0.0 (0.0-0.0)	2.9 (1.0-7.0)	4.4 (1.5-6.0)	5.0 (2.0-8.5)	2.7 (0.5-7.0)	0.9 (0.0-2.0)	0.5 (0.0-3.5)	0.0 (0.0-0.0)	0.0 (0.0-0.0)	0.0 (0.0-0.0)	0.0 (0.0-0.0)
Other LF	0.9 (0.0-2.5)	1.0 (0.0-4.5)	1.2 (0.0-4.5)	4.5 (1.5-7.0)	5.7 (2.0-10.5)	3.0 (0.5-8.0)	1.3 (0.0-3.5)	1.4 (0.0-3.0)	1.8 (0.0-9.5)	1.5 (0.0-4.0)	0.2 (0.0-1.0)	0.6 (0.0-2.0)
Total	**3.5**	**1.0**	**6.4**	**16.4**	**18.5**	**11.5**	**8.1**	**9.3**	**9.6**	**6.3**	**1.8**	**4.9**

Feeding habits and primary production phenology

In our study areas, the start of the growing season was situated on day 122 (2nd of May; sd=9.5) and the peak of production on the Julian day 199 (18th of July; sd= 10.9). As shown in Fig 5.2.3, the most consumed plants for chamois were NLW except during the green-up season, where graminoids took over NLW in synchrony with plant phenology. Graminoids took over NLW on day 120, very close to the start of the growing season and this process was reversed on day 202, very close to the peak of production (day 199).

The same synchrony appeared with forbs as the peak of consumption occurred on day 189 for NLF and 193 for LF, just a few days before the peak of production. Furthermore, NLW reached its lowest consumption on day 180. Noticeably, the increase in graminoid consumption during the growing season was stopped and reversed just after the arrival of livestock in the alpine and subalpine meadows, but one month before the primary productivity reached its peak (Fig. 5.2.3).

Our GAM analysis revealed that 32.4% and 41% of the observed variability of LF (edf=2.63; F= 11.47, $p<0.005$) and NLF (edf=3.05; F= 14.46, $p<0.005$) were positively associated with NDVI in the study area. In contrast, NLW consumption was negatively associated with NDVI (edf=1.85; F= 13.11, $p<0.005$), which explained 27.7% of the observed variability. We failed to detect any association between NDVI and either LW (variance explained: 13.3%; edf=3.64; F=2.22; p=0.07) or graminoid feeding (variance explained: 1.12%; edf=1; F=0.95; p=0.33).

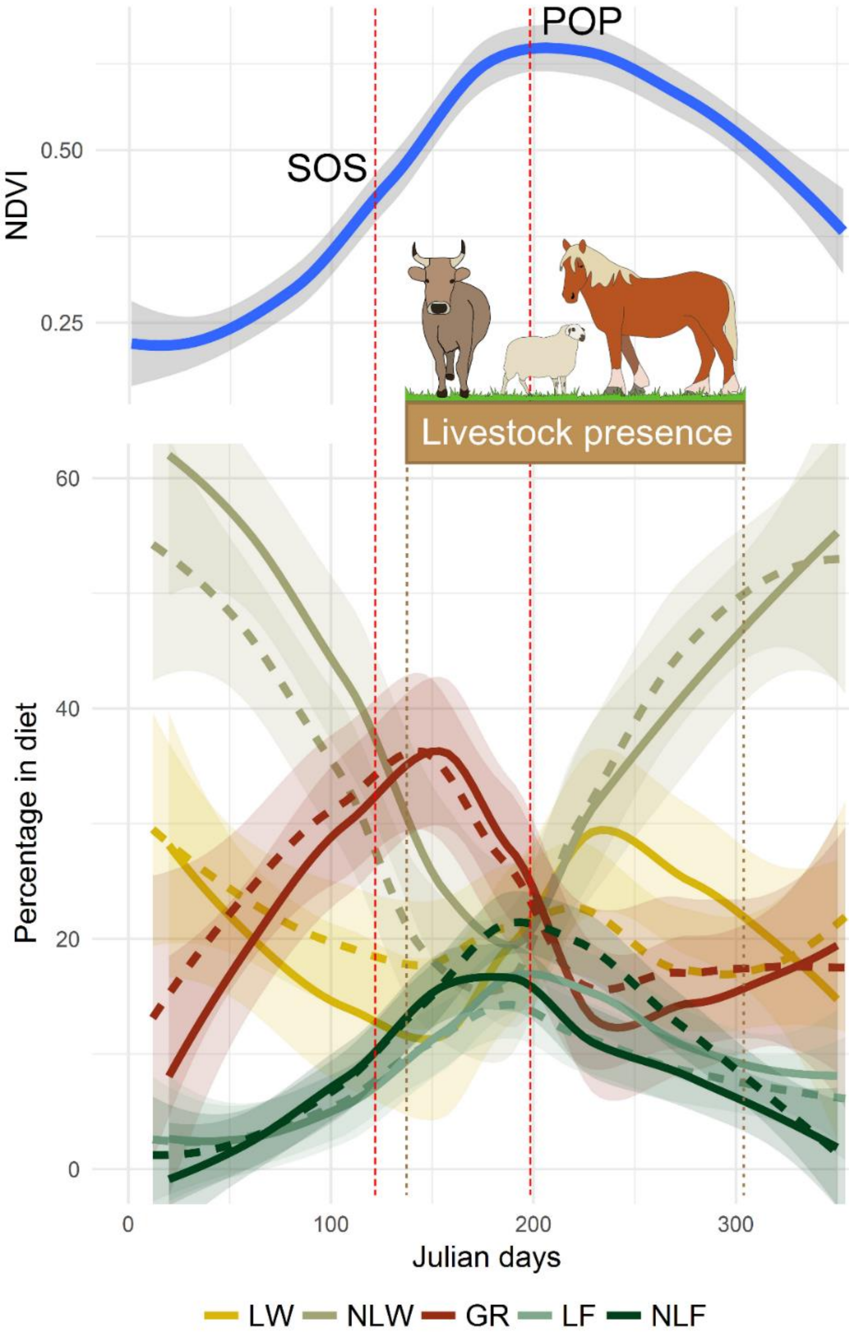

Fig 5.2.3. Annual relative distribution of Pyrenean chamois diet (bottom part) in Fontalba (dashed line) and Costabona (solid line) and annual NDVI trends (top part). Diet composition was assessed by cuticle microhistological analysis of faecal samples and NDVI data was extracted from the MOD13Q1 repertory. Plants are grouped into Non-leguminous woody (NLW), Leguminous woody (LW), Graminoids (GR), Non-leguminous forbs (NLF) and Leguminous forbs (LF). Start of the growing season (SOS), peak of production (POP) and presence of livestock in the area are also indicated.

Resource selection of Pyrenean chamois

As shown in Fig 5.2. 4, Pyrenean chamois in June markedly preferred LW plants (Wi= 2.69, se=0.57), showed a light preference for NLF (Wi=1.32, se=0.08) and LF (Wi=1.17, se=0.13), and a light avoidance of NLW (Wi=0.75, se=0.08) and GR (Wi=0.74, se=0.08). When plant taxa were analysed individually, Pyrenean chamois showed a strong preference for *Rubus* sp. (Wi= 28.41, se=6.69), *Veronica* sp. (Wi= 19.55, se= 3.79), *Rosa* sp. (Wi= 6.11, se= 2.29) and *Cytisus* spp. (Wi= 2.83, se= 0.60) whereas *Calluna vulgaris* (Wi= 0.25, se= 0.09) and *Festuca* spp. (Wi=0.82, se= 0.10) were not positively selected.

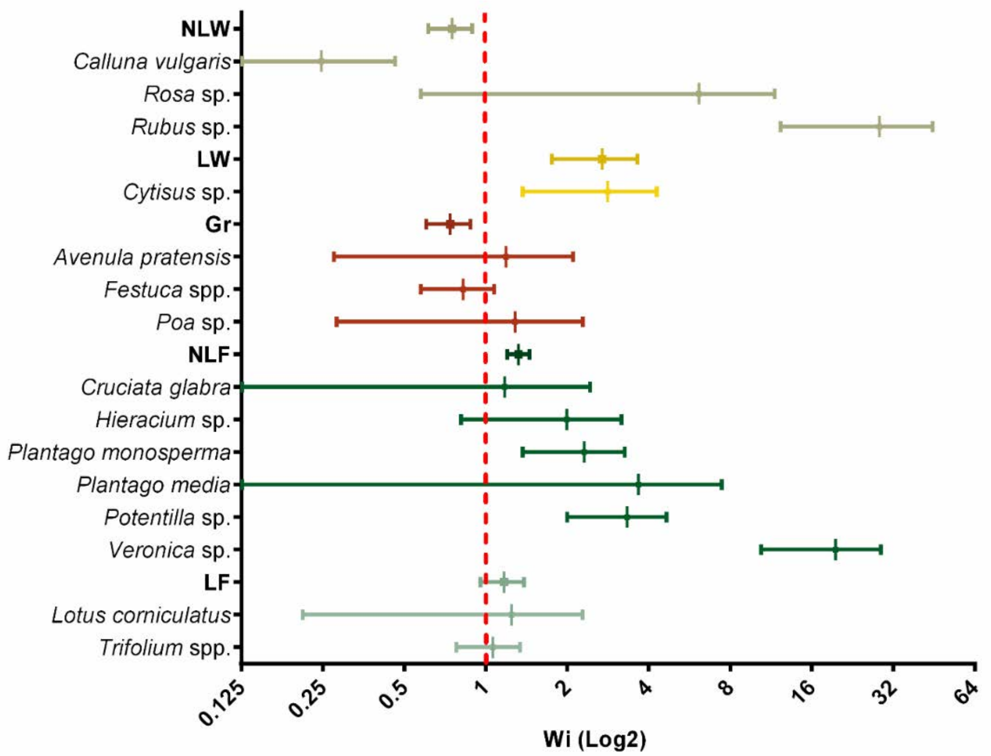

Fig 5.2.4. Plant selection indices (Wi) for Pyrenean chamois during June. A Type II selectivity analysis was used. Values under one (situated on the left of the red dashed line) indicate avoidance, values around one (situated around the red dashed line) indicate indifference and values above one (situated to the right of the red dashed line) indicate preference.

Diet selection of sheep and diet overlap with chamois

The diet of sheep was assessed during the cohabitation period in subalpine and alpine ecosystems and 33 plant taxa were identified. Only 19 of these plants appeared at levels of more than 1% of fragments, which represented 95.2% of the total diet composition. A mean of 0.18% of the observed fragments could not be identified (min=0.0%, max=1.0%). As seen in Table 5.2.4, almost half of the diet was composed of graminoids (46.5%), with *Festuca* spp. the most important (41.1%). Forbs formed the other important part of the diet (41.4%) and were similarly divided between LF (21.7%) and NLF (19.7%), with *Trifolium alpinum* (7.4%) and *Trifolium pratense* (5.3%) the most consumed forbs. The rest of the diet was composed of woody plants (12.1%), with *Rubus* sp. (4.4%) and *Pinus uncinata* (2.7%) the most consumed. Unlike in chamois, *Calluna vulgaris* (2.1%) and *Cytisus* sp. (0.5%) were barely consumed by sheep (Fig 5.2.5). Sheep positively selected NLF and LF (Wi=1.22, se=0.02 and Wi=1.5, se=0.056) and negatively selected NLW and LW (Wi=0.67, se=0.17 and Wi=0.00), while graminoids were not actively sought (wi=0.98, se=0.03).

Table 5.2.4. Diet composition of sheep. Values represent mean percentage of fragment frequency (min-max). Only plants with a consumption greater than 1% are represented. *Faecal samples of sheep could not be collected during September 2012. Therefore, only results from September 2011 are shown.

	June	**July**	**August**	**September***	**October**
Non Leguminous Woody					
Calluna vulgaris	0.5 (0.5-0.5)	1.5 (1.0-2.0)	3.25 (3.0-3.5)	2.0	3.0 (0.0-6.0)
Pinus uncinata	5.5 (4.0-7.0)	2 (2.0-2.0)	1.25 (0.5-2.0)	0.5	4.25 (3.5-5.0)
Rosa sp.	1.75 (1.5-2.0)	0.75 (0.5-1.0)	2.5 (2.5-2.5)	3.0	1.75 (0.0-3.5)
Rubus sp.	2.25 (1.0-3.5)	2.25 (1.0-3.5)	1.75 (0.0-3.5)	10.0	5.5 (3.0-8.0)
Other NLW	0 (0.0-0.0)	0 (0.0-0.0)	0 (0.0-0.0)	2.5	0.75 (0.5-1.0)
Total	**10.0**	**6.5**	**8.75**	**18.0**	**14.5**
Leguminous Woody					
Total	**0**	**0**	**0**	**1.5**	**0.75**
Graminoids					
Festuca spp.	44.0 (42.0-46.0)	38.0 (33.5-42.5)	28.25 (27.5-29.0)	46.5	48.5 (45.0-52.0)
Agrostis sp.	2.5 (2.0-3.0)	2.75 (0.5-5.0)	3.0 (2.0-4.0)	0	1.75 (1.0-2.5)
Avenula pratensis	3.0 (1.5-4.5)	1.5 (0.5-2.5)	1.75 (0.0-3.5)	3.5	1.25 (0.0-2.5)
Other Gr	1.0 (0.5-1.5)	2.0 (1.5-2.5)	0.5 (0.5-0.5)	2.0	1.0 (1.0-1.0)
Total	**50.5**	**44.25**	**33.5**	**52.0**	**52.5**
Non Leguminous Forbs					
Hieracium pilosella	1.0 (0.5-1.5)	3.25 (1.0-5.5)	1.5 (1.0-2.0)	3.0	1.5 (1.5-1.5)
Plantago monosperma	4.0 (3.0-5.0)	4.5 (3.5-5.5)	6.75 (3.5-10.0)	4.0	6.0 (6.0-6.0)
Plantago media	0.75 (0.0-1.5)	1.0 (1.0-1.0)	0.75 (0.5-1.0)	0	3.0 (0.5-5.5)
Potentilla spp.	4.5 (3.5-5.5)	6.0 (4.0-8.0)	6.25 (5.0-7.5)	2.5	3.0 (1.5-4.5)
Ranunculus bulbosus	5.0 (3.0-7.0)	3.75 (2.0-5.5)	3.0 (2.5-3.5)	4.0	3.0 (2.5-3.5)
Veronica sp.	3.0 (2.0-4.0)	1.0 (0.5-1.5)	1.5 (0.5-2.5)	0	4.25 (2.5-6.0)
Other NLF	1.0 (1.0-1.0)	1.75 (1.0-2.5)	2.25 (2.0-2.5)	1.0	0.75 (0.0-1.5)
Total	**19.25**	**21.25**	**22.0**	**14.5**	**21.5**
Leguminous Forbs					
Anthyllis vulneraria	2.75 (2.0-3.5)	4.75 (4.5-5.0)	5.75 (5.0-6.5)	6.5	1.75 (0.5-3.0)
Chamaespartium sagittale	0.5 (0.5-0.5)	1.75 (0.0-3.5)	2.25 (0.0-4.5)	1.0	0.75 (0.0-1.5)
Hippocrepis comosa	1.75 (0.0-3.5)	1.25 (0.0-2.5)	2.0 (0.0-4.0)	0	0.25 (0.0-0.5)
Lotus corniculatus	1.0 (1.0-1.0)	1.0 (0.0-2.0)	3.25 (0.5-6.0)	0	1.25 (0.0-2.5)
Trifolium alpinum	7.0 (6.5-7.5)	10.0 (9.5-10.5)	14.25 (13.5-15.0)	1.0	4.75 (4.5-5.0)
Trifolium pratense	6.25 (6.0-6.5)	7.0 (5.5-8.5)	6.5 (6.5-6.5)	5.0	1.5 (1.0-2.0)
Other LF	1.0 (0.5-1.5)	2.25 (1.5-3.0)	1.25 (0.5-2.0)	0.5	0.5 (0.0-1.0)
Total	**20.25**	**28.0**	**35.25**	**14.0**	**10.75**

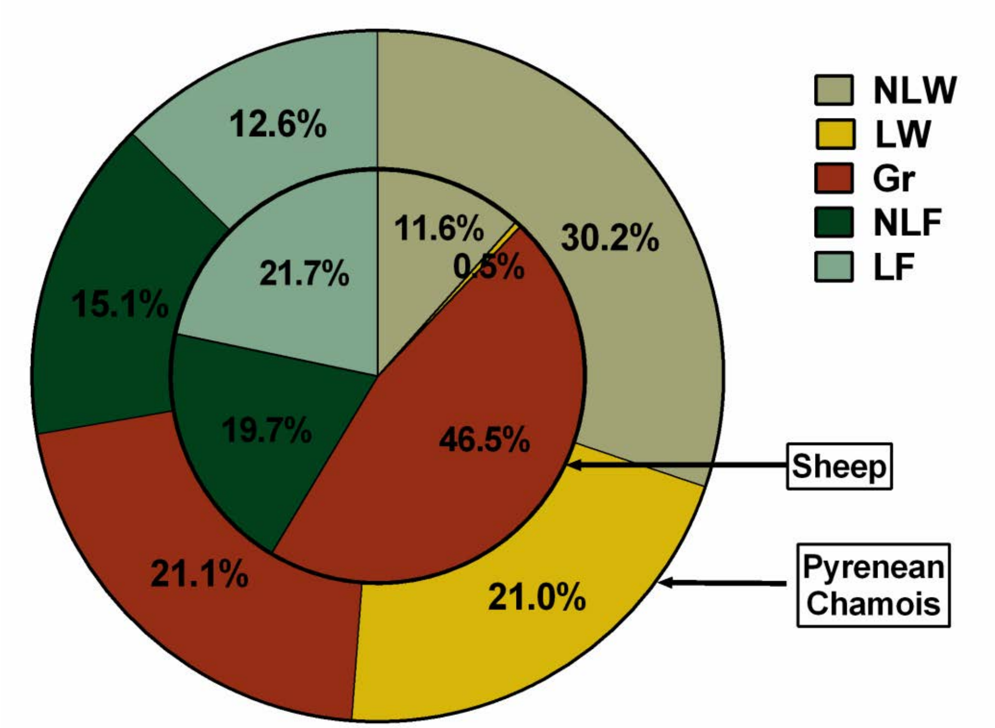

Fig 4.2.5. Total diet composition (in %) of sheep and Pyrenean chamois during the cohabitation period in subalpine and alpine ecosystems (from June to October). Diet composition was estimated by cuticle microhistological analysis of faecal samples collected in the Freser-Setcases National Game Reserve, Catalonia, north-eastern Spain.

During the cohabitating period, chamois consumed 31 out of the 33 plants consumed by sheep. The Horn's indices of diet overlap between these two species were high (R_o=0.85) at the beginning of the cohabitation period (June) and slowly decreased until September (R_o=0.49), followed by a light increase in October (Ro=0.58).

Discussion

Our results show a clear intra-annual variation in the diet composition of Pyrenean chamois and demonstrate a strong relationship between plant consumption and phenology dynamics in our alpine area. Besides, we couldn't detect a specific effect of sheep flocks on the diet of chamois despite a moderate to high diet overlap. However, we mark out the possibility that multi-specific livestock, and not only the presence of sheep, affect the diet of chamois during the co-habitation period.

In our study, we found that Pyrenean chamois eat a wide variety of plants and adapt their diet to seasonal changes in forage phenology, confirming that in a subalpine-alpine climate under Mediterranean influence, chamois also act as intermediate feeders. This intermediate opportunistic foraging behaviour of *Rupicapra* species has been confirmed by abounding studies (Bertolino *et al.*, 2009; García-gonzález & Cuartas, 1996; Hofmann, 1989; Lovari *et al.*, 2014). In fact, depending on the floristic characteristics of their living area, they can exhibit extremely opposite dietary behaviours. Thus, chamois mainly depend on woody plants during the summer and behave as browsers in the New Zealand Alps (Yockney & Hickling, 2000), whereas they show a completely woody-free summer diet acting as grazers in the western Italian Alps (La Morgia & Bassano, 2009). This characteristic has been attributed to an anatomo-physiological adaptation of the digestive tract of this species (Hofmann, 1984).

