

**Czech University of Life Sciences Prague**  
**Faculty of Forestry and Wood Sciences**  
**Department of Game Management and Wildlife Biology**



Doctoral Dissertation

**Environmental, morphophysiological, and individual factors  
affecting faecal indices: application of near-infrared reflectance  
spectroscopy to nutritional studies**

Environmentální, morfofyziologické a individuální faktory ovlivňující faecal indices: aplikace  
spektroskopie v blízké infračervené oblasti na nutriční studie

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I hereby confirm that this Ph.D. thesis, “Environmental, morphophysiological, and individual factors affecting faecal indices: application of near-infrared reflectance spectroscopy to nutritional studies”, was elaborated independently with the use of quoted literature and based on consultations and the recommendations of my supervisor and co-supervisors. I agree with publishing this Ph.D. thesis according to Czech law n. 111/1998 Sb. about the universities in its current valid wording. This agreement is independent from the result of the official defence of this thesis.

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## List of published academic papers

Following published academic papers serve as a core to this doctoral dissertation thesis.

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- Čupić, S., Cassinello, J., Kušta, T. and Ceacero, F., **2023**. Differences in Faecal Nutritional Components in Three Species of Saharan Gazelles on Standard Diets in Relation to Species, Age and Sex. *Animals*, 13(21), p.3408.
- Čupić, S., Ježek, M. and Ceacero, F., **2023**. Are they both the same shit? Winter faeces of roe and red deer show no difference in nutritional components. *Journal of Forest Science*, 69, pp.114-123.
- Bernátková, A., Paříková, A., Cisneros, R., Čupić, S. and Ceacero, F., **2021**. Ecological effects on the nutritional value of bromeliads, and its influence on Andean bears' diet selection. *Ursus*, 2021(32e21), pp.1-8.

Following published academic papers do not form part of this doctoral diertation thesis, but mark the author's contribution to the area of ecology.

- Begović, K., Rydval, M., Mikac, S., Čupić, S., Svobodova, K., Mikoláš, M., Kozak, D., Kameniar, O., Frankovič, M., Pavlin, J. and Langbehn, T., **2020**. Climate-growth relationships of Norway Spruce and silver fir in primary forests of the Croatian Dinaric mountains. *Agricultural and Forest Meteorology*, 288, p.108000.
- Ceacero, F., Ny, V., Kotrba, R., Bartoň, L., Čupić, S., Bureš, D., Turek, J., Komárková, M. and Needham, T., **2023**. Combined effects of supplementation of amino acids and immunocastration in first antler growth of farmed fallow deer (*Dama dama*). *Animal Production Science*, 63(16), pp.1583-1593.

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## Abstract

The dissertation addresses the enduring and relevant topic of food quality evaluation in wild animals. Its primary objective is to assess the factors that influence the efficacy of near-infrared reflectance spectroscopy (NIRS) in faecal analyses, focusing on its practical application.

The first part of the thesis research focuses on comprehensive analyses of red deer and gazelles in captivity, with a particular emphasis on the distinct responses of each species to nutrition provided. The initial studies on captive red deer demonstrate that factors such as sex, age, and environmental conditions, such as pasture availability, significantly impact the variability of nutrients in their faeces. These findings emphasise the need for initial controlled experiments to standardise methods for interpreting faecal data from wild populations.

Three closely related species of gazelles were the focus of further investigation, which revealed how variations in faecal nitrogen and fibre levels express differences in digestive efficiency between species. This study emphasises the specifics of digestive processes in different species and highlights the influence of individual characteristics such as age and sex on nutritional intake. It suggests that comparing distinct species requires a detailed and careful approach.

Further use of NIRS in a wild setting entailed comparing faecal samples from roe and red deer in the Bohemian forests. This endeavour aimed to determine the specific dietary patterns of two different specialists by analysing the nutrients found in their faeces. However, the presence of external factors, such as supplementary feeding and predation, impacted the clear identification of species-specific dietary patterns. This emphasises the difficulties faced when conducting field studies in uncontrolled environments.

Lastly, the dissertation offers a succinct summary of the research on Andean bears, utilising NIRS to explore the nutritional makeup of bromeliads in southern Ecuador. This addition demonstrates NIRS's adaptability to various ecological studies and species, including non-ruminant herbivores, thereby expanding the scope of dietary analysis in conservation science.

Together, these studies contribute to our comprehension of herbivore nutrition and ecological interactions, providing valuable insights for managing wildlife and conservation endeavours. This dissertation connects controlled experiments and field applications, laying the groundwork for future environmental research using advanced analytical techniques such as NIRS.



## Abstrakt

Tato disertační práce zkoumá způsoby výběru potravy volně žijících sudokopytníků s využitím spektroskopie blízké infračervené oblasti (NIRS) pro analýzu vzorků trusu. Tato metoda poskytuje cenné informace o nutriční ekologii mnoha druhů v různých prostředích.

První část výzkumu se zaměřuje na komplexní analýzy jelenů a gazel, s důrazem na odlišné reakce každého druhu na poskytovanou výživu. Počáteční studie na jelenech chovaných v zajetí ukázaly, že faktory jako pohlaví, věk a environmentální podmínky, jako je dostupnost pastvy, významně ovlivňují variabilitu živin v jejich trusu. Tato zjištění zdůrazňují potřebu počátečních kontrolovaných experimentů, které by standardizovaly metody interpretace dat o trusu z volně žijících populací.

Další výzkum se soustředil na tři úzce příbuzné druhy gazel, který odhalil, jak rozdíly v úrovních dusíku a vlákniny v trusu vyjadřují rozdíly v účinnosti trávení mezi jednotlivými druhy. Tato studie zdůrazňuje specifika trávicích procesů u různých druhů a poukazuje na vliv individuálních charakteristik, jako jsou věk a pohlaví, na příjem potravy. Naznačuje, že srovnání jednotlivých druhů vyžaduje detailní a opatrný přístup. Další použití NIRS ve volné přírodě zahrnovalo porovnání vzorků trusu srnců a jelenů v českých lesích. Cílem této snahy bylo určit specifické stravovací návyky různých druhů pomocí analýzy živin nalezených v jejich trusu. Avšak přítomnost vnějších faktorů, jako je doplňkové krmení a predace, ovlivnila jasnou identifikaci druhově specifických stravovacích návyků. To zdůrazňuje obtíže, kterým čelí provádění terénních studií v nekontrolovaných prostředích.

Závěrem disertace nabízí stručné shrnutí výzkumu andských medvědů s využitím NIRS pro prozkoumání nutričního složení bromélií v jižním Ekvádoru. Tento dodatek ukazuje přizpůsobivost NIRS různým ekologickým studiím a druhům, včetně nepřežvýkavých býložravců, čímž rozšiřuje rozsah analýz stravy ve vědě o ochraně přírody.

Společně tyto studie významně přispívají k pochopení výživy býložravců a ekologických interakcí, poskytující cenné poznatky pro řízení volně žijících živočichů a pro snahu o ochranu přírody. Tato disertace spojuje kontrolované experimenty a terénní aplikace, čímž klade základy pro budoucí environmentální výzkum s využitím pokročilých analytických technik, jako je NIRS.

# 1. INTRODUCTION

Examining the dietary patterns and feeding behaviours of large herbivorous vertebrates, especially ungulates, is crucial for understanding and tackling ecological and socio-economic challenges that are worsened by the rapid changes in climate. These challenges have a significant effect on the variety of species and the interactions within ecosystems at various levels, ultimately affecting the populations of hooved mammals that play a crucial role in maintaining ecosystem stability (Naiman, 1988; Hodgman et al., 1996; Searle and Shipley, 2008). Nutrition is a crucial factor in determining the health of animals, impacting various aspects such as the growth rate of young individuals, the survival of adults, and their reproductive success (Simard et al., 2008; Monteith et al., 2014; Lamb et al., 2023). Although recognised as crucial, the incorporation of nutritional information into wildlife management and conservation strategies frequently lacks sufficiency (Wisdom et al., 2020; Morgan et al., 2021).

This thesis in its part discuss the intricate interaction of various factors that affect the choice of diet among ungulates. Dietary choice is not only important for their survival and ability to reproduce, but also for the maintenance of population dynamics and ecological balance in forest habitats (Christianson & Creel, 2007; Felton et al., 2017). Food quality, environmental changes, and physiological diversity among species have a direct influence on their dietary selection behaviours and broader ecological consequences.

Ungulates demonstrate advanced food selection behaviours, which indicate their ability to respond to a wide range of chemically diverse resources. The selection of food is influenced by various factors, such as the nutritional quality, chemical defences of plants, and overall food availability. These factors are further complicated by variables like the specific part of the plant, the timing of plant growth and development, and variations within and between different species (Cook et al., 1962; Hanley, 1982; Zweifel-Schielly et al., 2009; Severson et al., 2021).

This research is not solely focused on academic pursuits, but rather it is a crucial undertaking that seeks to improve the effectiveness of management and conservation efforts. This is particularly important due to the substantial socio-economic and ecological impacts associated with the dietary patterns of ungulates, especially in the face of current climate changes (Gordon et al., 2004; Milner et al., 2014; Champagne et al., 2021). The study utilises advanced methodologies, specifically near-infrared reflectance spectroscopy (NIRS) and detailed faecal

analysis, which are considered significant advancements in ecological research. NIRS is a highly advantageous technique for rapidly and inexpensively estimating the composition of plant and animal tissues. Additionally, it facilitates ecological studies by enabling rapid, non-invasive quantitative analyses (Foley et al., 1998; Counsell & Vance, 2016; Vance et al., 2016).

Finally, this dissertation seeks to understand how these external factors and internal physiological processes influence ungulate feeding preferences, as well as their implications for ecosystem health and conservation efforts. By combining NIRS and faecal analysis, the study not only fills a gap in our understanding, but also emphasises the importance of looking at these issues from multiple angles in order to improve conservation efforts and management practices. The findings will shed light on ungulates' complex dietary patterns and their critical role in ecosystems.

## 2. AIMS AND OBJECTIVES

This doctoral dissertation aims to deepen our understanding of animals dietary patterns, both in captive and free-ranging contexts, leveraging near-infrared reflectance spectroscopy (NIRS) and adding knowledge to the research applying faeces as a proxy. It elucidates how environmental, morphophysiological, and individual factors affect faecal nutritional composition and discusses NIRS's effectiveness and limitations in quantifying content of nutritional components in faeces and discuss it further in the context of its suitability to explain complex dietary patterns.

The particular objectives are:

(i) To measure the impact of factors such as pasture availability, season, and individual-specific characteristics, such as sex, age, reproductive status, body mass, and body condition, on the nutrient composition of red deer faeces. This is done in controlled settings to improve the accuracy of ecological assessments of wild populations.

(ii) To measure the influence of sex and age, along with morphophysiological specificities, on the faecal nutrient profiles of three captive gazelle species using NIRS, and to demonstrate the method's precision and applicability in controlled experimental conditions.

(iii) To determine the applicability of faecal near-infrared reflectance spectroscopy (fNIRS) in distinguishing nutritional components in the faeces of free-ranging roe and red deer in the winter season.

iv) To see how well and how flexible NIRS combined with faecal microhistological analysis is at predicting the nutritional makeup of bromeliads and answering the complex ecological question of species nutrition-induced habitat selection patterns. A study focusing on this objective, while significantly deviating from the primary focus on ungulate dietary analysis, provides valuable insights into the broader applications of NIRS in various ecological settings..

### 3. SHORT LITERATURE REVIEW

#### 3.1. Overview of Herbivory and Large Herbivores

Herbivory significantly impacts ecological and evolutionary processes, playing a crucial role in the complex interactions within the biosphere. Richard Owen coined the term "herbivore" in 1854, derived from the Latin words *herba*, which refers to small plants, and *vora*, which means to consume or to eat. This term expands upon the fundamental concepts proposed by Charles Lyell, highlighting the essential function of herbivores in the ecosystem. Herbivory, the act of consuming autotrophs, which are organisms that can produce their food through photosynthesis, is a necessary evolutionary adaptation that originated around 470 million years ago, coinciding with the colonisation of land by plants (Kenrick & Crane, 1997).

Herbivory occurs in many organisms, such as protists, bacteria, and fungi, frequently resulting in plant diseases or harm. Phytophagy refers to the plant-consuming behaviour observed in insects, particularly those in the Coleoptera order. This behaviour reflects their specialised ecological niches, as observed in groups such as the Phytophaga (Strong, 1984). Animals of various sizes, including small rodents and large mammals like moose and elephants, have essential ecological functions. They notably affect the process of nitrogen cycling and contribute to the formation of ecosystem structures (Ritchie & Olff, 1999; Lovegrove & Haines, 2004). Recent research has highlighted that herbivory plays a crucial role in expanding ecological niches and promoting intricate interactions between plants and animals. This process drives evolutionary changes and contributes to the overall diversity of species. The emergence of arthropods in the Carboniferous period, as well as the development of new feeding strategies by tetrapods, took advantage of these ecological connections, which are vital for maintaining environmental equilibrium and deepening our understanding of biodiversity.

Large herbivores are critical elements and primary consumers in terrestrial ecosystems. They facilitate necessary energy transfers that impact the dynamics of plant communities and the overall structure of food webs (Owen-Smith, 1988). They have a role beyond just consuming; as ecosystem engineers, they change habitats in ways that can significantly impact biological diversity and ecosystem function. This involves the generation of physical disruptions by trampling, which can improve soil and water infiltration aeration, and selective grazing, which

can enhance plant diversity by reducing the dominance of competitive plant species (Liu et al., 2015).

Ruminants, found in the Artiodactyla order, possess specialised digestive systems that efficiently break down fibrous plant materials, thus obtaining nutrients inaccessible to other animals (Hofmann, 1989; Flint et al., 2008). Their unique capability emphasises their essential role in the cycling of nutrients, which is crucial for preserving the health and resilience of ecosystem processes (Augustine & McNaughton, 1998). In addition, large herbivores play a vital role in indicating the overall health of an ecosystem. Their population dynamics and habitat preferences reflect their environment's underlying conditions and long-term viability (Beschta & Ripple, 2009).

### **3.1.1. Nutritional needs and ecological impacts**

The nutritional welfare of large herbivores is vital for their physical well-being, reproductive efficacy, and ability to adapt to environmental pressures. Optimal nutrition underpins crucial aspects of their lives, including growth, development, immune function, and resilience against diseases. Parker et al. (1999) and Barboza and Parker (2008) highlight that the availability of nutrients directly impacts energy reserves and survival strategies, effectively linking dietary intake to overall physiological health.

Proteins, as Robbins (2012) emphasised, are particularly crucial during the growth and reproductive stages. Millán et al. (2022) also emphasise the connection between consistent access to high-quality forage and maintaining optimal body condition and reproductive rates. The diversity of the gut microbiome also plays a significant role in determining these animals' health and ecological adaptability, influencing their home range sizes and habitat usage, which in turn impacts their survival capabilities (Wolf et al., 2021). Herbivores require a balanced intake of energy, proteins, minerals, water, and fibre to sustain health and critical physiological functions that affect behaviours necessary for survival and adaptation. For example, Barboza et al. (2024) describe how caribou modify their migration and foraging behaviours in response to seasonal changes in high-energy forage availability, a strategy crucial for accumulating fat reserves for winter survival. Findings from Blum et al. (2023) and Darker (2022), which emphasise the significance of minerals like calcium and phosphorus for bone health and metabolic processes that affect movement and habitat selection, support this point.

Schmied née Stommel (2024) showed that water scarcity significantly impacts territorial behaviours and population densities, highlighting the need for efficient water management in conservation strategies. Moreover, the fibre content of the diet is critical for ruminants, who rely on microbial fermentation to break down cellulose-rich plant material. Dietary changes can substantially alter the gut microbiota, affecting these animals' overall health and well-being, as shown in the study by Fresno Rueda et al. (2023).

Ruminants like deer and elk, with complex multi-chambered stomachs, and non-ruminant herbivores like horses and zebras, which have simpler digestive systems, exhibit varied nutrient absorption efficiencies due to their specialised digestive adaptations (Hofmann, 1989; Duncan & Gordon, 1999). These adaptations necessitate flexible management and conservation strategies that respond to different species' changing environmental conditions, life stages, and ecological demands (Kartzinel & Pringle, 2020; Tyler et al., 2020).

Understanding the interactions between large herbivores and plant communities is critical for effective wildlife and habitat management, especially in the face of climate change and anthropogenic pressures. Conservation strategies should consider the specific needs of herbivore types within their climatic contexts to support natural feeding behaviours and sustain biodiversity (Orr et al., 2022; Gordon et al., 2004).

### **3.1.2 . The overall socio-ecological importance**

Large herbivores, including giraffes, elephants, bison, and deer, play a vital role in preserving ecological equilibrium and shaping habitats worldwide. Their discerning foraging behaviours have a crucial influence on the regulation of plant communities, subsequently affecting the structure of vegetation and fostering biodiversity (e.g., Rivadeneira-Canedo, 2008; Lendrum et al., 2014; Åkesson et al., 2021). These activities are vital in managing plant succession, determining the types of species present, and promoting efficient nutrient cycling. These processes ultimately improve the ability of ecosystems to withstand and recover from disturbances (Rooney & Waller, 2003; de Boer et al., 2015). In addition to managing vegetation, these herbivores significantly impact soil fertility by affecting the nitrogen cycle and other biological interactions. This contribution greatly influences the structuring of habitats (Owen-Smith, 1988; Augustine & McNaughton, 1998). Their role in seed dispersal and plant regeneration is crucial for the long-term viability of forest and grassland ecosystems, bolstering

these habitats' ability to adjust to environmental fluctuations (Janzen, 1984; Ratajczak et al., 2022). Large herbivores have a crucial impact on fire prevention by their grazing behaviour. This leads to a decrease in the occurrence and intensity of wildfires, which is particularly important for global climate change (Archibald et al., 2005). Nevertheless, they make a substantial contribution to the process of carbon sequestration, which involves capturing and storing carbon dioxide. This leads to increased plant biomass growth and carbon storage in the soil. These actions are crucial in reducing the impact of climate change (Teague et al., 2016; Berzaghi et al., 2023).

Large herbivores play a crucial role in indicating the quality of a habitat. These umbrella species play a vital role in safeguarding a wide variety of biodiversity in their habitats (Carranza & Mateos-Quesada, 2001; Forbes, 2021; Afonso et al., 2023). Contemporary conservation strategies emphasise protecting large herbivores to uphold their crucial ecological services, which are indispensable for the health of natural ecosystems and human societies (Putman, 2012; Pringle et al., 2023). We must implement comprehensive strategies to ensure these critical species' effective administration and conservation. These measures include protecting the natural environments where they reside, revitalising the ecological pathways connecting different habitats, and involving nearby communities to reduce conflict between humans and wildlife. Using these strategies is essential to protecting herbivore populations and the ecosystems they support, encouraging peaceful coexistence, and making natural systems better able to handle environmental changes (Reimoser & Putman, 2011; Ahrestani et al., 2012; Puri et al., 2019).

### **3.1.3. Challenges in conservation and management**

The conservation and management of large herbivores pose significant challenges, primarily due to habitat degradation, fragmentation, urban expansion, and the changing climate (e.g., Asner et al., 2009; Løvschal et al., 2017; Bardgett et al., 2021). These factors collectively jeopardise their survival and ecological functions. Human activities worsen these challenges by speeding up habitat destruction and disrupting critical environmental networks that support these species' ecological functions. The changes in vegetation patterns caused by climate change significantly impact the eating habits and migration behaviours of large plant-eating animals. This highlights the need for adaptive management strategies to address these changing



ecological landscapes effectively (Bissonette and Storch, 2003; Gordon et al., 2004; Parker et al., 2009; Ripple et al., 2015; Rudnick et al., 2012).

Nutrition plays a critical role in preserving large herbivores, as sufficient forage availability is required for their survival and reproductive success. Alterations in the habitat that influence the accessibility and excellence of food resources can directly impact these animals' well-being and population dynamics. Urbanisation and the construction of roads and fences disrupt migration routes and alter animals' natural behaviours. This also affects their ability to access traditional feeding grounds, making it challenging for them (Ascensão et al., 2019). Due to closer contact between humans and wildlife populations, these barriers lead to higher mortality rates, lower genetic variation, and increased chances of diseases (Mackenzie et al., 2013; Muehlenbein, 2016; Esposito et al., 2023).

The connection between the migratory patterns of animals and their food search emphasises the significance of incorporating nutritional factors into conservation frameworks. Preserving and repairing migratory corridors is critical for allowing animals to freely move and access areas that provide essential nutrients. These corridors play a significant role during critical life stages, such as calving and rutting (Holechek, 1989; Barboza et al., 2008; Post et al., 2008; Berg et al., 2019).

To effectively conserve large herbivores, a comprehensive strategy is necessary that tackles the diverse challenges they encounter. This encompasses safeguarding crucial habitats, regulating herbivore populations to minimise their effects on biodiversity, and conserving ecosystem services like carbon and nitrogen cycling in grasslands (Speed et al., 2020; Sitters et al., 2020). Moreover, to enhance the resilience of herbivore populations and preserve ecological balance, conservation efforts need to be flexible enough to accommodate different global conditions and incorporate ecological, social, and cultural aspects (Gosnell et al., 2020; Zhang et al., 2020; Li and Pim, 2020).

The practical preservation of large herbivores relies on comprehending their ecological functions and nutritional requirements and having the capacity to monitor these factors proficiently. In this context, technological advancements and data collection are crucial. Our approach harnesses real-time data and non-invasive techniques to effectively monitor large herbivores, ensuring their sustainable management and conservation (e.g., Arnon et al., 2021). By integrating advanced technologies like GPS tracking and unmanned aerial vehicles, we

optimize environmental monitoring to support the long-term viability of these species across diverse ecosystems (Prosekov et al., 2020).

#### **3.1.4. The significance of dietary monitoring**

Monitoring techniques are critical in wildlife conservation to effectively manage and preserve large herbivores. The presence of giraffes, elephants, bison, and deer is essential for safeguarding ecological equilibrium and improving the health of habitats. They achieve this through their interactions with plant communities and involvement in nutrient cycling (Rooney & Waller, 2003; de Boer et al., 2015). Precisely observing these animals is crucial for comprehending their behaviour and requirements and guaranteeing the long-term viability of the ecosystems they uphold.

Contemporary conservation initiatives greatly profit from technological advancements like GPS tracking and satellite imagery, which enable meticulous monitoring of animal movements across vast landscapes. This ability is essential for the cartography of migration patterns, the examination of habitat utilisation, and the administration of spatial utilisation to avert conflicts between animals and human activities. GPS telemetry has examined elephants' migration patterns and habitat preferences. This data is vital for creating wildlife corridors that help reduce conflicts between humans and wildlife (Beier et al., 2008; Wittemyer et al., 2008).

Conservationists are increasingly using non-invasive methods to minimise the impact of monitoring on animals. Techniques such as near-infrared reflectance spectroscopy (NIRS) are increasingly being employed to evaluate the nutritional composition of herbivore diets using faecal samples (e.g., Rodríguez-Hernández et al., 2023; Vera-Velez et al., 2023). This method offers valuable insights into the quality of forage animals consumed, removing the need for direct interaction (Staaland & White, 2001). Furthermore, the analysis of DNA found in faecal matter is being used increasingly to assess genetic diversity and the overall health of populations. This method provides a significant amount of valuable information essential for effectively managing populations, all without the need to capture and handle animals, which can be stressful and invasive (Kohn & Wayne, 1997, Zemanova, 2021).

Integrating data from multiple sources is critical in conservation to understand wildlife well-being and habitat needs comprehensively. Conservationists can develop sophisticated management strategies by combining movement data with genetic, nutritional, and ecological information. These strategies can effectively address both immediate conservation needs and long-term sustainability goals. We can link spatial movement data with botanical surveys by identifying critical foraging areas essential for species survival throughout the seasons. This enables the implementation of more targeted conservation measures (e.g., Tédonzong et al., 2018).

The ecosystem's health is intricately linked to the welfare of large herbivores, making these approaches critical for their preservation. By utilising these sophisticated techniques, conservationists are more prepared to create successful plans that uphold the sustainable existence of these species and the broader range of ecological benefits they offer, thereby advancing the preservation of biodiversity and the resilience of ecosystems.

### **3.2. Faecal Analysis Using Near-infrared Reflectance Spectroscopy (NIRS)**

Faecal analysis has been a fundamental aspect of ecological research for a considerable period, providing valuable information about large herbivores' eating habits, well-being, and environmental relationships. This method yields essential information regarding the nutrients ingested, digestion efficiency, parasites' presence, and stress indicators, all obtained from faecal matter. Diverse biochemical assays and microbiological techniques are employed to extract and analyse this abundant information, accurately representing the animals' overall health condition in their native environments (Landau et al., 2022).

Near-infrared reflectance spectroscopy (NIRS) is a non-invasive analytical method that improves the capabilities of conventional faecal analysis. NIRS employs the near-infrared portion of the electromagnetic spectrum, specifically ranging from 800 nm to 2500 nm. The mechanism behind its function is that the absorption of near-infrared light triggers molecular vibrations, resulting in unique spectral patterns. These patterns offer a quick and thorough analysis and measurement of different organic compounds in faecal samples. This method enables the rapid evaluation of food's nutritional value and digestion effectiveness without the need for complicated and time-consuming chemical analysis.