The detection of 39 different plants in the chamois diet is within the range of other studies using the same methodology (Andreoli *et al.*, 2016; Ferrari *et al.*, 1988; Garcia-gonzalez, 1984; Homolka & Heroldová, 2001; La Morgia & Bassano, 2009). A study using DNA barcoding (Rayé *et al.*, 2011), on the other hand, showed that chamois can consume up to 110 different species. This difference has been attributed to the high level of resolution obtained with the DNA approach but also to the higher plant biodiversity of the study area (more than 1.500 species recorded; Thuiller *et al.*, 2017). In fact, it is necessary to highlight the limitations related to dietary studies based on faecal cuticle microhistological analyses, such as ours. Even though this technique can provide reliable estimates of diet composition for herbivores (Anthony & Smith, 1974; Johnson & Pearson, 1981), it has been noted that it tends to overestimate the less digestible species in the diet. Usually, grasses and shrubs are overdetected and easily digested forbs are underdetected (Bartolome *et al.*, 1995; Chapuis *et al.*, 2001; Havstad & Donart, 1978; McInnis *et al.*, 1983). Some forb species sporadically consumed could even be unnoticed (McInnis *et al.*, 1983). Even if this results can vary depending on the experimental variables and the herbivore species (Alipayo *et al.*, 1992; Gill *et al.*, 1983), one must take into account the possibility of estimation bias. At the same time, when maturing, some species can become difficult to identify (Havstad & Donart, 1978), however this was not the case in this study as the rate of unidentified species was low, even in autumn and winter. For practical purposes, microhistological analysis appears to be one of the best techniques to evaluate diet composition of large herbivores but its accuracy could be enhanced by the determination of digestibility coefficients or corrector factors at the consumer-plant level (Bartolome *et al.*, 1995; Brand, 1978).

In our study area, a high use of woody species was observed in winter as was a high use of forbs and graminoids in spring and summer. Specifically, chamois are able to track changes in plant quality with an increasing use of NLF, LF and graminoids, in concert with the timing of spring. As a capital breeder, chamois should rely mostly on the most nutritious plants during the growing season in order to accumulate body reserves to cope with winter food scarcity and reproduction needs. By their seasonal phenology, perennials forbs and graminoids have a high protein content as early phenological growth stages of plants have a much higher nitrogen/fiber ratio than older senescent ones (Körner, 2011; Marinas *et al.*, 2003). However, the dry and hot summers of our areas makes perennial plants available for only a short time. Furthermore, throughout this season, the fibre content of forbs and graminoids increases, while protein content and organic matter digestibility decrease, driving a decline in their nutritive value (Bovolenta *et al.*, 2008). These results are in agreement with the findings of Galvez-Cerón et al. (Gálvez-Cerón *et al.*, 2013) who described a unimodal seasonal pattern of faecal nitrogen in Pyrenean chamois with a peak between May and July. This preference for herbaceous vegetation (forbs and graminoids) has also been described in several studies (García-gonzález & Cuartas, 1996; La Morgia & Bassano, 2009).

During spring and summer, chamois still consume a significant percentage of non-leguminous woody plants, mainly *Calluna vulgaris*. Except from May to August, this perennial dwarf-shrub is the plant most consumed by Pyrenean chamois in our area. The consumption of this plant is not new as it has been detected in the diet of chamois from the Cantabrian Mountains to the Italian central Alps (Andreoli *et al.*, 2016; Pérez-Barberia *et al.*, 1997; Rayé *et al.*, 2011). In other areas, it even provides a permanent food source for red grouse (Moss, 1969) and red deer (Staines *et al.*, 1982). Despite having a low protein content and a high phenolic compound content compared to other plants (Tolera *et al.*, 1997), this species is rich in antioxidant compounds (Rieger *et al.*, 2008) and is annually available in the snow-free patches of grass. The quality of the winter foliage of

Calluna vulgaris has been studied and results suggest that it has a relatively high nutritive value (Armstrong & Thomas, 1953). Furthermore, its total phenol content is lower during winter than in summer (Jalal *et al.*, 1982).

Leguminous woody plants from the *Cytisus* genus are constantly consumed across all seasons, are positively selected by chamois during summer and are the second most consumed plant taxa by chamois. *Cytisus* spp. can be found in most of the Pyrenees, Cantabrian Mountains and some parts of the Alps, where chamois is present. However, a thorough search of the relevant literature yielded that the consumption of plants in the *Cytisus* genus (and synonyms like *Genista* or *Sarothamnus*) by chamois has only been described anecdotally in a single study (Rayé *et al.*, 2011). Although these perennial shrubs are not abundant in our areas, they form dispersed patches in the subalpine and alpine pastures and thickets in the montane abandoned meadows, and can be browsed throughout the year due to their green branches and their high height that allows them to remain uncovered by snow (de Bolòs & Vigo, 1984). As with other leguminous shrubs in the Iberian mountains, even when herbaceous plants are available and despite their relatively high lignin content (up to 6% and 15% in leaves and green stems respectively (Ammar *et al.*, 2004)), *Cytisus* spp. can also represent a highly digestible and high-protein meal (up to 29% and 24% of crude protein in leaves and green stems, respectively, at the beginning of spring (Ammar *et al.*, 2004)) resulting in an interesting nutritious plant for chamois. *Cytisus spp.* are considered colonising plants (Prévosto *et al.*, 2006), and thus chamois could play an important role in shrub encroachment control in open habitats caused by the abandonment of traditional farming practices (Poyatos *et al.*, 2003).

Calluna vulgaris, *Cytissus* spp. and *Festuca* spp. are therefore key forage species for Pyrenean chamois throughout the year but forbs are equally important during the growing season. For this reason, population management programmes should focus on the preservation of mixed grasslands composed of patches of shrubs and herbs. Shrub expansion is causing a shift from herbaceous to woody plant dominance in mountainous habitats worldwide, mainly due to land use (Mottet *et al.*, 2006; Prévosto *et al.*, 2011). By reducing the availability of forbs and graminoids during summer, this phenomenon could affect chamois population dynamics and therefore deserves attention. Furthermore, as Pyrenean chamois adapt their diet to primary production trends, the current and future effects of climate change on the alpine environment (e.g., snow cover diminution

(Christensen *et al.*, 2013), shifts in the seasonal timing of plants (Arft *et al.*, 1999) or a shortening growing season (Ernakovich *et al.*, 2014)) could affect the diet of chamois and therefore their population dynamics as a result of a desynchronization between resource availability and reproduction periods or lactation needs. Pettorelli *et al.* (2007) already identified that changes in plant spring phenology negatively affected the juvenile growth of other alpine ungulates.

The analysis of faeces from sheep indicated a high presence of graminoids and forbs and a moderate to high diet overlap with chamois. Sheep diet in summer has been studied in the central and western Pyrenees using faecal microhistological cuticle analysis and these other studies showed even a higher consumption of graminoids (up to 77.3%) and a lower preference for forbs and woody species than our findings (Aldezabal *et al.*, 2002; García-González & Montserrat, 1986). This difference could be attributed to a different vegetation composition in these specific study areas but these data were not available. Surprisingly, there were no significant differences in the diet of chamois between the two study areas despite the presence of sheep in Costabona. In fact, modification of feeding habits and spatial segregation of chamois when flocks of sheep are present have been observed in numerous areas (Rüttimann *et al.*, 2008; La Morgia & Bassano, 2009 but see Fankhauser *et al.*, 2008). Even if a high diet overlap can suggest competition, it can also only indicate an overabundance of resources or consumer-specific plant part selection at the bite scale, allowing the coexistence of relatively similar large herbivores (Redjadj *et al.*, 2014). Still, in open landscapes, the real extent of resource availability at the population level can be complex to assess and some bias may occur when diet composition is compared with vegetation availability.

This study shows that, despite a high consumption of graminoids during the green-up season by chamois, there was a marked reduction in the use of this resource right after the green-up season started. This fact concurred with the arrival of livestock in the shared meadows and point out the possibility of an over-use of graminoids in the meadows where this resource is abundant. In fact, the preference for graminoids and forbs by sheep has also been described in horses and cattle (Celaya *et al.*, 2011; Schoenecker *et al.*, 2016) and spatial disturbance caused by large livestock flocks has been suggested by Chirichella *et al.* (2013). It is possible that the spatial aggregation of high densities of livestock in the meadows depleted the availability of graminoids or forced chamois to move to suboptimal patches. However, a similar study on diet composition in an area free of livestock would be necessary to exclude that the feeding behaviour of chamois is not driven by other factors such as temperature constrains or even a depletion in graminoids quality (Marinas & García-González, 2006; Mason *et al.*, 2017).

To conclude, chamois are well-adapted to the variations in the seasonal phenology of plants in alpine habitats. The key forage species in its diet suggest that population management plans should focus in the preservation of mixed grasslands composed of patches of shrubs and herbs. Nevertheless, climate change and shrub expansion are variables that could potentially affect chamois population dynamics through changes in habitat composition and temporal shifts in forage availability.

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Supporting Information

S1 Table. Maximum number of Pyrenean chamois and livestock observed in a single day during the different seasons. During monthly faecal sample transects, herbivore groups on the two study areas were located using 10 x 42 binoculars and 20-60 x 65 spotting scopes. Size and composition of groups was recorded and the total of animals from the same species observed during the day was also calculated. Maximum number of observed animals from one species in a day (in bold) was used to extrapolate population numbers.

		Fontalba				Costabona			
		Winter	Spring	Summer	Autumn	Winter	Spring	Summer	Autumn
Chamois	2009	-	186	70	149	-	66	59	24
	2010	**224**	76	67	157	16	56	5	85
	2011	145	138	126	123	57	**101**	59	58
	2012	133	108	-	-	15	19	-	-
Sheep	2009	-	0	0	0	-	0	257	54
	2010	0	0	0	0	0	150	206	204
	2011	0	0	0	0	0	328	**352**	28
	2012	0	0	-	-	0	0	-	-
Cattle	2009	-	227	225	109	-	0	426	164
	2010	0	15	179	146	0	235	224	185
	2011	0	37	**309**	171	0	74	**647**	143
	2012	0	111	-	-	0	0	-	-
Horse	2009	-	5	20	31	-	0	33	63
	2010	12	17	38	14	0	23	31	18
	2011	19	12	22	**47**	0	36	23	**71**
	2012	11	11	-	-	0	0	-	-

5.3 Study 3:

Assessing the impact of climate change and population density on the diet composition and quality of Pyrenean chamois.

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Abstract

The performances of large herbivores populations are primarily affected by resource availability and quality, which in turn are influenced by climatic conditions and intraspecific competition. Despite the fundamental role of resource-use strategies on life-history traits, very few studies have explicitly assessed the mechanistic impact of these factors on the dietary composition and quality of herbivores. We aimed at filling this gap by assessing the effects of climate change and population densities on the dietary quality and composition of a large herbivore during spring and estimating the relationship between forage consumption strategies and diet quality. Using a long-term (24 years) direct individual-based dietary monitoring of a Pyrenean chamois population (*Rupicapra p. pyrenaica*), coupled with multiple environmental databases, we showed that earlier onsets of spring lead to an earlier access to high-quality forage and therefore a higher diet quality at a fixed date of sampling, without affecting the dietary composition. On the other hand, by driving to a diet richer in woody plants (negatively related to dietary nitrogen) and poorer in forbs (positively related to dietary nitrogen) and graminoids (negatively related to lignin content), intraspecific competition deteriorated the quality of the diet. Our results also indicate that older animals feed less on graminoids and had a higher diet quality than younger ones, suggesting an enhanced foraging experience with age. By assessing the mechanical effects of climate change and population densities on the foraging selection of a large herbivores this study is an essential step to understand the consequences of global trends on the on the life-history traits of large herbivores.

Introduction

Animal populations are dynamic through time and many demographic parameters are influenced by density-dependent and density independent processes (Bonenfant *et al.*, 2009; Bowyer *et al.*, 2014; Brown, 2011). In large herbivores, high densities negatively affect several life history traits such as growth, survival or reproduction and particularly affect recruitment (Fowler, 1987; Putman *et al.*, 1996). At the same time, climatic fluctuations also shape herbivores populations via changes in the plant - and thus resources - phenology (Battin, 2004; Mysterud *et al.*, 2003). Ultimately, resource availability and quality are assumed to be the central pieces of this regulatory mechanism. The quantity and quality of forage available shapes herbivore populations through the so-called "Bottom-up" regulation (Gandiwa, 2013) and in general, plants with high nutritive quality or digestibility are primarily selected to maximize the energy gain (Albon & Langvatnt, 1992; Zweifel-Schielly *et al.*, 2012). Indeed, in large herbivores, diet quality affects body condition (Pettorelli *et al.*, 2002; Simard *et al.*, 2008), reproductive rates (Hempson *et al.*, 2015) and juvenile survival (Long *et al.*, 2016), among others life-history traits. Diet quality is even more influential than quantity on the performances of ungulates (White, 1983). Therefore, studying the mechanistic relationship between environmental conditions and diet quality is an important prerequisite for a better understanding of evolutionary and demographic processes in ungulate populations, especially in the context of the ongoing climate change (Gaillard *et al.*, 2013; Nielsen *et al.*, 2012).

Alpine environments appear to be highly susceptible to climate change (Ernakovich *et al.*, 2014) and species inhabiting these habitats are already impacted by climatic-related alterations (Büntgen et al., 2017; Mysterud & Austrheim, 2014). In fact, anthropogenic variations in climate are important drivers of populations dynamics (Forchhammer *et al.*, 2001; Gaillard *et al.*, 2013). Large herbivores living in temperate habitats, and especially in alpine ecosystems, must cope with strong seasonal changes in environmental conditions and resource availability (Bliss, 1962; Post & Stenseth, 1999). They are adapted to give birth during the vegetation green-up period in order to maximize the access to highly nutritious forage during late gestation and lactation (Clutton-Brock *et al.*, 1989; Rutberg, 1987). Therefore, the access to high-quality resources after the winter scarcity is key for reproductive success, rebuilding body reserves, juvenile growing and survival and finally population dynamics (Herfindal *et al.*, 2006; Pettorelli *et al.*, 2005b, 2007). In alpine ungulates, protein content peaks during growing season, following plant phenology (Gálvez-Cerón *et al.*, 2013; Hamel *et al.*, 2009). However, the ongoing increases in spring temperatures advance the timing of plant phenology in temperate regions and therefore also advance the access to high-quality vegetation during a strategical period for herbivores (Cleland *et al.*, 2007; Garel *et al.*, 2011; Pettorelli *et al.*, 2005b).

Nevertheless, climatic variability is not the only process affecting the dietary quality of large herbivores. In that way, population density has a clear impact on resources availability due to intraspecific competition. High densities alter the habitat use (Clutton-Brock *et al.*, 1987; Mobaek *et al.*, 2009), shape the diet composition (Kausrud *et al.*, 2006; Seto *et al.*, 2015) and therefore affect the diet quality (Nicholson *et al.*, 2006). Furthermore, density-dependent processes often interact with climatic variations in a complex interplay (Bonenfant *et al.*, 2009; Gaillard *et al.*, 1997; Loison & Langvatn, 1998). Usually, intraspecific competition is stronger when climatic conditions are unfavourable and harsh climatic conditions impose sharper constraints in highly densified populations (Coulson *et al.*, 2001; Sauer & Boyce, 1983). In the contest of mountainous environments, however, resource availability and diversity is not a constant factor. Due to the land-use and climatic changes during the 20th century, mountains have experienced an expansion and densification of woody plants habitats (Mottet *et al.*, 2006; Tasser & Tappeiner, 2002). Thus, it is important to evaluate concurrently the demographic evolution of herbivores and the changes in habitat diversity in order to think in terms of relative density (Horne, 1983).

At the same time, individual traits also interact with climatic and demographic fluctuations and can lead to variation in the strength of these associations (Coulson *et al.*, 2001). Age-related variation in foraging efficiency has been documented in several vertebrates, especially birds (Daunt *et al.*, 2007; Zimmer *et al.*, 2011). However, a new body of evidence reveals similar patterns of increased experience over life in ungulates (Froy *et al.*, 2018), potentially resulting in higher protein intake at old ages. At the same time, sex also influence foraging behaviour, as females tend to select a diet higher in protein than males [(Miranda *et al.*, 2012; Staines *et al.*, 1982); but see (Jiang *et al.*, 2009)].

Thus, it is important to take into account all these processes when studying the effect of environmental variation on dietary composition, and therefore, quality (Putman *et al.*, 1996). However, to our knowledge, very few studies have accomplished to connect climate change, intraspecific competition, individual characteristics and diet quality, through individual foraging choices. We aimed at filling this gap by studying the effect of both variables on the dietary composition and quality of a large herbivore during spring, taking into account individual characteristics. We benefit from a long-term dietary monitoring (24 years) of a Pyrenean chamois population (*Rupicapra p. pyrenaica*), a mountain ungulate highly selective for high-quality plants, experiencing large variation in population density and environmental conditions. Working with a direct individual-based measure of diet quality monitored over more than 250 individuals, coupled with multiple databases on climatic conditions, population abundance and habitat evolution, we were provided with a unique opportunity to assess consistent mechanistic links between environmental changes and dietary consequences, while accounting for individual characteristics.

Our objectives were to estimate (*i*) the effects of environmental and biological variables on the dietary quality of Pyrenean chamois. Specifically, and taking into account individual characteristics, we hypothesized that an earlier onset of spring will advance the access to higher quality vegetation. Conversely, population abundance would impair the access to this high-quality vegetation due to intra-specific competition. In addition, we assessed (*ii*) the relationship between diet quality and diet composition in order to understand which type of plants enhance the quality of the diet of this highly selective herbivore. We expected the dietary quality during that season to be reduced by the consumption of woody plants, and enhanced by the consumption of highly nutritious plants, such as forbs (Holechek, 1984; Marinas & García-González, 2006). Finally, we determined (*iii*) the effects of environmental and biological variables on the dietary composition of Pyrenean chamois. In that sense, we hypothesized that earlier springs will advance the snowmelt and therefore the access to short nutritious and digestible plants, enhancing their consumption (Klein, 1990; Menzel & Fabian, 1999). High population abundance, however, would force individuals to feed on resources with lower nutritional quality due to intra-specific competition (Stewart *et al.*, 2011).

Materials and methods

Study area and chamois data collection

The study was carried out in the National Game and Wildlife Reserve of Orlu (NGWR-O), Eastern Pyrenees, France (42.66°N, 1.97°E). The reserve is an area of 4243ha ranging from 950 to 2765 m.a.s.l. spanning from mountain to nival ecosystem. The area presents a mountainous oceanic climate with annual mean temperatures of 9.2ºC and mean yearly-accumulated rainfall of 1260 mm (data from Meteo France weather station of Ascou-Pailhères, 1120 m.a.s.l., 42.72°N 1.89°E). The mountain stage is mainly composed of beech forests (*Fagus sylvatica*) with substracts of *Vaccinium*, *Galium* and *Luzula* among others. The subalpine stage is mainly composed of heathlands dominated by *Rhododendrum* and *Vaccinim* spp., grasslands of *Festuca* and Nardus spp. and scarce mountain pine (*Pinus uncinata*) forests. Finally, grasslands, rocks and cliffs are the prevailing habitats in the higher slopes.