Herbivores' ability to swiftly assess the nutritional composition of forage exemplifies the efficacy of NIRS in ecological research. Villamuelas et al. (2017) utilised NIRS to effectively assess the nutritional status of herbivores through faecal indicators, notably faecal nitrogen. Their study developed a multispecies NIRS calibration that demonstrated high accuracy across various herbivore species, significantly enhancing our ability to evaluate the diet quality and overall health of these animals efficiently and cost-effectively. Furthermore, NIRS was utilised to analyse faecal nitrogen and phosphorus across a diverse dataset from multiple ruminant species including cattle, bison, deer, elk, goats, and sheep. While NIRS calibrations for faecal nitrogen showed high accuracy, the development and validation of faecal phosphorus calibrations demonstrated varying levels of effectiveness, indicating a need for further research (Tolleson & Angerer, 2021). These findings highlight the potential of NIRS as a versatile tool for non-invasive nutritional monitoring in wildlife management and ecological studies. This information is essential for comprehending how herbivores adjust to changes in food availability

throughout different seasons. It is valuable for managing conservation efforts that align with natural feeding behaviours and ecological dynamics.

Furthermore, incorporating NIRS alongside conventional faecal analysis techniques provides a comprehensive method for comprehending an animal's dietary ecology and overall health. This integrated method can significantly enrich and enlighten conservation strategies. Barbero-Palacios et al. (2023) successfully utilised faecal NIRS to assess stress and gut health by examining hormonal and microbiota profiles in faecal samples. This methodology yielded significant knowledge about the physiological reactions of herbivores to environmental pressures, emphasising the efficacy of NIRS for faecal analysis as a potent instrument in wildlife management and conservation endeavours.

### **3.2.1. Nutritional information obtained from faeces**

Analysing faecal matter is crucial in nutritional ecology, as it offers valuable information about large herbivores' diets and ecological relationships. This method is beneficial for evaluating essential dietary elements like nitrogen (N), acid detergent fibre (ADF), and neutral detergent fibre (NDF). These components provide a comprehensive overview of the animal's nutritional intake and its impact on ecosystem health.

The nitrogen content found in faeces serves as a fundamental measure of protein intake, providing insight into the nutritional value of consumed forage. Examining the nitrogen content in faeces allows for measuring both utilised and leftover nitrogen, giving insight into the amount of protein accessible in the herbivore's diet. Awuma (2003) demonstrates the effectiveness of using NIRS combined with geostatistical techniques to predict diet quality in livestock across sub-Saharan Africa (SSA). By analyzing the diet and fecal samples from multiple species and covering vast geographical, the study developed robust NIRS equations that accurately predicted crude protein and digestible organic matter in the animals' diets, with the precision matched with the one of traditional wet chemistry methods. This capability enables the creation of detailed diet quality maps across landscapes, providing a valuable tool for improvement of management and conservation through nutrition in the region. A precise understanding of these values is essential, particularly given the influence of plant secondary metabolites such as tannins, which can attach to proteins and significantly impact their bioavailability. Although there are difficulties, faecal nitrogen remains a dependable indicator of dietary quality, especially when

studying how herbivores adapt their diets to counteract the impact of tannins (Palo & Robbins, 1991; Leslie et al., 2008; Robbins, 2012; Landau et al., 2022).

Examinations of ADF and NDF offer valuable information about the fibre makeup and digestibility of the plant matter ingested by herbivorous animals. ADF primarily quantifies cellulose and lignin levels, which are critical for assessing the energy that herbivores can obtain from their diet. On the other hand, NDF encompasses hemicellulose and acts as an indicator of the overall fibre mass that influences the speed of digestion in the gut. The combined evaluations of ADF and NDF aid in assessing the effectiveness of herbivores in extracting energy from their diets. This is crucial for maintaining their ruminal health and overall well-being (Van Soest et al., 1991; Van Soest, 1994). In addition to N, ADF, and NDF, faecal analysis can also detect the existence of parasites, stress hormones, and even small amounts of environmental toxins. Each of these findings offers additional information about the health and ecological challenges experienced by herbivores. Cortisol, a stress hormone, can reveal an animal's response to environmental stressors such as habitat disruption or the threat of predation. At the same time, parasites can serve as an indicator of the health difficulties faced by a population. We can monitor the presence of environmental toxins, such as heavy metals, in herbivores using faecal samples. This method allows assessing herbivores' exposure to polluted environments (Clausen et al., 2003b).

By incorporating a range of different measurements of faecal matter, researchers can evaluate the appropriateness of habitats in terms of their ability to sustain herbivores' populations. Conservationists can develop strategies to optimise habitat management, improve forage quality, and reduce potential dietary stressors by examining the relationship between dietary fibre and protein intake and broader ecological data. This all-around approach makes it easier to come up with conservation plans that are both reactive and proactive, since it addresses nutritional deficiencies before they hurt herbivore populations (Felton et al., 2021; Merems et al., 2020). These analyses provide crucial knowledge for effectively managing and conserving herbivore populations, ensuring their continued role in maintaining ecological equilibrium and biodiversity.

### 3.2.2. Enhancing wildlife nutrition study and conservation with NIRS

Near-infrared reflectance spectroscopy (NIRS) has become essential in wildlife nutrition studies and conservation efforts, providing a non-intrusive and accurate technique for analysing dietary components in large herbivores. NIRS is based on its capacity to identify molecular vibrations which is valuable for analysing the chemical makeup of biological substances. This spectral data is highly efficient in quantifying the bonds of organic compounds, such as C-H, N-H, and O-H, which are prevalent in plant and animal tissues (see Appendix N.5: Principles of NIRS). It enables a thorough analysis of dietary intake, allowing for detailed examination (Foley et al., 1998; Shenk and Westerhaus, 1991). The potential of NIRS was initially investigated in agricultural contexts, where it played a crucial role in analysing the chemical composition of pastures, feedstuffs, and food products (Offer et al., 1998; Kays et al., 2000). This method has grown to include wildlife research, particularly in studies that analyse the nutritional value of ruminants' diets using faecal samples. These studies evaluate parameters such as crude protein, diet digestibility, and energy values (Lyons and Stuth, 1992; Leite and Stuth, 1994; Pumomoadi et al., 1996). In addition, NIRS has been used to determine the mineral content and botanical composition of diets, which has improved our knowledge of dietary habits in various species (Petersen et al., 1987; Volesky and Coleman, 1996; Walker et al., 1998).

Within the realm of conservation, NIRS plays a crucial role in examining the nutritional condition and dietary choices of herbivores through the analysis of their faecal samples. This analysis helps monitor health indicators and nutritional trends at individual and population levels. This capability is essential for developing well-informed conservation policies and efficient strategies for managing wildlife (Mahipala et al., 2010; Decruyenaere, 2015; Schiborra et al., 2015; Righi et al., 2017). NIRS can detect pathogens and monitor health, making it valuable for non-destructive biological research (Ezenwa, 2004; Tolleson et al., 2007; Dixon et al., 2013; Morgan et al., 2021; Qin et al., 2021; Kho et al., 2023).

The efficacy of NIRS relies on developing precise calibration equations that establish a correlation between spectral data from faecal samples and known dietary compositions (Andueza et al., 2017). The creation of extensive spectral databases, backed by the meticulous calibration process, ensures the dependability of dietary analyses and improves the model's accuracy in various species and ecosystems (Walker et al., 2007; Glasser et al., 2012; Serrano et al., 2020; Berauer et al., 2020). The databases are crucial for enhancing NIRS calibration

models and broadening their use in ecological research (Tigabu and Felton, 2018; Herrera-Sánchez et al., 2023).

NIRS and its faecal application (fNIRS) demonstrate how technological innovation can be smoothly incorporated into ecological research, greatly enhancing our comprehension of the connections between large herbivores and their surroundings. This technology helps maintain the quality of habitats and improves our ability to manage and protect biodiversity effectively. It promotes conservation strategies based on data, which are crucial for the sustainable management of wildlife populations (Jenks et al., 1994; Lahoz-Monfort and Magrath, 2021).



### 3.2.3. Faecal NIRS – a multilevel ecological perspective

Using Near-Infrared Reflectance Spectroscopy (NIRS) in examining faecal samples has significantly transformed our methodology in investigating large herbivores at the individual, population, and ecosystem scales. This method's non-invasive and precise nature makes it essential for modern wildlife ecology and conservation strategies.

Individually, faecal near-infrared spectroscopy (fNIRS) is a valuable tool for understanding herbivores' health and nutritional condition. Through the analysis of faecal samples, faecal near-infrared spectroscopy (fNIRS) can accurately assess nutrient consumption and detect possible deficiencies or imbalances. It is essential to monitor reintroduced or captive animals to ensure their optimal health, which is crucial for their survival and ability to reproduce. For instance, research has employed fNIRS to modify diets in captive breeding programmes, improving reproductive success and overall health by customising nutritional provisions to the specific requirements of various species (Prather et al., 2020; González et al., 2018; Jayne, 2020; Morgan et al., 2021).

Faecal NIRS (fNIRS) enables researchers to observe herbivore populations' well-being and eating habits from a broader perspective. This tool can identify differences in the composition of diets among a population (e.g., Jarque-Bascuñana et al., 2021). It provides data on how environmental factors, such as habitat quality and food availability, affect nutrient intake (Petit Bon, 2020). By examining these patterns, conservationists can detect populations susceptible to nutritional strain or decline, enabling prompt interventions. Redjadj et al. (2014) and Bison (2015) demonstrated the application of fNIRS in assessing herbivore populations' overall health and nutritional sufficiency across diverse habitats. This information can help guide management decisions to reduce the impact of environmental changes.

At the ecosystem level, functional near-infrared spectroscopy (fNIRS) enhances our comprehension of food web dynamics and trophic interactions. The dietary information derived from herbivore faecal samples provides insight into their function within the ecosystem, including their influence on the composition of plant communities and the cycling of nutrients. This information is essential for effectively managing ecosystems while preserving biodiversity and ecological function. Corlatti (2020) and Landau et al. (2021) employed fNIRS to investigate the impact of herbivore foraging behaviours on plant species diversity and structure, with the findings providing important insights for habitat conservation and restoration.

By incorporating fNIRS at various levels, a comprehensive conservation approach considers the intricate interactions between herbivores and their surroundings (e.g., Tuomi, 2020). fNIRS enables comprehensive and expandable evaluations of dietary health, thereby aiding conservation policies based on solid ecological data. Furthermore, as changing climate conditions affect the quantity and quality of food sources, fNIRS becomes essential in adjusting management strategies to ensure the long-term viability of herbivore populations (Garnick et al., 2018; Landau et al., 2021; Petit Bon, 2023). The utilisation of fNIRS, combined with comprehensive data on dietary patterns and health markers, enables the implementation of proactive conservation strategies that proactively tackle potential ecological imbalances (Schlägel et al., 2020; Schweiger et al., 2015). Overall, the utilisation of fNIRS showcases the integration of technological progress with ecological studies, offering a powerful instrument for enhancing our comprehension of the intricate connections within ecosystems (Stolter et al., 2006; Vasseur et al., 2022). This approach contributes to the broader objectives of conserving biodiversity and strengthening the resilience of ecosystems. By conducting meticulous and non-intrusive examinations of diets, fNIRS enables researchers and conservationists to make well-informed choices that align with both immediate conservation requirements (e.g., Ward et al., 2022) and long-term ecological sustainability (e.g., Ludwig et al., 2002; Jean et al., 2014; Berauer et al., 2020).

## 4. METHODOLOGY

(Summarised key methodological steps; for the full methodology, see Appendices)

### 4.1. Research Planning

The thesis presents a thorough investigation into the nutritional patterns of various species, both in controlled captive settings and in natural habitats, arranged into two phases. The first phase focused on captive populations of red deer and gazelles at the University of Castilla-La Mancha's deer experimental facilities in Albacete and "*La Hoya*" Experimental Farm of Arid Zones in Almería, Spain, respectively. This section of the study sought to determine the effect of specific individual and environmental factors, such as pasture presence and animal physiological states, on the digestibility of dietary components. Red deer samples were collected over four trials along the year to investigate seasonal effects, while gazelle samples were collected in a single trial to highlight species-specific, age-, and sex-related dietary differences. The second phase of the research involved moving the study into natural settings. This included studying free-ranging roe and red deer in the Bohemian Forest, Czech Republic, and Andean bears in the cloud forests of Ecuador, to understand more about their dietary habits and how it affects their ecological strategies. This phase was intended to capture the nuances of nutritional adaptation and efficiency in uncontrolled environments, employing NIRS in distinct ways to dissect dietary compositions and the role of environmental factors in shaping nutritional intake.

### 4.2. Research Organisation and Sample Collection

In our first comprehensive study, faecal sample collection from captive Iberian red deer was meticulously planned across four seasons - February (late gestation), May (births), July (mid-lactation), and September (late lactation and rut) - to ensure the highest data accuracy while minimising animal stress and adhering to ethical standards for animal research. During biweekly routine handling sessions, samples were collected directly from the rectums of 43 calves and yearlings, 23 stags (aged 3 to 7), and 30 hinds (aged 3 to 21, with 22 pregnant), ensuring comprehensive coverage within a 7-day window for each designated season. This study focused on the dietary habits of these deer, using a Total Mixed Ration (TMR) that varied seasonally to mimic natural food transitions closely. The TMR was composed of up of oats, barley, alfalfa meal, cereal straw and citrus pulp, and was intended to meet the deer's altering nutritional needs.

In addition to the TMR, stags were given a high-protein pellet diet during all seasons except September to meet their specific dietary requirements. To keep the animals hydrated, water was provide *ad libitum*. In addition to managing dietary intake, the study documented animal weight and body condition scores alongside with biweekly handling sessions. This detailed monitoring process was critical for gathering a large dataset on individual health and nutritional status, which improved the study's analytical capabilities. Furthermore, the preparation of four subsamples of TMR and feed pellets for each season, ground but not dried, and frozen for later analysis demonstrated the thorough approach taken. Such a methodical approach to dietary management, combined with precise data collection practices, demonstrated the study's commitment to investigating the intricate effects of diet on faecal nutrients in a strictly controlled environment, providing invaluable insights into the nutritional ecology of captive Iberian red deer.

For the second study, we employed a nuanced approach to analyse the dietary patterns of 193 individuals belonging to three species of gazelles: dama, Cuvier's, and dorcas. The animals were fed a carefully planned diet of fresh lucerne, wheat straw and herbivore-specific feed pellets, supplemented by unrestricted access to water and mineral licks, to closely mimic their natural dietary intake and ensure their health and well-being in captivity. This regimented nutritional management was critical, especially in the month preceding faecal sample collection, to maintain dietary consistency and accurately assess the impact of various factors on digestive efficiency. To minimise stress and contamination, faecal samples were meticulously collected directly from the rectum during routine veterinary inspections, and then processed according to a standardised protocol.

Collection of faecal samples from free-ranging roe and red deer in the Bohemian Forest, Czech Republic, was designed to investigate these species' winter dietary habits. The forest, with its mixed vegetation of Norway spruce, European beech, silver fir, and larch, as well as a bramble understory, provided an ideal habitat for both deer species. The study area of approximately 2,500 hectares outside the Šumava National Park (Bohemian Forests) was chosen for its biodiversity and the presence of meadows and forested areas conducive to the different dietary preferences of our target species. We collected samples in December 2016 and January 2017, taking advantage of the snow cover to identify and collect fresh faecal pellet groups. A total of 156 pellet groups were initially collected along predefined transects, with snow reducing soil contamination and ensuring sample freshness. The morphological characteristics of the faeces,

as well as nearby animal tracks, aided in distinguishing between the two deer species, with a senior expert validating the identification to ensure the dataset's accuracy. This process yielded 94 confirmed samples for analysis: 59 from red deer and 35 from roe deer. This careful selection and collection process was critical to achieving our study's goal of investigating species-specific dietary patterns and their ecological implications. Given the differences in feeding strategies between roe deer (concentrate selectors) and red deer (intermediate feeders), the Bohemian Forest's mixed forest environment provided a unique opportunity to observe how available vegetation influences dietary preferences. Our study sought to shed light on how these feeding specialists navigate their environment, particularly during the winter, while also contributing valuable data to the larger field of ungulate nutritional ecology.

The ecological study, which took place in southern Ecuador in June and July 2017, used meticulous sampling and analysis methodologies to investigate the ecological impacts on the nutritional value of bromeliads and their influence on Andean bear diet selection. The study area included six sites in montane forest and páramo ecosystems, providing a diverse range of bromeliad species, which are an important food source for Andean bears. To assess the abundance and nutritional value of bromeliads, 550-m x 50-m plots were created, each with two 10-m x 10-m subplots for detailed analysis. The most abundant bromeliad species in each subplot were identified and quantified, allowing us to better understand the availability of this critical food source. Furthermore, all faeces found in these plots were collected to highlight the bears' presence and dietary preferences using faecal microhistology. This analysis distinguished between the bromeliad species consumed by the bears, providing insights into their foraging behaviours in relation to bromeliad availability and nutritional value. The nutritional analysis of bromeliads concentrated solely on their meristematic components, using NIRS in conjunction with traditional wet chemistry analyses. This comprehensive approach allowed for the determination of protein, fat, starch, fibre, acid detergent fibre (ADF), neutral detergent fibre (NDF), and ash content, revealing the nutritional profile of bromeliads from various ecosystems and species. This methodological framework, which combined the study of bromeliad abundance, nutritional analysis, and faecal microhistological examination, provided a comprehensive understanding of Andean bear dietary habits and preferences. The nuanced understanding of how ecological factors such as ecosystem type, flowering, and recent fire events affect the nutritional value of bromeliads adds to the overall research design.

### **4.3. Sample Processing and Data Acquisition with NIRS**

In our studies, the use of NIRS was critical for the integrity and uniformity of sample analysis, which included a wide range of materials, from the faeces of captive and free-ranging species to plant specimens such as bromeliads, which are essential for dietary evaluation of the Andean bear. To prevent contamination and degradation, samples were immediately placed in plastic or paper bags and then frozen to preserve their composition. This preparation phase was critical for protecting the samples' natural state, followed by a meticulous drying process at a controlled temperature to optimise moisture levels before grinding. The goal was to maintain the nutritional and chemical integrity of the samples so that the NIRS analysis would accurately reflect their true nature.

Grinding the samples to a fine, uniform particle size was critical for ensuring homogeneity, a key factor in NIRS analysis, because it increased the interaction between infrared light and sample components, improving the accuracy of the spectral data collected. We then stored the samples in carefully labelled containers for NIRS scanning, where meticulous sample preparation aided in collecting high-quality data. This stringent sample processing protocol, applied to faeces and plant samples, demonstrated our commitment to methodological consistency and scientific rigour. It laid the groundwork for accurate nutritional composition analysis and enabled us to compare the examined materials.

Our study used the FOSS NIRS DS 2500 analyser, which used pre-built and already existing advanced calibration models to precisely determine nutritional constituents in both faecal and plant samples. The faecal calibration model, which are critical for accurate faecal nutrient estimation, used a diverse set of samples, including over 100 faecal samples from red deer. We improved the models' reliability by including more study samples, accounting for variables such as age, sex, and body condition, and validating them using wet chemistry analysis. This all-around method ensured that the calibration models were robust and accurate, with high prediction power, as evidenced by the ND and HD values. This demonstrated that the NIRS analytical strategy worked effectively in this large nutritional study.

In the captive red deer and gazelle experiments, as mentioned, NIRS was used to collect and analyse data, quantifying key nutritional components in faecal samples. The analysis, supported by calibration models designed specifically for red deer, resulted in high predictive accuracy ( $R^2 > 0.98$ ), allowing for detailed interpretations of faecal nutrient content regarding variables like

seasonality, diet, and physiological states. The gazelle study, as well as roe and red deer from the wild, also benefited from a calibration set refined with red deer faeces, which ensured the accuracy of nutritional content analysis.

#### **4.4. Statistical Analysis**

The statistical methodology employed in the study of captive red deer aimed to ascertain the manner in which diverse variables affect the variability of faecal nutrients. The study used General Linear Mixed Models to analyse data, with fixed factors such as season, age, reproductive status, pasture availability, body mass, and body condition score, and response variables faecal nitrogen (fN), neutral detergent fibre (fNDF), and acid detergent fibre (fADF). The analysis was tailored for different sex classes (stags, hinds, and young), and models were developed for each class to investigate the factors influencing faecal nutrients separately. The normality of the variables was confirmed using Kolmogorov-Smirnov tests, and a structure with ID as the subject and factor of season as a repeated measure was used. We refined the initial models, which did not include interactions, using a stepwise backward selection procedure to include only significant factors and interactions that had a significant influence. The Variance Inflation Factor was used to assess multicollinearity among independent variables, and it was found to be low, indicating that the models were robust. The models for each dataset and response variable were chosen using the Corrected Akaike's Information Criterion (AICc), with an emphasis on the models that provided the most plausible explanation for the observed data. This statistical methodology enabled a more nuanced understanding of the factors influencing faecal nutrient variability, shedding light on the complex interactions between various physiological and environmental factors and their impact on the nutritional ecology of captive red deer.

The statistical analysis for the research on three species of Saharan gazelles, focusing on inter-species differences in faecal nutritional components under a standard feeding regime, employed a Multivariate General Linear Model. This model aimed to elucidate the effects of species, sex, and age on the same faecal constituents as in previous research. Inclusion of the interactions between species and sex, and species and age, was informed by preliminary data inspection suggesting sex-related differences within at least one species. The analyses were carried out using IBM® SPSS® Statistics software, version 29.0.

In the study of roe and red deer winter faeces in the Bohemian Forest, Czech Republic, a statistical analysis approach based on the independent sample t-test was used to identify potential species-specific differences in nutritional components. This method sought to detect differences in faecal nutrients between and within species across different habitats (forest and meadow). Pearson correlations revealed relationships among the same faecal nutrients studied, with lignin added in this experiment.

To reduce dimensionality and better understand data variance, Principal Component Analysis (PCA) was performed on these faecal nutrients, with a focus on two components with eigenvalues greater than one. The PCA sought to investigate the overlap or discrimination between species and habitats, taking into account variables that had a strong correlation with the components for interpretation. Despite the initial hypothesis, the analysis found no significant differences in faecal nutritional components between roe and red deer, or between samples from different habitats. The PCA findings revealed a strong reliance on the quality of ingested nutrition, particularly fibrous components, highlighting the complexity of diet and nutritional intake during the winter. This method emphasises the difficulties in distinguishing the dietary habits of free-ranging animals using faecal analysis alone, given the numerous uncontrollable environmental and physiological factors in natural settings.

The impact of ecosystem type, fire frequency, bromeliad species, and flowering on the nutritional value of bromeliads and, by extension, the dietary preferences of Andean bears was investigated in a study. To accomplish this, a series of Generalised Linear Mixed Models were used to investigate the effects of various ecological variables on bromeliad nutritional composition. We chose this methodology to accommodate the data structure by treating sites as subjects and subplots as repeated measurements. We included ecological variables as factors in the models we used to test linear responses. We refined the models using a stepwise backward selection procedure, excluding non-significant variables and identifying the models that best explained the observed variation in nutritional composition. We used the Akaike Information Criterion to determine the best models. This statistical process revealed that ecosystem type, fire events, and flowering all significantly impacted bromeliad nutritional value, with notable differences between species. Mann-Whitney U-tests, Kruskal-Wallis tests, and Spearman's rank correlations were used to investigate the relationship between bromeliad abundance, nutritional quality, and Andean bear presence. These analyses addressed bear presence in different



ecosystems, the frequency of bear signs and bromeliad occurrence across study sites, and the relationships between bear signs and bromeliad abundance and nutritional composition.

## 5. RESULTS

### 5.1. Inter-Specific Induced Differences Of Faecal Nutritional Components Under Controlled Settings

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**OPEN** Evaluation of factors inducing variability of faecal nutrients in captive red deer under variable demands

Stipan Čupić<sup>1✉</sup>, Andrés J. García<sup>2</sup>, Michaela Holá<sup>3</sup> & Francisco Ceacero<sup>3</sup>

**Full Article in appendix 9.1.**

## Summary:

The study investigates the variability in the nutrient content of faeces in captive red deer (*Cervus elaphus*) across different seasons and under various physiological states. Utilizing near-infrared reflectance spectroscopy (NIRS), the research analyzed the content of nitrogen, neutral detergent fibres (NDF), and acid detergent fibres (ADF) in the faeces of 43 calves and yearlings, 23 stags, and 30 hinds (including 22 pregnant individuals). This comprehensive study was carried out at the experimental deer research facilities of the University of Castilla-La Mancha in Albacete, Spain.

The core objective of the research was to quantify the impact of several variables - including diet availability, season, sex, age, reproductive status, body mass, and body condition - on faecal nutrient content. These insights are crucial for the accurate interpretation of faecal analysis results, especially when such analyses are used to infer the diet and nutritional status of wild populations based on faecal samples, which often lack detailed background information on the sampled individual.