The Pyrenean chamois population size of the NGWR-O has been monitored by the Office National de la Chasse et de la Faune Sauvage (ONCFS) since 1984. Abundance was initially monitored by annual single block count in late spring from 1984 to 2008 and was progressively replaced in 2005 by helicopter survey replicated five to eight times each year to determine uncertainty around abundance estimates. Aerial counts were numerically scaled to block counts (see Gilot-Fromont et al., 2018). Although these approaches systematically underestimate the true population size to a variable extent (Morellet *et al.*, 2007), they provide a relatively fair overview of the long-term trend in population abundance due to the very contrasted demographic periods encountered (Beauneé *et al.*, 2015). Indeed, since the early 90s this population has been continuously infected by Border Disease, a virus that has caused dramatic decreases, and in some cases collapses, of Pyrenean chamois populations during the last 30 years along the Pyrenees (Lambert *et al.*, 2018; Marco *et al.*, 2015). As a consequence, the abundance of chamois in the NGWR-O has continuously decreased since the first contact with the virus and until the early 2010s when the local self-clearance of the virus occurred (Gilot-Fromont *et al.*, 2018).

Concurrently, a long-term capture-mark-recapture monitoring has been performed since 1984. The number of animals trapped each year varied from 2 to 66, using different methods as snares, corral nets, drive nets or tele-anaesthesia. The sex and age of animals were recorded since 1984 and faecal samples were collected since 1992 (except during 1994 and from 1996 to 1999). The age was determined by counting horn growth annuli (Corlatti *et al.*, 2015) and faecal droppings were stored frozen at -20ºC until laboratory analysis.

Diet quality

The faecal nitrogen (fN), acid-detergent fibre (fADF) and lignin (fL) estimations were carried out using Near-Infrared Spectrophotometry (NIRS), a method already calibrated for faecal samples of Pyrenean chamois (Gálvez-Cerón *et al.*, 2013; Villamuelas *et al.*, 2017). The fN is a reliable indicator of diet quality widely used in studies of herbivores diet (Hamel *et al.*, 2009; Robbins, 1983) while fL is a complementary indicator of low digestibility and low nutritive content of forage (Hatfield & Fukushima, 2005). The fADF will be used to correct fN values following the recommendations of Gálvez-Cerón et al. (2015).

All faecal samples collected in April-June from 1992 to 2015 were analysed (n=277). Frozen faeces were thawed and oven-dried at 60ºC for 24h and then ground with a 1-mm pitch laboratory mill (Cyclotec 1093, FOSS Tecator, Höganäs, Sweden). Ground faecal samples were packed in 35 mm diameter sample cups with a quartz glass cover on one side. Samples were scanned over a wavelength from 1100 to 2500 nm using a NIRSystems 5000 scanning monochromator (FOSS, Hillerød, Denmark). Reflectance was recorded at 2-nm intervals and each sample was scanned in duplicate by the same operator. Finally, WinISI III (v. 1.6) software was used to analyse the spectra and predict fN, fADF and fL values.

Diet composition

A subset (n = 155) of the previous faecal samples was selected to perform a cuticle microhistological analysis (Bartolome *et al.*, 1995; Storr, 1961) and obtain a representative summary of the different plant taxa ingested by each animal (Bartolomé *et al.*, 2011). Up to six samples (when available) were randomly selected each month.

Between 2 to 3 gr of each faecal sample was mixed with 3 ml of HNO_3. These test-tubes were placed for 1 min in a bath of boiling water and after that, diluted with 200 ml of water. This suspension was then passed through 1.00 mm and 0.25 mm filters. Later, 50% aqueous solution of glycerin was dispersed on a microscope slide. The fraction between 0.25 to 1.00 mm was then spread on these glass slides trying to avoid any overlapping of fragments. Finally, cover-slips (24 x 60 mm) were fixed to the slides with DPX microhistological varnish and left to dry overnight. For each sample, 3 slides were prepared.

Slides were examined by the same observer under an optical microscope at 400x magnification by viewing 2-mm- wide and 60-mm- long traverses, with 3 mm between traverses. Up to 200 fragments were identified to the genera level per sample based on the shape of epidermal cells, trichomes and stomata surrounding cells. The counting data of each plant genera were converted to percentages. Epidermal fragments of floral parts, stems, petioles, and midribs were not registered due to the difficulties in species identification. Recorded plants were pooled into five functional groups, namely: leguminous woody plants (hereafter LW), non-leguminous woody plants (NLW), graminoid plants (Gr), leguminous forb plants (LF) and non-leguminous forb plants (NLF).

Plant phenology

We used the accumulated growing degree day (GDD) during April to assess the changes in spring climate conditions since 1992 and therefore monitor the annual onset of spring (Langvatn *et al.*, 1996; McMaster & Wilhelm, 1997; Moyes *et al.*, 2011). GDD was estimated as the cumulative sum of the daily mean temperatures above 5ºC over the month of April (Studer *et al.*, 2005) and was calculated as follow:

$$GDD = \sum_{i=1}^{n} \left[\left(\frac{T_{max} - T_{min}}{2} \right) - 5 \right]$$

Where T_{max} and T_{min} are respectively the maximum and minimum daily air temperature (in ºC) and *n* are the days in April. Growing degree days are counted only if T_i is higher than the threshold temperature of 5ºC. High values of GDD were attributed to early springs while lower values were attributed to latter springs (Moyes *et al.*, 2011). Daily mean temperatures in our study area were obtained at the Météo France meteorological station of Ascou-Pailhères (42.72ºN 1.89ºE) situated at 1120 m.a.s.l.

Statistical analyses

We used a linear regression to investigate the influence of environmental and biological variables on the diet quality of chamois (objective *i*). Specifically, fN was considered as a response variable and the age, sex, fADF, the abundance of chamois in the reserve, the GDD during April and the Julian day of sampling were considered additive predictor variables. Because the protein content of growing plants peaks during the growing season in alpine environments (Fig. S1), the effects of climate change must integrate the temporal variation of the day of sampling. We standardized all the variables ($\acute{X}= 0$, SD = 1) so that coefficient estimates could be interpreted as the relative contribution of each variable to the model (Gelman & Hill, 2006). We tested all combinations of predictor variables and used the Akaike's information criterion corrected for small sample sizes (AICc) to select the best model. We then checked for multi-collinearity of the predictors on the selected model using the variance inflation factors (VIF). Following Zuur, Ieno, & Elphick (2010), if a predictor showed a VIF > 3, it would have been removed from the model due to collinearity with other predictors. In preliminary analyses, we used Generalized Additive Models (GAM) to explore potential non-linear relationships between our predictors and fN. A non-linear relationship would have justify the incorporation of a superior polynomial term but this was not the case here (results not shown).

Because the response variable and some of our predictors presented a trend over time (see Fig. S2 in the supplement material for an overview of temporal variation in the tested parameters), we were confronted with an inflated probability of detecting spurious correlations and confounding effects (Graham, 2003). Therefore, we also detrended these predictors (GDD, Abundance of chamois, Julian day of sampling and fADF) by regressing them on year and calculating the residuals (see Grosbois et al., 2008 for a detailed explanation). Again, we also standardized these variables. We then used linear regressions to assess the consistency of the previous model selection using the detrended variables in place of the raw covariates and by including the effect of year in all models to account for time trends in the variables. The results can be found in the Supplementary material (tables S5.3.3 and S5.3.4).

We studied the relationship between diet quality and diet composition (objective *ii*) by building predictive models using a partial least squares (PLS) multivariate regression approach (Carrascal *et al.*, 2009). The fN content was corrected by the ADF content (obtaining fNc), following Gálvez-Cerón et al. (2015). The proportions of LW, NLW, Gr, LF and NLF were used as the predictor variables and fNc and fL concentrations were used as the response variables.

Finally, we studied the influence of environmental and biological variables on the diet composition of chamois (objective *iii*) by using the same methodology as for objective *i* (i.e. raw and detrended analyses with the proportions of each plant group in the diet as response variables – namely woody plants, graminoids and forbs – and age, sex, the abundance of chamois in the reserve, the GDD during April and the Julian day of sampling as additive predictor variables). The results of the detrended models can be found in the Supplementary material (tables S5.3.5 and S5.3.6).

All statistical analyses were performed using R software version 3.4.2 (R Core Team, 2018) and the accepted significance level was fixed at $P<0.05$. The R-package "MuMIn" (version 0.1.17; Barton, 2018) was used for model selection and the package "car" (version 3.0-0; Fox & Weisberg, 2011) was used to calculate the VIF of the variables. The R-packages "mgcv" (version 1.8-23; Wood, 2006) and "plsdepot" (version 0.1.17; Sanchez, 2012) were used respectively to create the variable FNc and the PLS multivariate regression between diet composition and quality. The package "visreg" (version 2.5-0; Breheny and Burchett, 2017) was used to represent the effect of a predictor variable of interest on the response variable after correcting for the effect of the other model predictor variables (partial residual plots).

Results

Effects of environmental and biological parameters on diet quality

The most parsimonious model explaining the variation in the faecal nitrogen of Pyrenean chamois included effects of age, fADF, the abundance of chamois, GDD during April, and Julian day of sampling (Table 5.3.1, r^2= 0.64, $F_{271,5}$=94.38, p<0.001). The competing model (within ΔAICc<2) also included the effect of sex. Models including the abundance of chamois, GDD during April, and Julian day of sampling had a high support by the data as the sum of Akaike's weights>0.99. The variance inflation factor were <2 for every variable and therefore gave no evidence of correlation between them (Table S5.3.2).

Table 5.3.1. Model selection testing for the effects of biological and environmental variables on the fN of Pyrenean chamois. Age and sex of the animal, faecal acid-detergent fibre (fADF), the abundance of chamois (Abun), accumulated growing degree during April (GDD), and Julian day of sampling (JDay) were considered predictor variables while the faecal nitrogen (fN) was considered response variable. Ranking and selection was based on the AICc. K=number of models parameters. AICc = Akaike Information Criterion corrected for small sample sizes, ΔAICc = difference of AICc with respect to the best model, ωi = Akaike weight. We only present models with a ΔAICc<4 and the null model.

Biological model	**K**	**AICc**	**ΔAICc**	**ω_i**
Age + fADF + Abun + GDD + JDay	**7**	**352.6**	**0**	**0.516**
Age + Sex + fADF + Abun + GDD + JDay	8	353.8	1.05	0.287
fADF + Abun + GDD + JDay	6	355.2	2.55	0.144
Null	2	621.6	268.9	0.000

The faecal nitrogen was positively related with GDD in April, Julian day of sampling and the age of the animal whereas it was negatively related with the abundance of chamois and the fADF (Table 5.3.2 and Fig. 5.3.1). The size effect of the Julian day of sampling was two times higher than climatic and density-dependent predictors, which both had similar effects sizes.

Table 5.3.2. Estimates of the standardized parameters for the selected biological model (see Table 5.3.1). SE=standard error, fADF= faecal acid-detergent fibre, GDD= accumulated growing degree during April, JDay= Julian day of sampling.

Parameter	Estimate	SE	t-statistic	p-value
Intercept	2.785	0.027	102.91	<0.001
Age	0.060	0.028	2.14	0.032
fADF	-0.191	0.029	-3.86	<0.001
Abundance	-0.142	0.037	-6.69	<0.001
GDD	0.153	0.031	5.01	<0.001
JDay	0.368	0.033	11.07	<0.001

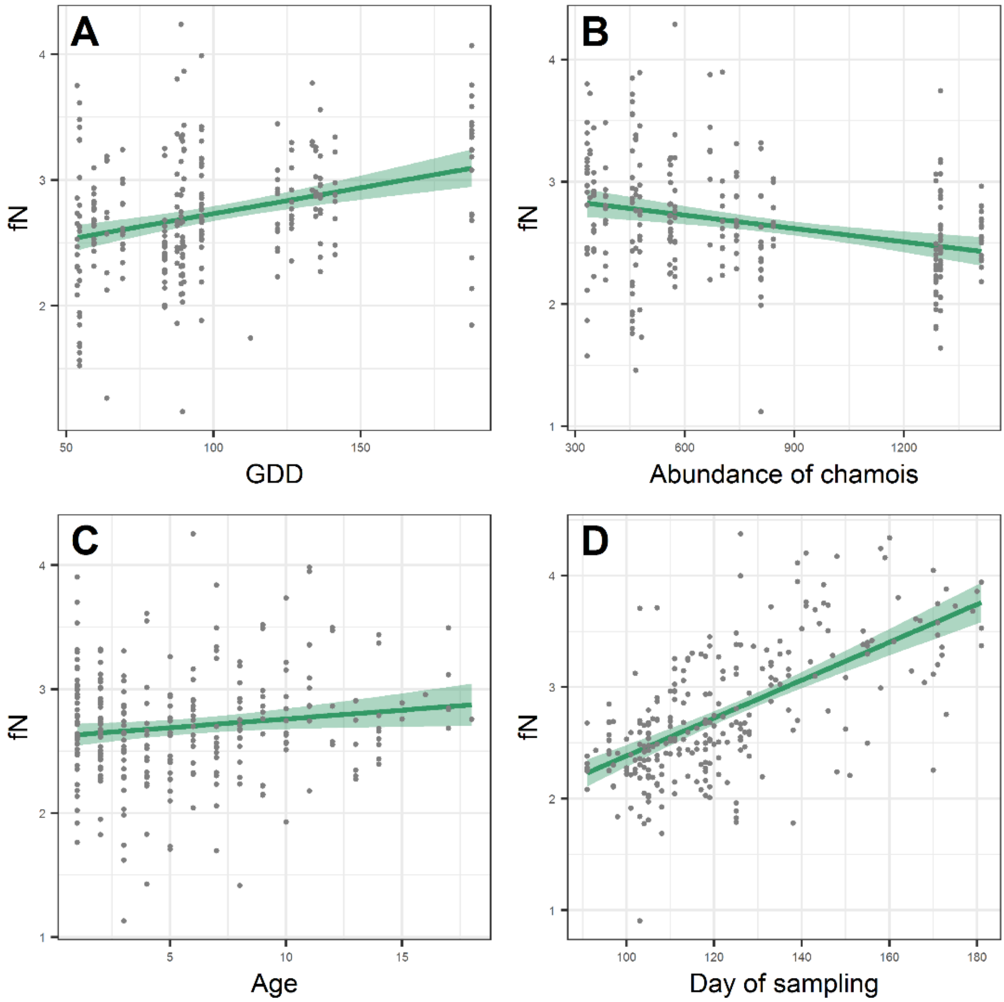

Figure 5.3.1. A) Effect of the Growing degree day (GDD) on the faecal nitrogen of chamois (adjusted for Age, fADF, abundance and day of sampling). B) Effect of the abundance of chamois on the faecal nitrogen of chamois (adjusted for Age, fADF, GDD and day of sampling). C) Effect of the age on the faecal nitrogen of chamois (adjusted for fADF, GDD, day of sampling and abundance). D) Effect of the day of sampling on the faecal nitrogen of chamois (adjusted for Age, fADF, GDD and abundance). Lines and surrounding coloured areas show the linear regression and their confidence intervals at 95%.

Relationship between diet quality and diet composition

In all the studied period, 39 plant genus were detected in Pyrenean chamois faeces sample but none of these genus were detected in all the samples. Overall, the most consumed group were graminoids (40.5%, CI_{95}= 36.9-44.2), mainly composed by *Festuca* spp. (26.1%, CI_{95}=23.3-28.9) and *Carex* spp. (7.2%, CI_{95}=6.2-8.3) and followed by the non-leguminous woody plant group (NLW; 25.3%, CI_{95}= 22.1-28.6) represented by *Calluna* spp. (8.7%, CI_{95}=6.6-10.8) and *Rhododendron* spp. (6.5%, CI_{95}=5.0-8.0). Non-leguminous forbs (NLF; 15.6%, CI_{95}=13.1-18.1), leguminous forbs (LF; 9.1%, CI_{95}=7.3-11.0) and leguminous woody (LW; 8.3%, CI_{95}=6.4-10.2) corresponded to the rest of the diet, mainly represented by the LW *Cytisus* spp. (8.2%, CI_{95}=6.3-10.0) and the LF *Trifolium* spp. (5.2%, CI_{95}=5.0-6.5).

The PLS multivariate regression was built using 2 latent vectors which explained 72.9% of the variance in diet composition and 45.6% of the variance in diet quality. The examination of the 2-dimensional regression coefficients (B_{PLS}, see Table 4.1.4) showed that faecal nitrogen was positively related to forbs consumption and negatively related to graminoids and woody plants. However, faecal lignin was positively related to woody plant consumption and negatively related to graminoids.

Table 4.1.4. Matrix of the 2-dimensional regression coefficients (B_{PLS}) between diet composition and diet quality of Pyrenean chamois. fNc= faecal nitrogen corrected by fADF, fL= faecal lignin, LF= Leguminous forbs, NLF= non-leguminous forbs, GR= graminoids, LW= leguminous woody, NLW= non-leguminous woody

	fNc	fL
LF	0.241	0.079
NLF	0.281	0.071
GR	-0.141	-0.453
LW	-0.119	0.190
NLW	-0.151	0.283

Effects of environmental and biological parameters on diet composition

All the most parsimonious models explaining the variation in diet composition of Pyrenean chamois included the effects of the abundance of chamois, and Julian day of sampling (Table 5.3.5). The selected model explaining the variation in the consumption of graminoids also included the effects of the age of the animal. The GDD in April was not included in any of the most parsimonious models but was present in a highly competing model for graminoids consumption. However its effect in this model was not significant (Estimate = -0.027, t.value= -1.37, p.value= 0.164).

Table 5.3.5. Model selection testing for the effects of biological and environmental variables on the diet composition of Pyrenean chamois. Age and sex of the animal, the abundance of chamois (Abun), accumulated growing degree during April (GDD), and Julian day of sampling (JDay) were considered predictor variables while the faecal nitrogen (fN) was considered response variable. Ranking and selection was based on the AICc. K=number of models parameters. AICc = Akaike Information Criterion corrected for small sample sizes, ΔAICc = difference of AICc with respect to the best model, ωi = Akaike weight. We only present models with a ωi>0.075 and the null model

Biological model	K	AICc	ΔAICc	ω_i
Woody plants				
Abun + JDay	**4**	**-34.8**	**0**	**0.293**
Age + Abun + JDay	5	-34.5	0.31	0.251
Sex + Abun + JDay	5	-32.8	1.96	0.110
Abun + JDay + GDD	5	-32.7	2.13	0.101
Age + Abun + JDay + GDD	6	-32.4	2.41	0.088
Age + Sex + Abun + JDay	6	-32.4	2.42	0.087
Null	2	36.4	71.18	0.000
Graminoids				
Age + Abun + JDay	**5**	**-29.1**	**0**	**0.299**
Age + Abun + JDay + GDD	6	-29.0	0.16	0.275
Age + Sex + Abun + JDay	6	-27.0	2.16	0.101
Age + Sex + Abun + JDay + GDD	7	-26.8	2.36	0.092
Null	2	-16.9	12.24	0.001
Forbs				
Abun + JDay	**4**	**-113.6**	**0**	**0.185**
Age + Abun + JDay	5	-113.0	0.67	0.132
Abun + JDay + GDD	5	-112.2	1.43	0.090
Sex + Abun + JDay	5	-112.0	1.67	0.080
JDay + GDD	4	-111.8	1.79	0.076
Null	2	-27.4	86.23	0.000

The consumption of woody plants was positively related with the abundance of chamois and negatively related with the Julian day of sampling (r^2= 0.38, $F_{152,2}$= 47.59, p<0.001). The consumption of graminoids was negatively related with the age of the animal, the abundance of chamois and the Julian day of sampling (r^2= 0.11, $F_{151,3}$= 6.40, p<0.001). Finally, the consumption of forbs was negatively related with the abundance of chamois and positively related with the Julian day of sampling (r^2= 0.44, $F_{152,2}$= 60.2, p<0.001; Table 5.3.6 and Fig 5.3.3).

Table 5.3.6. Estimates of the standardized parameters for the selected biological models (see Table 5). SE=standard error, JDay= Julian day of sampling.