The findings reveal significant variability in faecal nutrient content, attributed to individual animal factors such as pregnancy (affecting variability by around 4%), age, and weight (inducing up to 18% variability). Environmental factors, notably the presence or absence of pasture, contributed to approximately 13% variability. Seasonal changes were associated with an average variability of 17%, peaking at 21% under certain conditions. These variabilities underscore the complex interaction between the deer's physiological needs, environmental factors, and dietary availability across different times of the year.

This study highlights the significant challenge in interpreting faecal nutrient data from wild samples without detailed knowledge of the animals' individual characteristics and environmental context. We advocate for conducting preliminary investigations using captive animals of the species of interest before embarking on wild sample collection. Such preparatory work can enhance the understanding of potential variabilities in faecal nutrient content, leading to more accurate interpretations of dietary and nutritional assessments in wildlife ecology and management studies.

Given research underscores the necessity of considering a broad range of factors when using faecal analysis to assess the nutritional ecology of wild herbivores. The study's findings are particularly relevant for species experiencing significant seasonal and physiological changes in dietary requirements, offering valuable insights for future research in wildlife nutrition and ecological management.

## 5.2. Intra-Specific Induced Differences Of Faecal Nutritional Components Under Controlled Settings



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*Communication*

### Differences in Faecal Nutritional Components in Three Species of Saharan Gazelles on Standard Diets in Relation to Species, Age and Sex

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**Full Article in appendix 9.2.**

## Summary:

The study explores digestive efficiencies across three Saharan gazelle species - dama gazelle (*Nanger dama*), Cuvier's gazelle (*Gazella cuvieri*), and dorcas gazelle (*Gazella dorcas*) - housed under similar feeding conditions. Utilizing near-infrared reflectance spectroscopy (NIRS) for the analysis, this study meticulously examines faecal samples from 193 captive individuals to assess the concentrations of faecal nitrogen (fN) and fibres (ADF and NDF), thereby shedding light on the nutritional absorption and digestive process efficiencies within these species.

The primary motivation behind this research is rooted in the understanding that faecal component analysis can serve as a reflective measure of diet quality and digestibility in herbivorous mammals. The investigation reveals pronounced inter-specific variations in faecal nitrogen and fibres, indicating differing digestive efficiencies among the gazelle species studied. Notably, Cuvier's gazelle exhibited a markedly lower faecal nitrogen content in comparison to the dama and dorcas, suggesting a reduced digestive efficiency. Such findings are pivotal, as they underscore the influence of species-specific physiological and ecological traits on digestion.

Furthermore, the research delves into the roles of age and sex as moderating factors influencing faecal nutrient content. Although these factors exhibited a moderate effect on the nutritional components, particularly faecal nitrogen, their impact was not uniformly observed across the three species. This points to the complexity of digestive strategies and the necessity of considering species-specific factors in ecological and conservation studies.

Consistent across species, the study found that faecal fibre content remained relatively stable, indicating that diet quality was not a variable factor. This stability suggests that the observed variations in faecal nitrogen content likely stem from differences in digestive efficiency rather than dietary composition.

This study emphasizes the critical need for species-specific considerations when analyzing faecal nutritional components to understand feeding ecology and digestive efficiency. The findings offer valuable insights into the dietary management and conservation strategies for these gazelle species, highlighting the nuanced differences that exist even among closely related species under similar dietary conditions. This research not only contributes to our understanding

of gazelle physiology and ecology but also enhances the applicability of faecal analysis in wildlife nutrition and management studies.

### 5.3. Free Ranging Roe And Red Deer Settings Beyond Power Of NIRS

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### **Are they both the same shit? Winter faeces of roe and red deer show no difference in nutritional components**

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**Full Article in appendix 9.3.**

## Summary:

The study delves into the dietary habits of roe deer (*Capreolus capreolus*) and red deer (*Cervus elaphus*) within the Bohemian forests of the Czech Republic. Utilizing near-infrared reflectance spectroscopy (NIRS), the research aims to detect species-specific differences in faecal nutrients, reflecting the animals' dietary choices and digestive efficiency under natural winter conditions.

This investigation was prompted by the understanding that faecal composition can offer insights into ungulate nutritional ecology, potentially influenced by various environmental, physiological, and species-specific factors. Roe and red deer, differing in morpho-physiological traits and ecological preferences, were expected to exhibit distinct faecal nutrient profiles, particularly in terms of nitrogen and fibres. A total of 94 faecal samples from both species were analyzed for nitrogen, acid detergent fibre (ADF), neutral detergent fibre (NDF), and lignin content.

Contrary to expectations, the study found no significant differences in the winter faecal nutrient content between the two deer species, challenging the hypothesis that NIRS could differentiate between their dietary habits. The analysis suggested that both species relied heavily on the quality of ingested nutrition, particularly fibrous components, during the winter. The study hypothesizes that supplementary feeding practices common in the area, designed to support ungulate populations during nutritionally challenging periods, might have contributed to the observed dietary overlap.

The findings highlight the complexity of interpreting faecal nutrient data without detailed knowledge of individual animals' diets, physical condition, or environmental factors. This study underscores the need for caution in using faecal nutrient analysis as a standalone tool for assessing dietary preferences and nutritional status of free-ranging ungulates, especially in areas where human management practices, such as supplementary feeding, may influence natural feeding behaviors.

While NIRS and faecal nutrient analysis (fNIRS) hold promise for studying wildlife nutrition, its application in uncontrolled natural settings requires careful consideration of external factors. This research adds valuable insights into the feeding ecology of roe and red deer, with implications for wildlife management and conservation practices, particularly regarding supplementary feeding strategies during winter.



## 5.4. Integration of Methodologies for Dietary Patterns of Free-Ranging Herbivorous Bear

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SHORT COMMUNICATIONS

### Ecological effects on the nutritional value of bromeliads, and its influence on Andean bears' diet selection

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**Abstract:** Previous studies have recognized bromeliads as a key food resource for Andean bears (*Tremarctos ornatus*) throughout their range. However, it is still not clear how abundance and especially the nutritional value of bromeliads influence habitat and diet selection. Understanding this is essential because conflicts such as crop damage occur when available natural resources cannot of-

pueden ofrecer un aporte nutricional adecuado. Se estudiaron seis áreas de bosque y páramo en el sur de Ecuador en busca de signos de presencia de osos, abundancia y valor nutricional de bromelias, y selección de dieta por microhistología fecal. La composición nutricional de las bromelias se vio afectada por la floración y los incendios recientes, pero además encontramos una alta variabilidad entre especies y ecosistemas, con más proteína y menos fibra en las bromelias de páramo. Sin embargo, los signos de presencia de osos fueron más abundantes en el ecosistema forestal, y además la presencia no se vio afectada por la abundancia ni la composición de las bromelias. Otros estudios similares deben centrarse en otros recursos clave, como los frutos temporales.

**Palabras clave:** bosque montano, bromelias, composición nutricional, dieta, NIRS, oso andino, paramo, *Tremarctos ornatus*, uso del hábitat

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**Full Article in appendix Nr. 9.4.**

## Summary:

The research explores the interplay between Andean bears, bromeliads, and their ecological contexts in southern Ecuador. Conducted in June and July 2017 across six areas within forest and *páramo* ecosystems, this study aimed to elucidate how environmental variables such as ecosystem type, plant flowering stages, and fire incidents impact the nutritional attributes of bromeliads and subsequently affect the foraging behaviors and habitat preferences of Andean bears (*Tremarctos ornatus*).

Employing a comprehensive methodological framework, the researchers set out to document bear activity and bromeliad availability within designated plots in each study area. Signs of bear presence, including feces and footprints, were recorded to gauge bear activity. Simultaneously, the team quantified the prevalence of bromeliads within the plots, collecting specimens of the dominant species for nutritional analysis. The nutritional analysis focused on the meristematic parts of the plants, which bears are known to consume, assessing their content of proteins, fats, starch, fiber, acid detergent fiber (ADF), neutral detergent fiber (NDF), and ash through a combination of near-infrared reflectance spectroscopy (NIRS) and wet chemistry.

Additionally, fecal microhistology techniques were applied to further scrutinize the bears' diet, specifically identifying the consumption rates of *Puya* sp. and *Tillandsia* sp. bromeliads. Despite finding that the nutritional content of bromeliads varied across ecosystems and species, likely influenced by the investigated environmental factors, these variations did not directly correlate with the observed bear presence or their dietary choices. Notably, even though *páramo* ecosystems presented bromeliads with higher nutritional value, evidence of bear presence was predominantly found in forest areas. This suggests that other factors beyond the immediate nutritional value of bromeliads might be guiding the bears' habitat selection and foraging decisions.

This study sheds light on the complex dietary preferences and adaptability of Andean bears, underscoring the need for a holistic approach in conservation efforts that considers a wide range of ecological and nutritional dynamics. The findings imply that while bromeliads are a significant component of the Andean bears' diet, their foraging behavior and habitat use cannot be solely explained by the nutritional quality of these plants. The research emphasizes the importance of integrating ecological and nutritional perspectives in developing effective conservation strategies for Andean bears, aiming to mitigate human-wildlife conflicts and safeguard the rich biodiversity of the Andean region.

## 6. DISCUSSION

### 6.1. Insights From Captive Populations

#### 6.1.1. Intra-specific factors inducing differences in faecal nutrients of red deer

Red deer's faecal constituents exhibited substantial variation, even in a controlled environment with healthy animals and comparable food sources. Age/age class, body mass, sex, reproductive status, body condition, season, and pasture availability all contributed to the diversity in faecal nutritional content, which occasionally surpassed 25%. These findings are critical for interpreting ecological studies of wild faeces in the absence of individual data.

Faecal nitrogen is assumed to correlate with dietary nitrogen (Holechek et al., 1982; Robbins 2012). In young animals, faecal N matches dietary N, but not always in adults. While hinds choose quality, stags prefer low-quality food at nutritionally challenging times (Staines et al., 1982). Even when given with small amounts of high-protein feed pellets in all seasons except September, stags' fN levels matched seasonal forage nitrogen concentrations in all four measurement periods. This suggests that the fN did not reflect the increased protein intake. However, the dietary-fN mirroring in stags is more complex than it appears. Larger stags have higher requirements than pregnant or lactating hinds (Dryden, 2011). Stags eat more in proportion to their size to compensate for their mouth's ability to retain larger, more fibrous food particles with less nitrogen on a dry weight basis (Staines et al., 1982). High-fiber diets impair digestibility and increase retention time, leading in more dry matter and nitrogen loss (Hungate, 2013; Thompson & Barboza 2017). Stags had higher fNDF (Thompson & Barboza, 2017) than hinds in our sample, with the exception of July, possibly due to their high lactation demands and increased selectivity for high-quality food. Stags did not gain weight on a reduced diet over the summer, unlike lactating hinds on identical diets (Thompson & Barboza 2017). Stags' winter rumen nitrogen levels are lower than hinds' (Staines et al., 1982). These findings are consistent with ours, indicating that stags had lower fN concentration in February than hinds, despite ingesting protein-rich pellets. In our study, stags on pasture had lower fN levels, indicating that they ate more low-quality food to balance their N intake (Timmons et al., 2010). Stags use feed pellets to exceed their daily protein and calorie requirements, rebuilding body reserves and compensating for the low-quality food they consume in abundance. High digestive efficiency

could have prevented excess N absorption from impacting fN during the three supplemented periods that corresponded to antler growth (Gaspar-López et al., 2010).

Unlike males, hinds showed higher fN than predicted based on dietary N. This may seem odd given that reproduction is most active in February and May (Oftedal, 2000; Dryden, 2016), therefore digesting efficiency should be higher. However, from February to May (late pregnancy), red deer hinds reduce rumen volume (Bruinderink & Hazebroek, 1995), resulting in decreased food consumption. This may have enhanced the need for protein-rich meals (Ceacero et al., 2012). Hinds consume the least from February till parturition (Bruinderink & Hazebroek, 1995), indicating a preference for high-quality food. Lactation-related nutrient demands increase following parturition (Moen, 1973), resulting in increased rumen, abomasum, gut, and liver weight and size. Summertime, large herbivores eat more and gain weight to cover breeding energy expenses (Thompson & Barboza, 2017). Following weaning, hinds rebuild their digestive tracts and increase rumination (Monteith et al., 2014), which may have aided them in obtaining nitrogen from grass. Reindeer consume more and digest more efficiently while breastfeeding (Barboza & Parker, 2008). Forages with high digestible energy (e.g., high nonstructural carbohydrates) ferment quickly (Robbins, 2012; Schwarm et al., 2009), hence consuming high-quality forages increases faecal nitrogen through fermentation, microbial bio-mass absorption, and nitrogen bypass (Van Soest, 1994). Later, protein excretion was reduced between July and September, when hinds' rumen volume is usual (Bruinderink & Hazebroek, 1995).

The lack of rumen nutrient content data complicates fN interpretation for hinds and stags. Rumen nutrition would have shown undigestible nitrogen, which fermentative processes can only digest. Future studies could look into N bound to ADF in faeces (NDF-N, Van Soest 1994). Using this index, fN minus fNDF-N represents faecal metabolic nitrogen (MFN).

Secondary metabolites such as tannins may slow protein digestion (Hobbs 1987; Barbehenn & Constabel 2011). Because the study only employed typical livestock feeds with minimal plant secondary component (PSC) content, this issue is unlikely to have influenced our findings. Other researchers have noted that deer's tolerance to low diets restricts fN as a nutritional measure (Monteith et al., 2014). Lactating hinds might absorb more plant proteins from cell wall surfaces and digest smaller particles more efficiently by mastication (Gross et al., 1996). Lactation-induced gastrointestinal tract remodelling (Zimmerman et al., 2006) improves N absorption

while decreasing N excretion (Monteith et al., 2014). For these reasons, fN may misrepresent dietary quality. It is worth noting that the rumen microbiota and nitrogen recycling into the rumen are difficult to control in wild investigations, which may explain the diversity in faecal nutrients (particularly fN) and sex variations in operating processes (Staines et al., 1982).

Faecal fibre fractions (fNDF and fADF) also indicate diet and food quality differences (Robbins, 1987). Dietary N is difficult to detect in wild research, thus it should be investigated to support fN interpretation. ADF and NDF lower voluntary food intake and digestion (Van Soest, 1994). Dietary N and fibres in our principal feedstuff (TMR) have an inverse effect on our study, as expected. However, neither fNDF nor fADF adequately reflected diet. NDF and ADF levels fluctuated on a regular basis, but fNDF and fADF varied very slightly across seasons and age/sex classes. Food fibres, like fN, match faeces in calves but not in adults.

The findings also reveal that several particular factors influence fN for each age/sex group. We also discovered a significant effect on body condition: stags in poor health had a lower fN, indicating that they exploited meal protein more effectively. We found considerably lower fN values in hinds during reproductive limits (pregnancy or lactation), albeit this effect was minor, as we hypothesised that reproductive constraints would only affect fN by 4%. Age and other factors showed different variability between hinds and stags, corroborating previous findings. In hindsight, age class explains just 4% of faecal nutrients, whereas age accounts for 10% of total fN, fNDF, and fADF in stags. In stags, older people had lower fN, which supported previous findings. Antler investment and requirements increase with age (Gomez et al., 2011; Dryden, 2016), whereas hind reproductive investment remains constant.

The findings are significant for wild research that use this strategy. Faecal nitrogen is a popular food quality measure in nutritional ecology research (Leslie et al., 1994; Hamel et al., 2009). However, the variability found for individual parameters such as age, body mass, body condition, and reproductive and antler growth restrictions cannot be quantified in wild faeces samples, which may lead to incorrect conclusions based on data that do not account for these factors. Our findings also indicate that pasture and season influence physiological status. First, as previously stated, faecal fibres might be imprecise, resulting in mistakes in estimating food quality. In our investigation, pasture generated a 13% change in fN and fNDF, primarily in hind faeces. The seasonal effect is significant across all nutrients and sex/age groups. The season created a 10-21% variation in a highly consistent feeding regimen. Faeces samples from wild

animals from different seasons and locations should be compared with caution due to seasonal variations in diet selection (Dixon and Coates, 2009; Palacios et al., 1989; Alvarez et al., 1991; García-González & Cuartas, 1992; Bugalho et al., 2001; Villamuelas et al., 2017). The previously reported fact that tannins or other PSCs increase fN (Barbehenn & Constabel, 2011) may be significantly greater in natural diets, raising the uncertainty of the findings.

### **6.1.2. Inter-specific factors inducing differences in faecal nutrients of gazelles**

In a controlled research context, we observed inter-specific variations in the faecal content of three nutrients (N, ADF, and NDF) across three closely related species (dama, dorcas, and Cuvier's gazelles) under a regulated feeding regime. The Cuvier's gazelle had a notably reduced quantity of fN compared to the other species, indicating a lower level of digestive efficiency. The faecal level of nutrients, particularly fN, was moderately influenced by individual characteristics such as sex and age. However, it is important to note that these effects were not consistent among the three species that were tested. In contrast, faecal fibres exhibited a high degree of constancy, indicating their dependence on the quality of the diet. Given that the feed was uniformly administered to all the animals under investigation, the findings indicate variations in digestive efficiency rather than variations in diet choices.

The previous intra-specific study conducted on red deer revealed that various individual factors, such as sex, age, reproductive status, body mass, body condition, season (which is associated with distinct nutritional needs for each sex), and the presence or absence of natural pasture, had a significant impact on faecal nutrients within a comparable experimental context involving captive red deer. In the aforementioned study, distinct examinations were undertaken for males and females owing to the significant sexual dimorphism associated with varying nutritional needs within the species during different stages of the annual cycle. However, the disparities in faecal nutrients between animal of different sex can be verified as distinct reasons that account for these variations. Sex did not have significant importance in gazelles. Nevertheless, the interaction with the species was noteworthy: there were no disparities between the sexes of Cuvier's gazelle; females of dorcas gazelle exhibited higher fN and fNDF; and males of dama gazelle had higher fADN and fNDF. Overall, the observed disparities were minimal when compared to the findings of the prior investigation on red deer. This disparity could potentially be attributed to the relatively lower sexual dimorphism in body size observed in gazelles in

comparison to deer (Loison et al., 1999; Pérez-Barbería et al., 2002). Remarkably, there were no observed disparities between sexes in Cuvier's gazelle, a species characterised by sexual dimorphism and high reproductive productivity (with twins being prevalent). Therefore, it is reasonable to anticipate greater efficiency in females of this species, even if pregnant or breastfeeding females were not included in this study. Therefore, additional research is required to comprehensively comprehend the disparities in digestive efficiency between sexes in gazelles and other ungulates, as well as its association with sexual dimorphism.

The variable of age was also examined as an individual factor. Faecal nitrogen levels rose as individuals aged, while fibre levels remained unchanged. This outcome diverges once further from the one documented in red deer. In the aforementioned species, a decline in fN was seen as individuals aged, indicating an increase in efficiency. Additionally, alterations in faecal fibres were noted. Typically, the protein needs of ruminants decrease as they get older (Owens and Bergen, 1983), which appears to be the most straightforward reason for the observed increase in fN in our study.

The primary objective of this study was to examine the variations in faecal nutrients among species that share similar ecological traits, while being subjected to a controlled feeding regime. The observed variation in digestive efficiency among the studied species was evident in the case of fN. However, the impact on faecal fibres was found to be weak, as indicated by the low R<sup>2</sup> values in the models. Conversely, faecal fibres were found to be strongly influenced by diet quality, which was consistent across all three species. Therefore, the emphasis was placed on the outcomes achieved for fN.

Dorcas and dama gazelles exhibited similar characteristics, however Cuvier's displayed lower values, indicating a reduced digestive efficiency in this particular species. The higher fNDF provides additional evidence in comparison to the other species. This result may be attributed to the reproductive performance of Cuvier's gazelles, as previously mentioned. Undoubtedly, the dama gazelle would have exhibited the lowest fN. The retention duration, a species-specific measure influenced by body mass (Van Soest, 1996), is closely linked to digestive efficiency. This relationship enables larger species to endure with food supplies of lower quality (Clauss and Hummel, 2005). Although the three species are classified as browsers with some ability to choose their diet, dama gazelle exhibits a greater inclination towards grazing (Groves et al., 2015), which could potentially account for the variations in digestive efficiency reported among

the species. Water requirements could possibly be an additional ecological element that accounts for the observed outcomes. Certain species or individuals may have employed diverse tactics in water utilisation, varying the degree to which they combine it with water derived from food. This approach is frequently adequate for gazelle species in their natural habitat to meet their requirements. Cuvier's gazelle has the highest water requirements among the three species. Given that the majority of these needs are met by the water content of plants in their natural habitat, it can potentially impact the selection of the natural diet. Hence, it is plausible that the species exhibits a preference for the protein content of the plants over the water, perhaps leading to the observed decline in digestive efficiency.

## **6.2. Insights From Free-Ranging Animals**

### **6.2.1. Free-ranging roe and red deer show no difference in faecal nutritional constituents by NIRS**

Despite numerous environmental, species-specific, and animal-specific factors (susceptible to continuous spatiotemporal changes) directly or indirectly related to red and roe deer diet selection, the hypothesis of different winter faecal nutrients was not supported. Four faeces nutritional components clustered on two axes were utilised to compare roe and red deer, but no difference was found. Animals depend heavily on nourishment, since fibrous components accounted 48% of variance. In the Czech Republic and elsewhere, winter supplementary feeding of large animal herbivores is a popular management practice to promote healthy populations, productivity, and trophy sizes. Hunters in the research region provided that, although minimal amounts given cervid density. Consequently, a significant effect was not anticipated; in fact, all prior investigations in this field furnished insights into this methodology. Given that both species' nutritional outputs are practically the same, which is mostly due to food quality, the role of that extra feed should be examined.

Long retention times should benefit ungulates in hard winters. Ruminants with higher body mass have larger relative gut fill, which increases mean retention time (Demment and Van Soest, 1985; Illius and Gordon, 1992; McNab, 2002). Browsers like roe deer have smaller digestive tracts and shorter retention durations than grazers or intermediate feeders (red deer have three times more rumen per body weight than roe deer; Prins and Geelen, 1971). Interspecific



differences also increase fibrous forage tolerance (Hofmann and Stewart, 1972; Hofmann, 1989; Clauss and Lechner-Doll, 2001; Clauss et al., 2003c). Since ungulates must spend more energy avoiding hunting and predation, this benefit is greater. Movement and stress might raise maintenance needs 200–300% (Weiner, 1977). Thus, huge ungulates must choose between refuge and nourishment. Due to its body size and morpho-physiological traits, red deer should be better at balancing that trade-off than roe deer. When animals must rely solely on natural feed sources, roe deer behaves as a typical browser, choosing high-protein diets and avoiding high-fibre diets. Due to the species' morpho-physiology, increased energy demands (e.g., winter heat production) lower locomotor activity and enhance hunting and predation. When more carbs are available as a supplementary feed, roe deer may ingest more since they are more vital than red deer. Thus, supplemental feed may assist both species, especially roe deer, survive the winter while eating on low-nutrition plants (Davis et al., 2016), resulting in more comparable diets and faeces.

Predators and hunting pressure affect the distribution, habitat preference, and nutritional ecology of roe and red deer in the study area, supporting the previous explanations. The area's major predator, the Eurasian lynx (*Lynx lynx*), relying on roe deer and young red deer (Heurich et al., 2012). Red deer are hunted more due of their appealing trophies, but both species are under risk. Stress may cause both species to dedicate less time to search for food and ingestion, and compensate with extra feed relatively easily accessible. Roe deer prefer supplementary feeders on forest and meadow margins in the research region (Heurich et al., 2015). This same study found that Bohemian forests roe deer prefer unprotected regions despite the substantial impact of shooting over protected parts of the park due to supplementary feeding by hunters outside the park. To the contrary, red deer prefer deep forests with 70% cover (Heurich et al., 2015). Red deer can exploit the winter shrub plant cover in certain regions. Red deer likely rely more on this resource than roe deer due to their big rumens requiring a particular fibre threshold (Bauchop, 1979; Gebert and Verheyden-Tixier, 2008), resulting in reduced dependency on additional diet.

Supplemental feeding, hunting, and predation are probably the main causes of both species' nearly comparable faeces production. That doesn't mean the two species' food composition or natural food quality were similar; rather, NIRS was unable to detect the protein source. In the captive red deer experiment, the inability to detect ruminal N made fN interpretation

challenging. N bound to ADF in faeces can be used to determine metabolic faecal nitrogen (MFN) and dietary N (Van Soest, 1994).

### **6.2.2. Determining dietary patterns of free-ranging Andean bear using NIRS**

Neither bromeliad availability nor nutritional value seemed to affect bear presence or food selection. Contrary to expectations, Andean bears were nearly entirely in the forest habitat and not in páramo during the study. The investigated bromeliads' nutritional value varied by habitat. It is widely known that all Ursidae prefer fruitful areas (Schoen, 1990). Bromeliads in the *páramo* were more nutritious than those in the woodland habitat, yet bears were most commonly found in the woodland areas. This suggests that factors other than the immediate nutritional value of bromeliads might influence the habitat preferences of Andean bears. Bromeliads are epiphytic in forests but terrestrial in páramo, hence their nutritional composition may vary due to their tactics.