Parameter	Estimate	SE	t-statistic	p-value
Woody plants				
Intercept	0.336	0.017	19.69	<0.001
Abundance	0.113	0.020	5.59	<0.001
JDay	-0.076	0.020	-3.76	<0.001
Graminoids				
Intercept	0.405	0.017	23.39	<0.001
Age	-0.039	0.017	-2.23	0.027
Abundance	-0.074	0.021	-3.63	<0.001
JDay	-0.059	0.020	-2.86	<0.005
Forbs				
Intercept	0.247	0.013	18.68	<0.001
Abundance	-0.033	0.016	-2.12	0.036
JDay	0.125	0.015	7.97	<0.001

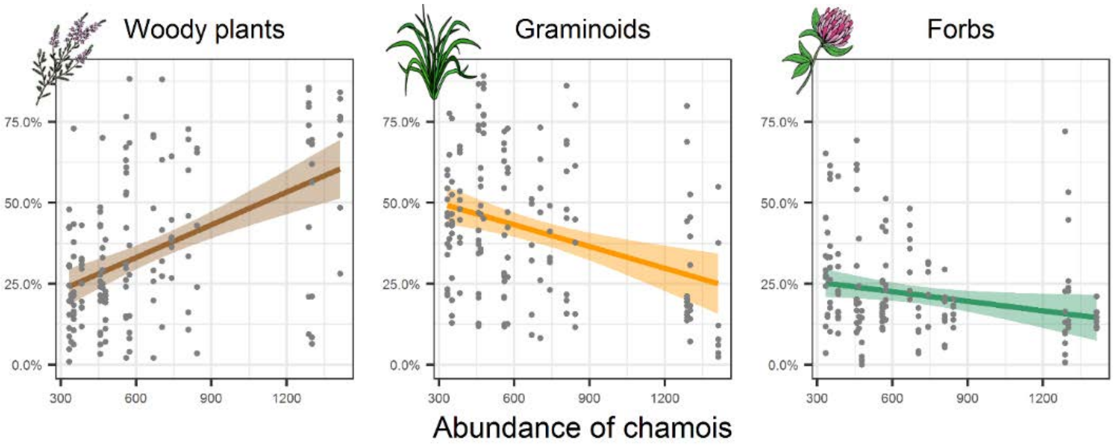

Figure 5.3.3. Effect of population abundance on the diet composition of Pyrenean chamois. The plots display the model's estimated relationships conditioned by the day of sampling. Lines and surrounding coloured areas show the linear regression and their confidence intervals at 95%.

Discussion

In agreement with other studies (Espunyes *et al.*, 2019; Gálvez-Cerón *et al.*, 2013) we found that Pyrenean chamois follow a seasonal pattern of diet quality and composition during spring. Our results also show that these patterns can be affected by environmental conditions. For instance, we found that earlier onset of spring advances the access to high-quality forage, without affecting the dietary composition. On the other hand, by driving to a diet richer in woody plants and poorer in forbs and graminoids, intraspecific competition deteriorate the quality of the diet. Therefore, while the effect sizes of these "bottom-up" regulating factors are proportionally similar on diet quality, they differ regarding diet composition.

Pyrenean chamois, like other species living in highly seasonal environments (Pettorelli *et al.*, 2007; Zweifel-Schielly *et al.*, 2012), follow a seasonal pattern of forage selection that increases during spring and peaks during summer, determined by primary production cycles (Espunyes *et al.*, 2019). In this study, we observed that chamois increased their consumption of forbs along spring but decreased the consumption of graminoids and woody plants. This feeding pattern is directly related to the pattern of dietary quality. Our results show an increase of faecal nitrogen content along the season and the percentage of faecal nitrogen was positively related to the consumption of forbs but negatively related to the consumption of graminoids and woody plants. In highly seasonal environments, growing plants present seasonal changes in nitrogen concentrations, particularly in leaves. Deciduous plants allocate substantial quantities of nitrogen into new leaves during early growing stages (i.e. spring). However, evergreen plants allocate it gradually throughout the growing season and in lower quantities, making deciduous plants more appetizing at the biomass-quality level (Jaeger *et al.*, 1999; Körner, 2011). In fact, the consumption of forbs along spring vary much more than the other plant groups, supporting the importance of phenology in its consumption. Because plant growing season is a key period for the reproduction and survival of alpine herbivores, any factor affecting these seasonal patterns of forage quality and availability may have long-lasting effects on their life-history traits (Gaillard *et al.*, 1996; Solberg *et al.*, 2008).

In fact, through our results we show that diet quality of Pyrenean chamois in spring was positively affected by earlier onsets of vegetation green-up. We do not imply that earlier springs enhances the quality of the diet but rather advances the access to nutritious forage. Earlier springs lead to an earlier access to highly nutritious early growing stages and therefore a higher diet quality at a fixed date of sampling. However, these earlier onsets of vegetation green-up had a lower effect on the diet composition. Thus, consuming the same proportions of plant groups, chamois obtained a higher nutritious diet during earlier springs, confirming the higher nutritious quality of early growing stages (Jaeger *et al.*, 1999). In our study, we also detected an earlier onset of the growing-season over the last decades, in line with several studies that provide evidences about climate warming and advances in phenological traits (Menzel & Fabian, 1999; Moyes *et al.*, 2011; Parmesan, 2006). By allowing individuals more time to grow and create reserves, this phenomenon could lead to an enhancement of the populations performances, as reported in other populations and species (Garel *et al.*, 2011; Ozgul *et al.*, 2010; Tveraa *et al.*, 2013). However, this is not always the case as Pettorelli et al. (2007) determined that warmer springs also tended to be associated with fast plant growth and shortened periods of high quality forage, that in turn negatively affected juveniles growth. Similar counterproductive effects may occur in kids growth and survival if the species is unable to adjust its parturition time to the earlier springs, causing a mismatch between parturition and the optimal foraging period (Post & Forchhammer, 2008). Kourkgy *et al.* (2016) already questioned the ability of Pyrenean chamois to adjust their gestation length or conception dates to the earlier onsets of spring. Their results showed that females had a much lower adaptive potential to the spring conditions than to autumn conditions.

Pyrenean chamois, as a capital breeder, should meet the high cost of early lactation mainly through maternal capital, potentially reducing the adverse effects of this mismatch (Gustine *et al.*, 2017; Richard *et al.*, 2017).

Our findings also show that population density negatively affected diet quality. As densities increased, it is expected that intraspecific competition for resources also increases, affecting the access to the most nutritious plants at the individual level (van Beest *et al.*, 2014; Kie *et al.*, 1980). The abundance of chamois in the area was negatively related with the consumption of forbs and graminoids, while positively related to the consumption of woody plants. In fact, the consumption of woody plants was mainly driven by the abundance of chamois and the effect was two and four times stronger than for the consumption of graminoids and forbs, respectively. Lignin is not only indigestible, but also interferes with digestion by reducing the rumen turnover rate and the intake capacity (Van Soest, 1994). Thus, the consumption of graminoids, while negatively related with nitrogen intake, can be interesting due to their highly negative relationship with faecal lignin content. Therefore, intraspecific competition forces chamois to have a lower digestible and protein-rich diet. As the abundance of chamois presented an important negative trend over time (see supplementary material Table S1 and Figure S2), we also checked the evolution of the habitat in order to think in terms of relative abundance of resources (supplementary material S3). As we observed a modest reduction of 11.8% of grasslands over woody habitats, we suggest that the total availability of forbs and graminoids has not increased over time but that the increased consumption is due to a higher density-dependent availability of these resources. Even with a lower availability of grassland species, the lower abundance of animals along the years compensated for the lower availability of resources.

This study also provides additional support to the theory of increased experience in plant selection for herbivores along their life (Froy *et al.*, 2018). We observed that older animals consume lower proportions of graminoids (negatively related to faecal nitrogen content) and had a higher diet quality than younger ones. A similar pattern of lower consumption of graminoids by older Pyrenean chamois was also suggested by García-González (1984). However, contrary to the predictions, sex was not included in the most parsimonious models explaining the variance in faecal nitrogen or diet composition, despite a sexual difference in spatial behaviour and habitat selection (Dalmau *et al.*, 2013;

Loison *et al.*, 1999b). Sexual differences in diet quality and composition has been rarely studied in any chamois species, but Pérez-Barberia *et al.* (1997) observed a similar diet composition in both sexes, at least in spring in Cantabrian chamois (*Rupicapra p. parva*). Being highly selective for high-quality palatable plants (Corlatti *et al.*, 2013; García-gonzález & Cuartas, 1996) chamois species may be able to selectively feed on high-quality forage and compensate for their sexual morphological (at least in the case of Alpine chamois; Mendoza *et al.* 2002) and behavioural differences.

In the current context of climate change (Steffen *et al.*, 2018) and increasing densities of large European wild herbivores (Apollonio *et al.*, 2010), understanding the mechanical effects of these variations on the dietary selection of individuals is an essential step to understand their consequences on life-history traits of wild populations (Sinclair & Parkes, 2008). We advise the possibility to use faecal nitrogen to monitor long term changes in density-dependent and –independent environmental conditions through changes in the nutritional condition of a given population (Blanchard *et al.*, 2003; Gálvez-Cerón *et al.*, 2013). Furthermore, because we detected few sex and age effects, the collection of fresh unidentified droppings during seasonal field surveys coupled with NIRS analysis could be an inexpensive option to monitor the population condition as a complement to other indicators of ecological change (Morellet *et al.*, 2007; Santos *et al.*, 2014). This study also strengthen the fundamental need of long-term monitoring programs to understand the ecological processes affecting herbivores performances in the context of global change (Festa-Bianchet *et al.*, 2017).

Acknowledgments

We are thankful to all the field assistants and volunteers for their amazing work in trapping Pyrenean chamois and collecting faeces during such a long period. This study was funded by the by the research partnership programme "Approche Intégrée de la Démographie des Populations d'Isard" (N°2014/08/6171) between the Office de la Chasse et de la Faune Sauvage (ONCFS) and the Servei d'Ecopatologia de la Fauna Salvatge (SEFaS)

Supporting Information

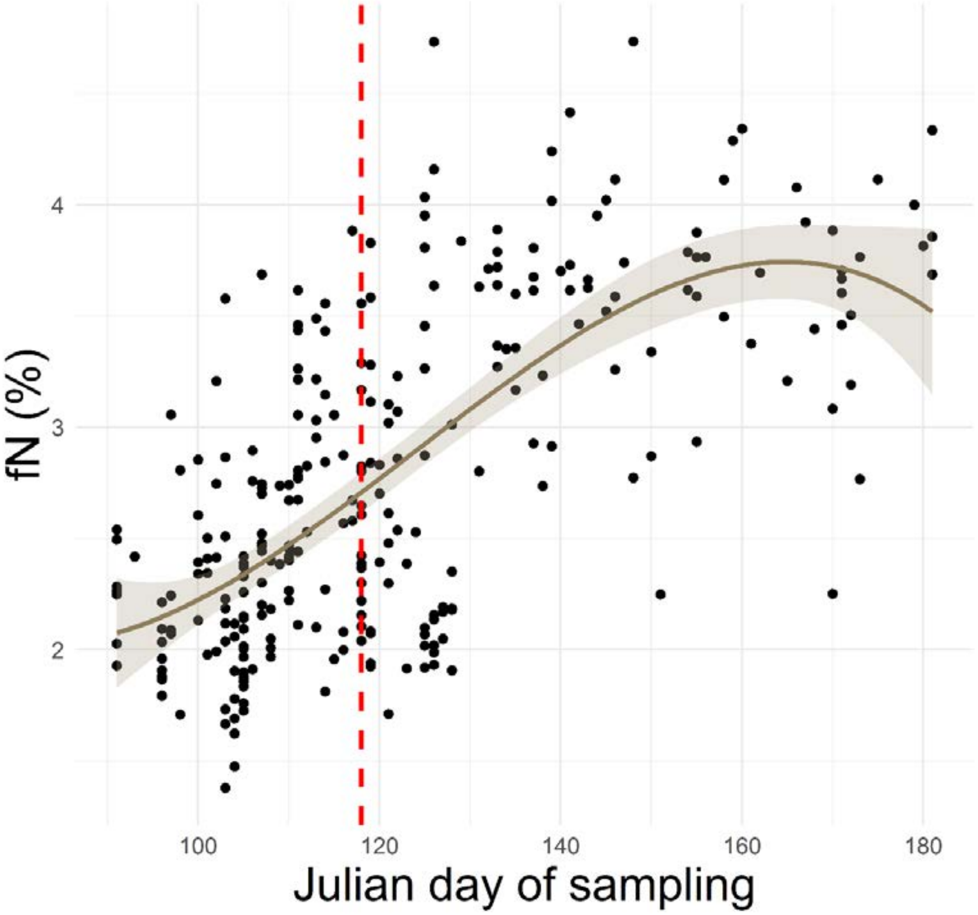

Figure S5.3.1. Relationships between the day of sampling and the faecal nitrogen (fN) of chamois. Brown line was obtained with a cubic smoothing spline and coloured areas show the confidence intervals at 95%. The red dashed line represents the median of the days of sampling.

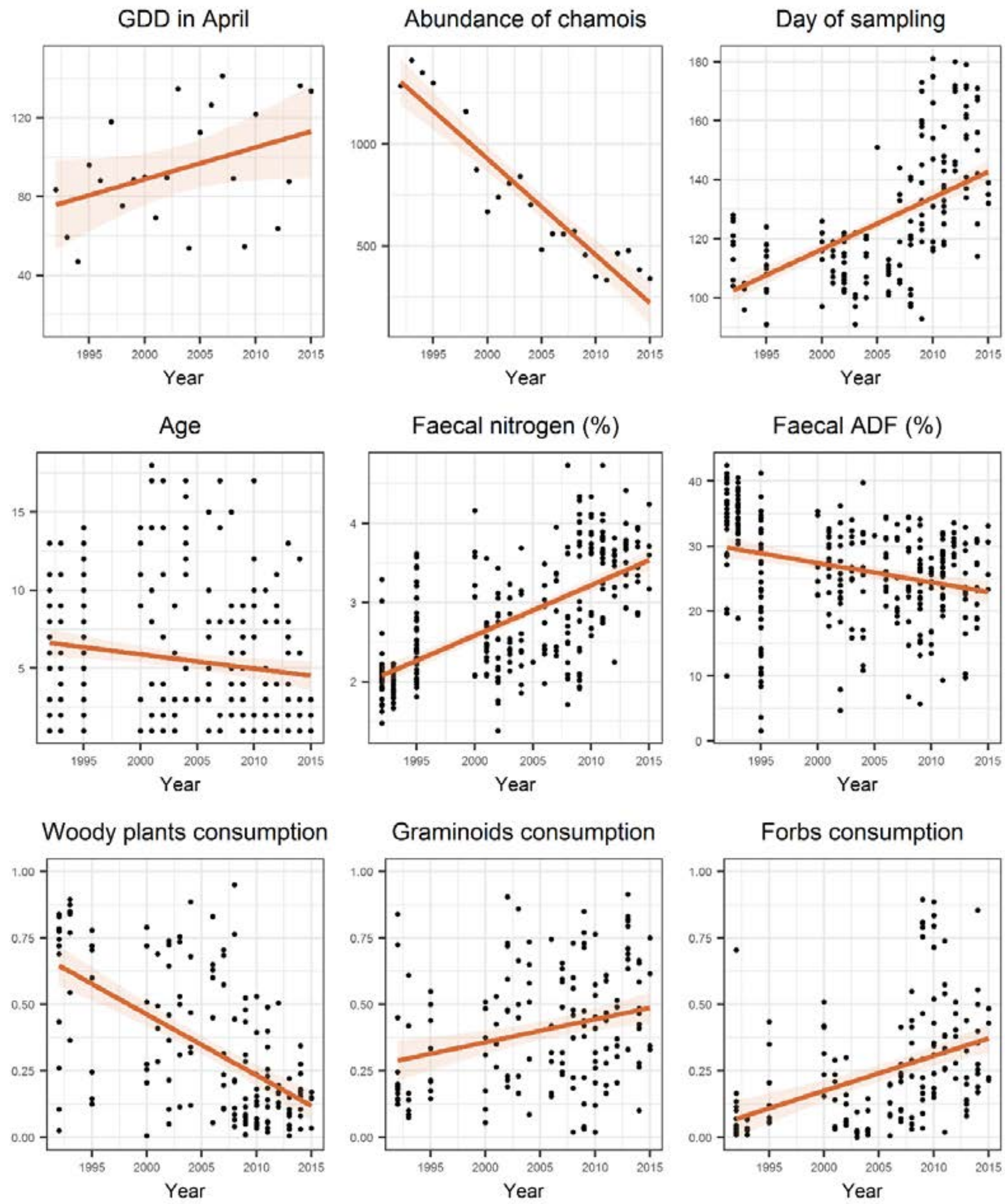

Figure S5.3.2. Trends over time of the parameters of our study. The red lines and surrounding coloured areas show the linear regression and their confidence intervals at 95%. GDD= accumulated growing degree during April.

Table S5.3.1. Estimates of the linear regression between the studied parameters and the year. SE=standard error, GDD= accumulated growing degree during April, ADF= Acid-detergent fiber.

Parameter	Estimate	SE	r^2	F value	p-value
GDD	2.15	0.93	19.6%	5.36	0.030
Abundance of chamois	-47.19	3.92	87.9%	145.0	<0.001
Day of sampling	1.75	0.029	36.8%	159.8	<0.001
Age	-0.09	0.034	2.6%	7.35	<0.01
Faecal ADF	-0.29	0.061	7.9%	23.9	<0.001
Faecal Nitrogen	0.06	0.004	41.4%	194.2	<0.001
Forbs consumption	0.017	0.002	17.5%	32.46	<0.001
Woody plants consumption	-0.023	0.003	35.2%	83.0	<0.001
Graminoids consumption	0.008	0.002	7.0%	11.57	<0.001

Table S5.3.2. Variance inflation factors (VIF) values for the selected model (see Table 5.3.2). Abun= Abundance of chamois. fADF= faecal ADF. GDD= accumulated growing degree during April. Jday= day of sampling.

Covariate	**VIF**
Age	**1.068**
Abun	**1.846**
fADF	**1.106**
GDD	**1.265**
Jday	**1.501**

S5.3.1. Detrended effects of environmental and biological parameters on diet quality

In overall, performing model selection with the detrended variables had no significant qualitative impact on our results (See Table S5.3.3 and Table S5.3.4) with Age, fADF, GDD during April, and Julian day of sampling still consistently selected among all the best supported models. Only the effect of population abundance detracted from this general pattern by being replaced in the two first best models by year effect. This result was probably due to the very high correlation between the abundance of chamois and the covariate year (r= 0.93 $F_{20,1}$= 145, p<0.001; see Table S5.3.1 and Figure S5.3.2) which limit our ability to disentangle abundance effects from any other factors that would exhibit linear trend over the study period. However, when present in a model along with the year effect, chamois abundance still had a negative effect on faecal nitrogen (beta =-0.0058 and see Table S5.3.3) as observed in the non-detrended analysis. At the view of these results, we felt confident in the biological meaning of selected effects in our non-detrended models.

Table S5.3.3. Detrended model selection testing for the effects of biological and environmental detrended variables on the fN of Pyrenean chamois. The sex of the animal, the detrended age (Ager), the detrended faecal acid-detergent fibre (fADFr), the detrended abundance of chamois in the reserve (Abunr), the detrended accumulated growing degree during April (GDDr), the detrended Julian day of sampling (JDayr) and the year were considered predictor variables while faecal nitrogen (fN) was considered response variable. Ranking and selection was based on the AICc. K=number of models parameters. AICc = Akaike Information Criterion corrected for small sample sizes, ΔAIC = difference of AIC with respect to the best model, ωi = Akaike weight. We only present models with a ΔAICc<4 and the null model.

Biological model	**K**	**AICc**	**ΔAICc**	ω_i
Ager + fADFr + GDDr + JDayr + Year	**7**	**351.1**	**0**	**0.442**
Ager + Sex + fADFr + GDDr + JDayr + Year	8	352.7	1.61	0.198
Ager + fADFr + GDDr + Abunr + JDayr + Year	8	353.2	2.08	0.157
Ager + Sex + fADFr + GDDr + Abunr + JDayr + Year	9	354.7	3.65	0.071
fADFr + GDDr + JDayr + Year	6	354.8	3.71	0.069
Null	2	621.6	270.51	0.000

Table S5.3.4. Estimates of the standardized parameters for the selected detrended model (see Table S5.3.3). SE=standard error, Ager= detrended age , fADFr= detrended faecal acid-detergent fibre, GDDr= detrended accumulated growing degree during April, JDayr= detrended Julian day of sampling.

Parameter	Estimate	SE	t-statistic	p-value
Intercept	-127.2	7.32	-17.35	<0.001
Ager	0.066	0.027	2.39	0.017
fADFr	-0.179	0.027	-6.51	<0.001
GDDr	0.139	0.027	5.06	<0.001
JDayr	0.282	0.027	10.31	<0.001
Year	0.065	0.004	17.74	<0.001

S5.3.2. Detrended effects of environmental and biological parameters on diet composition

In overall, performing model selection with the detrended variables had no significant qualitative impact on our results (See Table S5.3.5 and Table S5.3.6) with Age, abundance of chamois and Julian day of sampling still consistently selected among all the best supported models. However, in the case of woody plants, the effect of population abundance was not present in the best model probably due to the same reasons as in the case of diet quality. When present in a model along with the year effect, the abundance of chamois still had the same effect on diet composition as observed in the non-detrended analysis in the case of woody plants and forbs. In the case of graminoids, though, the effect is reversed but not so significant ($p=0.04$, see Table S5.3.6). At the view of these results, we felt confident in the biological meaning of selected effects in our non-detrended models.

Table S5.3.5. Detrended model selection testing for the effects of biological and environmental detrended variables on the diet composition of Pyrenean chamois. The sex of the animal, the detrended age (Ager), the detrended abundance of chamois in the reserve (Abunr), the detrended accumulated growing degree during April (GDDr), the detrended Julian day of sampling (JDayr) and the year were considered predictor variables while woody plant, graminoids and forbs consumption were separately considered response variable. Ranking and selection was based on the AICc. K=number of models parameters. AICc = Akaike Information Criterion corrected for small sample sizes, ΔAICc = difference of AICc with respect to the best model, ωi = Akaike weight. We only present models with a ωi>0.75 and the null model.

Biological model	K	AICc	ΔAICc	ω_i
Woody plants				
Year + JDayr	**4**	**-36.5**	**0**	**0.234**
Ager + Year + JDayr	5	-35.3	1.25	0.125
Year + Abunr + JDayr	5	-34.9	1.60	0.105
Sex + Year + JDayr	5	-34.4	2.06	0.083
Year + JDayr + GDDr	5	-34.4	2.14	0.080
Null	2	36.4	72.88	0.000
Graminoids				
Ager + Year + Abunr + JDayr	**6**	**-40.9**	**0**	**0.148**
Ager + Year + Abunr + JDayr + GDDr	7	-40.9	0.04	0.145
Year + Abunr + JDayr	5	-40.6	0.30	0.128
Year + Abunr + JDayr + GDDr	6	-40.0	0.91	0.094
Ager + Sex + Year + Abunr + JDayr	7	-39.8	1.16	0.083
Ager + Sex + Year + Abunr + JDayr + GDDr	8	-39.8	1.17	0.083
Null	2	-16.9	24.04	0.000
Forbs				
Year + Abunr + JDayr	**5**	**-122.2**	**0**	**0.333**
Year + Abunr + JDayr + GDDr	6	-120.9	1.28	0.175
Ager + Year + Abunr + JDayr	6	-120.4	1.79	0.136
Sex + Year + Abunr + JDayr	6	-120.2	2.02	0.121
Ager + Year + Abunr + JDayr +GDDr	7	-119.2	2.93	0.077
Null	2	-27.4	94.79	0.000

Table S5.3.6. Estimates of the standardized parameters for the selected detrended model testing for the effects of biological and environmental variables on the consumption of forbs by Pyrenean chamois (see Table S5.3.5). SE=standard error, Abunr=the detrended abundance of chamois in the reserve, JDayr=the detrended Julian day of sampling.

Parameter	Estimate	SE	t-statistic	p-value
Intercept	-28.009	3.849	-7.28	<0.001
Year	0.014	0.002	7.34	<0.001
Abunr	-0.049	0.013	-3.86	<0.001
JDayr	0.105	0.012	8.82	<0.001

S5.3.3. Land-cover evolution

In order to characterize the diachronic evolution of habitats extension in our study area, we used historic aerial photographs taken in 1995, and orthophotos made in 2003 and 2013. The 1995 colour-scale aerial photographs were georeferenced and orthorectified using at least 30 ground control points per photograph. In this approach of classifying images, polygons delimitating ecological cover types were manually traced on top of the raster image using QGIS version 2.18.10 (QGIS Development Team, 2014). The area measurements of all the polygons falling under a single category were added. The habitat categories used for the classification were: water, shrubland, grassland, forests and rocks.

Our results showed that from 1995 to 2013, a land cover change occurred in the NGWR-O, characterized by an expansion of forested areas and shrubland at the expense of grassland. Grasslands were reduced from 765.5ha to 674.9ha while forest expanded from 1083.1ha to 1180.6ha. At the same time, shrubland increased from 1287.5ha to 1294.7ha. As observed between studied years, grassland slowly turned into shrubland, which then converted to forest. Some rocky areas were also covered by shrubs.

5.4 Study 4:

Different effects of alpine woody plant expansion on domestic and wild ungulates

Global Change Biology (10.1111/gcb.14587)

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Abstract

Changes in land-use and climate affect the distribution and diversity of plant and animal species at different spatiotemporal scales. The extent to which species-specific phenotypic plasticity and biotic interactions mediate organismal adaptation to changing environments, however, remains poorly understood. Woody plant expansion is threatening the extent of alpine grasslands worldwide, and evaluating and predicting its effects on herbivores is of crucial importance. Here, we explore the impact of shrubification on the feeding efficiency of Pyrenean chamois (*Rupicapra p. pyrenaica*), as well as on the three most abundant coexisting domestic ungulate species: cattle, sheep and horses. We use observational diet composition from May to October and model different scenarios of vegetation availability where shrubland and woodland proliferate at the expense of grassland. We then predicted if the four ungulate species could efficiently use their food landscapes with their current dietary specificities measuring their niche breath in each scenario. We observed that the wild counterpart, due to a higher trophic plasticity, is less disturbed by shrubification compared to livestock, which rely primarily on herbaceous plants and will be affected 3.6 times more. Our results suggest that mixed feeders, such as chamois, could benefit from fallow landscapes, and that mountain farmers are at a growing economic risk worldwide due to changing land-use practices and climate conditions.

Introduction

Environmental and climatic changes are affecting biological and ecological systems across the globe at alarming rates (Steffen *et al.*, 2005). These trends influence fauna and flora in many ways, from habitat degradation to distributional range shifts, as well as phenological mismatch (Parmesan & Yohe, 2003; Pereira *et al.*, 2010; Root *et al.*, 2003). In fact, global land-use and climatic changes, through their influence on different aspects of the biology and ecology of species, have caused numerous extinctions (Vitousek *et al.*, 1997), with models predicting an intensification of these trends over the next century (Loarie *et al.*, 2009). Specialist species are predicted to decline at a faster rate due to their limited adaptive potential within their narrow environmental tolerances (Morrison *et al.*, 2018; Thuiller *et al.*, 2005b). Understanding the species-specific potential and limitation to cope with global change is thus a central aspect of timely conservation studies (Charmantier *et al.*, 2008; Nussey *et al.*, 2005).

In this context, evaluating and predicting the impact of global change on wild herbivores has become a conservation priority globally, since their protection and management has been deemed crucial for the long-term conservation of ecosystems (e.g., Büntgen *et al.*, 2014). Indeed, herbivores fulfil key roles in the terrestrial trophic cascades and the maintenance of ecosystem health by affecting nutrient cycles and maintaining the diversity and stability of predators and primary producers (Bardgett & Wardle, 2010). They are also considered "environmental engineers" due to their fundamental role in the structure, composition, and functioning of ecosystems (Schmitz, 2008). Numerous questions regarding the effects of global change must be answered including whether or not herbivores will be able to maintain their role in a particular ecosystem subject to change.

European mountains are a paradigmatic representation of a changing ecosystem, host of a wide variety of wild and domestic herbivores. As in other mountain ranges around the world, they have undergone a biological shift since the mid-20th century due to profound agricultural land-use and climatic changes (Mottet *et al.*, 2006; Sanz-Elorza *et al.*, 2003; Steinbauer *et al.*, 2018). The dramatic decline in rural populations and agropastoral activities have led to a general decline in livestock densities (Didier, 2001; Gartzia *et al.*, 2016). Temperatures have simultaneously increased (IPCC, 2007), which have affected these ecosystems, albeit to a lesser degree, for example by stimulating shrub development or by upward shifting the tree line (Ameztegui *et al.*, 2016; Peñuelas *et al.*, 2007). Consequently, plant succession at varying rates leads to woody plant expansion (Mod & Luoto, 2016; Prévosto *et al.*, 2011), resulting in a shift in dominance from herbaceous to woody plants in one of the richest habitats in the world: alpine grasslands (Wilson *et al.*, 2012). However, this so-called shrubification (Martin *et al.*, 2017) generally decreases the diversity of plant species (Koch *et al.*, 2015; Tasser & Tappeiner, 2002), the productivity of the environment (Lett & Knapp, 2003) and the diversity and total abundance of mammals (Stanton *et al.*, 2018). Community composition and ecological interactions between species, including herbivore-plant interactions, are being importantly restructured (Lurgi *et al.*, 2012). These trends will likely continue as models predict a continuous increase in temperature (López-Moreno *et al.*, 2008) and a decrease in rural agropastoral activities (Mann, 2013; Verburg *et al.*, 2010).

The Pyrenees, in southwest Europe, have experienced a major expansion and densification of shrubland and forested areas over the last century. For this reason, they constitute an ideal study case of the effects of land-use and climate change on natural communities. Not only have tree line ecotones increased on average by 35m (Ameztegui *et al.*, 2016), with forest cover in some areas expanding by at least two-thirds (Lasanta-Martínez *et al.*, 2005; Poyatos *et al.*, 2003), but they have also experienced an increase in recent summer temperatures occurring at an unprecedented rate (Büntgen *et al.*, 2008, 2017a). These locally detected changes are consistent with a larger-scale trend across most (or even all) of the European mountain systems (Fig. 5.4.1).

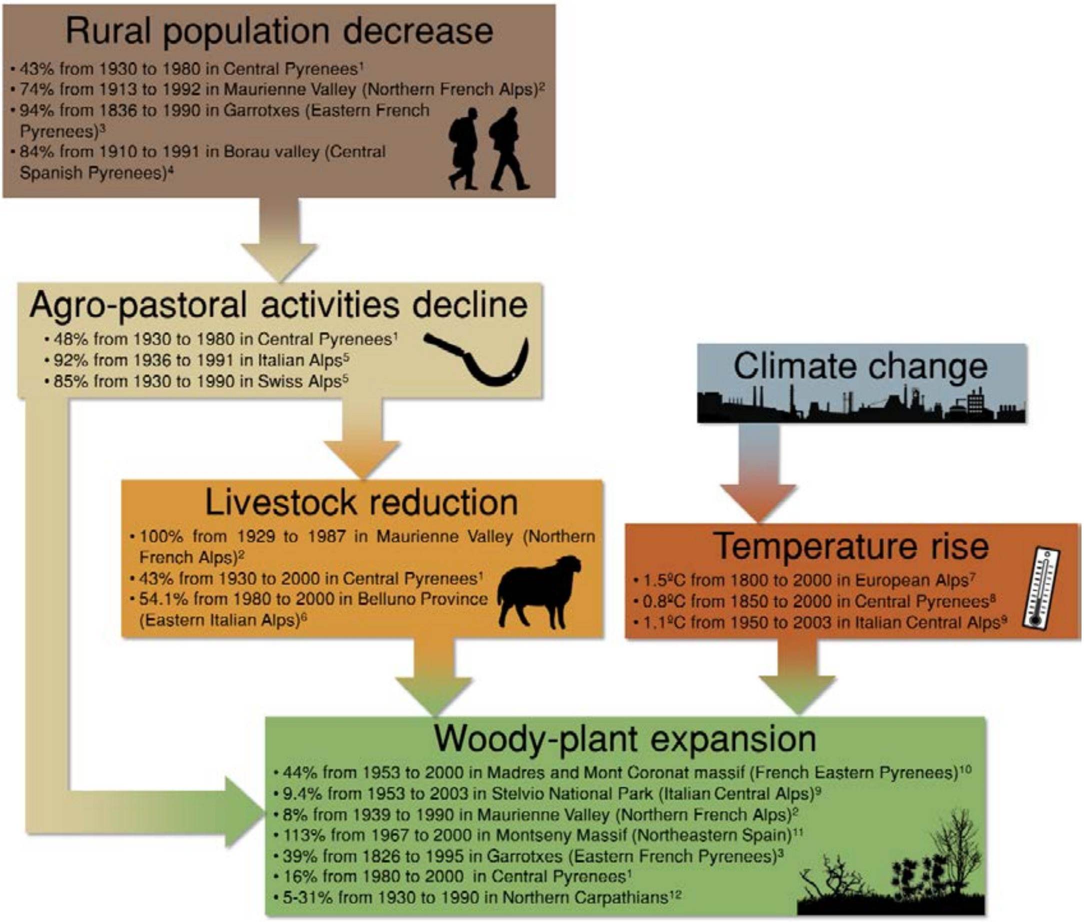


Figure 5.4.1. Schematic representation of the primary causes of woody plant expansion in European alpine ecosystems. The decrease in mountain rural populations causes a decline in agropastoral activities and therefore a reduction in livestock densities. At the same time, temperatures increase due to climate change. The combination of these factors leads to the expansion of woody plants in alpine grasslands (1. Gartzia *et al.*, 2016; 2. Didier, 2001; 3. Metailié & Paegelow, 2005; 4. Lasanta-Martínez *et al.*, 2005; 5. Collantes, 2006; 6. Sturaro *et al.*, 2005; 7. Büntgen *et al.*, 2006; 8. Büntgen *et al.* 2017; 9. Cannone *et al.*, 2007; 10. Roura-Pascual *et al.*, 2005; 11. Bartolomé *et al.*, 2005; 12. Kozak *et al.*, 2007)

Through evolution, and in some cases further domestication, large herbivores present in the Pyrenean grasslands display a wide range of body sizes, digestive systems and feeding behaviours adapted to specific diets. They are consequently expected to respond differently to habitat change (Somero, 2010) and are thus good models for the study of species-specific effects of woody plant expansion.

The aim of this study is to explore the potential impact of the expansion of woody plant coverage on four herbivore species in the eastern Spanish Pyrenees - the wild Pyrenean chamois (*Rupicapra p. pyrenaica*), as well as seasonal domestic cattle, sheep and horses - that inhabit the same alpine habitats. Traditional farming is based on livestock freely living and grazing on alpine grasslands during the summer to reduce the economic costs involved in livestock maintenance. Once the yield of these human-created grasslands decay, livestock is then moved to the hay meadows in the lower parts of the valleys until the following spring (Montserrat & Fillat, 1990). The Pyrenean chamois is a medium-sized wild caprinae well adapted to mountain environments. They are considered mixed feeders, which consume a wide variety of resources depending on the habitat and season, following the annual cycle of primary productivity (Berducou, 1974; García-gonzález & Cuartas, 1996). Together, these four species represent a contrasting collection of energetic requirements (large- vs medium-sized herbivores), feeding behaviours (grazers vs mixed feeders), digestive physiologies (rumen vs hindgut fermenters), and origins (livestock vs wildlife).

We devised various scenarios of shrubification based on the current vegetation cover, where shrubland and woodland proliferate at the expense of grassland following models of projected woody plant expansion. We then use these shrubification scenarios and the diet composition of the ensemble of herbivores to predict how phenotypic plasticity and biotic interactions mediate the effects of shrubification. Given the diverse dietary requirements of our four species, we hypothesise that changes in land cover would affect each species differently, with grazers being more severely and rapidly affected than mixed feeders.

Materials and methods

Study area

The study was carried out in the Freser-Setcases National Game Reserve (FSNGR), in the eastern part of the Spanish Pyrenees (42°22'N, 2°09'E). This area of 410 ha is known as Costabona and ranges from 1500 to 2400 m.a.s.l. It belongs to the sub-humid subalpine and alpine bioclimatic belts of the southern slopes of the Pyrenees, with a noticeable Mediterranean climatic influence (Vigo, 2008). Mean annual temperature for 2009-2012 was 5.7 ºC (daily min=-18.2, max=26.6), and mean yearly accumulated rainfall for the same period was 1042.4 mm (yearly min=762.6, max=1282.8). These data were obtained from the Nuria meteorological station at 1971 m a.s.l. in the core of the FSNGR (Servei Meteorològic de Catalunya).

The vegetation cover of our study area was assessed in June 2011 following the line-intercept method proposed by Cummings and Smith (2000). The cover of all plant species present was recorded along six randomly selected transects of 10×0.1 m at different altitudes.

Collection and analysis of faeces

Fresh faecal samples from each of the four ungulates considered in this study (namely Pyrenean chamois, cattle, horses and sheep) were collected monthly from June to October 2011 and 2012 (except in September 2012 when sampling was not possible due to adverse meteorological conditions), coinciding with the presence of the four species in the area. Once every month, two observers walked a transect of about 5 km, covering the entire altitudinal range and main vegetation communities of the study area. Fresh faecal samples from at least five individuals per species were collected and pooled together before being transported to the laboratory and frozen at -20 ºC after every session. A total of nine pooled samples was obtained per species. This sampling procedure was used to obtain a general overview of the variability of feeding in the field during the three periods of plant phenology in our study area (namely: green-up, plateau greenness and senescence periods; Villamuelas *et al.*, 2016).

A faecal cuticle microhistological analysis was used to determine dietary composition, adapted from a protocol described by Stewart (1967). The samples were thawed, washed and ground to separate the epidermal fragments. Ten grams of sample were then placed in a test tube containing 5 ml of 65% concentrated nitric acid, boiled in a water bath for 1 min, and diluted with 200 ml of water. This suspension was passed through 1.00 and 0.25 mm filters. The 0.25-1.00 mm fraction was spread on glass microscope slides in 50% glycerol, and cover-slips were fixed with DPX microhistological varnish. Two slides were prepared from each sample. The slides were microscopically examined by the same operator at magnifications of 100× and 400×, and 200 fragments of plant epidermis were identified per sample. An epidermis collection of the 55 main plant species in the study area were collected and used for fragment identification. Plant cuticles were identified to the species or genus level depending on the difficulty of the task.

Simulation of woody plant expansion

Patterns of expansion of woody plants into grasslands have been studied worldwide (Bartolomé *et al.*, 2005; Eldridge *et al.*, 2011; Falcucci *et al.*, 2007; Olsson *et al.*, 2000). This plant succession can proceed at different speeds and with different numbers of stages depending on land-use patterns, initial state, altitude, or topography (Tasser & Tappeiner, 2002; Vacquié *et al.*, 2016). This process can nevertheless be synthesised in a first successional stage when herbaceous species are replaced by shrubs, followed by a second successional stage when shrubs are replaced by trees (Améztegui *et al.*, 2010; Gellrich *et al.*, 2007; Tasser *et al.*, 2007; Wallentin *et al.*, 2008). Succession can be fast; descriptive and predictive studies have demonstrated that woody plant cover can increase by 0.5-5% per year (Barger *et al.*, 2011; Komac *et al.*, 2013).