Fibre content, which was higher in forest bromeliads, should be prioritised. Foods high in fibre have little nutritional value because herbivorous mammals like bears lack enzymes to degrade fibre. To meet their nutritional needs, they can form a symbiosis with cellulose-hydrolyzing microbes in their gut. Bacteria families *Lachnospiraceae* and *Ruminococcaceae* discovered in Andean bear faeces indicate their gut microbiota may break down cellulose, hemicellulose, and lignocelluloses (Borbón-García et al., 2017).

Ecosystem changes altered bromeliad nutrition. Fire negatively impacts bromeliads (Rocha et al., 2004), as shown by this study's decreased fat, fibre, ADF, and NDF. However, burned bromeliads have more starch, which should boost their nutritional value for an omnivorous forager like the Andean bear. Ariani et al. (2004) suggest that the plant's water may help protect the meristem, which stores soluble carbohydrates (Paisley, 2001; Rivadeneria-Canedo, 2008), and aid in vegetation recovery after a fire.

Andean bears may change habitats due to food source changes (Cuesta et al., 2003). Andean bears use *páramo* and forest settings year-round, however their frequency varies. Both environments had bromeliads, but contrary to our assumptions, bears were unaffected by their nutritional content or quantity. This shows that bromeliads were not a major food source during our study period, as in prior studies in this area (Ontaneda & Armijos, 2012). The relative

homogeneity of forest research locations may help explain that outcome. Seasonal fluctuations in bromeliad nutrition, not ecological factors, may explain this outcome and warrant more study. In the vibrant tapestry of ecological research, the application of NIRS in this study stands out not only for its exploration into the dietary habits of wildlife but also for its application in analyzing plant material, a notable pivot from its use in evaluating faecal samples in other studies. This innovative approach underscores the versatility of NIRS, offering unprecedented insights into the complex interplay between fauna and flora within uncontrolled natural settings. By examining the nutritional content of bromeliads across different habitats, NIRS reveals the nuanced dietary strategies of Andean bears and their remarkable adaptability to ecosystem changes, such as those induced by fire.

### **6.3. Brief General Discussion**

An important step forward in ecological study has been the use of near-infrared reflectance spectroscopy (NIRS) in the quest to understand the herbivorous ungulates' nutritional ecology. Dietary preferences, physiological adaptations, and environmental consequences have been revealed by NIRS's painstaking integration of controlled research in captivity with the dynamic uncertainty of the wild. Dryden (2003), Tolleson et al. (2005), and González et al. (2018) conducted groundbreaking studies on ungulate species in controlled environments. These studies proved that NIRS was accurate in detecting dietary subtleties and highlighted the importance of these types of studies in identifying factors that affect ungulate diets. Insights into the role of age, sex, reproductive state, and seasonality in ungulate nutrition ecology have laid the groundwork for subsequent studies in ecological and conservation biology. After examining the dietary patterns of Andean bears and free-ranging roe and red deer, the application of NIRS in the wild has broadened our understanding of its capabilities and limitations in the face of the capriciousness of nature. Carefully planned research objectives are crucial when using NIRS in uncontrolled environments due to the presence of unexpected dietary overlaps and complex challenges caused by environmental changes (e.g., Kamler et al., 2004; Kamler & Homolka, 1995; Corlatti, 2020), such as forest fires influencing the diet of Andean bears. Transforming the conversation to a more systemic level, NIRS is an essential tool for group investigations into intricate ecological systems, helping us make sense of things like

species richness, trophic relationships, and the dynamics of plant communities (Landau et al., 2021).

Studying animals in captivity and in their natural habitats side by side highlights the necessity of a holistic strategy that takes into account the complex character of ecological systems. In order to fully utilise NIRS, which can provide invaluable information about herbivores' dietary patterns and nutritional status, these types of research are essential. This, in turn, can improve species management in their natural habitats and inform more targeted conservation efforts. In order to preserve our natural legacy for future generations, it is crucial to use extensive analysis and predictive modelling in this effort to integrate insights for large herbivore conservation and management. Schimann et al. (2007), Serrano et al. (2020), and Charmley et al. (2023) all agree that keeping herbivorous ungulates and their ecosystems healthy and diverse for future generations is our shared responsibility.

## 7. CONCLUSIONS AND FUTURE PROSPECTS

This dissertation has advanced our understanding of the nutritional ecology of large herbivores, particularly ungulates, through the adept application of near-infrared reflectance spectroscopy (NIRS). It bridges the gap between controlled environments and the multifaceted dynamics of the wild, underscoring the technology's invaluable role in unraveling the complex dietary preferences and nutritional strategies of these species. Through meticulous research spanning red deer, gazelles, roe deer, and bear, this work unveils a landscape rich in dietary variability, intricately woven from individual, physiological, and environmental threads.

At the heart of our findings is the validation of NIRS as an important tool for exploring ungulate diet and nutrition. However, our journey has underscored the critical need for comprehensive preliminary studies in controlled settings to refine this innovative technology. Such preparatory efforts are crucial for isolating the myriad factors - particularly those unique to each species - that influence the interpretation of data from free-ranging animal and wild populations. This preliminary phase is essential not just for honing NIRS calibration models but also for developing effective sampling strategies and improving the analysis of wild populations.

Moreover, our investigations spotlight the urgent necessity for further controlled experiments involving captive animals. By examining a wider array of species in such conditions, we can better assess how faecal indices detected by NIRS can serve as reliable indicators for extensive studies in natural habitats.

In sum, this dissertation not only contributes profoundly to the scholarly discussion on ungulate nutritional ecology but also lays the groundwork for future exploration in this field. Leveraging NIRS technology, this research not only fills existing knowledge voids but also heralds the advent of innovative conservation tactics aimed at supporting the sustainability of ungulate populations. Looking forward, the ongoing refinement of NIRS, paired with a holistic approach to studying wildlife, will be crucial for dissecting the intricacies of ecological systems and safeguarding natural legacy. The promise of NIRS in contributing to a deeper understanding of ungulate nutritional ecology and the broader ecological implications stands as a testament to the power of integrating novel technology with ecological research, highlighting a path toward more informed and effective conservation strategies in the complex web of natural ecosystems.

Key future prospects:

- Developing methodologies to accurately assess metabolic faecal nitrogen (MFN), aiming to surmount the present constraints in detecting protein sources. This refinement is crucial for improving the diagnostic precision of NIRS, such as in the context of supplementary feeding practices or (nutritional) habitat alteration by forest fire.
- Enhancing the application of NIRS in wildlife conservation and management by establishing it as a routine analytical tool. This advancement will enable conservationists to formulate more targeted interventions, underpinned by detailed insights into the dietary composition and nutritional status of wildlife populations.
- Integrating NIRS with traditional conservation strategies, thereby crafting a holistic approach that addresses the dietary needs of ungulates amidst escalating human-induced environmental changes.
- The future of ungulate conservation and management lies in the innovative application of technologies like NIRS to accurately assess and process large amounts of data, coupled with a deepened understanding of processes determining herbivores nutritional ecology. As we progress, the adoption of tracking technologies and the continuous refinement of analytical methodologies will be critical in developing comprehensive and scientific-based conservation policies.

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## 9. SEPARATE APPENDICES

9.1. Čupić, S., García, A.J., Holá, M. and Ceacero, F., 2021. Evaluation of factors inducing variability of faecal nutrients in captive red deer under variable demands. *Scientific Reports*, 11(1), 2394.

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# Evaluation of factors inducing variability of faecal nutrients in captive red deer under variable demands

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Based on the assumption that dietary and faecal nitrogen correlate, the number of studies using faecal samples collected in the wild to understand diet selection by wild herbivores and other ecological patterns has been growing during the last years, especially due to the recent development of cheap tools for analysis of nutrients like Near-Infrared Reflectance Spectroscopy (NIRS). Within the annual reproductive cycle, cervids (members of the family Cervidae) face strong seasonal variations in nutritional demands, different for hinds (gestation and lactation) and stags (antler growth) and reflected in differential patterns of seasonal diet selection. In this study we aimed to quantify how pasture availability, season and individual factors like sex, age, reproductive status, body mass and body condition affect faecal nutrients in captive red deer with the goal of understanding how these factors may influence the interpretation of results from samples obtained in the wild with little or no information about the animals who dropped those faeces. We used NIRS for analysing nitrogen, neutral and acid detergent fibres in faeces. The relative influence of some individual factors like pregnancy was low (around 4%), while age and weight may induce a variability up to 18%. The presence or absence of pasture contributed to a variability around 13%, while the season contributed to an average variability around 17% (and up to 21% in certain situations). This high variability in faecal nutrients was observed in a controlled setting with captive animals and controlled diets. Thus, in natural situations we suspect that there would be even greater variation. According to the results, we recommend that preliminary research with captive animals of the species of interest should be conducted before collecting samples in the wild, which should help in the interpretation of results.

Faecal samples are commonly used to estimate the nutritional value of the diet<sup>1</sup>. The nutritional composition of feces is related to the quality of the ingested diet<sup>2,3</sup>, and thus faecal N is commonly used as an index of dietary quality in nutritional ecology studies<sup>4,5</sup>. On the other hand, knowing the nutritional value of the available food, the analysis of faecal samples may reveal the digestive efficiency of an animal; i.e., sometimes high FN may not mean high diet quality but low digestive efficiency. For example, low quality diet may wash down the rumen microbiome and lead to a higher faecal. However, this fact is surprisingly not considered in most studies. The content of neutral detergent fibre (NDF) in forage and faeces may bring some light about it, since fibre fractions inform about feed quality of ungulates<sup>6</sup> and influences their performance<sup>7</sup>. However, in studies in the wild researchers often have little or no information about the individuals who deposited a sample, and sources of variability related to it. Moreover, food quality and availability are constantly changing, which makes difficult to accurately interpret faecal nutrient values. On the contrary, working with semi-captive or captive animals ensures that diets are controlled, and variations of feed quality are less pronounced, allowing to study the confounding factors affecting faecal nutrients. Furthermore, handling and collection of accurate individual data is easier.

The red deer (*Cervus elaphus*) is a versatile feeder in Mediterranean habitats<sup>8</sup>, with grasses being commonly consumed when the availability is high from late autumn to early summer. In southern Europe during the summer, when grasses are scarce, deer mostly become browser<sup>9–13</sup>. Different seasonal nutritional demands by hinds

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and stags (driven by different physiological needs) also affect the differential seasonal use of resources between sexes, as well as by young animals<sup>14,15</sup>.

The species is also a seasonal breeder. Hinds enter oestrus between September and February<sup>16</sup>. Gestation and lactation place the greatest nutritional demands on females<sup>17</sup>, and thus, births occur in the period with greater food availability<sup>18</sup>. During this period, growing mothers (often primiparous ones<sup>19</sup>) have higher nutritional requirements since they have to invest energy both for reproduction and for their own growth. Births occur around May and lactation demands are maximum during the first month, decreasing thereafter and becoming low around September<sup>20</sup> when the new reproductive cycle starts. In males, antler growth is nutritionally expensive<sup>21</sup> with increased nitrogen demands<sup>22</sup>. However, the period of high nutritional demands in males (early stages of antler development) is not synchronized with that of females (gestation and lactation).

This study aims to examine how the faecal nutrients vary in captive Iberian red deer with very similar feeding regime along the seasons (connected to their annual reproductive cycle and physiological status), and how sex, age, reproductive status, pasture availability, and body traits affect the observed seasonal variations in faecal nutrients, in order to highlight the variability explained by these factors and discuss the adequacy of the technique in studies in the wild when this information is missing.

## Materials and methods

This study was carried out at the experimental deer research facilities of the University of Castilla-La Mancha in Albacete (Spain) during four sampling trials (thereafter *Seasons*) distributed along the reproductive cycle of the species: February (late gestation), May (births), July (mid-lactation) and September (late lactation and rut), during 2017. Forty-three calves and yearlings, 23 stags ranging from 3 to 7 years of age, and 30 hinds were studied, out of which 22 were pregnant. The hinds ranged between 3 and 21 years of age, and were divided into three categories: subadults (aged <4 at mating; n = 11), adults (aged 4–15 at mating; n = 12), and senescent (aged >15 at mating, n = 7). All the animals used in this experiment (except one founder female) were born in captivity. Animals were kept in seven 1-ha paddocks, either with bare soil or with irrigated pasture based on tall fescue (*Festuca arundinacea*, 52.4%), cocksfoot (*Dactylis glomerata*, 28.6%), lucerne (*Medicago sativa*, 14.3%), and white clover (*Trifolium repens*, 4.8%). All paddocks used were similar in size and kept a similar (low) density of animals. Irrigation and adequate densities ensured that the pasture quality varied insignificantly along seasons. Animals were assigned to different paddocks and rotated according to management needs (i.e., a given animal could be in a pasture with paddock in one season, but in one with bare soil in another season). All animals were ad libitum fed a Total Mixed Ration (TMR), in order to avoid strong competition for feed<sup>23</sup>. TMR tried to imitate the transition of food in natural conditions, and the ratio of feedstuffs included in the TMR (based on oats, barley, alfalfa meal, cereal straw and citrus pulp) was changing according to the animals' different nutritional requirements along the year. Stags were also supplemented during all seasons except September with a small daily amount of high protein pellets. Water was provided ad libitum for all animals.

**Data collection and processing.** Faecal samples were collected directly from the rectum during routine handling in indoor handling premises<sup>24</sup> and afterwards stored in labelled paper bags. Routine handling occurs every week, being every animal handled every second week. Thus, samples from each studied animal were collected within two handlings (i.e., all animals were sampled within 7 days during each season). Simultaneously, body mass and body condition score<sup>25</sup> were recorded. In order to achieve uniformity of the faecal samples, they were dried to a constant weight in a hot air dryer at 40 °C for 48 h. Afterwards, they were ground with a mill to pass through a 1-mm sieve. Thereafter the samples were thoroughly mixed to achieve maximum homogeneity and dispensed into sample cups for analysis through near-infrared reflectance spectroscopy (NIRS). Four subsamples of the TMR, and feed pellets were obtained for each season. Feed samples were similarly ground (but not dried) and frozen until further analysis (n = 4 for each feedstuff and season).

All the samples were scanned with the NIRS DS 2500 FOSS analyser under the ISIScan Routine Analysis Software (Foss, Hillerød, Denmark). For studies based on faecal samples, an extensive sampling of feed and faeces is inevitably needed. Traditional wet-chemistry methods are time-consuming and expensive, and thus, NIRS technology has become a widely used method that overcomes these drawbacks and allows rapid, low-cost, chemical-free, and non-destructive analysis of a large number of samples<sup>26</sup>. Thus, the technique is already commonly used for measuring food quality through faecal indices in ungulates<sup>1,27,28</sup>. The analyser automatically calculates the average of 8 successive scans at a resolution of 0.5 nm which gives the spectrum of each sample, recorded as the logarithm of the reciprocal of reflectance (amount of radiation reflected from the sample<sup>29</sup>). The software displays the curve of the reciprocal logarithm of reflectance and a curve of absorbing component in a close-to-linear relationship. The peak values of the two curves occur at wavelengths that correspond to absorption bands in the sample (i.e., lower reflectance<sup>30,31</sup>). By this method, the contents of fN (faecal Nitrogen), fNDF (faecal Neutral Detergent Fibre), and fADF (faecal Acid Detergent Fibre) were calculated with WinISI 4 Calibration Software (Foss, Hillerød, Denmark) according to a calibration set previously developed for red deer faecal samples<sup>32</sup> based on 100 samples, and showing very high predictive power  $R^2 > 0.98$ . TMR calibration from the *Ruminant Feed Package* (Foss, Hillerød, Denmark) was used for every set of subsamples, and mean value was calculated. All the values obtained with these calibrations showed adequate GH (distance to the population average) and NH (distance to the closest sample) values.

Protein, ADF and NDF in pellets for stags were analysed through standard wet chemistry methods in a specialized lab<sup>33</sup>. Pasture was not collected for this study, since it has been analysed for several previous studies in the experimental facilities. The nutritional composition of pasture (average from previous analyses), TMR, and pellets is shown in Table 1.

	Pasture	Feed pellets*	TMR February	TMR May	TMR July	TMR September
Protein (%)	19	20.264	13.052	16.186	15.791	14.024
Crude Fibre (%)	22	–	–	–	–	–
ADF (%)	–	14.946	43.918	38.896	35.587	43.415
NDF (%)	–	36.879	66.819	58.904	54.291	57.095

**Table 1.** Nutritional composition (dry matter) of the supplemented total mixed ration (TMR), feed pellets and pasture used along the study. \*Supplemented only to stags during February, May, and July.

Sampling frequencies and handling procedures were designed to reduce the stress of the animals, according to the European and Spanish laws and current guidelines for ethical use of animals in research<sup>34</sup>, and Spanish and European guidelines and laws in the use of animals in research. The research protocols were approved by the Committee of Ethics in Animal Experimentation from the University of Castilla-La Mancha.

**Selected faecal indices.** fN is an important parameter for research on diet quality since it shows positive linear relationship with dietary nitrogen<sup>33,35–37</sup>. fN is also stable for few weeks post-defecation in well preserved samples<sup>38</sup>. However, other authors warn about its limitations because the presence of secondary metabolites like tannins decrease protein digestion in ruminants<sup>39,40</sup>, which may lead to misinterpretation of diet quality based on fN.

Fibre fractions (NDF and ADF) are among the faecal constituents that inform about food quality of ungulates<sup>36</sup>. The faecal fibre fractions (fADF and fNDF) are sensitive to fluctuations in food quality, so they should be included to support fN as a proxy for nutritional analyses, especially when diets are expected to contain high amounts of tannins<sup>36</sup>. NDF (which consists predominantly of hemicellulose, cellulose and lignin) reduce voluntary food intake when in high dietary levels. ADF is a subset of NDF which includes the least digestible compounds for herbivores: lignin, cellulose and cutin. As the content of ADF in a diet increases, digestibility and available energy decrease, while, on the other hand, sufficient fibre levels are required in the diet to maintain normal rumen function<sup>6</sup>.

**Statistical analysis.** Analyses were performed using IBM SPSS Statistics (version 25.0 for Windows, IBM, USA). Since factors affecting faecal nutrients are different among sex classes, three datasets were created and analysed individually: stags, hinds, and young (calves + yearlings).

General Linear Mixed Models (GLMM; normal distribution with identity link) were used for each dataset. Normality of the variables used for each of the models were previously confirmed by Kolmogorov–Smirnov tests. A data structure based on ID as subject and Season as repeated measure was used. Season, Age (young and stags' datasets), Age Class (hinds' dataset), Reproductive status (hinds' dataset), Pasture, Body Mass, and Body Condition Score entered the model as fixed factors; fN, fNDF and fADF were the response variables of the models (response variables). For hinds it was possible to use both variables Age and Age Class; thus, preliminary models for the three faecal nutrients were created using the whole set of independent variables plus Age or Age Class, and the models with Age Class had always lower Corrected Akaike's Information Criterion (AICc). Thus, Age Class was used in the models for hinds. Absence of multicollinearity was tested for the independent variables used through the Variance Inflation Factor, which was always low. Initial models were built as described, without including interactions, and were subsequently solved through a stepwise backward selection procedure. Thereafter, a number of interactions were identified as potentially interesting for at least one of the datasets: Reproduction\*Age Class, Reproduction\*Body Mass, Reproduction\*Body Condition Score, Reproduction\*Pasture, Reproduction\*Season, Season\*Age Class, Season\*Body Mass, Season\*Body Condition Score, Season\*Pasture and Season\*Sex. Since the sample size did not allow to include all of them at once in the models for any of the datasets, each one was individually tested together with the variables previously described. Only those interactions significant in these preliminary models ( $p < 0.05$ ) were selected for further analyses. For hinds, the selected interactions were: Reproduction\*Pasture ( $p = 0.018$ ), Reproduction\*Season ( $p = 0.017$ ), Season\*Body Mass ( $p < 0.001$ ) and Season\*Body Condition Score ( $p = 0.013$ ) for fN; Reproduction\*Season ( $p = 0.048$ ), Season\*Age Class ( $p = 0.021$ ) and Season\*Body Mass ( $p < 0.001$ ) for fNDF; Reproduction\*Pasture ( $p = 0.009$ ), Reproduction\*Season ( $p = 0.003$ ), Season\*Body Mass ( $p = 0.001$ ) and Season\*Pasture ( $p = 0.003$ ) for fADF. For males, Season\*Age was selected for fN ( $p = 0.004$ ) and fNDF ( $p = 0.003$ ), while no interaction was selected for fADF. For calves, only Season\*Sex was selected for fADF ( $p = 0.045$ ), while no interaction was selected for fN and fNDF. New GLMMs were built including these selected interactions, and solved as previously indicated. Thus, for each dataset and response variable three models were built: full initial model without interactions (with no backward selection procedure implemented), solved initial model (with backward selection but without interactions), and solved model including the previously selected interactions. AICc was used for selecting the most plausible models. Thereafter, only the selected models are shown and discussed.

Finally, the coefficients of variation for each faecal nutrient and dataset was calculated in order to understand the variability of the data collected. Thereafter, and considering the mean values for each variable, ranges and the coefficients obtained in the GLMMs, the variability induced by each significant independent variable studied was also calculated for each faecal nutrient and dataset.

	Hinds	Stags	Young
<b>fN</b>			
Initial model	40.678	2.457	-43.495
Solved model (without interactions)	<b>33.940</b>	<b>-17.858</b>	<b>-62.797</b>
Solved model (with interactions)	37.381	*	*
<b>fNDF</b>			
Initial model	503.869	266.304	505.405
Solved model (without interactions)	531.816	303.811	513.589
Solved model (with interactions)	523.144	280.607	*
<b>fADF</b>			
Initial model	467.402	254.428	508.482
Solved model (without interactions)	481.230	289.930	515.968
Solved model (with interactions)	453.708	*	498.698

**Table 2.** Corrected Akaike's Information Criterion (AICc) values of each of the three models prepared for each dataset and faecal nutrients (nitrogen—fN; neutral detergent fibre—fNDF; acid detergent fibre—fADF). The selected models, those with lower AICc value, are highlighted in bold. Only these models are described and discussed. \*The solved model was the same as the solved model without interactions.

## Results

For each dataset (hinds, stags and young), one model was selected for each dependent variable according to AICc (Table 2). The significant variables in the selected models (Table 3) are described below.

**Faecal nutrients in hinds.** The fN was lower in hinds under reproductive constraints ( $t = -2.359$ ,  $\beta = -0.119$ ). Hinds living in paddocks with pasture had higher fN ( $t = 4.549$ ,  $\beta = 0.399$ ). Body mass also had a negative effect on fN ( $t = -3.693$ ,  $\beta = -0.006$ ). Season was an important source of variability (Fig. 1): fN was significantly higher in February compared to July ( $t = 2.199$ ,  $\beta = 0.280$ ,  $p = 0.030$ ), in February compared to September ( $t = 2.941$ ,  $\beta = 0.366$ ,  $p = 0.004$ ), in May compared to July ( $t = 8.488$ ,  $\beta = 0.506$ ,  $p < 0.001$ ), and in May compared to September ( $t = 11.039$ ,  $\beta = 0.592$ ,  $p < 0.001$ ).

The fNDF was higher in heavier hinds ( $t = 2.664$ ,  $\beta = 0.109$ ). Hinds in paddocks with pasture had lower fNDF ( $t = -4.858$ ,  $\beta = -6.134$ ). Adult hinds had higher fNDF than subadults ( $t = 2.151$ ,  $\beta = 1.342$ ,  $p = 0.034$ ) and senescent ( $t = 2.453$ ,  $\beta = 1.879$ ,  $p = 0.016$ ). Season was also a source of variability (Fig. 2): fNDF was significantly higher in July compared to February ( $t = 3.846$ ,  $\beta = 9.102$ ,  $p < 0.001$ ), in July compared to May ( $t = 6.086$ ,  $\beta = 7.125$ ,  $p < 0.001$ ), in July compared to September ( $t = 5.415$ ,  $\beta = 4.968$ ,  $p < 0.001$ ), and in September compared to May ( $t = 2.542$ ,  $\beta = 2.157$ ,  $p = 0.013$ ).

The fADF was lower in hinds under reproductive constraints ( $t = -2.171$ ,  $\beta = -1.392$ ). As for fNDF, adult hinds had higher fADF than subadults ( $t = 2.123$ ,  $\beta = 1.268$ ,  $p = 0.037$ ) and senescent ( $t = 5.530$ ,  $\beta = 4.038$ ,  $p < 0.001$ ), and subadults had higher fADF than senescent ( $t = 4.008$ ,  $\beta = 2.771$ ,  $p < 0.001$ ). Contrary to fN and fNDF, season was a weaker source of variability (Fig. 3): even if it was overall significant, no pairwise comparison was significant itself. This is probably due to the significant interaction *Season\*Pasture*. When the animals were in paddocks with pasture, significantly lower fADF was found in September compared to all the other seasons; on the contrary, in bare-soil paddocks the fADF was higher in September compared to February and May. The interaction *Season\*Body Mass* showed a significant positive correlation of body mass and fADF in February, but a significant negative correlation between them in May.