To simulate the effects of woody plant expansion, we devised eight hypothetical scenarios where shrubland and woodland proliferate at the expense of grassland without any agricultural practices or forestry management. Based on the original vegetation availability in the study area, we designed shrubification scenarios where woody plants increased and graminoids and forbs decreased proportionally. The initial state of the system (i.e. original scenario) comprised a relative abundance of woody species of 21.4% (19.6% shrubs and 1.8% trees). Total relative abundance of woody plants was then increased by intervals of 10% per scenario until reaching 100% of woody plant cover (except the first scenario which increased by 8.6% to achieve 30% woody plant cover). This procedure yielded nine scenarios of woody plant cover (the original scenario plus eight hypothetical): 21.4%, 30%, 40%, 50%, 60%, 70%, 80%, 90% and 100%, respectively. The increase in woody plant cover was distributed proportionally across the plant species included in this category according to their relative abundance. For example, if woody plant cover increased by 20% and the plant *Juniperus communis* represents 50% of the total woody plant cover then a 10% increase of that plant was simulated. Conversely, the cover of forbs and graminoids was decreased by the same fraction of woody plant increase. This decrease was also distributed proportionally between the species of these categories (see Supplementary Table S5.4.1).

Landscape-use efficiency

The breadth of a resource niche can be used as a proxy for species performance (Rotenberry & Wiens, 1980) or to quantify the extent to which organisms are able to exploit their environment (Krebs, 1999). We used the measure of niche breadth proposed by Smith (1982) as a proxy for the efficiency in the use of resources by the herbivores (i.e. landscape-use efficiency, LUE).

LUE for each herbivore in each shrubification scenario was calculated as:

$$LUE = \sum \left(\sqrt{P_j \times A_j} \right)$$

where P_j is the proportion of plant j in the diet of the herbivore, and A_j is the proportion of plant j available in the study area. This index ranges from nearly zero, for the narrowest possible niche when a species is specialised in eating the rarest resources, to one, for the broadest possible niche when a species uses resources in proportion to their availability. This index is thus low when a species inefficiently uses the resources of its habitat and is high when a species uses them efficiently (i.e. proportionally to the availability).

Statistical analysis

After describing the diets of our studied species by basic statistics, we performed a non-parametric multivariate analysis of similarity (ANOSIM; Clarke, 1993) to check for differences in diets between herbivores. The ANOSIM statistic *R* is based on the difference of mean ranks between groups and within groups and a high value of *R* in this analysis indicates a high dissimilarity between groups. A nonmetric multidimensional scaling (NMDS) plot based on Bray–Curtis dissimilarity indices was created to visually identify the patterns in dietary similarities between species. Stress, a measure of goodness of fit should be <0.2 in order to have a good representation with no prospect of misinterpretation (Clarke & Warwick, 2001). Our NMDS stress was 0.0985 so our representation was considered to be sufficiently well-described in two dimensions. The ANOSIM and the NMDS plot were performed using the R vegan package (version 2.4-5 Oksanen *et al.*, 2017).

We then evaluated the impact of woody plant expansion (i.e. woody plant abundance in the scenarios) on the LUE of each species by a linear model (LM). LUE of each species was the response variable in our LM whereas the interaction between animal species and degree of woody plant expansion were our fixed explanatory factors. Interspecific differences of LUE values were analysed with a pairwise Mann-Whitney U test using the FSA package (version 0.8.17, Ogle, 2017). Normality of residuals and homogeneity of variance assumptions were checked previous to the performance of any analysis. All statistical analyses were performed using R version 5.4.3 (R Core Team, 2018).

Results

Initial state of the system and herbivore diets

We recorded 65 plant species in our study area. Graminoids represented half of the vegetation cover (51.6%), where *Festuca* spp. was clearly dominant (32.3%), followed by *Carex cariophyllea* (12.4%). Forbs covered almost one-third of the area and were dominated by *Trifolium alpinum* (7.5%), followed by *Trifolium repens* (1.5%) and *Hippocrepis comosa* (1.4%). The other plants were woody species (shrubs and trees, 21.2%), where dwarf shrubs (*Calluna vulgaris* and *Juniperus communis*) and legumes *Cytisus* spp. were the most common (See Table S5.4.1).

The ANOSIM indicated that the differences in dietary composition were higher between Pyrenean chamois and the livestock than amongst the livestock species (Table 1). Among the livestock diets, horse diet differed the most from the rest, while cattle and sheep showed more similar dietary compositions. The NMDS plot supported these interspecific dietary differences (Fig. 5.4.2).

Table 5.4.1. Differences in dietary composition between alpine ungulates in the Pyrenees. Summary of the pairwise ANOSIM of the differences in dietary composition between Pyrenean chamois, cattle, horses, and sheep from June to October 2011 and 2012 in the eastern Spanish Pyrenees. A high value of R in this analysis indicates a high dissimilarity between groups.

	Cattle	Horses	Sheep
Chamois	R=0.506 p= 0.002	R=0.692 p=0.001	R=0.569 p=0.001
Sheep	R=0.246 p=0.013	R=0.427 p=0.001	
Horses	R=0.386 p=0.002		

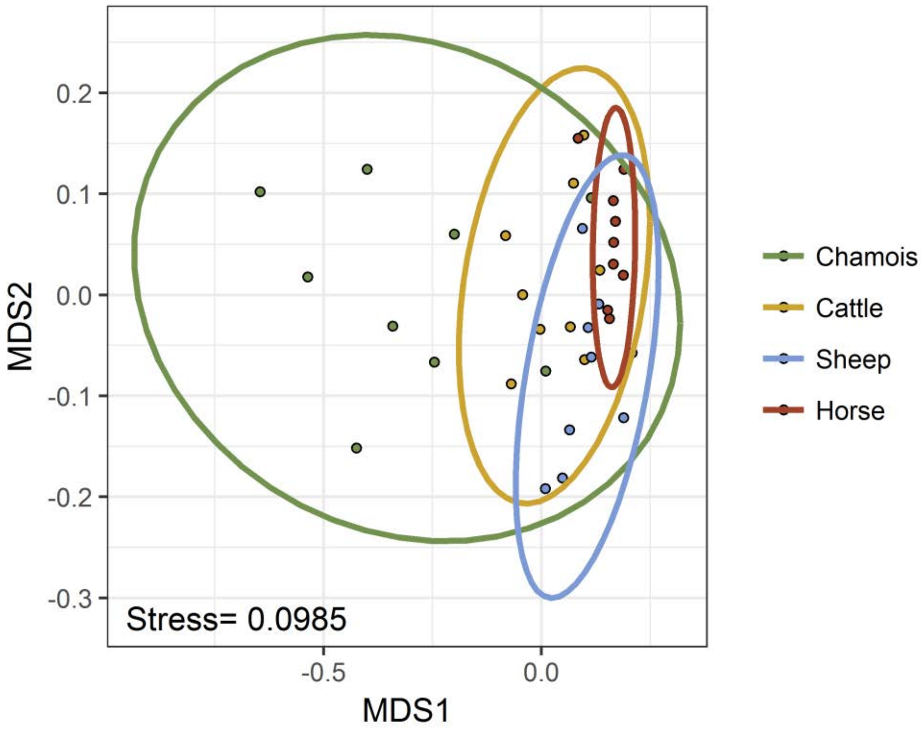


Figure 5.4.2 Diet dissimilarity among alpine ungulates in the Pyrenees. Nonmetric multidimensional scaling (NMDS) plot representing dietary dissimilarity for seasonal livestock (cattle, horses, and sheep) and Pyrenean chamois from June to October 2011 and 2012 in eastern Spanish Pyrenees. When stress, a measure of goodness of fit, is <0.2, NMDS reproduces an adequate depiction of the groups.

Analysis of diet composition for livestock during summer and autumn showed a larger overall content of graminoid and forb fragments compared to woody plants. Horses were the most extreme livestock species with the highest consumption of graminoids (63.7%) and the lowest consumption of woody plants (5.9%; see Table 5.4.2). On the contrary, Pyrenean chamois faeces had the highest content of woody plant fragments (48.6%) and the lowest content of graminoids (25.8%) and forbs (25.3%). Cattle and sheep had similar diets (*R*=0.246, see table 1), but cattle showed a higher content of graminoids (cattle: 49.8%; sheep: 45.9%) and woody plants (cattle: 16.2%; sheep: 12.4%) and a lower content of forbs (Cattle: 34.1%; sheep: 41.6%).

Table 5.4.2. Dietary composition of Pyrenean chamois and seasonal cattle, horses, and sheep in the Pyrenees. Data from June to October 2011 and 2012 in the Freser-Setcases National Game Reserve (eastern Spanish Pyrenees). Values represent mean percentages of fragment frequency (min-max).

	Chamois	**Cattle**	**Horses**	**Sheep**
Woody plants				
Calluna vulgaris	24.6 (0.0-6.0)	10.9 (1.0-22.5)	2.2 (0.0-12.0)	2.1 (0.0-29.5)
Cytisus spp.	17.3 (3.5-51.5)	0.1 (0.0-0.5)	0.0 (0.0-0.0)	0.3 (0.0-6.0)
Other woody plants	7.1 (0.0-17.5)	5.2 (2.0-8)	3.7 (0.5-9.5)	10.1 (3.0-17.0)
Total	**48.6**	**16.2**	**5.9**	**12.4**
Graminoids				
Festuca spp.	22.0 (8.5-50.0)	40.4 (26.5-52.0)	52.4 (43.0-63.5)	40.4 (27.5-52.0)
Avenula pratensis	1.8 (0.0-6.5)	3.2 (0.0-6.5)	1.8 (0.0-6.0)	2.1 (0.0-4.5)
Other graminoids	2.1 (0.0-6.5)	6.2 (3.0-11.5)	9.5 (5.0-13.0)	3.4 (2.0-7.0)
Total	**25.8**	**49.8**	**63.7**	**45.9**
Forbs				
Anthyllis vulneraria	1.3 (0.0-5.0)	2.2 (0.0-7.0)	1.5 (0.0-4.5)	4.1 (2.0-6.5)
Plantago monosperma	3.6 (0.0-9.0)	3.5 (2.0-5.0)	3.9 (2.0-8.0)	5.2 (3.0-10.0)
Potentilla spp.	2.5 (0.0-6.0)	3.9 (2.0-6.0)	2.2 (0.5-4.5)	4.7 (1.5-8.0)
Trifolium spp.	11.2 (2.0-17.5)	13.4 (5.5-21.0)	13.8 (7.0-20.0)	14.2 (6.0-21.5)
Other forbs	6.7 (2.5-10.5)	10.9 (4.0-17.0)	9.1 (5.0-19.0)	13.5 (8.5-20.0)
Total	**25.3**	**34.1**	**30.4**	**41.6**

Simulation of woody plant expansion and LUE

Changes in LUE through the hypothetical scenarios of woody plant expansion suggested that horses would be most affected by the disappearance of grasslands in the Pyrenees. Pyrenean chamois, on the other hand, could even benefit during the early stages of expansion (Fig. 5.4.3). Current LUE is lower for chamois (median=0.72, min=0.57, max=0.75) than livestock (cattle: median=0.76, min=0.70, max=0.79; horses: median=0.74, min= 0.71, max=0.79; sheep: median=0.73, min=0.69, max=0.77; significantly different only between cattle and chamois, w=73, *p*=0.0028). Our LM revealed that 76.8% of the observed LUE variability was explained by the interaction between woody plant expansion and ungulate species ($F_{3,316} = 149.2$, $p<0.001$).

The LUE of all the herbivores studied decreased to some degree from the initial to the final scenario (Fig. 4.3.3). However, this decrease was sharper in livestock species. In fact, the LUE of chamois remained practically stable until woody plant cover reached 90%. It even increased slightly in scenarios of initial shrubification and then began to decrease moderately from the fourth hypothetical scenario (60% woody plant abundance) onwards, acquiring at the same time the highest values relative to the other species. Livestock, however, lost their foraging resilience at very early degrees of shrubification (Fig. 5.4.3). The LUE of cattle ($\beta = -0.005$, SE = 0.0005, $p < 0.001$), sheep ($\beta = -0.004$, SE = 0.0005, $p < 0.001$) and horses ($\beta = -0.005$, SE = 0.0005, $p < 0.001$), steadily decreased from the first scenario of shrubification. The LUE of sheep and horses were significantly smaller than chamois from the third hypothetical scenario onwards (50% woody plant cover; w= 74, $p<0.005$ and w==75, $p<0.005$, respectively) and cattle differed from the fourth scenario onwards (60% woody plant cover; w=14, $p<0.05$).

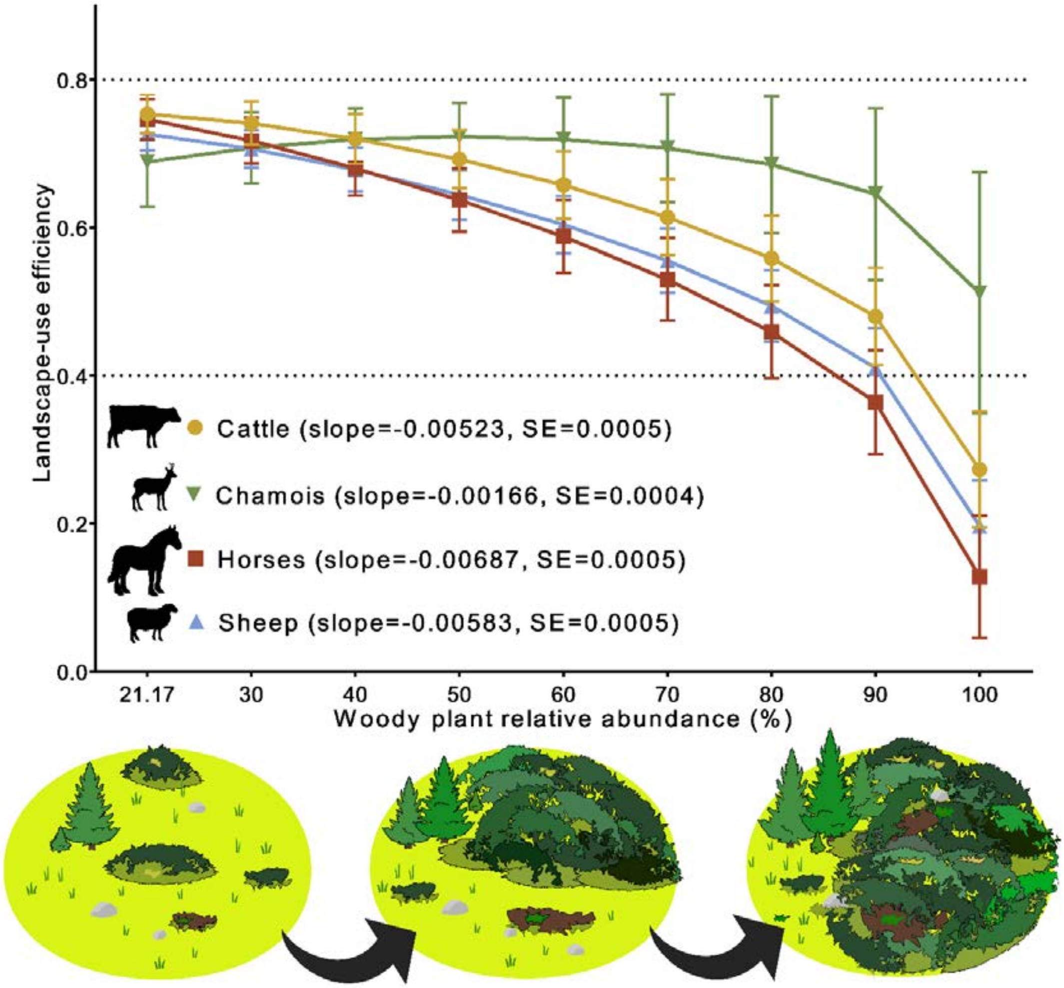


Figure 5.4.3. Evolution of the landscape-use efficiencies (LUE) of Pyrenean chamois and seasonal livestock along different scenarios of woody plant expansion. Slopes of the linear regression are also reported for each species.

The lowest LUE values were detected in our final scenario of shrubification, where woody plants occupied the entire area, notably decreasing livestock LUE (cattle: median=0.29, min=0.14, max=0.41; sheep: median=0.17, min=0.06, max=0.29; horses: median=0.09, min=0.14, max=0.29). LUE was significantly higher for chamois (median=0.56, min=0.21, max=0.70) than cattle (w=13, *p*=0.014), sheep (w=76, *p*<0.0001), and horses (w=79, *p*<0.001) due to its moderate decrease in niche breadth along the scenarios. Livestock LUEs differed significantly only between cattle and horses (w=71, *p*=0.006).

Discussion

Our results suggest that woody plant expansion in an unmanaged environment will affect herbivores in alpine grasslands during summer and autumn but that the magnitudes and direction of these effects will vary between species. Animals with a preference for herbaceous plants will have difficulties to follow the same diet and they will need to acclimatise to a higher consumption of woody plants in order to remain in these areas. At the same time, competition for the most consumed plants, such as *Festuca* spp. or *Trifolium* spp., would lead to overgrazing, one of the main causes of rangeland degradation worldwide (Hilker *et al.*, 2014; Du Toit *et al.*, 2010).

Changes in woody plant cover importantly restructure the wild herbivore assemblage as grazer densities decrease when woody cover increases (Smit & Prins, 2015). The increasing woody plant expansion in alpine environments will render grazers less efficient users of their landscape, as our predictions suggested, therefore decreasing their density in response to food availability. Livestock are highly dependent on the availability of montane grasslands, and the number of livestock grazers will have to decrease to prevent a reduction in productivity. In fact, increases in woody plant cover are already having repercussions on livestock production and reproduction (Anadon *et al.*, 2014). The need to maintain sustainable levels of production will force farmers to move livestock to more suitable areas. Habitat diversity will consequently decline even faster in alpine areas, because plant species richness is maintained by grazing in these human-created herbaceous communities (Bakker, 1998; Boulanger *et al.*, 2018).

Horses feed mostly on graminoids and, to a lesser extent, on forbs and thus would be more quickly and broadly affected by woody plant expansion. This strong dependence on herbaceous plants has been widely described in feral and free-ranging horses (Celaya *et al.*, 2011; Olsen & Hansen, 1977; Patrick, 1992; Salter & Hudson, 1979). The consumption of grasses (50% of total consumption) can be lower in some extreme environments, such as the Chihuahuan Desert (Hansen, 1976), but animal growth is usually restricted when high-quality pastures are not readily available (Andreyev, 1971; Celaya *et al.*, 2011; Dawson *et al.*, 1945). The production of horse meat in the Pyrenees, as in other parts of Europe, is exclusively free-range. Animals make use of grasslands at different altitudes according to the season and, as a consequence, depend highly on montane pastures to subsist (Martin-Rosset & Trillaud-Geyl, 2015). Woody plant expansion is therefore a real threat to horse meat production.

Our results support those of several studies that found that free-ranging cattle generally consume higher proportions of forbs and woody plants than horses (Celaya *et al.*, 2011; Krysl *et al.*, 1984; Menard *et al.*, 2002; Scasta *et al.*, 2016). Woody plant expansion would thus affect cattle less than horses. Diets can be more variable and contain more woody species in free-ranging cattle than horses, although diets of cattle can also be high in graminoids and forbs (Aldezabal *et al.*, 2002; Scasta *et al.*, 2016). The high content of plant secondary metabolites in shrubs, such as tannins, can affect intake, digestion, and metabolism in herbivores and can be toxic if consumed in large amounts (Burrit & Provenza, 2000; Hanley *et al.*, 1992). Cattle can consume a relatively high proportion of woody plants in specific habitats and conditions, but this rusticity and adaptability are only observed in some local breeds (Bartolomé *et al.*, 2011; Guevara, 1996). However, local breeds have been gradually abandoned in recent decades for the benefit of highly productive commercial breeds (Taberlet *et al.*, 2008) and consequently, many locally adapted breeds have already become extinct (Scherf, 2000). At the same time, the use of these breeds is impaired by important inbreeding situations and small effective population sizes (Taberlet *et al.*, 2008), highlighting future challenges of livestock farming in areas were local adaptations will be needed.