**Faecal nutrients in stags.** The fN was lower in older stags ( $t = -2.993$ ,  $\beta = -0.051$ ). Stags living in paddocks with pasture had lower fN ( $t = 2.001$ ,  $\beta = 0.193$ ). Body condition had a positive effect on fN ( $t = 2.037$ ,  $\beta = 0.159$ ). Season was also a source of variability (Fig. 1): fN was significantly higher in May compared to February ( $t = 4.426$ ,  $\beta = 0.280$ ,  $p < 0.001$ ), and September ( $t = 4.494$ ,  $\beta = 0.302$ ,  $p < 0.001$ ), and in July compared to February ( $t = 2.151$ ,  $\beta = 0.157$ ,  $p = 0.036$ ), and September ( $t = 2.888$ ,  $\beta = 0.178$ ,  $p = 0.006$ ).

The fNDF was higher in older stags ( $t = 2.305$ ,  $\beta = 1.104$ ) and marginally lower in heavier ones ( $t = -1.980$ ,  $\beta = -0.066$ ,  $p = 0.054$ ). Season was an important source of variability (Fig. 2): fNDF was significantly higher in September compared to February ( $t = 4.962$ ,  $\beta = 7.544$ ,  $p < 0.001$ ), May ( $t = 2.467$ ,  $\beta = 4.057$ ,  $p = 0.018$ ) and July ( $t = 2.389$ ,  $\beta = 3.538$ ,  $p = 0.021$ ), higher in July compared to February ( $t = 2.547$ ,  $\beta = 4.006$ ,  $p = 0.014$ ), and higher in May compared to February ( $t = 2.291$ ,  $\beta = 3.488$ ,  $p = 0.027$ ).

The fADF was higher in older stags ( $t = 2.752$ ,  $\beta = 1.009$ ,  $p = 0.009$ ) and lower in heavier ones ( $t = -2.391$ ,  $\beta = -0.068$ ,  $p = 0.021$ ). Season was also a source of variability (Fig. 3): fADF was significantly higher in September compared to February ( $t = 4.889$ ,  $\beta = 7.102$ ,  $p < 0.001$ ), May ( $t = 3.691$ ,  $\beta = 5.248$ ,  $p = 0.001$ ) and July ( $t = 4.826$ ,  $\beta = 5.922$ ,  $p < 0.001$ ).

**Faecal nutrients in young.** The fN was higher in calves compared to yearlings ( $t = 7.620$ ,  $\beta = 0.406$ ). Season was also a source of variability (Fig. 1): fN was significantly higher in May compared to February ( $t = 12.400$ ,  $\beta = 0.541$ ,  $p < 0.001$ ) and September ( $t = 6.740$ ,  $\beta = 0.314$ ,  $p < 0.001$ ), in July compared to February ( $t = 10.081$ ,

	Hinds	Stags	Young
<b>fN</b>			
Age	–	F = 8.956, <i>p</i> = 0.004**	F = 58.061, <i>p</i> < 0.001****
Age class	**	–	–
Sex	–	–	**
Reproduction	F = 5.564, <i>p</i> = 0.021*	–	–
Season	F = 42.374, <i>p</i> < 0.001****	F = 10.842, <i>p</i> < 0.001****	F = 60.695, <i>p</i> < 0.001****
Pasture availability	F = 20.697, <i>p</i> < 0.001****	F = 4.002, <i>p</i> = 0.050*	**
Body mass	F = 13.641, <i>p</i> < 0.001****	**	**
Body condition score	**	F = 4.149, <i>p</i> = 0.046*	**
Season*age	–	**	–
<b>fNDF</b>			
Age	–	F = 5.313, <i>p</i> = 0.026*	F = 0.200, <i>p</i> = 0.656**
Age class	F = 3.667, <i>p</i> = 0.030*	–	–
Sex	–	–	F = 1.210, <i>p</i> = 0.274**
Reproduction	F = 0.549, <i>p</i> = 0.461**	–	–
Season	F = 14.630, <i>p</i> < 0.001****	F = 8.244, <i>p</i> < 0.001****	F = 13.380, <i>p</i> < 0.001****
Pasture availability	F = 23.604, <i>p</i> < 0.001****	F = 0.975, <i>p</i> = 0.329**	F = 6.694, <i>p</i> = 0.011*
Body mass	F = 7.098, <i>p</i> = 0.009**	F = 3.919, <i>p</i> = 0.054**	F = 0.016, <i>p</i> = 0.900**
Body condition score	F = 2.752, <i>p</i> = 0.101**	F = 0.060, <i>p</i> = 0.808**	F = 0.166, <i>p</i> = 0.684**
<b>fADF</b>			
Age	–	F = 7.574, <i>p</i> = 0.009**	F = 19.294, <i>p</i> < 0.001****
Age class	F = 15.446, <i>p</i> < 0.001****	–	–
Sex	–	–	**
Reproduction	F = 4.713, <i>p</i> = 0.033*	–	–
Season	F = 8.475, <i>p</i> < 0.001****	F = 9.642, <i>p</i> < 0.001****	F = 25.756, <i>p</i> < 0.001****
Pasture availability	**	F = 0.273, <i>p</i> = 0.604**	**
Body mass	**	F = 5.715, <i>p</i> = 0.021**	**
Body condition score	**	F = 0.117, <i>p</i> = 0.734**	**
Season*body mass	F = 6.342, <i>p</i> < 0.001****	–	–
Season*pasture	F = 4.906, <i>p</i> = 0.001**	–	–
Season*sex	–	–	F = 2.463, <i>p</i> = 0.050**

**Table 3.** General linear mixed models explaining the content of faecal nutrients in red deer hinds, stags and young (nitrogen—fN; neutral detergent fibre—fNDF; acid detergent fibre—fADF; see Table 2 for more details about the selection process). Dashes indicate variables not included in a certain model. \*\*Indicate variables included in a certain model, but not significant. Statistically significant differences at 0.05, 0.01, and 0.001 are indicated by \*, \*\*, and \*\*\*\*, respectively.

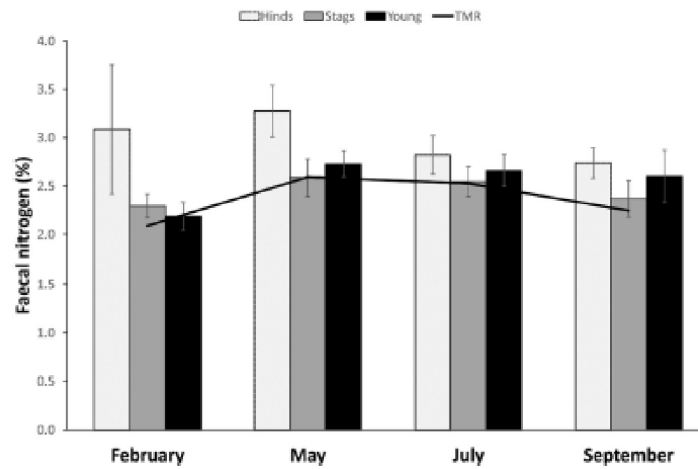
$\beta = 0.474$ ,  $p < 0.001$ ) and September ( $t = 4.957$ ,  $\beta = 0.247$ ,  $p < 0.001$ ), and in September compared to February ( $t = 4.718$ ,  $\beta = 0.227$ ,  $p < 0.001$ ).

The fNDF was lower in young living in paddock with pasture ( $t = -2.587$ ,  $\beta = -6.967$ ). Season was an important source of variability (Fig. 2): fNDF was significantly higher in September compared to February ( $t = 2.872$ ,  $\beta = 2.838$ ,  $p = 0.005$ ), May ( $t = 5.978$ ,  $\beta = 5.597$ ,  $p < 0.001$ ) and July ( $t = 4.355$ ,  $\beta = 3.489$ ,  $p < 0.001$ ), in February compared to May ( $t = 3.131$ ,  $\beta = 2.759$ ,  $p = 0.002$ ), and in July compared to May ( $t = 2.431$ ,  $\beta = 2.107$ ,  $p = 0.017$ ).

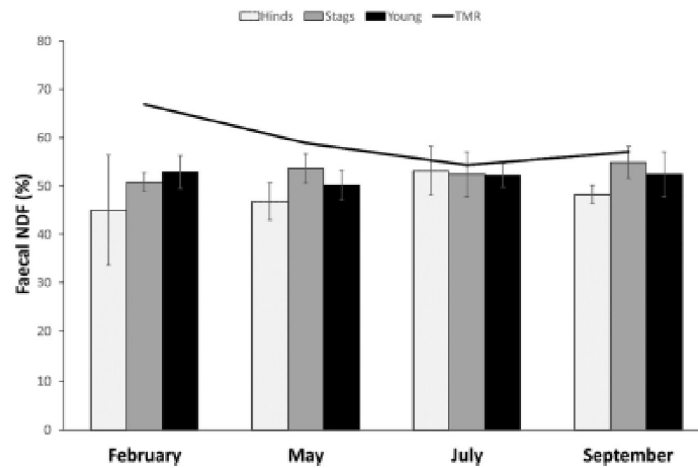
The fADF was lower in calves than in yearlings ( $t = -4.392$ ,  $\beta = -4.082$ ,  $p < 0.001$ ). Season was also a source of variability (Fig. 3): fADF was significantly higher in September compared to February ( $t = 5.076$ ,  $\beta = 3.732$ ,  $p < 0.001$ ), May ( $t = 6.148$ ,  $\beta = 5.223$ ,  $p < 0.001$ ) and July ( $t = 8.511$ ,  $\beta = 6.550$ ,  $p < 0.001$ ), and in February compared to July ( $t = 4.136$ ,  $\beta = 2.818$ ,  $p < 0.001$ ).

**Variability induced by the studied factors on the observed faecal nutrients values.** The coefficients of variation (c.v.) of the faecal nutrients analysed in hinds, stags and young showed low values (most of them below 15%; Table 4). These were much lower in young than in adults (7.3% vs. 12.8% on average), and much higher in February (15.4%) than in the other seasons (9.6%, 9.7%, and 9.2% for May, July and September respectively). On the other hand, fN in hinds and fADF in hinds and stags during February even exceeded a 20% c.v.

According to the average faecal nutrient values recorded and the coefficients ( $\beta$ ) obtained in the models for the significant variables, we calculated the variability induced by them (Table 5). While the relative influence of some factors like pregnancy is low (around 4% variation between pregnant and not pregnant hinds), other individual characteristics like age and weight induce a variability up to 18%; e.g., it can be expected that, even



**Figure 1.** Faecal nitrogen content for red deer hinds, stags, and young along the 4 studied periods (seasons). The line indicates the percentage of nitrogen found in the feedstuff (*TMR* total mixed ration) that animals were provided during the study. Males were supplemented with extra small amounts of 20% protein feed pellets in February, May and July.

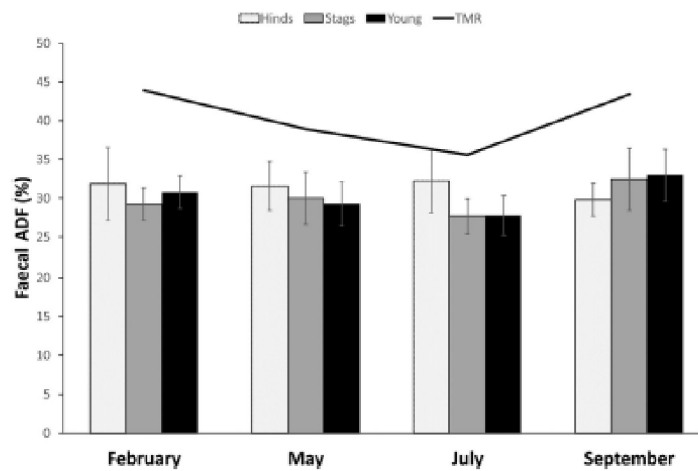


**Figure 2.** Faecal NDF content for red deer hinds, stags, and young along the 4 studied periods (seasons). The line indicates the percentage of NDF found in the feedstuff (*TMR* total mixed ration) that animals were provided during the study.

under the same diet, the heavier and the lighter hinds in a population will vary in their fN values with 16%. The presence or absence of pasture induce a variability around 13%, while the season induce a variability around 17% (on average) and in certain situations up to 21%.

### Discussion

Even under a controlled environment with healthy animals and very similar feeding sources, red deer showed a relatively high variability in the analysed faecal nutrients: fN, fNDF and fADF. This variability jumped in some situations above 25%, and all the individual factors studied (age/age class, body mass, sex, reproductive status, body condition) as well as season and pasture availability significantly contributed to explain such variability in the faecal nutrient content to a certain degree. These results have important implications for the interpretation



**Figure 3.** Faecal ADF content for red deer hinds, stags, and young along the 4 studied periods (seasons). The line indicates the percentage of ADF found in the feedstuff (TMR total mixed ration) that animals were provided during the study.

	Hinds	Stags	Young
<b>fN</b>			
February	21.72	18.64	6.56
May	8.12	13.25	4.97
July	7.09	16.33	6.19
September	5.81	12.60	10.34
<b>fNDF</b>			
February	14.76	13.78	6.75
May	10.01	8.87	9.60
July	12.81	10.56	9.15
September	7.08	8.04	10.15
<b>fADF</b>			
February	25.34	26.08	4.54
May	8.30	17.41	6.03
July	9.52	11.34	4.62
September	4.03	16.36	8.79

**Table 4.** Coefficients of variation (c.v.) of the studied faecal nutrients (nitrogen—fN; neutral detergent fibre—fNDF; acid detergent fibre—fADF) for each dataset (hinds, stags and young) and each sampling period.

of ecological studies based on faecal nutrients conducted in the wild, with low or any information about the individuals being sampled.

Faecal N has been widely accepted to correlate with the N content of the diet<sup>35,36</sup>. However, while faecal N appear to mirror dietary N in young individuals (see Fig. 1), this seems to not always be the case for adults. Stags and hinds are known to adopt different feeding strategies which certainly reflect their physiological adaptations, especially during nutritionally demanding periods: while hinds select for quality, stags opt for a greater amount of poorer quality food<sup>41</sup>. In stags, the variation in fN appears to mirror the seasonal variation in forage nitrogen content in all the four sampling periods even if stags were additionally supplemented with small amounts of high protein feed pellets in all seasons except September. This suggests that the extra protein ingestion was not reflected in the fN. However, this mirroring between dietary and fN observed in stags is more complex than it seems at first glance. Because of their larger size, stags have greater total requirements compared to pregnant or lactating hinds<sup>42</sup>. With their mouths, stags can ingest larger, more fibrous food particles with less nitrogen on a percentage dry weight basis, and they compensate this lower-quality food by eating more relative to their size<sup>41</sup>. High fibre diets decrease the digestibility<sup>43</sup> and increase the retention time<sup>44</sup>, which leads to increased faecal loss of dry matter and N. But also to increased fNDF<sup>43</sup>, which in our study was always higher for stags

	Hinds (%)	Stags (%)	Young (%)
<b>fN</b>			
Reproduction	4.0		
Pasture availability	13.4		
Age		10.4	15.8
Weight	16.3	7.9	
Season	19.8	12.3	21.1
<b>fNDF</b>			
Age Class	3.9		
Pasture availability	12.7		13.4
Age		10.4	
Weight	18.1	14.2	
Season	18.9	14.2	10.8
<b>fADF</b>			
Reproduction	4.4		
Age Class	12.9		
Age		9.5	13.3
Weight	4.0	17.9	
Season	19.3	13.4	21.3

**Table 5.** Variability induced by the individual and nutritional factors studied in the faecal nutrients (nitrogen—fN; neutral detergent fibre—fNDF; acid detergent fibre—fADF; see Materials and Methods for more details about the calculations).

compared to hinds, except in July (probably due to their high lactation demands and increased selectivity of good quality food). It has been reported that when placed on a poor-quality diet, stags did not increase their body mass during the summer which was in contrast to lactating hinds, despite consuming diets of identical quality<sup>41</sup>. Lower contents of N in rumen of stags in winter compared to those of hinds have also been reported<sup>41</sup>. These are consistent results with ours and support lower concentration of fN in February for stags compared to hinds, despite stags being provided feed pellets rich in protein. In our research, stags on pasture had lower fN which supports the hypothesis of increased intake of low-quality food in order to balance the final N intake<sup>45</sup>. Feed pellets seems to be important source for stags to exceed daily maintenance requirements for protein and energy to replenish body reserves and to compensate for the inability to do so from the lower-quality food which they eat abundantly. Alternative explanation is that extra N ingestion was not reflected in the fN due to a high digestive efficiency at least during the three supplemented periods which match with the antler growth period<sup>46</sup>.

Contrary to males, in hinds fN was always higher than expected based on the amount of dietary N. This result might seem surprising since February and May are periods with highest demands because of reproduction<sup>17,21</sup>, and thus, greater digestive efficiency would be expected. On the other hand, from February to May (late pregnancy) red deer hinds tend to decrease the volume of rumen<sup>47</sup>, which means decreased food intake. This may have led to a greater selectivity for the feedstuff items with greater protein content<sup>23</sup>. Lowest ingestion by hinds from February until the approximate time of parturition has been reported<sup>47</sup>, indicating higher selectivity for good-quality food. After parturition, demands for nutrients associated with lactation rise<sup>48</sup> which is followed by increased weight and size of the rumen, abomasum, intestines, and liver<sup>49,50</sup>. Large herbivores tend to eat more and increase body mass during the summer months to support breeding energy expenses<sup>43</sup>. Furthermore, after weaning hinds remodel the digestive tract and increase rumination<sup>51</sup> which may have enhanced their ability to extract nitrogen from their forage. Reindeer have been reported to have increased digestibility and food intake during increased lactation demands<sup>52</sup>. Forages with high levels of digestible energy (like high non-structural carbohydrates) generate high rates of fermentation<sup>36,53</sup> therefore, high rates of intake and consumption of high-quality forages result in high values of faecal nitrogen via increased fermentation, absorption of microbial biomass, and bypass of nitrogen<sup>6</sup>. Later on, the elevated protein excretion was not pronounced in July and September as much as in previous seasons, which correspond to the period of normal volume of hinds' rumen<sup>47</sup>.

The difficult interpretation of fN for hinds and stags is mostly associated with the lack of information about concentration of nutrients in rumen. The nutritional value of rumen content would have allowed calculation of undigestible nitrogen that can only be digested by fermentative processes. Analysing N bound to ADF in faecal samples (NDF-N<sup>6</sup>) may be an interesting alternative for future studies. This index allows to calculate metabolic faecal nitrogen (MFN) as fN minus fNDF-N.

Some authors have warned about the influence of secondary metabolites like tannins, which may decrease protein digestibility<sup>39,40</sup>. This factor is unlikely to have affected our results since only common feedstuffs for livestock with low content of plant secondary compounds (PSCs) were used in the study. Other authors have also warned of limitations of fN as dietary index due to adaptations by the deer to relatively poor diets<sup>51</sup>. They showed that lactating hinds have an ability to more thoroughly process the food via mastication, extracting more plant proteins from cell wall surfaces and increasing digestion of finer particles<sup>54</sup>. Also, the ability to remodel the gastrointestinal tract during lactation<sup>50</sup> contribute to increase N absorption, resulting in additional reduction

in N excretion<sup>51</sup>. For these reasons, fN may provide an incorrect impression of the relative quality of the diet. At this stage, it is necessary to highlight that the rumen microbiome and nitrogen recycling into the rumen are factors difficult to control in studies in the wild that may explain the variability in faecal nutrients found in this study (especially fN), but also even the differences in functioning mechanisms among sexes<sup>41</sup>.

Similarly, faecal fibre fractions (fNDF and fADF) are considered to reflect diet quality and to be sensitive to fluctuations in food quality<sup>36</sup>. They are suggested to be analysed in order to support the interpretation of fN, since in studies in the wild it would be difficult to determine the dietary N. Both NDF and ADF reduce voluntary food intake and digestibility<sup>6</sup>. Indeed, as expected, dietary N and fibres in our main feedstuff (TMR) are inversely related in our study (see Figs. 1, 2 and 3). Nevertheless, neither fNDF nor fADF fairly reflected dietary levels: while dietary levels of NDF and ADF varied seasonally, very low variability across seasons and age/sex classes were observed for fNDF and fADF. Similarly to fN, dietary fibres seem to match faecal ones just for calves, but not in adults.

Our results also highlight the influence of several individual factors on fN for each of the age/sex class studied. Further than the previously discussed low fN with high dietary N during antler growth, we found a significant effect of body condition: stags in low condition had lower fN; that is, they were more efficient using the dietary protein. Indeed, we also found a significantly lower fN values in hinds under reproductive constraints (pregnancy or lactation); however, this effect was quite low since we estimated that the variability in fN due to reproductive constraints is just 4% (Table 5). Other studied factors like age also showed sources of variability different for hinds and stags, which further support the previous observations. Age is an important source of variability in stags, around 10% for all fN, fNDF, and fADF, while age class was not a significant factor explaining faecal nutrients in hinds, just around 4% for faecal fibres. In stags, fN was lower in older individuals, which fits with the results previously discussed: while antler investment and requirements increases with age<sup>14,55</sup>, the reproductive investment is relatively constant for hinds, independently of their age.

Our results have important implications for studies using this technique in the wild. Faecal N is commonly used as an index of dietary quality in nutritional ecology studies<sup>4,5</sup>. However, the variability observed for some individual factors like age, body mass, body condition, and reproductive and antler growth constraints can hardly be estimated in faecal samples collected in the wild, and thus may mislead the conclusions drawn from the results obtained without considering these factors. Furthermore, our results also show important influence of the presence of pasture and the season (physiological status). First, as already explained, diet quality determination through faecal fibres in certain situations (if not approached carefully) can be quite inaccurate and induce errors. Moreover, the presence of pasture in our study induced a variability around 13% in fN and fNDF, especially in faecal samples from hinds. The seasonal effect is also evident, being significant for all the studied nutrients and sex/age classes. The variability induced by the season ranged from 10 up to 21% in a set up with relatively stable feeding regime. This fact suggest that one should be cautious when comparing faecal samples from wild animals from different seasons and locations, especially considering the important seasonal variations in the diet selection by the species in the wild<sup>19–12,56</sup>. Further consideration is also necessary for the already highlighted fact of increased fN when diets are rich in tannins or another PSCs<sup>40</sup>: these may be much higher in natural diets, increasing the degree of uncertainty of the results obtained.

Our results also confirm that NIRS is a powerful tool to investigate feeding and nutrition of herbivorous ungulates. However, our results clearly suggest that thorough preliminary studies with the target species of interest under controlled conditions are necessary in order to previously validate the technique and determine the degree how multiple factors (mainly linked to individual characteristics) may affect the interpretation of data obtained from samples collected in the wild. That may help for defining sampling strategies in the wild and to interpret the results obtained. Definitely, the results highlight that more studies with captive animals (other taxa) under controlled conditions are needed to evaluate if faecal indices can be used as a proxy for studies in the wild. It would be also interesting to perform a similar study in the same species in different latitudes: in Mediterranean habitats, summer is commonly the season with low feed availability, while it is winter in temperate climates. That may greatly influence the results in experimental settings similar to ours.

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### Author contributions

S.C. and E.C. designed the study, S.C., A.G. and M.H. collected the data, F.C. and S.C. analysed the data and drafted the manuscript. All authors participated in critical revision and approval of the manuscript.

### Competing interests.

The authors declare no competing interests.

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Communication

### Differences in Faecal Nutritional Components in Three Species of Saharan Gazelles on Standard Diets in Relation to Species, Age and Sex

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**Simple Summary:** The study examines how different factors influence the nutritional content of faeces from three gazelle species, with particular interest in the inter-specific factor. Through the contents of nitrogen and fibre, faeces can tell us about their digestive process. The research focuses on 193 captive individuals of three gazelle species and applied Near InfraRed Spectroscopy technology. The results show that different species have varying faecal nutrient levels. Cuvier's gazelle had lower nitrogen content, suggesting less efficient digestion than other gazelles. Factors like sex and age also played a role, but their effects were not the same for all species. Fibre content, related to diet quality, remained consistent. This study shows that factors affecting faecal nutrients are species-specific.

**Abstract:** Various environmental, individual, and species-specific factors may affect digestive efficiency in wild ruminants. The study of faecal nutritional components is a commonly used technique to understand these effects, assuming that faecal nitrogen and fibre contents reflect the diet's nutritional quality and digestibility. Recent studies have highlighted the relatively high influence of factors like sex, age, weight or body condition on digestive efficiency. This manuscript is focused on the inter-specific variability in faecal nutritional components under the same feeding regime, using three captive populations of closely related gazelles as model species. Faecal samples from 193 individuals were analysed through Near InfraRed Spectroscopy. Species, sex and age influence on faecal nitrogen and fibres (ADF and NDF) were investigated. We found inter-specific differences in the faecal content of the three studied nutritional components. Cuvier's gazelle showed lower faecal nitrogen content, suggesting lower digestive efficiency than dorcas and dama gazelles. Sex and age also had a moderate effect, especially in faecal nitrogen, but these effects were not constant across the three studied species. On the contrary, faecal fibres were highly constant (i.e., dependent on diet quality). These results confirm that individual factors affecting faecal nutritional components are also species-specific.