Medium-sized herbivores, such as sheep, tend to have a proportionally higher maintenance cost per body weight unit (Kleiber, 1961). They therefore need to forage on plants higher in nutritional value compared to larger herbivores, such as cattle or horses. Sheep can select preferred components in fine-scale mixtures due to their smaller size, which determines gape size, and can therefore feed on the more nutritional parts of plants (Gordon & Illius, 1988). Studies on the composition of diets have reported higher contents of forbs and woody plants by sheep than by large herbivores (Karmiris & Nastis, 2010; La Morgia & Bassano, 2009). Still, the consumption of graminoids and forbs by sheep and cattle in our study was similar, probably due to the high availability of these resources in our study area, generating a strong overlap in the use of resources.

The societal demand for livestock products is increasing the development of research programmes focusing on animal behaviour and genetics for developing animals able to use shrubs more efficiently (Estell *et al.*, 2012). The productivity of these breeds, however, is currently relatively low (Verrier *et al.*, 2005), and animals in mountainous areas have adaptations and functional traits of interest for the montane farming system but a lower production of muscle or milk than commercial breeds (Verrier *et al.*, 2005). The use of shrub-dominated areas for meat or milk production does not presently meet animal requirements (Casasús *et al.*, 2005). These practices appear unsustainable due to the necessity of management intervention (e.g. thinning and spraying) and intensive supplementary feeding (Brosh *et al.*, 2006; Gutman *et al.*, 2000). As a consequence, livestock farming in areas suffering from woody plant expansion will be at high economic risk due to the impossibility of maintaining sustained economic incomes.

Livestock farming in mountainous areas is important to local economies and cultural heritages and is often essential for the livelihood of rural populations worldwide (Mann, 2013). In fact, 32% of Kenyans inhabiting mountains mainly depend on livestock farming to subsist and in Nepalese mountains, where 59% of the population lives below the poverty line, livestock contribute to 21.2% of total household incomes (Abington, 1992; Golicha *et al.*, 2012). Besides a direct nutritional income through meat or milk, livestock also play a vital role in supporting farming systems providing wool, manure, working traction, transportation, cash income and risk diversification (Sherman, 2005). In rural areas where subsistence agriculture is prevalent, the loss of pasture land would have a dramatic socio-economic impact, regardless of the causes of the local shrubification.

We found that Pyrenean chamois during summer and autumn would be favoured by a moderate to high expansion of woody plants due to their ability to balance their feeding behaviour between grazing and browsing. In fact, chamois can have extremely diverse dietary preferences depending on the habitat and season (Herrero *et al.*, 1996; La Morgia & Bassano, 2009) and can even be exclusively dependent on woody species (Yockney & Hickling, 2000). This high phenotypical plasticity is due to their capacity to alternate between ruminal and hindgut fermentation depending on forage quantity and quality (Hofmann, 1989). Because chamois evolved during thousands of years in unmanaged environments (Masini & Lovari, 1988) it is not surprising that the reversion to unmanaged conditions could favour them. The chamois diet in our study area differed from the diets of all the livestock species and showed an evenly distributed consumption of herbaceous and woody plants, despite a lower availability of the latter. This behaviour will allow the chamois to have a higher theoretical LUE than the other herbivores in scenarios of future woody plant expansion. Furthermore, mixed feeders may even be able to slow shrub expansion (Olofsson *et al.*, 2009; Schulze *et al.*, 2018), highlighting the importance of the conservation of these herbivores to maintain open habitats. Our results suggest that the LUE of chamois would be impaired in scenarios of extreme shrubification, despite their adaptation to a high consumption of woody plants. However, the phenotypic capacity of chamois could allow them to consume more woody plants than observed in our study area but further studies would be necessary to assess the effect of these dietary adaptations on the performance of this species.

Our data also suggest that extensive land management and human intervention (e.g. manual shrub clearance), will be necessary for maintaining semi-natural grasslands and free-range farming systems. Keeping in mind that half of the European network of Natura 2000 sites are associated with farming, agricultural land abandonment may have important impacts on landscape and biodiversity in Europe (MacDonald *et al.*, 2000). Moreover, in a kind of vicious circle, the capital investment needed and the time and effort of the demanding work to reverse woody plant expansion on agricultural land is leading to an intensification of the abandonment of farmland and rural areas, accelerating shrubification (MacDonald *et al.*, 2000). Being a driver of woody plant expansion, temperature increase in mountainous environments will also impair biodiversity and efforts to limit global warming will be capital for the future of these environments (Steffen *et al.*, 2018).

Finally, the consequences of our results are not only representative of a Pyrenean scenario or a European montane ecosystem. Considering that shrubification is a global issue affecting other habitats and ecosystems worldwide, from the African savannah to the arctic tundra (Naito & Cairns, 2011; Tape *et al.*, 2006), lessons learned from this paradigmatic case example can be extrapolated to a global scale.

Acknowledgements

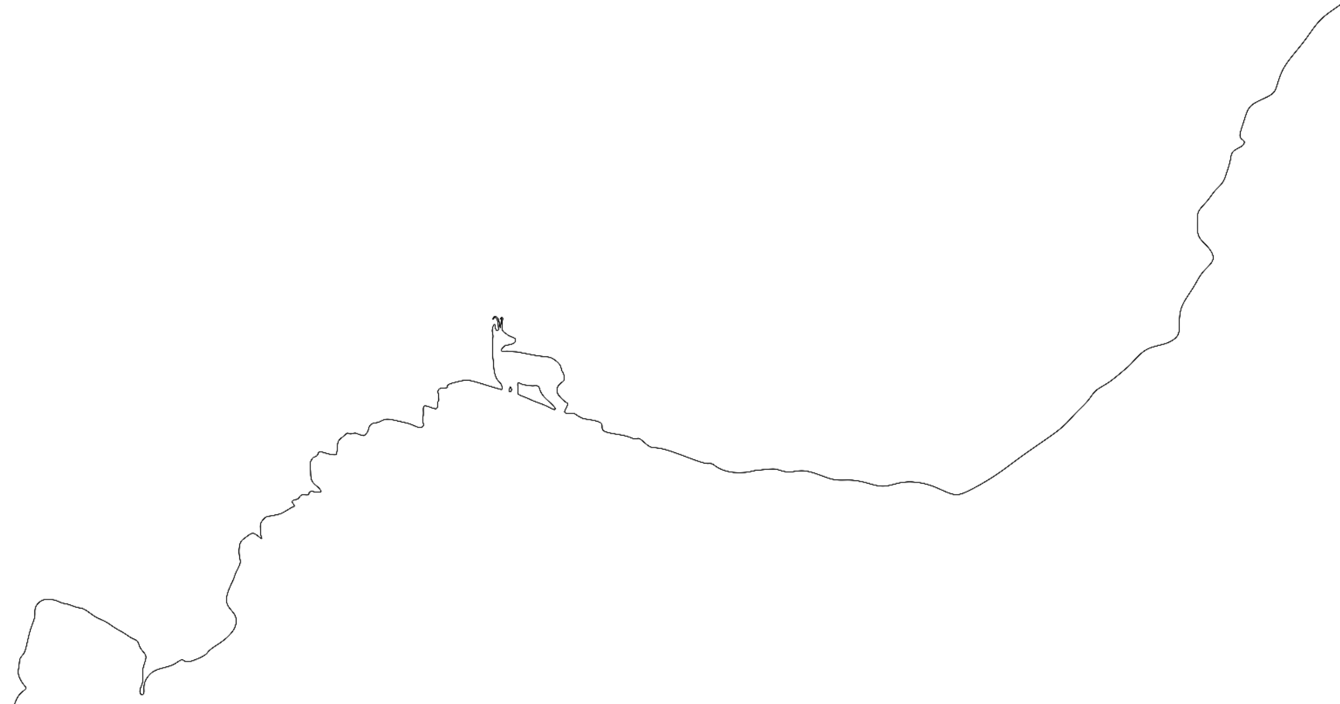
We would like to thank the National Game Reserve of Freser-Setcases personnel for their authorization to carry out this study. We also thank Marco Apollonio and an anonymous reviewer for valuable feedback on the early draft of this manuscript.

Supporting Information

Table S5.4.1. Change in the relative vegetation availability (in percentage) over the different scenarios of woody plant expansion. The initial state represents the actual vegetation availability in our study area. Based on the initial state, we designed eight possible scenarios where woody plants increased on a ten-percentage-point basis (except the first scenario which was increased by 8.6 percentage points to achieve 30% relative abundance of woody plants). This increase was passed on every plant species included in this category proportionally to their relative abundance. At the same time, the total relative abundance of forbs and graminoids was decreased on an exact percentage point basis and this decrease was also distributed proportionally between the species of these categories.

	Initial state	Designed scenarios							
		S1	**S2**	**S3**	**S4**	**S5**	**S6**	**S7**	**S8**
Woody plants									
Calluna vulgaris	7.92	11.09	14.79	18.48	22.18	25.88	29.57	33.27	36.97
Juniperus communis	4.62	6.47	8.63	10.78	12.94	15.09	17.25	19.41	21.56
Cytisus spp.	4.31	6.04	8.06	10.07	12.09	14.10	16.11	18.13	20.14
Pinus uncinata	1.73	2.42	3.22	4.03	4.83	5.64	6.45	7.25	8.06
Other woody plants	2.84	3.98	5.31	6.64	7.96	9.29	10.62	11.94	13.27
Total	21.4	30.00	40.00	50.00	60.00	70.00	80.00	90.00	100.00
Herbs									
Festuca spp.	32.28	28.83	24.72	20.60	16.48	12.36	8.24	4.12	0.00
Carex cariophyllea	12.44	11.11	9.52	7.93	6.35	4.76	3.17	1.59	0.00
Trifolium alpinum	7.51	6.71	5.75	4.79	3.83	2.88	1.92	0.96	0.00
Nardus stricta	2.49	2.22	1.90	1.59	1.27	0.95	0.63	0.32	0.00
Plantago monosperma	2.13	1.90	1.63	1.36	1.09	0.82	0.54	0.27	0.00
Avenula pratensis	1.88	1.68	1.44	1.20	0.96	0.72	0.48	0.24	0.00
Other herbs	19.64	17.55	15.04	12.53	10.03	7.52	5.01	2.51	0.00
Total	78.38	70.00	60.00	50.00	40.00	30.00	20.00	10.00	0.00

6. General Discussion



In this thesis, we have emphasised the importance of feeding ecology in understanding the impact of global change on alpine ecosystems, and have improved the methodology to assess diet composition of ungulates. In this chapter, we discuss these results in a more integrative way.

Comparing the accuracy of methods to assess food composition of ungulates

A systematic review of the most common techniques to assess the diet composition of wild herbivores (**Annex**) revealed the need to optimise current methods to improve their reliability and feasibility. The PCR-CE methodology described in **study 1** is simple and affordable, but strongly conditioned by the freshness of samples. The collection of fresh samples is not a drawback in our study system but can be a limitation when studying rare species. However, and above all, the main shortcoming of this technique is that it only provides information about the occurrence of the consumed plants (*i.e.* qualitative assessment), hardly transposable to a reliable quantitative data (see **Annex**). FCMA, on the other hand, provides quantitative information; however this methodology is also not problem-free due to its tendency to over-detect woody species compared to herbs. Further experimental research will be more than welcome to evaluate the potential improvement of FCMA. In any case, we chose to assume the drawbacks of this technique due to our requirement for a quantitative measure to describe and understand the fine scale variations of diet selection in our target species.

Seasonal diet composition of Pyrenean chamois

Because it is important to understand the natural history of species before inferring their response to global change, we previously described the monthly changes in diet composition of Pyrenean chamois. This type of work is rare, particularly in mountain ungulates, due to limited access to faecal samples during winter (but see García-gonzález & Cuartas, 1996 who described the annual diet of Pyrenean chamois on a bi-monthly basis).

As seen in **studies 2** and **3**, there is a strong relationship between plant consumption and plant phenology in alpine areas. That fact reinforces the key role of vegetation green-up in the performance of alpine herbivores as previously pointed out by Herfindal *et al.*

(2006) and Mysterud *et al.*, (2001). In fact, the increasing consumption of high-quality forage (such as forbs) when animals recover their body reserves after winter (Parker *et al.*, 2009) and when females bear the cost of final gestation and subsequent lactation (Richard *et al.*, 2017) appear to be crucial for ungulates living in such extreme environments (Leader-Williams & Ricketts, 1982). Thus, variations in forage quality and availability, through changes in environmental conditions during that key period will play an important driving role in population performances through multiplier effects (White, 1983)(White 1983; Cook et al. 2004). Indeed, the weight gain during summer impacts the posterior conception rates and the survival of ungulates, which, in turn, determine other demographic aspects (Clutton-Brock *et al.*, 1989; White, 1983).

By being well adapted to the dynamics of their environment, Pyrenean chamois are revealed as an excellent model to study the effects of global change in alpine habitats. This opens the question of whether feeding behaviour of chamois will be resilient to the expected changes in the timing of vegetation onset caused by global warming. At the same time, the importance of forbs during a capital period, such as spring, also call attention to the possible impact of shrub expansion in alpine meadows.

Global change and Pyrenean chamois

Global change is affecting biological and ecological systems across the globe and Pyrenean chamois are no exception. As seen in **study 3,** earlier vegetation onsets are leading chamois to earlier access to high-quality forage after winter scarcity. The analyses in **study 4** suggest that Pyrenean chamois during summer and autumn should be favoured by a moderate to high expansion of woody vegetation. Overall, our results suggest that global changes have no negative impacts on Pyrenean chamois and that this species may even be favoured by environmental changes. However, these results must be interpreted with caution and placed into a more global context. During the green-up period, alpine ungulates recover their body reserves from winter and prepare for a new year (Parker *et al.*, 2009). Females also bear the cost of late gestation and early lactation, and kids experience intense body growth (Clutton-Brock *et al.*, 1989; Parker *et al.*, 2009). Therefore, modifications in the synchrony between the phenology of alpine vegetation and the seasonality of life-history traits may affect not only body growth and reproductive success but also the performances and dynamics of alpine herbivore populations.

Douhard *et al.* (2018), for example, observed how warm springs reduced the mass gain of bighorn sheep at all ages. They consider that the duration of access to high-quality forage is shortened due to a rapid and homogeneous snowmelt over the landscape that reduces the spatial heterogeneity in the timing of green-up. A similar trend in the negative influence of early springs on lamb growth was detected in other species by Pettorelli *et al.* (2007). However, we may expect a positive influence on body weight gain and the performance of alpine herbivores if this earlier onset of vegetation is related to prolonged growing periods as observed by Stöckli & Vidale (2004).

In ungulates living in highly seasonal environments, births are synchronised with the period of higher forage availability and quality. Some species (Moyes *et al.*, 2011), including Pyrenean chamois (Kourkgy *et al.*, 2016), show a relative phenotypic plasticity in gestation length and conception date in response to environmental conditions. The onset of autumn shapes the timing of oestrus while the onset of spring shapes the gestation length and therefore parturition date. However, at least in Pyrenean chamois, the effect of the onset of autumn has a markedly more important effect on birth timing than the onset of spring (Kourkgy *et al.*, 2016). Females (but also males) may be less constrained in adjusting the conception date than the gestation length (Clements *et al.*, 2011) and therefore may be unable to advance parturition as fast as the plant growing season is advancing. As a capital breeder, Pyrenean chamois may be able to use its maternal capital to meet the high cost of early lactation if parturition takes place later than the peak availability of high quality resources. However, late births may mean less time to grow before the period of plant scenecence. Indeed, late-born bighorn sheep lambs—considered capital breeders (Festa-Bianchet *et al.*, 1998)—are smaller than early-born lambs at the onset of winter, potentially lowering winter survival (Feder *et al.*, 2008; Festa-Bianchet, 1988). That being said, and knowing that the timing of birth also has important consequences on the survival of the mother (Clutton-Brock *et al.*, 1983), we can assume that counterproductive effects in species performances will occur if the species is unable to adjust its parturition time to an earlier spring over the long term (Post & Forchhammer, 2008).

According to **study 3**, the consumption of woody plants is related to a lower nitrogen intake than forb consumption. Nitrogen is the most limiting nutrient for wild herbivores. It is required to produce proteins and amino acids and is therefore essential for the body's metabolic processes and the structure and growth of animals (White, 1993). When facing a long period of quality-forage shortage, animals may not obtain sufficient energy and nitrogen to satisfy their metabolic rate and will enter a negative energy balance despite consuming adequate quantities of available low-quality forage. During these periods, animals use endogenous fat and protein built up during the previous green-up period to meet their energy requirements and sustain foetal development (Parker *et al.*, 1999; Torbit *et al.*, 1985). While buds from deciduous shrubs can have higher crude protein than dormant forbs, their levels are still generally lower than growing forbs (Holechek, 1984). Therefore, the results of **study 4** may also mask counterproductive effects. While we show that chamois could thrive in scenarios of future woody plant expansion, this effect only relates to their dietary composition during summer and autumn. Indeed, the low nutritive quality of these shrubland may restrict the replenishment of body reserves during spring and therefore affect the reproduction and survival of Pyrenean chamois (Morano *et al.*, 2013).

In fact, this thesis examined the link between environmental conditions and foraging behaviour of a mountainous species. However, future research should also focus on the link between these dietary variations and the life-history traits of the same populations (*i.e.* fat recovery, reproductive success or survival). An introduced population of Alpine chamois in New Zealand has been shown to consume almost only woody plants (Yockney & Hickling, 2000) but there is no information on their life-history traits or performances. In our case, in showing that Pyrenean chamois in Orlu have earlier access to high-quality forage, we should further explore if, for example, the body weight in autumn was positively related to this tendency (Garel *et al.*, 2011).

Competition for resources

Because diet selection is directly linked to forage availability, it is important to take into account the possibility that competition issues between herbivores may bias studies on feeding behaviour. In **study 2**, we took advantage of an opportunity to compare two populations of Pyrenean chamois to assess the impact of livestock on their feeding behaviour. We focussed on the impact of sheep because they have similar morphologies to chamois and it has been shown that sheep are able to modify the feeding habits and spatially segregate chamois groups (La Morgia & Bassano, 2009; Rüttimann *et al.*, 2008).

However, we could not detect a specific effect of sheep flocks on the diet of chamois. Furthermore, we suggest the possibility that livestock in general (cattle and horses were present in both areas) may affect the diet of chamois during the co-habitation period. We based our hypothesis on the fact that chamois in both areas markedly reduced their consumption of graminoids in concurrence with the arrival of relatively large flocks of livestock. In fact, and contrary to our assumptions, results from **study 4** indicated that cattle had a slightly higher similarity in dietary composition to chamois than sheep in our area. Cattle consumed lower quantities of forbs and higher quantities of woody plants than sheep, approaching the diet of chamois. In any case, the tree species of livestock consumed high quantities of graminoids, providing support to our hypothesis that high densities of livestock in the meadows may deplete the availability of this resource.

A similar conclusion can be drawn regarding the consumption of woody plants. In **study 2,** we suggest that chamois still consume a significant proportion of woody plants during the growing season. While we can speculate on the potential nutritional benefits of consuming certain species, such as *Calluna vulgaris* or *Cytisus* spp, the results from s**tudy 3** indicate that the consumption of woody plants is density-related. This outcome also supports the hypothesis that increased inter-specific interactions affect the feeding behaviour of Pyrenean chamois in the Freser-Setcases National Game Reserve.

As well as taking into account the degree of interaction between different species, studies should also account for the local density of the focal species. Because individuals from the same population are more similar in their ecological requirements, intraspecific competition may be stronger than interspecific competition in regulating biological communities. In fact, body mass and demographic parameters, such as reproduction or survival, are greatly influenced by population density in large herbivores (Bonenfant *et al.*, 2009).