**Keywords:** body size; digestive efficiency; feeding ecology; fibre; nitrogen



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#### 1. Introduction

Animal feeding ecology is a complex field due to the numerous factors affecting it and the countless interactions among them. These can be divided into environmental (habitat-specific), individual (animal-specific) and species-specific (morpho-physiological) factors [1]. Various techniques are used to understand the feeding ecology of wild species. Among them, the study of faecal nutritional components has been an essential approach

to studying the nutritional quality of animal diets for decades, herbivorous mammalian species in particular [2], under the assumption that faecal nitrogen (fN) and fibre (acid detergent fibre (fADF), and neutral detergent fibre (fNDF)) contents reflect dietary ones [3,4], and thus food quality can be estimated. Even if this view is widely accepted [2,5], other studies have suggested that fN measures feed digestibility [6]. Nevertheless, under controlled settings with fixed and equal diets, individual and specific differences in food digestibility arise, informing about digestive efficiency [1]. For samples collected in the wild, researchers often have little or no information about the individual and the feed [7] and, by extension, about the variability related to environmental, individual or species-specific factors as described above, making it challenging to reach sound conclusions [8].

In a previous study on captive red deer [1], we demonstrated how environmental and individual factors strongly affect fN, fADF and fNDF under a controlled feeding regime. In this manuscript, we focus on inter-specific variability in faecal nutritional components under a common feeding regime, using three captive populations of gazelles from the Sahel-Saharan region as model species: dama gazelle (*Nanger dama*), Cuvier's gazelle (*Gazella cuvieri*), and dorcas gazelle (*Gazella dorcas*). Even if closely related, these three species inhabit ecologically distinct habitats (Figure 1), use different food sources, and experience different nutritional demands and challenges due to their different body size and life history traits [9–11]. Thus, predicting species-specific strategies in their digestive function and efficiency is reasonable. For example, large body size ungulates may have lower relative energy requirements due to their increased gastrointestinal tract capacity and longer ingesta passage rates [12] or as an adaptation to the feed quality and availability under different ecological conditions [13]; on the contrary, it has been suggested that forage quality plays an especially critical role in the nutritional regulation of small-herbivore species [14] (but see [15]). The reproductive effort is another factor affecting faecal nutritional components at the individual level [1].



**Figure 1.** Distribution map of the studied species: *Gazella cuvieri*, *Nanger dama* and *Gazella dorcas* (source: IUCN SSC Antelope Specialist Group [16–18], respectively).

Dama gazelle selects a mixed diet based on grazing herbaceous plants and browsing the foliage of woody species in close association with acacia woodlands [19–21]. It is the largest of the three studied species. Cuvier's gazelle favours grasses, young leaves of leguminous plants, perennials and plants associated with maquis [22–24]. Among the three studied species, it is the only one that may deliver twins [25]. Moreover, it is the only diurnal one, and thus, may have increased water requirements than the other two species. Dorcas gazelle is distributed along a wider variety of habitats across the region, and their needs for food and water vary significantly across that range. It can survive in areas with

no surface water throughout the year. They prefer habitats with trees and shrubs, browsing on acacia groves [26–29].

NIRS technology has become a widely used method that allows for the rapid, low-cost analysis of the nutritional content of large amounts of samples and is already commonly used for measuring food quality through faecal indices in ungulates [30]. The three study species are threatened in the wild. Thus, this study and the validation of the technique in captivity can lay the foundations for further studies on these species' feeding and nutritional ecology in their areas of origin. Considering all the previously stated differences between the three species described, we aimed to study inter-specific differences in digestive efficiency under the same feeding regime by analysing faecal nutritional components after controlling for individual factors like sex and age.

## 2. Materials and Methods

### 2.1. Data Collection and Processing

The study was carried out in May 2017 during the yearly handling of the animals for regular health control at “La Hoya” Experimental Farm (FEH) of the Experimental Station of Arid Zones (EEZA-CSIC) in Almería, Spain. One hundred and ninety-three healthy animals were studied, out of which 100 were dama gazelles (37 males and 63 females), 21 Cuvier's gazelles (7 males and 14 females) and 72 dorcas gazelles (38 males and 34 females). The animals used in this research ranged from 1 to 14 years old for dorcas and Cuvier's, and 1 to 17 for dama gazelle. Pregnant individuals were not considered for this research to avoid causing eventual stress.

All animals were kept in spacious paddocks with bare soil and no pasture provided, minimising soil ingestion and the transmission of nematodes, which can be a confounding factor in nutritional studies [6]. Animals of different species were assigned to separate paddocks and subdivided according to population management needs, from isolated animals to small breeding groups. Animals were fed daily with a combined diet of fresh alfalfa (*Medicago sativa*), wheat and feed pellets for herbivores (composition shown in Table 1). Each feedstuff, water and mineral licks were provided ad libitum to avoid competition and selection [31]. This combination of feedstuff has been successfully used for many years at FEH, ensuring constant protein availability and an adequate source of fibre, which is important for proper gut function. The ratio of provided feedstuffs changed slightly over the year according to seasonal needs. Still, it was constant for the previous month before the samples for this research were collected. No further measuring of leftovers and the exact amount of each feed component in each paddock was possible since the husbandry protocols are designed to minimise contact with the animals to reduce stress.

**Table 1.** Nutritional content of the feedstuff provided to the study animals in percentage of dry matter.

	Protein (%)	ADF (%)	NDF (%)
Fresh alfalfa	19.7	45.2	51.5
Wheat	26.4	20.3	28.9
Pellets	17.9	16.3	34.6
Dry silage	9.5	47.3	71.9

Handling and sampling procedures carried out at the farm were designed during the routine yearly handling of the animals for veterinary inspection under the expertise and supervision of the veterinarian in charge, who complies with the authorisations established by Spanish regulations on animal welfare. Animals were hand captured by net, immobilised in the ground with covered eyes, identified, and visually inspected as described and advised in the studbook of Cuvier's gazelle [32]. To reduce contamination, the faecal samples were collected from the rectum while animals were immobilised, just after routine blood sampling. Samples were dried to a constant weight in a hot air dryer at 40 °C for 48 h, ground with a mill to pass through a 1 mm sieve, and thoroughly mixed to achieve maximum homogeneity. The same approach was used for four subsamples of

each feedstuff previously described. All the samples were subsequently scanned with the NIRS™ DS 2500 FOSS analyser under the ISIScan™ 4.10 Routine Analysis Software (Foss, Hillerød Denmark), which is a rapid, low-cost, chemical-free, and non-destructive analysis method rapidly developing [7,33]. By this method, the contents of fN, fNDF and fADF were calculated using WinISI 4 Calibration Software (Foss, Hillerød, Denmark) according to a calibration set previously developed from a subset of the main sample set, which was analysed using conventional wet chemistry methods (NEN-ISO 5983-2 for protein; EN-EN-ISO 16472:2006 for NDF; NEN-EN-ISO 13906:2008 for ADF; [34]). For the calibration, we chose 34 samples out of the 193 samples collected (14 from dama gazelle, 12 from dorcas gazelle and 8 from Cuvier's gazelle), which is representative of the dataset regarding animals' body weight, age and sex. The wet chemistry confirmed that neither sand nor any other contaminants or dust affected the purity of the samples. Also, the accuracy of the calibration set was strengthened by adding faecal samples from red deer [35], reaching adequate goodness-of-fit indicators for the samples analysed (average  $GH1 = 0.912$ ;  $NH1 = 0.168$ ). The nutritional content of feedstuffs was calculated using standard calibration packages (Foss, Hillerød Denmark).

## 2.2. Statistical Analyses

The normality of the continuous variables studied was confirmed through Kolmogorov–Smirnov tests, and the homogeneity of variances was confirmed through Levene's test. A multivariate general linear model was conducted to understand the effects of Species, Sex and Age on the studied faecal nutritional components: fN, fADF and fNDF. The interactions Species\*Sex and Species\*Age were also included in the model since the preliminary inspection of the data suggested sex-related differences in at least one species. Analyses were performed using IBM® SPSS® Statistics (version 29.0 for Windows, IBM, USA).

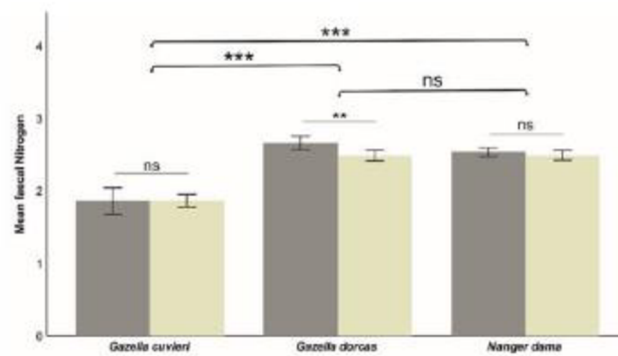
## 3. Results

Species (Wilks'  $\lambda = 0.667$ ;  $F_{6,364} = 13.592$ ;  $p < 0.001$ ), the interaction Species\*Sex (Wilks'  $\lambda = 0.844$ ;  $F_{6,364} = 5.374$ ;  $p < 0.001$ ), and Age (marginally; Wilks'  $\lambda = 0.962$ ;  $F_{3,182} = 2.397$ ;  $p = 0.070$ ) showed a significant influence in the studied faecal nutritional components, while Sex (Wilks'  $\lambda = 0.995$ ;  $F_{3,182} = 0.307$ ;  $p = 0.820$ ) and the interaction Species\*Age (Wilks'  $\lambda = 0.965$ ;  $F_{6,364} = 1.091$ ;  $p = 0.367$ ) were not.

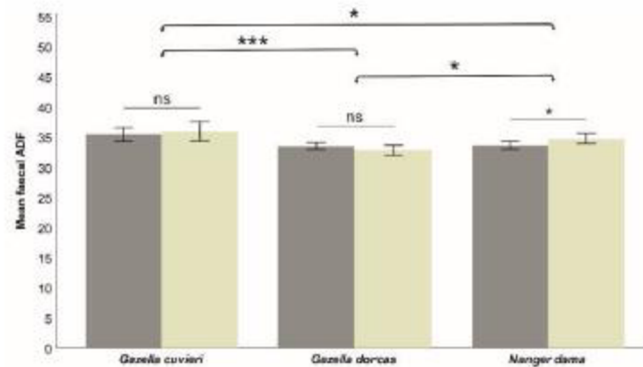
The model was quite robust for fN ( $R^2 = 0.493$ ), which was affected by Species, Age and the interaction Species\*Sex. However, the models were relatively weak for fADF ( $R^2 = 0.125$ , significantly affected only by Species) and fNDF ( $R^2 = 0.111$ , significantly affected by Species—marginally—and the interaction Species\*Sex). That indicates that individual factors moderately influence faecal nitrogen, while faecal fibres are weakly influenced by individual characteristics but strongly dependent on diet quality. The effects of these factors on each of the studied faecal nutritional components are shown in Table 2. Species significantly affected fN (lower in Cuvier's gazelle than in dorcas and dama gazelle; Figure 2). Species also affected the faecal fibres (fADF and fNDF; Figures 3 and 4), although the differences were much smaller (6.9% for fADN and 2.2% for fNDF; differences between the largest and smaller average values across the three studied species) compared to fN (27.9%). The effect of Sex was different across species. In dama gazelle, faecal fibres were lower in females ( $t = -2.010$ ,  $p = 0.046$  for ADF;  $t = -2.771$ ,  $p = 0.006$  for NDF). In dorcas gazelle, fN ( $t = 3.380$ ,  $p = 0.001$ ) and fNDF ( $t = 2.528$ ,  $p = 0.012$ ) were higher in females. In Cuvier's gazelle, no sex-related differences in faecal nutritional components were found. Faecal nitrogen significantly increased with Age ( $t = 2.921$ ,  $p = 0.004$ ), while faecal fibres were not affected.

**Table 2.** Influence of the selected factors on each of the studied faecal nutritional components. Significance is indicated at  $p < 0.001$  (\*\*\*),  $p < 0.010$  (\*\*),  $p < 0.050$  (\*) and  $p < 0.100$  (†) levels.

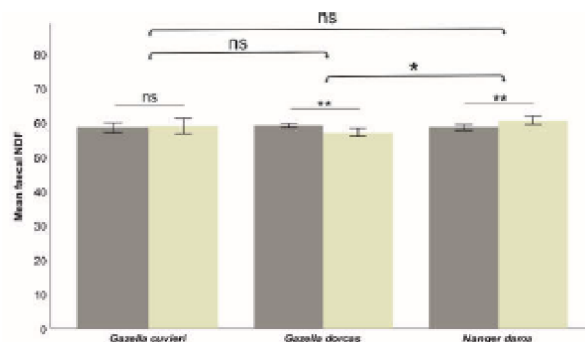
	fN	fADF	fNDF
R <sup>2</sup>	0.493	0.125	0.111
Intercept	F = 4669 ***	F = 10344 ***	F = 17067 ***
Species	F = 34.530 ***	F = 6.606 **	F = 2.637 †
Sex	F = 0.769	F = 0.086	F = 0.001
Age	F = 6.355 *	F = 0.681	F = 0.360
Species*Sex	F = 3.459 *	F = 2.200	F = 6.613 **
Species*Age	F = 0.398	F = 0.622	F = 0.064



**Figure 2.** Influence of species and sex (dark bars correspond to females) on the measured faecal nitrogen (% dry matter) was lowest in Cuvier’s gazelle with respect to the other two studied species. Sex differences were found only for dorcas gazelle. Means  $\pm$  SD (bars) are shown. Significance is indicated at  $p < 0.001$  (\*\*\*) and  $p < 0.010$  (\*\*) levels; ns indicates a lack of significance.



**Figure 3.** Influence of species and sex (dark bars correspond to females) on the measured faecal ADF (% dry matter), which was significantly different for each species. Sex differences were found only in dama gazelle. Means  $\pm$  SD (bars) are shown. Significance is indicated at  $p < 0.001$  (\*\*\*) and  $p < 0.050$  (\*) levels; ns indicates a lack of significance.



**Figure 4.** Influence of species and sex (dark bars correspond to females) on the measured faecal NDF (% dry matter), which was only significantly different between dorcas and dama gazelles. Sex differences were found in the same two species but not in Cuvier’s gazelle. Means  $\pm$  SD (bars) are shown. Significance is indicated at  $p < 0.010$  (\*\*) and  $p < 0.050$  (\*) levels; ns indicates a lack of significance.

#### 4. Discussion

In a controlled research setting with three closely related species (dama, Cuvier’s and Dorcas gazelles) under the same feeding regime, we found inter-specific differences in the faecal content of three studied nutritional components: N, ADF and NDF. Cuvier’s gazelle showed a significantly lower amount of fN than the other species, suggesting lower rumen microbial activity and thus lower digestive efficiency. Individual factors like sex and age also moderately influenced the faecal content of nutritional components, especially fN, but these effects were not constant across the three studied species. On the contrary, faecal fibres were highly constant (i.e., highly dependent on diet quality). Since the diet was the same for all the studied animals, the results show differences in digestive efficiency but not diet selection nor digestibility.

Recent intra-specific research [1] found that individual factors, like sex, age, reproductive status, body mass, body condition, season (linked to different nutritional requirements for each sex) and presence/absence of natural pasture, significantly affected faecal nutritional components in a similar experimental setting with captive red deer. In that study, separate analyses were conducted for males and females due to the large sexual dimorphism linked to different nutritional requirements in the species at different periods of the yearly cycle. Still, differences in faecal nutritional components between sexes could be confirmed since these were explained by different factors. In gazelles, sex was not an important factor per se. However, it was significant in interactions within each species: no differences between sexes of Cuvier’s gazelle; higher fN and fNDF in females of dorcas gazelle; and higher fADN and fNDF in males of dama gazelle. In general, these significant differences were low compared with our previous study on red deer, which may be related to the smaller sexual dimorphism in body size among gazelles compared with deer [36,37]. Surprisingly, no sex differences were found in Cuvier’s gazelle, which is a species with certain sexual dimorphism and greatest reproductive outputs (twins are common), so greater efficiency in females of this species could be expected even if we did not use pregnant or lactating females in this study. Thus, further studies are necessary to fully understand sexual differences in digestive efficiency in gazelles and other ungulates, and how it is linked to sexual dimorphism.

Age was the other individual factor studied. Age had a significantly positive effect on fN but not on faecal fibres. This result is again different to the one observed in red deer [1]. In that species, fN decreased with age (i.e., lower efficiency), and changes in faecal fibres



were observed. In general, the protein requirements in ruminants decrease with age [38], which seems to be the easiest explanation for the fN increase observed in this study. It may be argued that these differences may be due to the presence of tannins and other plant secondary compounds in the feed, which may decrease protein digestibility and increase its excretion [39,40]. However, this is unlikely in our setting since only common feedstuffs for livestock with low content of plant secondary compounds were used.

This study aimed to investigate species-specific differences in faecal nutritional components in related species with different ecological characteristics under the same feeding regime. This was clearly observed for fN, which indicates different digestive efficiency among the studied species but not for faecal fibres, confirming that they are weakly affected by specific or individual factors (low  $R^2$  in the models) but strongly dependent on diet quality, which was the same for the three species. Thus, we will focus on the results obtained for fN. These were similar for dorcas and dama gazelles but lower for Cuvier's, suggesting a lower ruminal activity and digestive efficiency in this species. This is further supported by the greater fNDF observed compared to the other species. The previously commented greater reproductive performance of Cuvier's gazelles may explain this result. Indeed, the lowest fN would have been expected in dama gazelle. Digestive efficiency is directly related to retention time, a species-specific parameter determined by body mass [41]. While the three species are considered browsers with a certain flexibility in diet selection, the dama gazelle shows a higher degree of grazing [42], which may also explain the different digestive efficiency observed among species. Finally, water requirements may be another ecological factor explaining the results. Among the three species, Cuvier's gazelle has greater water requirements. In the wild, since most of these requirements are satisfied by the water content of plants, it may affect the natural diet selection. Thus, the species may prioritise the water over the protein content of the plants, which may explain the decreased digestive efficiency that the results suggest.

## 5. Conclusions

These results confirm our previous finding on individual factors affecting faecal nutritional components but also show that these individual factors may work differently for different ungulate species, even if taxonomically closely related. Moreover, the results show that while faecal fibres are a reliable indicator of diet quality across species, faecal nitrogen is not because of species-specific differences in digestive efficiency. Thus, comparative studies based on faecal nutritional components for different species sharing distribution may be considered carefully and may benefit from preliminary studies with captive individuals and controlled diets. That seems the only reasonable way to interpret samples collected in the wild adequately.

**Author Contributions:** Conceptualization, S.C. and E.C.; methodology, S.C. and E.C.; software, S.C.; validation, S.C. and E.C.; formal analysis, E.C.; investigation, S.C., J.C. and E.C.; resources, S.C., J.C., T.K. and E.C.; data curation, S.C. and E.C.; writing—original draft preparation, S.C., J.C. and E.C.; writing—review and editing, S.C., J.C., T.K. and E.C.; visualisation, S.C. and E.C.; supervision, E.C.; project administration, S.C.; funding acquisition, S.C., T.K. and E.C. All authors have read and agreed to the published version of the manuscript.

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**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** The data used to support the findings of this study are available from the corresponding author upon request.

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**Conflicts of Interest:** The authors declare no conflict of interest.

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## Are they both the same shit? Winter faeces of roe and red deer show no difference in nutritional components

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**Abstract:** Herbivorous ungulate diets affect population performance and overall forest health through balanced interactions on plant-herbivore relations; therefore, understanding them is critical. Faeces are frequently used in ungulate nutritional ecology because they can provide information about animals' digestive efficiency. Roe deer (*Capreolus capreolus*) and red deer (*Cervus elaphus*) have different morpho-physiological and ecological constraints, and these differences should be reflected in their faeces. On the other hand, the lack of information about the animal (sex, age, reproductive status, diet selection, etc.) may be challenging for such studies. This study aimed to detect species' different susceptibility to these factors reflected in animals' faeces. Thus, we hypothesised that near-infrared reflectance spectrometry (NIRS) could distinguish between the faecal nutrients of two cervids. We collected 94 usable faeces from both species along the forest transect in Bohemian forests in the Czech Republic, covering 2 500 ha. Roe and red deer overlap was determined using the four faecal nutritional components on two axes. No discrimination occurred, refuting our hypothesis and highlighting that out-of-control variables are critical for faecal studies in uncontrolled settings. Fibrous parts explained the most variance (48%), indicating animals' strong reliance on nutrition quality. Apparently, uncontrolled supplementary feeding produced similar faecal nutrient outcomes during the nutrition-limiting winter, which was theoretically supported by the animal's response to predation and hunting pressure. The inability of NIRS to identify the source of N in faeces may also explain the lack of discrimination.

**Keywords:** *Capreolus capreolus*; *Cervus elaphus*; diet overlap; faecal nutrients; fibre; nitrogen; nutritional ecology

The nutritional quality of the feed ingested determines wild ungulate populations' performance and well-being, which is, at the same time, essential for maintaining healthy forest habitats (Parker et al. 1999, 2009; Christianson, Creel 2007; Felton et al. 2017). Understanding the ungulates' feeding behaviour and the drivers of diet selection regarding nu-

tritional quality, chemical defence, and availability has been of high interest to scientists (Naiman 1988; Hodgman et al. 1996; Barboza et al. 2009; Lambert, Rothman 2015; Corlatti 2020) but is often overlooked during management and conservation decision-making (Morgan et al. 2021). After Raymond (1948) and Lancaster (1949) described how the or-

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ganic matter digestibility of pasture could be calculated from the nitrogen content of the faeces, the use of faecal nitrogen (fN) as a research proxy has been extensively applied in ecological research, and especially in studies related to the nutrition of wild ruminants (Putman 1984; Leslie Jr, Starkey 1987; Osborn, Jenks 1998; Dryden 2003; Leslie Jr et al. 2008). Certainly, there are circumstances in which fN is limited as a nutritional quality indicator for wildlife ungulates in natural settings due to numerous interacting factors that directly or indirectly affect animal nutrition. For instance, high parasite load alters N metabolism and increases fN output (Gálvez-Cerón et al. 2013), or tannins can directly or indirectly affect food intake, digestive efficiency, or protein digestibility through binding to digestive enzymes (Robbins et al. 1987). Furthermore, recent studies have shown that faecal nutritional components can also be influenced by individual factors at the intra-specific level [factors that cannot be controlled for in studies in the wild (Čupić et al. 2021)] and by species-specific differences in digestive capability (Mould, Robbins 1982; Redjadj et al. 2014). Nevertheless, a simultaneous study of other faecal nutritional fractions like lignin (fLig) and acid (fADF) and neutral detergent fibre (fNDF) may help to draw a better picture of the diet quality. Nowadays, this can be achieved through a fast and cheap technique like near-infrared reflectance spectrometry (NIRS) (Putman 1984; Leite, Stuth 1995; Foley et al. 1998; Dryden 2003; Tolleson et al. 2005; Landau et al. 2006; Showers et al. 2006; Gálvez-Cerón et al. 2013; Villamuelas et al. 2017). Indeed, the technique has already been successfully used for estimating the diet quality of roe and red deer in the Czech Republic (Kamler et al. 2004).

These large ungulates are commonly classified along the browser-intermediate-grazer continuum in the context of botanical diet composition (Clauss et al. 2008, 2010; Codron et al. 2019). Roe deer (*Capreolus capreolus*) and red deer (*Cervus elaphus*) are the two most widely distributed cervid species in Europe (Tixier, Duncan 1996; Burbaitė, Csányi 2009, 2010). The roe deer is a small-bodied concentrate selector (browser) that selectively ingests the vegetative parts of herbaceous and woody plants (leaves, buds, and twigs), fruits, and forbs (Hofmann 1988; Tixier, Duncan 1996). As predicted from the digestive morphology and body size of this concentrate selector, they tend to depend on high-quality low-fibre food items (Illius,

Gordon 1992) and consume plants with low cell wall contents (Jung, Allen 1995). Grasses usually do not form a large part of the roe deer diet due to the large volume of poorly digestible fibre (Danell et al. 1994). On the contrary, the red deer is classified as an intermediate feeder (generalist). They can adapt to either browsing or grazing, shifting according to plant availability (Hofmann 1989; Langvatn, Hanley 1993). Their general patterns of diet selection focus on maximising the energy intake rate and minimising the intake of antinutritional or toxic compounds (Hanley 1997). Red deer select concentrate food items when the overall browse quality and availability are high (during the vegetation season) and switch to a grass-based diet in response to the decline of concentrate food availability which usually occurs during winter (Dumont et al. 2005; Verheyden-Tixier et al. 2008). In the Bohemian Forest (Central Europe), both deer species display their typical feeding strategies (Barančeková et al. 2010; Krojerová-Prokešová et al. 2010). Meadows are the favourite sites providing a diversity of protein-rich plants significant for the winter diets of both species (Zweifel-Schielly 2005; Hewison et al. 2009; Bonnot et al. 2013), but spruce (*Picea abies*) also constitutes an important portion of their diets (Homolka 1995; Mysterud et al. 1997, 2002; Barančeková et al. 2010; Krojerová-Prokešová et al. 2010). Furthermore, meadows, as a part of the contemporarily frequent fragmented mosaic natural habitats across Europe, are particularly favourable sites for roe deer (Hewison et al. 2001; Jepsen, Topping 2004), which often visit them in search of plants or plant parts that are indispensable for their more selective diet when compared to the one of red deer.