Results from **studies 2**, **3** and **4** have thus reinforced the importance of taking into account the variations in population size of the whole herbivore assemblage as well as their biotic interactions when assessing the effects of global change using long-term studies on animal populations (Montoya *et al.*, 2006). Identifying the pathways via which inter- and intra-specific density-dependence factors affect population dynamics is key to disentangling the interactions and confounding effects of density-dependent and -independent variables.

Management recommendations and future perspectives

Through our studies, we can highlight the importance of forbs in spring for the dietary quality of Pyrenean chamois (**study 3**). In fact, the consumption of these plants is related to the green-up season, when their quality peaks (**study 2**). At the same time, woody plants also play a central role in chamois feeding behaviour throughout the year but especially in winter (**study 2**). Therefore, we suggest that habitat management programmes for Pyrenean chamois should focus on the preservation of mixed grasslands. In view of current land abandonment processes and the speed of woody plant encroachment, urgent human intervention will be necessary to maintain the semi-natural grasslands we currently know.

Our results also suggest that faecal nitrogen can be used as an index of forage quality to monitor long-term changes in environmental conditions through changes in the nutritional condition of a given population (Blanchard *et al.*, 2003; Gálvez-Cerón *et al.*, 2013). Therefore, the long-term faecal sampling of a population could provide useful information on density-dependent and independent changes affecting the population. Because the age and sex of the animals had a low or null effect on dietary quality, we recommend the on-field collection of fresh unidentified droppings. Managers, by seasonally collecting samples and analysing them by NIRS, could use dietary quality as a practical and inexpensive option to monitor population condition as a complement to other indicators of ecological change (Morellet *et al.*, 2007; Santos *et al.*, 2014).

We further strengthen the fundamental need of long-term monitoring programs to understand the ecological processes affecting herbivore populations (**study 3**). The majority of current and past long-term studies of ungulates have focussed on assessing the consequences of climate change at the individual and population levels (Festa-Bianchet *et al.*, 2017). However, understanding the pathways by which global change is affecting species performance is fundamental. Therefore, we suggest that investigators running or initiating long-term studies also focus on the nutritional pathways by which global change is affecting their population. At the same time, by assessing the link between nutrition and performance, we can achieve a better understanding of the effects of early springs or shrubification on the life history traits of large herbivores.

Finally, we could not sufficiently assess the wildlife-livestock compatibility issue in this thesis. We highlight the possibility of resource or space competition between herbivores in our study area (**studies 2** and **4**) but we were unable to confirm these hypotheses. We recommend future research on herbivore-plant interactions on populations with no livestock contact to gain an unbiased overview of the feeding behaviour of the focal herbivore. At the same time, it is crucial to take into account resource availability through total availability but also through intra-specific interactions. As we showed in **study 3**, animal densities are important drivers of diet composition and quality.

7. Conclusions

1. PCR-CE combining two complementary genes is a suitable method that can replace complex massive-sequencing technologies in routine studies of diet composition. However, semi-quantitative determinations can only be obtained with cuticle microhistological analysis.

2. Pyrenean chamois are well adapted to the variations in the seasonal phenology of plants in alpine habitats but could be disturbed by the presence of livestock during summer.

3. Earlier onsets of spring are leading to earlier access to high-quality forage and therefore a higher diet quality at a fixed date of sampling, without affecting the dietary composition of Pyrenean chamois.

4. 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.

5. Mixed feeders, such as Pyrenean chamois, are less disturbed by fallow landscapes, due to a higher trophic plasticity. However, mountain livestock, which rely primarily on herbaceous plants, will be greatly affected by the current shrubification.

8. Annex: Assessing food habits

The initial evaluations of food habits of herbivores were of a qualitative nature where diet preference was just described, providing information on the kind of food consumed. To understand the complex relationships between herbivores and plants, research has afterwards focussed on a quantitative assessment of the diet composition. When quantitatively studying the diet of herbivores, a range of procedures have been developed, from the direct field-evaluation of eaten plants to the complex DNA metabarcoding assessment. All of them present shortcomings but also different advantages. The conclusions of dietary studies are affected by the method used and the material analysed. The appropriate methodology depends on several factors discussed hereunder. However, be warned that currently there is no method that can quantify exactly the intake of a wild herbivore.

Forage utilization

Utilization techniques are one of the oldest approaches to evaluate the diet of herbivores (Beruldsen & Morgan, 1934). It relies on the identification of herbivory impact directly on plants and is usually expressed as the percentage of forage removed from a specific plant (Smith *et al.*, 1962). There are various utilization techniques such as the measurement and comparison of forage availability in a specific plot before and after the consumption by herbivores. Another technique implies the comparison between fenced (or caged) and grazed plots after the presence of an herbivore. The temporary or spatial difference between plots or periods represent the amount and type of forage consumed by animals (Heady, 1949; Martin, 1970).

While this technique can provide indications of species preferences it is not commonly used nowadays, especially in wildlife species (but see, for example, Muya and Oguge 2000). One of its several drawback is the extensive labour of clipping, weighing and estimating the biomass of each plant species but also the difficulty in assigning a specific bite to a specific herbivores. Indeed, this technique is considered unsuitable when more than one herbivore is in the area, such as in the majority of terrestrial ecosystems. Furthermore, when plants are actively growing, species–specific differences in regrowth after defoliation can confound the estimations of utilization (Holechek *et al.*, 1982).

Direct observation

The direct observation of the animal behaviour in the field is also a widely used technique to determine diet composition. Using binoculars or spotting scopes, an observer simply observe and time the animal as it bites off each plant. The length of time spent (feeding-minute approach) or the number of bites realized (bite-counts approach) when grazing each plant species are the usual noted information (Bjugstad *et al.*, 1970).

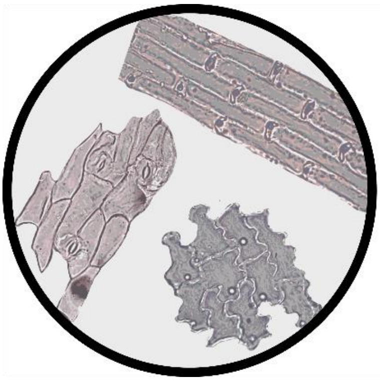
This method is simple and requires a small material investment. It can determine the species and plant parts that are consumed, the seasonal and spatial foraging preferences and also the feeding behaviour (Bjugstad *et al.*, 1970). However, the accuracy of this procedure strongly depends on the degree of training of the observer to identify plant species from the distance. At the same time, bite size is plant-species dependent as herbivores apprehend different forage types differently (Wilson & Kerley, 2003), hindering the quantification of how much of a plant is consumed. It is also a time consuming method because only one animal can be observed at a time and unfavourable weather can lengthen the field work. But above all, this method is hampered in wild herbivores due to the difficulty to locate and approach such species (Holechek *et al.*, 1982) and their tendencies to have a marked crepuscular feeding behaviour, difficult to observe (Forester *et al.*, 2007). Concurrently, the accuracy of the observer can be limited in short grass pastures (difficulty to distinguish between plant species) or high shrub and forested areas (difficulty to observe the animal). Some of these difficulties are overcome using tame animals (e.g. Tixier *et al.*, 2009) but their potential artificial food use due to previous foraging experience or lack of hunger has been criticized (Litvaitis, 2000). Recent developments of new technologies is allowing to partly overcome these limitations. For example, the recent use of high resolution video cameras attached to radio collar in caribous increased dietary information on a species hardly observable in the wild, due to low population densities and avoidance of open areas (Thompson *et al.*, 2015).

Rumen or stomach content analysis

This approach can be done by two different methodologies. The first involves the cannulation of the animal and the posterior hand-collection of a sample of the forage eaten directly from the rumen or the stomach through the fistula (Rice, 1970). Other researcher have also used oesophageal-fistula cannulas and a collecting bag attached to the neck (Cook *et al.*, 1958). These methods have been widely used in domestic herbivores but rarely applied to wild species due to obvious handling difficulties (Although tame specimens have sporadically been used: Gaare & Skogland, 1975).

In wildlife studies however, the alternative is to examine stomach or rumen content obtained on dead animals, therefore needing the culling of the specimen. Samples are later washed, air-dried, manually separated and visually analysed under a microscope. Observed leaf fragments are then assigned to a plant species by comparing with botanical collections (Lesperance *et al.*, 1960). This method is commonly used by wildlife researchers (Marchand *et al.*, 2013; Redjadj *et al.*, 2014; Seto *et al.*, 2015) as it provides a good estimation of resources consumed. Some difficulties may be encountered to identify partially digested or too finely masticated food items (Anderson *et al.*, 1965; Cook *et al.*, 1958). However, the method is strongly limited by its restriction to legal hunting periods and game species as the culling of animals only for studying diet composition may not be possible. As a consequence, it is rarely used in the long term monitoring of a population through seasons and years (but see Seto et al., 2015) and impossible with endangered species.

Faecal cuticle microhistological analysis



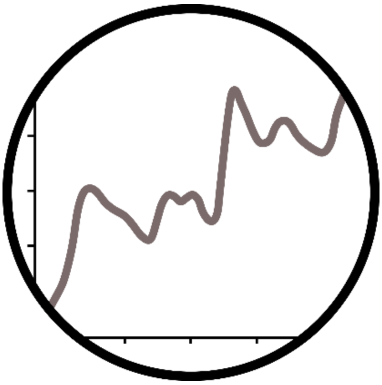
The microhistological examination of faecal samples is a popular method in dietary studies based on the microscopic recognition of cellular structures on plant epidermal fragments persisting in the faeces (Stewart, 1967). The original method was proposed by Baumgartner and Martin (1939) and further improved by various authors (Alipayo *et al.*, 1992; Holechek, 1982). It has been widely used by researchers because it presents numerous advantages over other techniques. Faecal pellets can be easily obtained without any special equipment and without disrupting the behaviour of animals or culling them. At the same time it is not hampered by the topography or the vegetation cover, and can be used in endangered species and for long-term studies.

However, even if large sample sizes can be collected easily, the afterward laboratory work is extremely labour-intensive. Samples must be clean-washed with HNO3 or NaOH, optionally bleached, filtered and then spread out on a variable number of slides (usually 3-5). Each slide is examined under a microscope by identifying and counting fragments or by assessing their cover allow systematic traverses. Once all fragments are identified in each traverse, the results are pooled and converted to percentages (Bartolome *et al.*, 1995; Holechek, 1982; Scotcher, 1979). At the same time, it is crucial to prepare a detailed reference collection of permanent epidermal mounts of the potential food plants for each herbivore species and study area (Ahmed *et al.*, 2015; Carrière, 2002).

Despite this tedious process, microhistological methodology is well established and probably one of the most popular methods to estimate food habits of wild herbivores (Ferretti *et al.*, 2015; Häsler & Senn, 2012; Puig *et al.*, 2011; Wingard *et al.*, 2011).

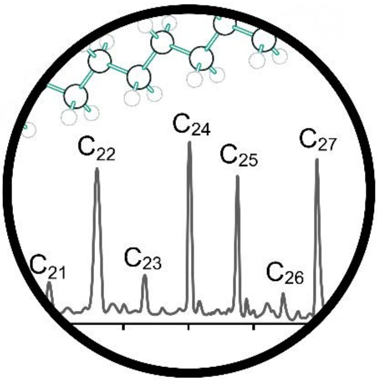
However, several limitations of this technique have been raised such as the important training needed for an accurate cuticle identification (Alipayo *et al.*, 1992) or the difficulty to precisely identify the fragments to the species level in several plant groups (La Morgia & Bassano, 2009; Soininen *et al.*, 2009). Nevertheless, the most argumentative aspect of faecal analysis is that it is based on the assumptions that the frequency of occurrence of identified fragments in faeces is proportional to the ingestion rates. This variation in the digestibility and survival of epidermal cuticles in the digestive tract may bias the result of preference estimation for some plant species (Holechek *et al.*, 1982). Indeed, grasses and woody species are usually overdetected and easily digested forbs are underdetected to the point that sporadically consumed species could even be undetected (Bartolome *et al.*, 1995; Chapuis *et al.*, 2001; McInnis *et al.*, 1983). In that sense, some researchers have developed correction factors to adjust the contribution of specific plant fragments to the actual biomass consumed (Alipayo *et al.*, 1992; Bartolome *et al.*, 1995). However, to be reliable, correction factors should be herbivore-specific but also plant species-specific (Bartolome *et al.*, 1995). Unfortunately, these factors almost always rely on experimental approaches with domestic herbivores and cannot adequately simulate the highly diversified diets of wild species (but see (Dearden *et al.*, 1975). Despite its limitations, faecal cuticle microhistological analysis is by now, the more reliable method to quantify diet composition of wild herbivores ounce its limitations are understood and taken into account.

Near-infrared spectroscopy



Near-infrared spectroscopy (or simply NIRS) is a relatively new methodology widely used to measure the nutritional composition of forage and feeds for livestock but also the diet quality of wild species using faecal pellets (Showers *et al.*, 2006; Tellado *et al.*, 2015). Summarizing, an infrared spectrum can be obtained by directing a range of near-infrared light onto a sample and determining what fraction is absorbed by the sample. The degree of absorption depends on the occurrence of different chemical bonds (C-H, N-H and O-H) present in the analysed material. Using mathematical algorithms, the spectral features are converted to information regarding the physico-chemical composition of the sample such as its nitrogen content, moisture or fibre content. The mathematical algorithms are obtained through a calibration model specific to each component analysed (Williams & Norris, 2001). Following these premises, in 1979, Shenk *et al.* showed that NIRS have the potential to determine the botanical composition of simple mixed forages and posterior studies have attempted to apply it on more complex pastures (Locher *et al.*, 2005; Pitman *et al.*, 1991) (e.g., Coleman et al. 1990; Pitman et al. 1991; Atkinson et al. 1996), opening the possibility to adapt the methodology to faecal samples (Foley *et al.*, 1998; Walker *et al.*, 2002). Compared to other methodologies such as faecal cuticle microhistology, NIRS requires little sample preparation, no chemicals and allows an extremely rapid analysis of the sample. However, until now, NIRS method had only offered acceptable precision and accuracy when predicting proportions of botanical groups (legumes, grass, etc...). Discriminating between species is hampered by the species-specific degree of detectability by NIRS added to the fact that valid predictions couldn't be made for components with a low frequency of occurrence in diet (Volesky & Coleman, 1996; Walker *et al.*, 2002). At the same time, feeding trials have shown that NIRS predictions are too sensitive to the plant part of each component (Foley *et al.*, 1998). Nowadays, the application of NIRS is not yet suitable to predict the botanical composition of diets of wild species in natural conditions because calibrations are not sufficiently robust. A greater effort will be needed to understand the factors that affect the robustness of the predictive equations in these situations (Foley *et al.*, 1998).

Plant wax components



The surface of plant cuticles are covered with a layer of wax composed by a complex chemical mixture of fatty constituents including n-alkanes, long-chain fatty alcohols and long-chain fatty acids (Dove & Mayes, 1996). The composition of this mixture of marker compounds greatly differs between plant species and, to some extent, between parts of the same plant (Dove *et al.*, 1996). The first studies to use these compounds as faecal markers were carried out by Body and Hansen (1978) using long-chain fatty acids but nowadays the n-alkanes with between 25 and 35 carbon atoms are the compounds most widely assessed.

The plant-wax compounds are extracted from faeces using a process involving solvent extraction and purification to separate the crude extract into particular compound fractions. Finally, the purified extracts are identified by gas chromatography and their concentrations are calculated by a peak-integration software (Dove & Mayes, 2006). Using a matrix of simultaneous equations or algorithms, researchers are able to calculate the diet composition comparing the faecal profile to the known concentrations of known plant and forages. Simultaneously, a correction factor must be case-specifically calculated because faecal recovery of these compounds is incomplete (Dove & Mayes, 2005).

Several studies have demonstrated that wax compounds patterns effectively estimate the diet composition of livestock when animals consume simple dietary mixtures of up to four components. However, when studies want to focus on the diet of wild herbivores, they face numerous constrains due to the complexity of the diets and the need to know the wax-compounds composition of all the ingested plants. Nowadays, studies focus on the search for additional markers or combine the use of plant wax analysis with other methods such as faecal cuticle microhistological analysis (Seri *et al.*, 2018). Decreasing the number of plant components to be discriminated by grouping species according to their taxonomy or type (browse and herbs) is also a possible option (Ferraz de Oliveira *et al.*, 2013). Still, this technique is not widely used in wildlife due to the numerous drawbacks mentioned and the extreme case-specificity of the correction factors (Ferreira *et al.*, 2017).

DNA-barcoding

DNA-barcoding is a novel technique that uses a short marker from a standard part of the genome of an organism's DNA, known as barcodes, to provide an accurate species identifications (Hebert *et al.*, 2003). It is an increasingly used methodology in forensic science, taxonomy or the food industry, for example in cases of food mislabelling (Barcaccia *et al.*, 2015; Valentini *et al.*, 2009b). Alongside, it is now widely used to determine the diet composition of wildlife species from linyphiid spiders to giant squids (Agusti *et al.*, 2003; Deagle *et al.*, 2005). The use of this DNA-based methodology has also been extensively applied in dietary studies of wild herbivores, using their faeces or rumen content (Czernik *et al.*, 2013; Kartzinel *et al.*, 2015; Rayé *et al.*, 2011).

DNA is extracted from the sample and then amplified through polymerase chain reaction (PCR), usually using universal primers capable of binding with the target barcode. The ideal barcode should have highly conserved priming sites and be sufficiently variable between species, but identical between plants from the same species (Taberlet *et al.*, 2007). Different chloroplastic genes have been extensively used as barcodes for plants, such as the matK region, the P6 loop of the trnL (UAA) intron or the rbcL - either alone or combined with nuclear genes, such as Internal Transcribed Spacer regions of ribosomal DNA (Chen *et al.*, 2010; Hollingsworth *et al.*, 2011; Rayé *et al.*, 2011). After that step, two options are available. The most widely used methodology is sequencing the barcodes by next-generation sequencing technology (NGS) and identifying the species by comparing the sequences with a public database (such as GenBank or the European Molecular Biology Laboratory - EMBL) or a database made for this purpose. Even if this option is well adapted to the assessment of diets, NGS is still a relatively expensive technology that could hold back low-funded research.

A "low-cost" alternative option, optimized for diet analysis in this thesis, implies the determination of the amplicon lengths by automated capillary electrophoresis (PCR-CE). The subsequent botanical adscription of each amplicon lengths is realized through matching the obtained data with those available in the public or personal databases. Usually, the resolution of this methodology is considered low for complex samples because plant species with similar fragment sizes cannot be identified (Czernik *et al.*, 2013). However, this limitation can be overcome by combining simultaneous PCR amplification of two complementary barcodes, as proposed in our study 2.

In general, DNA-barcoding is a powerful method to qualitatively assess the diet composition of herbivores but some limitations must be highlighted. As with other methodologies, it is strongly recommended to build a detailed DNA-barcoding reference database of the plants of the study region to limit the ambiguities in sequence assignment and improve the identification to the species level (Valentini *et al.*, 2009a). However, sequences are not always identified and assigned to a species because it is not reported in the study area or indexed in the researcher database (Quéméré *et al.*, 2013). Still, this fact will rapidly be overcome through international initiatives such as the Barcoding of Life initiative (Costa & Carvalho, 2007) which are inventorying the planet's biodiversity. Some researchers have also used the frequencies of sequences in the samples to quantitatively estimate diet compositions but this methodology is debatable because the number of copies is not always proportional to the original number of fragments in the sample (Deagle *et al.*, 2006). Due to variations in the original DNA content and the differential digestibility of plants, DNA-barcoding is limited in its ability to express relative quantities of forage species (Kartzinel *et al.*, 2015). Finally, PCR inhibition, DNA degradation due to issues in sample preservation or even sequencing errors can lead to wrong species identification or the inability to assign DNA sequences. Still, due to its high taxonomic resolution, DNA-barcoding is seen as a powerful tool for herbivores diet studies and is now regularly used in wildlife research (e.g. Kowalczyk *et al.*, 2011; Pompanon *et al.*, 2012; Bison *et al.*, 2015).

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