Despite the certain similarities in the winter diets of roe and red deer (Spitzer et al. 2020), partly due to the low food quality and availability, species-specific factors like differences in their digestive tract allow to predict the existence of differences in the faecal nutrients: in fibres due to the different quality of the selected diet, and in nitrogen because of the species-specific digestive efficiency (Hofmann et al. 1988; Hofmann 1989; Clauss, Rössner 2014). Furthermore, distinct life-history traits of these two species should be the source of variety regarding nutritional needs and the capability to fulfil them. Therefore, we hypothesise that the set of overall influencing factors will be clearly reflected in species' faecal samples – roe and red deer will excrete distinguishable faeces in their composition of fN, fADF,

fNDF, and fLig. Consequently, we will test the NIRS and provide an insight into its applicability level for wildlife, game, and forest management and whether it can depict these fluctuations that reflect the ungulate-feed interaction and the difference in their morphophysiological-induced differences. The potential differences in faecal nutrients between samples collected in meadows and forests would also be tested (Ossi et al. 2017). However, considering the size-scale of the area in this experiment, its associated mosaic landscape structure, the large home range of these species, and their long food retention time, we did not set our hypothesis based on previous arguments, but rather include this analysis as support to the main research hypothesis.

#### MATERIAL AND METHODS

The study area is situated in the Bohemian Forest, outside the Šumava National Park in the Czech Republic. This is a forested mountain area and the most continuous mountain range in Central Europe, approximately 130 km long and 60 km wide. Elevation ranges from 370 m a.s.l. to 1 456 m a.s.l., and the climate is continental with a light maritime influence. The mean annual temperature is 6.5 °C in the valleys and 2 °C at higher elevations. Annual precipitation ranges from 400 mm to 2 500 mm without a dry period, but a considerable amount of precipitation occurs as snowfall. Snow cover persists for 7–8 months

at higher elevations and 5–6 months in the valleys (this might have undergone certain changes due to the global climate conditions changes in the last years). Cold air pockets are often present in the valley bottoms, leading to an inversion of the thermal gradient, especially in winter. The coldest period is December and January, when temperatures could drop below –15 °C (Heurich et al. 2015).

The area is dominated by Norway spruce (*Picea abies*) with European beech (*Fagus sylvatica*), silver fir (*Abies alba*), and larch (*Larix* sp.). There are some other tree species, such as white birch (*Betula pendula*), sycamore maple (*Acer pseudoplatanus*), and common rowan (*Sorbus aucuparia*) (Wild et al. 2004). We also noticed the presence of aspen (*Salix* sp.) and poplar (*Populus* sp.) in the area. The understorey is dominated by brambles (*Rubus* sp.), which were found to be an important food resource for roe deer (Moser et al. 2006), common honeysuckle (*Lonicera eryclimenum*), ivy (*Hedera helix*) and butcher's broom (*Ruscus aculeatus*). Forest covered most of the study area (Figure 1), while the proportion of meadows was around one-third (Mašková et al. 2009; Voženílková et al. 2010).

The roe and red deer co-occur in the area, 0.6 and 2.9 individuals per km<sup>2</sup>, respectively (Košíňář, Rajnyšová 2012). Wild boar (*Sus scrofa*) is also widely distributed in the area, while moose (*Alces alces*) is found only in small numbers in the southern part. The main large predator is the Eurasian

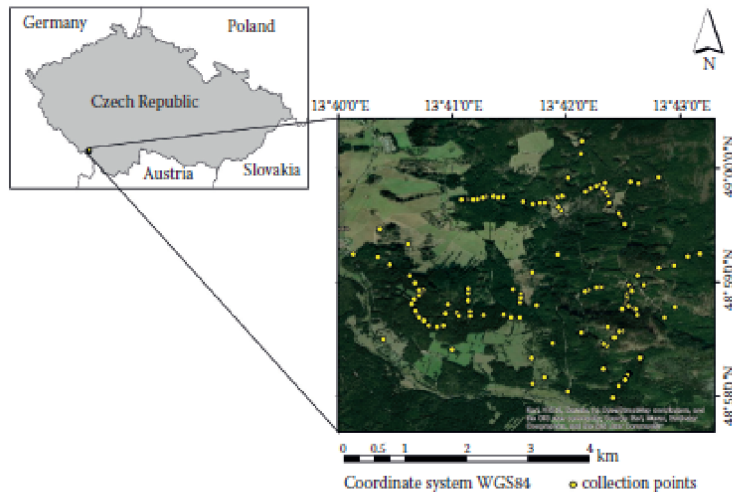


Figure 1. Study area, situated outside the protected zone in the Czech part of Bohemian forests, encompassing approximately 2 500 ha, with meadows comprising around one-third of the area (yellow dots represent sampling spots)

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lynx (*Lynx lynx*) which preys mainly on roe deer and, to a much lesser extent, on red deer calves (Heurich et al. 2012). Nevertheless, a wolf appearance has been reported in the area recently (Dvořák 2018; Janík 2020), although that happened after the samples for this study were collected. There is no significant agricultural activity nearby; crop feeding is therefore not common in the area.

We collected 156 faecal pellet groups from roe and red deer along 51.97 km of transects which covered an area of approximately 2 500 ha (Figure 1) at elevations between 782 m a.s.l. and 1 079 m a.s.l. The collection of the samples was conducted in December 2016 and the following January. Snow cover was present during the days of sampling, which facilitated the collection of fresh (recently exposed) faecal samples and avoided soil contamination. Samples in the close surroundings of previously collected samples were discarded to avoid repeated sampling of the same individuals. Discrimination between roe and red deer samples was done by *in situ* visual identification of morphological features (shape and size), further supported by animal tracks in the snow in the approximate vicinity of the sample group. Once in the lab, we calculated the length/width ratio to classify the samples according to their shape [following Chame (2003)] and discarded samples with outlier values (probably calves/fawns). The remaining samples were further confirmed by a senior expert researcher (Prof. Jaroslav Červený). All samples that were unclear to determine or did not pass the previous methodological criteria were discarded. Thus, we finally analysed 94 confirmed samples, 59 for red deer and 35 for roe deer.

The fresh faecal pellet groups were stored in plastic bags and labelled. Afterwards, we air-dried the samples at 50 °C for 48 h, ground them to pass a 1-mm sieve, and mixed them until being homogeneously distributed. We used NIRS™ DS 2500 FOSS analyser under the ISIScan™ Routine Analysis Software (Foss, Denmark) for scanning the samples and obtaining their near-infrared spectra, following Čupić et al. (2021). The contents of *fN*, *fNDF*, *fADF* and *fLig* were calculated with WinISI 4 Calibration Software (Foss, Denmark), according to a calibration set previously developed for red deer faecal samples (Holá et al. 2016) based on 100 samples, which showed a very high predictive power ( $R^2 > 0.98$ ). To increase the robustness of the results, 21 samples with at least one faecal nutrient showing

high global and neighbourhood distances (GH1 and NH1) were discarded. Thus, the final dataset consisted of 45 red deer and 28 roe deer samples.

**Statistical analyses.** The independent samples *t*-test was used to detect differences in faecal nutrients between the studied species and, within each species, between forest and meadow locations. Levene's test for equality of variances was applied in this procedure. Pearson correlations showed the relationships among the four faecal nutrients analysed (*fN*, *fADF*, *fNDF*, *fLig*). Since these were highly correlated, principal component analysis (PCA) was conducted based on these four faecal nutrients to obtain two axes. Varimax rotation with Kaiser normalisation was used as an extraction method to minimise the number of components extracted. Only those components with eigenvalues above 1 were selected. These axes were used to determine the overlap or discrimination between red and roe deer samples and between forest and meadow samples. For the interpretation of the selected axes, only the variables correlating  $> 0.7$  were considered.

## RESULTS

The *t*-test analyses failed to detect differences in the winter faecal nutrients between red and roe deer: *fN* (2.54% vs 2.50% respectively for red and roe deer;  $t = -0.637$ ,  $P = 0.526$ ), *fNDF* (56.4% vs. 56.3%;  $t = 0.043$ ,  $P = 0.965$ ), *fADF* (38.3% vs. 38.7%;  $t = -0.315$ ,  $P = 0.754$ ), *fLig* (29.3% vs 30.2%;  $t = -1.243$ ,  $P = 0.218$ ). Similarly, no differences were detected in the winter faecal nutrients of red and roe deer collected in meadow and forest habitats, with just a marginally significant difference in *fN* content in roe deer (2.48% in forest vs. 2.67% in meadow;  $t = 1.941$ ,  $P = 0.064$ ).

The four faecal nutrients analysed were significantly correlated in the 69 samples analysed, ex-

Table 1. Pearson correlations of the studied faecal nutritional components ( $N = 94$ )

Faecal nutritional components	<i>fN</i>	<i>fADF</i>	<i>fNDF</i>
<i>fADF</i>	-0.368**	-	-
<i>fNDF</i>	-0.658***	0.831***	-
<i>fLig</i>	0.577***	-0.083**	-0.328**

\*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ ; \*\*non significant; *fN* – faecal nitrogen; *fADF* – faecal acid detergent fibre; *fNDF* – faecal neutral detergent fibre

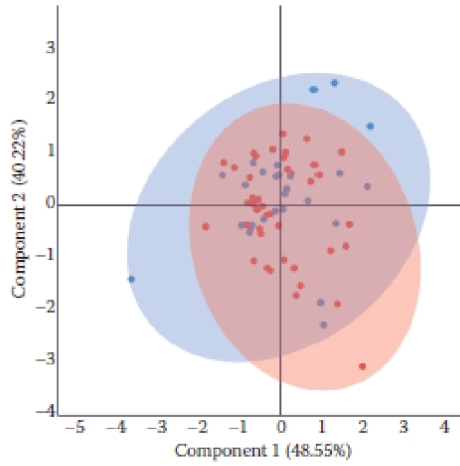


Figure 2. Graphical representation of the samples studied for red deer and roe deer along two axes based on faecal nutrients; component 1 is linked to the fibrous components *fADF* and *fNDF*, while faecal lignin and nitrogen are linked to component 2

Red – red deer; blue – roe deer; *fADF* – faecal acid detergent fibre; *fNDF* – faecal neutral detergent fibre

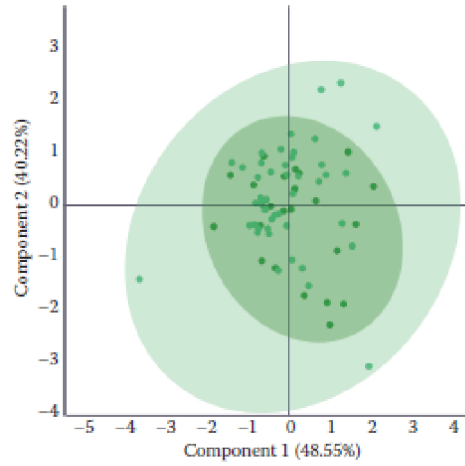


Figure 3. Graphical representation of the samples studied based on the collection habitat along two axes based on faecal nutrients; component 1 is linked to the fibrous components *fADF* and *fNDF*, while faecal lignin and nitrogen are linked to component 2

Dark green – forest; light green – meadows; *fADF* – faecal acid detergent fibre; *fNDF* – faecal neutral detergent fibre

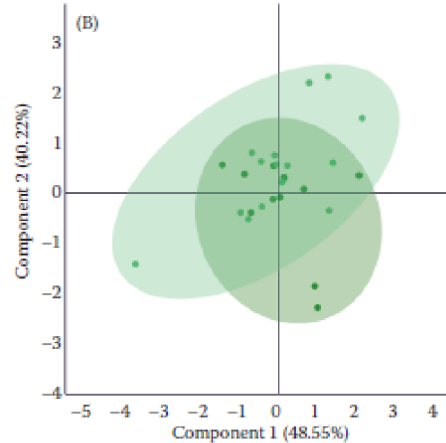
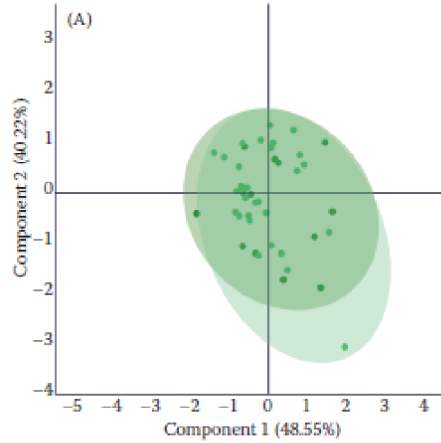


Figure 4. Graphical representation of the samples studied for (A) red deer and (B) roe deer along two axes based on faecal nutrients; component 1 is linked to the fibrous components *fADF* and *fNDF*, while faecal lignin and nitrogen are linked to component 2

Light green – samples collected in meadow habitats; dark green – samples collected in forest habitats; *fADF* – faecal acid detergent fibre; *fNDF* – faecal neutral detergent fibre



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cept  $fLig$  and  $fADF$  (Table 1). The PCA selected two components (axes) with eigenvalues above 1, well representing the original dataset of faecal nutrients. The first component (eigenvalue = 1.942) explained 48.55% of the variance and correlated with  $fADF$  ( $r = 0.963$ ) and  $fNDF$  ( $r = 0.904$ ). The second component (eigenvalue = 1.609) explained 40.22% of the variance and correlated with  $fLig$  ( $r = 0.934$ ) and  $fN$  ( $r = 0.781$ ). The plots of the samples studied on these two axes do not allow for discrimination between red and roe deer samples (Figure 2), and neither between samples collected from the forest and meadow habitats (Figure 3). When analysed separately for each species, it was not possible to discriminate between samples from the forest and meadow habitats neither in red (Figure 4A) nor roe deer (Figure 4B).

## DISCUSSION

Even under the numerous environmental, species-specific, and animal-specific factors (susceptible to continuous spatiotemporal changes) directly or indirectly connected to the diet selection of red and roe deer, our hypothesis of different winter faecal nutrients between both species was not supported. The four faecal nutritional components grouped in two axes were used for determining the overlap between roe and red deer, but no discrimination was observed. The component that explained the highest portion of variance (48%) correlated with fibrous components, indicating animals' strong reliance on the quality of ingested nutrition.

In this research, the wide variety of environmental, morphophysiological (species-specific), and animal-specific factors and the complexity of their interrelatedness are unknown, which is indeed a general characteristic of most research conducted in natural settings. According to our previous study (Čupić et al. 2021), factors such as pregnancy, pasture availability, and even physical condition or body weight can induce significant variations in faecal output, even when animals consume a similar diet. Thus, under controlled or captive environments, intra- and inter-specific differences in faecal nutrients are indeed observed. However, in nature, where all these factors are unknown, and animals have free access to a greater diversity of plant species, it is extremely improbable that their diet similarity will be even close to that of populations in a controlled environment.

Different rations of even the few sources available during the nutritionally-limiting winter season and the attendant specific morphophysiological response during the processes of ingestion, retention, digestion, and excretion should shape their final output. Tannins, already mentioned, could further support this interspecific diet dissociability. Simultaneously, in such a context, it is even more difficult to predict animals' energy expenditure, as well as inter- or intra-specific variation in required energy and, consequently, intraspecific dietary preference. Winter supplementary feeding of large mammalian herbivores is a common management tool in the Czech Republic (Conover 2001; Hothorn, Müller 2010; Möst et al. 2015) and elsewhere, mainly aiming at promoting healthy populations and increasing productivity and trophy sizes. Hunters in our research area provided that, but in low amounts considering the density of cervids. We were unable to obtain exact information either about the amount or about the ratio of supplementary feeding provided since it is a non-protected area. The decision-making process is in the hands of local hunters who do not have strict protocols to follow regarding supplementary feed or a defined law to comply with. Indeed, none of the previous research in this area provided information about this procedure. However, we did not expect a strong impact of supplementary feed, taking into consideration the entire set of previously mentioned potential influencing factors and experience from our previous research with captive animals. Given the almost total overlap observed in the nutritional outputs of both species, predominantly explained by the food quality ingested, the role of the supplementary feed should be further discussed.

The long retention times should be advantageous for ungulate species during harsh winter conditions. Ruminants with higher body mass are prone to having a larger relative gut fill, which leads to increased mean retention time (Demment, Van Soest 1985; Illius, Gordon 1992; Robbins 1993; McNab 2002). Moreover, browsers like roe deer have smaller digestive tracts and shorter retention times than grazers or intermediate feeders [three times larger rumen as a percentage of body weight in red deer compared to roe deer (Prins, Geelen 1971)]. Higher tolerance to fibrous forage has also been attributed to the same interspecific differences (Hofmann, Stewart 1972; Hofmann 1989; Clauss, Lechner-Doll 2001; Clauss et al. 2003). In habitats where un-

gulates must account for expensive activities such as avoiding hunting and predation, this benefit is increased as energy expenditures are higher. The maintenance requirements may increase with movement and stress by as much as 200–300% (Weiner 1977). Therefore, large ungulates often find themselves in a trade-off between shelter and food search. According to this, that trade-off should be easier to solve by red deer compared to roe deer, given its body size and previously discussed morpho-physiological characteristics. In a scenario where animals must rely exclusively on natural feed sources, roe deer acts as a typical browser, selecting diets with a higher nutritional value in terms of high protein content and avoiding high-fibre diets (Drescher-Kaden, Seifelnasr 1977; Hofmann 1989; Duncan et al. 1998). Given the morpho-physiology of the species, in times of increased energy demands (e.g. heat production during winter), this means reduced locomotor activity and higher exposure to hunting and predation. However, when increased amounts of carbohydrates are available in the form of supplementary feed, these may be mainly consumed by roe deer since that may be of higher vital importance for them than for red deer. The supplementary feed may thus help both species, but especially roe deer, to survive the winter while feeding on natural plant species with low nutritional value (Miranda et al. 2015), leading to more similar diets than initially expected and thus to similar faecal nutrients.

The above explanations can be supported by further incorporating the impact of predators and hunting pressure on the distribution, habitat preference, and nutritional ecology of roe and red deer in the study area. The Eurasian lynx (*Lynx lynx*) is the main predator in the area, predominantly preying on roe deer but also on young red deer individuals. As already commented, hunting pressure is relatively high in the area for both species, with greater exploitation of red deer due to their attractive trophies. That may induce stress in both species, and the consequence may be lower time searching for food, lower feed quality and increased compensation through the supplementary feed. In the study area, supplementary feeders are usually situated on the edges of forests and meadows, the habitat preferred by roe deer (Heurich et al. 2015). Indeed, this same study showed that roe deer in Bohemian forests prefer unprotected areas, despite the high impact of hunting, over protected areas of the park due to the supplementary

feeding provided by hunters out of the national park. In contrast, red deer prefer vegetation-dense forest habitats of around 70% of cover (Heurich et al. 2015). In these areas, the shrub vegetation layer, which red deer can use, stands during winter. Red deer probably keep feeding on this resource more extensively than roe deer due to the necessity to reach a certain threshold of fibres for supporting the proper functioning of their large ruminants (Bauchof 1979; Gebert, Verheyden-Tixier 2001), thus showing less dependence on supplementary feed.

## CONCLUSION

The inability of NIRS to identify the source of N in faeces may be another reason behind the lack of discrimination between free-ranging roe and red deer faecal samples. The difficult interpretation of  $f/N$  has been mentioned in previous research (Čupić et al. 2021). This problem might be solved in future studies by analysing the amount of N bound to ADF (Van Soest 2018), which would allow the calculation of metabolic faecal nitrogen (MFN). That would inform about the proportion of N being used by the animal cell function and not only excreted by it. Such analysis would confirm if there was an actual lack of differences in the diet consumed by both species or if our hypothesis was rejected just because of the inability of NIRS to detect the source of protein excreted. Even if the second reason was correct, certain discrimination at the axis explained by fibres could still have been found.

Altogether, the results thus suggest that NIRS and the analysis of faecal nutrients should not be employed for this type of research during periods when dietary overlaps across species can be expected, but especially when we have neither other sources of information about what the animals could have consumed (e.g. camera traps, direct observation, or GPS collars), nor information about each individual. The sum of uncontrolled factors may easily lead to non-significant results, which, theoretically, should have been expected. That may subsequently lead to making incorrect management decisions.

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SHORT COMMUNICATIONS

Ecological effects on the nutritional value of bromeliads, and its influence on Andean bears' diet selection

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**Abstract:** Previous studies have recognized bromeliads as a key food resource for Andean bears (*Tremarctos ornatus*) throughout their range. However, it is still not clear how abundance and especially the nutritional value of bromeliads influence habitat and diet selection. Understanding this is essential because conflicts such as crop damage occur when available natural resources cannot offer an adequate nutritional supply. During June and July 2017, we studied 6 forest and páramo areas in southern Ecuador for signs of bear presence, the abundance and nutritional value of bromeliads, and diet selection by fecal microhistology. The nutritional composition of bromeliads was affected by flowering and recent fire events but we found greater variability across species and ecosystems, with higher protein and lower fiber in páramo. However, bears were more often present in the forest ecosystem, and moreover, their presence was not affected by abundance nor composition of bromeliads. Further similar studies must focus on other key resources, such as temporary fruits.

**Key words:** Andean bear, bromeliads, diet, habitat use, montane forest, NIRS, nutritional composition, páramo, *Tremarctos ornatus*

**Resúmen:** Estudios previos han reconocido que las bromelias son un recurso clave para los osos andinos (*Tremarctos ornatus*) a lo largo de su área de distribución. Sin embargo, aún no está claro cómo la abundancia y especialmente el valor nutricional de las bromelias influyen en la selección de hábitat y dieta. Comprenderlo es fundamental, ya que conflictos como el daño a cultivos se producen cuando los recursos naturales disponibles no

pueden ofrecer un aporte nutricional adecuado. Se estudiaron seis áreas de bosque y páramo en el sur de Ecuador en busca de signos de presencia de osos, abundancia y valor nutricional de bromelias, y selección de dieta por microhistología fecal. La composición nutricional de las bromelias se vio afectada por la floración y los incendios recientes, pero además encontramos una alta variabilidad entre especies y ecosistemas, con más proteína y menos fibra en las bromelias de páramo. Sin embargo, los signos de presencia de osos fueron más abundantes en el ecosistema forestal, y además la presencia no se vio afectada por la abundancia ni la composición de las bromelias. Otros estudios similares deben centrarse en otros recursos clave, como los frutos temporales.

**Palabras clave:** bosque montano, bromelias, composición nutricional, dieta, NIRS, oso andino, paramo, *Tremarctos ornatus*, uso del hábitat

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The Andean bear (*Tremarctos ornatus*), also known as the spectacled bear, is the only species of the Ursidae family still present in South America. It is categorized as vulnerable and the population is decreasing (IUCN 2019), mainly because of habitat destruction and fragmentation, poaching, and human-wildlife conflict (Peyton 1999, Velez-Liendo and García-Rangel 2017). When there is not sufficient food in their native habitat, Andean bears may attack livestock or feed on crops, leading to conflict with local farmers (García-Rangel 2012).

Although the Andean bear has been described as an omnivorous species, switching between habitats and potential food sources (Peyton 1980, Paisley 2001), most of the literature on the diet of Andean bears states that bromeliads are one of the most important food sources (Table 1), if not the most important. Another very important food source is ripe fruit, which is consumed seasonally, with the time period varying among different regions of South America (Peyton 1980, Rodríguez et al. 1986). Although some authors claim that fruit is the most important part of Andean bear diet during specific seasons (Peyton 1980, 1986; Rodríguez et al. 1986), in the highlands of Ecuador, Andean bears feed almost exclusively on bromeliads throughout the year (Suárez 1988, Ontaneda and Armijos 2012). Similar diet preference of Andean bears was reported in Bolivia

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**Table 1. Summary of previous studies documenting the use of bromeliads by Andean bears (*Tremarctos ornatus*), including study area and period, reported use of bromeliads, and method used to study it.**

Reference	Location	Study period	Use of bromeliads (method)
Peyton 1980	Peru (whole country)	Year round	46.8% (feeding sign)
Goldstein and Yarena 1986	Venezuela	Not indicated	50% (feces, not indicated)
Suárez 1988	Ecuador (Antisana)	Year round	96% (feeding sign) 80% (feces—volume per scat)
Rivadeneira-Canedo 2008	Bolivia (Apolobamba)	Jul, Oct, Apr, Jun	57.5% (feeding sign and feces—frequency of feces with bromeliads)
Ríos-Uzeda et al. 2009	Bolivia (Apolobamba and Madidi)	Year round	47.3–76.9% (feces—frequency of occurrence in feces)
Ontaneda and Armijos 2012	Ecuador (Podocarpus NP)	Year round	70% (feces—frequency of occurrence in feces)
Figueroa 2013	Peru (whole country)	Year round	58.5% (feeding sign), 45.5% (feces—frequency of occurrence in feces)
Hernani-Lineros 2016	Bolivia (Cotapata)	Oct–Nov	38–60% (feces—frequency of occurrence in feces)
Ardila-Montaña 2020	Colombia (Chingaza)	Year round	34–37% (feces—frequency of occurrence in feces)
Cáceres-Martínez et al. 2020	Colombia (Tamá)	Year round	89% (feces—frequency of occurrence in feces)
Hernani-Lineros et al. 2020	Bolivia (Cotapata)	Oct–Nov	33–46% (feces—frequency of feces with bromeliads)

(Rivadeneira-Canedo 2008, Ríos-Uzeda et al. 2009, Hernani-Lineros et al. 2020). Moreover, Andean bears feed almost exclusively on bromeliads in Podocarpus National Park, Ecuador, during June and July (Ontaneda and Armijos 2012; time and place corresponding to our research). Although some authors have suggested that bromeliads are a good food source—high in carbohydrates, fat, and protein (Goldstein 1990)—other sources suggest that bromeliad meristems have low nutritional value in the form of soluble carbohydrates and small amounts of proteins and lipids (Paisley 2001, Rivadeneira-Canedo 2008). Nevertheless, substantial information on the nutritional value of bromeliads is lacking, and their chemical composition has been studied mostly in relation to their potential medical and mechanical properties (Vieira-de-Abreu et al. 2005, Xie et al. 2005, Errasti et al. 2018).

As a consequence of their feeding behavior, Andean bears are a keystone species, with a fundamental role in the dynamics of the ecosystems in which they live as dispersers of seeds (Rivadeneira-Canedo 2008) and by contributing to the formation of small illuminated spaces suitable for the growth of new vegetation (Oso Andino 2020). Thus, for the successful protection of the species, knowledge on habitat use, food preferences, and nutritional requirements is essential. Certain studies have focused on some of these topics in several localities (Peyton 1980, Paisley and Garshelis 2006, Ríos-Uzeda et al. 2006, Rivadeneira-Canedo 2008); however, there is a noticeable lack of research combining all these aspects, especially ecological studies considering the nutritional value

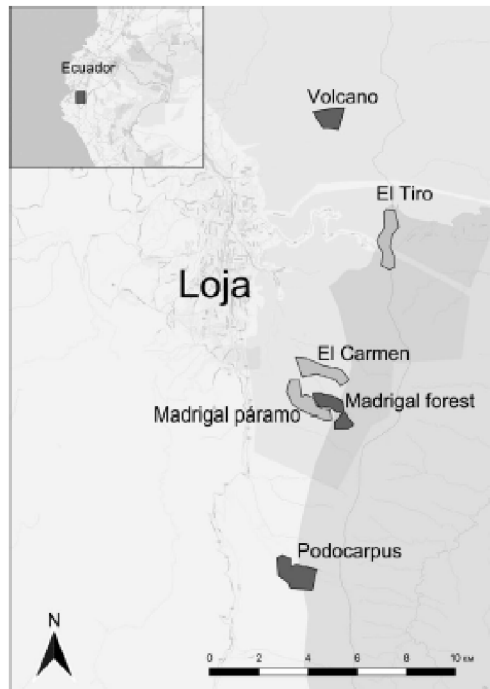
of bromeliads. In this study, we investigated the ecological factors affecting the nutritional value of bromeliad species used by Andean bears in southern Ecuador, and the influence of the availability and nutritional quality of bromeliads on the bears' habitat use and foraging preferences.

### Study area

We collected data at 6 study sites—3 in Andean páramo (a tropical alpine vegetation ecosystem above the tree line [Cleef 1979]) and 3 in montane forest ecosystems in Loja Province, southern Ecuador, during June and July 2017 (Fig. 1)—because Andean bears are active in both páramo and forest ecosystems during this period of the year, according to Cisneros-Vidal (2013).

Podocarpus National Park (montane forest) is located above 2,800 m above sea level between the provinces of Loja and Zamora Chinchipe. In this area, the centers of endemism of the Northern and Southern Andes overlap (Narvaez 2013). Podocarpus National Park covers approximately 1,460 km<sup>2</sup> and possesses an exceptional flora. A phytogeographic study in the area described up to 187 vascular plant genera (Lozano et al. 2009). Of these, Andean bears are known to consume only few species, such as *Puya eryngioides*, *Puya maculata*, *Bejaria mathewsii*, *Pernettya prostrata*, *Macleania salapa*, *Miconia* sp., *Macleania* sp., *Hesperomeles obtusifolia*, *Clusia duroides*, *Guzmania gloriosa*, and *Tillandsia* sp. (Ontaneda and Armijos 2012). Both páramo (ranging from 2,800 to 3,800 m; Lozano et al. 2009) and montane

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**Fig. 1.** Map of study sites in the area of Loja, southern Ecuador (forest is represented in darker color, páramo is represented in lighter color), where we studied 6 forest and páramo areas in June and July 2017 for signs of Andean bear (*Tremarctos ornatus*) presence, the abundance and nutritional value of bromeliads, and diet selection by fecal microhistology.

forest ecosystems are found there. The poor accessibility led us to stratify the study area following the touristic pathways and roads.

Madrigal is a private reserve of 800 ha, including montane forest and páramo ecosystems. The reserve is part of a microbasin located north of Podocarpus National Park, at elevations from 2,200 to 3,300 m. Andean bears are common in the area, according to previous research (Cisneros-Vidal 2013). In November 2016 there was a fire in the páramo area of the reserve.

Volcano is a montane forest located above 2,000 m. It is an open area bounded by meadows with no or insignificant human disturbance. This forest is very similar to forests in Podocarpus National Park and Madrigal in its plant composition, but there are no pathways and roads.

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The marginal parts of the forest are not very dense and there is a high abundance of bromeliads, whereas the core of the forest is almost impenetrable and less suitable for the bears.

El Carmen is a páramo located in the west slope of the Madrigal del Podocarpus border. It is an artificial páramo (man-made, fire-induced ecosystem [Holz and Gradstein 2005]) at a lower elevation than that at which natural páramo ecosystems are found. This area is surrounded by farmlands and meadows in the south and by forest on other sides. The plant community, elevation, climate, and slope are very similar to Madrigal, but there was no influence of fire for dozens of years. On the uppermost part, many bromeliad species are abundant in large numbers.

El Tiro is a natural páramo ecosystem located in the northern part of the Podocarpus National Park. Since 2009, industrial changes, such as power line construction and road extension, have started in this area and generated a great disturbance of the natural ecosystem. However, even after the construction of the road and continuation of the industrial management, Andean bears have been observed in El Tiro (Cisneros-Vidal 2013).

## Materials and methods

In each study site, we selected 5 50-m × 50-m plots. To ensure independency in the data collection, all the plots in each site were ≥250 m apart.

### Presence of Andean bears

Most studies focusing on habitat use of Andean bear were based on the evaluation of signs of presence (Cuesta et al. 2003). We did this in the plots in all 6 study sites. A team of 2–3 people searched every plot thoroughly. Signs recorded included feces, ground nests, partly eaten plants, scratch marks, hairs, and footprints. We counted data as number of signs per hectare.

### Abundance of bromeliads

In each of the plots, we placed 2 10-m × 10-m subplots in opposite corners. We evaluated only the most abundant bromeliad species at each subplot, and counted the exact number of each of these species and recorded them as bromeliads per hectare. We adopted this method because the most abundant species of each subplot always represented the vast majority of the bromeliad species at each place and other bromeliads were rather scarce.

### Collection of bromeliads

In each subplot, we collected 3 specimens of the most abundant bromeliad species. We collected 3 species from



subfamily *Pitcairnioideae*: *Puya eryngioides*, *P. nitida*, and *P. parviflora*; subfamily *Tillandsioideae* was represented by *Tillandsia* sp.

#### Diet selection

We collected all the feces that we found in the plots, placed them into zip-lock bags, and stored them in a freezer. Once the samples were unfrozen, we filtered them through a 1.5-mm sieve, so only the largest particles were obtained. We placed the retained particles into plastic vessels and preserved them in alcohol. We placed the samples into large plastic dishes and spread out all the particles equally. Using 2 measuring sticks, we selected every tenth particle and placed it in a dish, to obtain the percentage representation from the original sample. We measured the fragments in the feces using 10 nonoverlapping optical fields, in which we identified undigested particles on 10 slides. We carefully placed the selected fragments in the middle of the slide using sterile lab tweezers (100 optical fields per sample). We divided these particles into those with bromeliad origin and unknown by microhistological analysis (Carrière 2002). We distinguished bromeliad particles into *Puya* sp. and *Tillandsia* sp. by microscopical comparison with a reference collection of bromeliads found in the study area, which we made to identify the unique characteristics of the plant species. We made the reference collection by mashing *Puya* sp. and *Tillandsia* sp. into small particles in a blender, so they would resemble plants digested by Andean bears. We preserved the reference collection in alcohol. We counted the percentage representation of the bromeliad species in every sample (Davitt and Nelson 1980, Breuer 2005).

#### Nutritional composition of bromeliads

We only used the meristematic parts of plants for the nutritional analysis. We hot-air-dried all forage samples at 40°C for 48 hours, ground them with a mill to pass through a 1-mm sieve, and thoroughly mixed them to achieve maximum uniformity and homogeneity. Just before the nutritional analysis, we dispensed the samples into sample cups (Foley et al. 1998). We used the Near-Infrared Reflectance Spectroscopy (NIRS) method in combination with wet chemistry analyses, so we scanned all the samples with the NIRS™ DS 2500 FOSS analyzer under the ISIScan™ Routine Analysis Software (FOSS A/S, Hillerød, Denmark) and obtained spectra for all bromeliads. We predicted all spectra with existing calibrations for plant-based food ingredients: Vegetal Protein Meal v3000 and v2000, and Vegetal By-Products v2000 (FOSS). We discarded the latter as a result of inappro-

priate protein range. The output contained several inadequate GH (Global distances) and/or NH (Neighborhood distances) values, so we selected 18 samples for classical wet chemistry analyses and used the results to improve the calibrations. By this method, we calculated protein, fat, starch, fiber, acid detergent fiber (ADF), neutral detergent fiber (NDF), and ash contents for each sample. We used the mean of the 3 plants collected at each subplot in further analyses.

#### Statistical analysis

We conducted all analyses in IBM® SPSS® Statistics 25 (IBM, Armonk, New York). We evaluated normality by Shapiro–Wilk (S–W) and Kolmogorov–Smirnov (K–S) tests, depending on the sample size available for the different data sets. Nutritional composition (protein, starch, fiber, ADF, NDF, fat, and ash) of bromeliads showed a normal distribution (all K–S >0.200), whereas the abundance of bromeliads, signs of Andean bear presence, and percentage representation of *Puya* sp. and *Tillandsia* sp. in feces did not (all S–W <0.050). This conditioned the subsequent statistical approach.

We designed a set of generalized linear mixed models to test the influence of the recorded ecological variables (ecosystem, fire occurrence, bromeliad species, and flowering) on the nutritional composition of bromeliads used by the Andean bear. We determined data structure by site as subject and subplot as repeated measure. The other ecological variables entered the model as factors. We always used linear response. To discard nonsignificant variables, we used the traditional stepwise backward selection procedure, and we used the Akaike Information Criterion to detect the best model. We used analysis of variance with post-hoc Tukey test to determine differences in nutritional composition among the 4 studied bromeliad species.

We designed a second set of analyses to determine the influence of the occurrence and nutritional value of bromeliads on bear presence. We used the Mann–Whitney *U*-test for the analysis of bear presence across ecosystems and the Kruskal–Wallis test to detect differences in frequency of bear presence and occurrence of bromeliads among the different study sites. We used Spearman's rank correlation to detect correlation between signs of presence of Andean bears and abundance of bromeliads, between signs of presence of Andean bears and nutritional composition of bromeliads, and between percentage representation of bromeliads in feces and nutritional composition of bromeliads in the area.

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**Table 2. Multivariate analysis of factors affecting the nutritional value of the basal part of bromeliads, which is the part usually consumed by Andean bears (*Tremarctos ornatus*), in June and July 2017 in southern Ecuador. ADF = acid detergent fiber; NDF = neutral detergent fiber.**

Factor	F	$\beta$	P-value
<b>Protein</b>			
Ecosystem <sup>a</sup>	2.983	2.673	0.090
Species <sup>b</sup>	32.646		<0.001
Flowering <sup>c</sup>	2.882	2.101	0.095
<b>Fat</b>			
Ecosystem	6.453	1.726	0.014
Burned <sup>d</sup>	3.379	-1.747	0.072
Species	11.447		<0.001
<b>Starch</b>			
Ecosystem	8.484	1.215	0.005
Burned	5.242	1.420	0.026
Species	33.349		<0.001
<b>Fiber</b>			
Ecosystem	5.403	-2.115	0.024
Burned	8.341	-3.906	0.006
Species	7.888	-4.580	<0.001
<b>ADF</b>			
Ecosystem	18.473	-5.884	<0.001
Burned	9.553	-5.828	0.003
Species	14.948		<0.001
Flowering	4.177	-2.810	0.046
<b>NDF</b>			
Ecosystem	14.909	-7.850	<0.001
Burned	5.867	-6.846	0.019
Species	19.273		<0.001
Flowering	4.300	-3.804	0.043
<b>Ash</b>			
Species	5.922		0.001

<sup>a</sup>Forest was used as category of reference. Therefore, positive  $\beta$  means higher content in páramo compared with forest.

<sup>b</sup> $\beta$  values are not indicated because the nutritional composition of each species is shown in Table 2.

<sup>c</sup>Nonflowering plants were used as category of reference.

<sup>d</sup>Unburned conditions were used as category of reference.

## Results

The effects of the ecological factors we examined on the nutritional value of the studied bromeliads are shown in Table 2. Bromeliads in páramo ecosystem had higher protein ( $\beta = 2.673$ ), fat ( $\beta = 1.726$ ), and starch ( $\beta = 1.215$ ) than in forest, but lower fiber ( $\beta = -2.115$ ), ADF ( $\beta = -5.884$ ), and NDF ( $\beta = -7.850$ ). In burned areas, bromeliads had lower fat ( $\beta = -1.747$ ), fiber ( $\beta = -3.906$ ), ADF ( $\beta = -5.828$ ), and NDF ( $\beta = -6.846$ ), but higher starch ( $\beta = 1.420$ ). Flowering bromeliads had higher protein content ( $\beta = 2.101$ ), but lower ADF ( $\beta = -2.810$ ) and NDF ( $\beta = -3.804$ ).

However, we found the main variability in nutritional composition was among bromeliad species, which was

always the most significant factor affecting all the studied nutrients (always  $P \leq 0.001$ ). *Tillandsia* and *Puya nitida* were nutritionally similar, with greater contents of protein, fat, fiber, and ash, but with lower content of starch compared with *Puya eringyoides* and *Puya parviflora* (Table 3).

During our study period, signs of the presence of Andean bear were significantly more abundant in forest than in páramo, where the presence was very low ( $U = 12.5$ ;  $P < 0.001$ ). Thus, the rest of analyses are focused on the bear's presence in the forest ecosystem. The presence (abundance of signs) was similar across the 3 forest study sites ( $H = 2.784$ ;  $P = 0.249$ ), as was the abundance of bromeliads ( $H = 2.215$ ;  $P = 0.330$ ). However, the presence of Andean bears in the forest was not correlated to the abundance of bromeliads ( $\rho = -0.070$ ;  $P = 0.811$ ), nor to the nutritional value of the bromeliads in that area (all  $P > 0.243$ ).

To investigate feeding preferences of Andean bears, we analyzed the occurrence of bromeliads in feces. The average percentage occurrence of bromeliads in feces samples differed between the 2 studied ecosystems. In forest the frequency of occurrence of *Puya* sp. was 1.38% ( $\pm 4.29$ ) and of *Tillandsia* sp. was 26.62% ( $\pm 13.59$ ). In páramo, we found *Tillandsia* sp. in 8% ( $\pm 8.00$ ) of the feces, but we found no *Puya* sp. The percentage of feces with bromeliads was not correlated with the abundance of bromeliads in the area ( $\rho = -0.800$ ;  $P = 0.104$ ), nor to the nutritional value of the bromeliads in that area (all  $P > 0.148$ ).

## Discussion

Our results show that ecological factors like ecosystem type, flowering, or recent fire events have a strong effect on the nutritional composition of bromeliads, which also differs among the bromeliad species used by Andean bears in southern Ecuador. However, neither the availability nor the nutritional value of bromeliads seemed to influence the presence or the diet selection of the bears. Moreover, contrary to what we expected, Andean bears were present almost exclusively in the forest ecosystem, but not in páramo during the study period.

The nutritional value of the studied bromeliads differed between ecosystems. It is well-known that all the members of family Ursidae favor habitats with the most productive conditions (Schoen 1990). However, in the forest ecosystem (where bears were most often present) the nutritional value of bromeliads was lower than in páramo. These differences in the nutritional composition may be driven by differences in strategies for the acquisition of

**Table 3. Nutritional value ( $\pm$  standard error) of the basal part of 4 species of bromeliads known to be consumed by Andean bears (*Tremarctos ornatus*; as derived from fecal samples) in the study area in southern Ecuador in June and July 2017.**

Nutrient	<i>Puya eringyoides</i> Mean $\pm$ SE	<i>Puya nitida</i> Mean $\pm$ SE	<i>Puya parviflora</i> Mean $\pm$ SE	<i>Tillandsia</i> sp. Mean $\pm$ SE
Protein (%)	5.92 $\pm$ 0.21 <sup>B</sup>	6.75 $\pm$ 0.32 <sup>A</sup>	4.40 $\pm$ 4.02 <sup>C</sup>	6.47 $\pm$ 0.41 <sup>AB</sup>
Fat (%)	5.60 $\pm$ 0.19 <sup>A</sup>	6.95 $\pm$ 0.49 <sup>A</sup>	4.47 $\pm$ 6.88 <sup>A</sup>	6.32 $\pm$ 4.48 <sup>B</sup>
Starch (%)	7.26 $\pm$ 0.39 <sup>B</sup>	5.62 $\pm$ 0.55 <sup>B</sup>	9.63 $\pm$ 1.49 <sup>A</sup>	3.42 $\pm$ 1.09 <sup>C</sup>
Fiber (%)	17.61 $\pm$ 0.48 <sup>B</sup>	20.66 $\pm$ 1.11 <sup>AB</sup>	18.72 $\pm$ 4.04 <sup>B</sup>	23.86 $\pm$ 1.92 <sup>A</sup>
ADF (%) <sup>a</sup>	19.08 $\pm$ 1.08 <sup>B</sup>	27.71 $\pm$ 0.90 <sup>A</sup>	20.83 $\pm$ 5.09 <sup>B</sup>	30.56 $\pm$ 3.61 <sup>A</sup>
NDF (%) <sup>b</sup>	46.28 $\pm$ 2.25 <sup>C</sup>	54.82 $\pm$ 2.38 <sup>B</sup>	44.69 $\pm$ 6.88 <sup>C</sup>	63.22 $\pm$ 4.48 <sup>A</sup>
Ash (%)	8.72 $\pm$ 0.46 <sup>AB</sup>	10.29 $\pm$ 0.51 <sup>AB</sup>	8.42 $\pm$ 1.44 <sup>B</sup>	10.80 $\pm$ 1.74 <sup>A</sup>

<sup>a</sup>ADF = acid detergent fiber.<sup>b</sup>NDF = neutral detergent fiber.<sup>ABC</sup>Superscripts indicate significant differences after analysis of variance with Tukey test.

nutrients by bromeliads (i.e., bromeliads are mainly epiphytic in forest, but terrestrial in páramo [Benzing 2000]).

Special attention should be given to fiber content, which was higher in bromeliads in the forest ecosystem. Herbivorous mammals, such as bears, do not possess enzymes for fiber degradation, which means that foods with high fiber content have low nutritional value for them. However, they can establish a symbiosis with cellulose-hydrolyzing microorganisms in their gastrointestinal tract to be able to meet their nutritional requirements (Zhu et al. 2011, Xue et al. 2015). Bacteria families Lachnospiraceae and Ruminococcaceae have been found in wild Andean bear feces, suggesting that the gut microbiota of Andean bears possess the ability to break down constituents such as cellulose, hemicellulose, and lignocelluloses (Borbón-García et al. 2017).

Alterations in the ecosystem also affected the nutritional value of bromeliads. Previous research has suggested a strong negative impact of fire (Rocha et al. 2004), which is supported by our study; bromeliads in burned areas had lower fat, fiber, ADF, and NDF. However, the starch content of bromeliads from burned areas was higher, which should also increase the nutritional value for an omnivore forager like the Andean bear. The meristematic part, in which soluble carbohydrates accumulate (Paisley 2001, Rivadeneria-Canedo 2008), is encircled by water; and according to Ariani et al. (2004), the water inside the plant may assist in the conservation of the meristem, and thus of the carbohydrates, helping in the vegetation recovery of the plant following the fire.

Andean bears may change their habitat use as a result of changes in the availability of different food sources (Cuesta et al. 2003). Although the frequency of presence

of Andean bears may vary between habitats, they use both páramo and forest ecosystems year-round. Bromeliads are available in both ecosystems but, contrary to our expectations, the presence of bears was not affected in either of them by the nutritional composition nor the abundance of bromeliads. This suggests that bromeliads were not a key food source during our study period, although that was found in previous studies in this same location and period (Ontaneda and Armijos 2012). The relative homogeneity among the forest study sites may also explain that result. Seasonal variations affecting the nutritional composition of bromeliads, rather than the ecological factors studied in this work, may be also behind this result and deserve to be studied in the future.

In summary, our results show that the nutritional composition of bromeliads is species-specific and affected by ecological factors like ecosystem, fire, and flowering period. Nevertheless, neither nutritional value nor abundance seem significant factors influencing the habitat use of Andean bears. Thus, the interpretation of potential links between the nutritional value of bromeliads and presence of Andean bears is complicated, perhaps as a result of the relatively high homogeneity of forest and páramo habitats, or due to the fact that bromeliads are indeed not a nutritionally key resource for Andean bears.

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## 9.5. Principles of NIRS

The foundation of NIRS is the creation of a calibration equation that depicts the relationship between the components of the sample and NIRS spectral data. It includes collecting the light reflected from or transmitted through a specific material in the near-infrared (NIR, 750 nm to 1000 nm) and short-wave infrared (SWIR, 1000 nm to 2500 nm) regions of the electromagnetic spectrum, which are often referred to as near-infrared (NIR, Pasquini, 2003). Because NIR light is frequently absorbed by C-H, N-H, and O-H chemical bonds, which compose the vast majority of organic compounds (plant and animal tissues), it is particularly useful for quantitative analysis of these materials (Foley et al., 1998). When radiated, chemical bonds vibrate in reaction to their atomic connections and energy level when they spin, flex, and stretch. These vibrations absorb electromagnetic radiation at wavelengths corresponding to their energy level, producing observable overtones and harmonics in the nearinfrared (NIR) range (Kokaly & Clark, 1999). Given that various materials have distinct chemical ingredient combinations, and therefore the patterns of reflected and absorbed light differ between materials, NIRS may be used to determine the chemical composition of a sample (Shenk & Westerhaus, 1991; Westerhaus, 1993; Foley et al., 1998; Shenk et al., 2007; Cen & He, 2007). Furthermore, NIRS and the accompanying chemometrics can identify correlations between complex features that are not determined by any single chemical compound but rather by several interacting effects and different wavelengths throughout the electromagnetic spectrum (Foley et al., 1998; Moore et al., 2010; Marsh et al., 2014). However, the underlying mechanism driving those relationships is not always known (Tolleson et al., 2005; 2012). For instance, the ability of NIRS to distinguish males from females in some species may be attributable to sex-based changes in diet choices that result in samples from males and females having a distinct chemical composition rather than wavelengths related to the chemical bonds of sex hormones (Dixon and Coates, 2009). However, the standard five-step procedure for NIRS consists of acquiring spectra, processing them to remove noises (chemometrics, thus common mathematical pre-treatments and statistical methods for the qualitative and quantitative analysis of NIRS data), creating a calibration model by analysing a subset of samples with known analysed concentration obtained via wet-chemistry methods, verifying/validating the calibration, and predicting unknown samples (Cen & He, 2007).

A collected spectral data (spectrum), in addition to chemical information, also contains noises or non-useful information that reflect the physical properties of the sample, such as particle size,

as well as the conditions under which samples were scanned, such as temperature and humidity (Stuth et al., 2003; Cen & He, 2007). The following step includes some common mathematical and statistical pre-processing treatments, such as standardisation (i.e., centring and scaling, Bro & Smilde, 2003), for qualitative and quantitative analysis of NIRS data contained by chemometrics. Furthermore, multiplicative scatter corrections (Geladi et al., 1985; Martens & Naes, 1991) and detrending, or standard normal variate transformations (Barnes et al., 1989) are among common pre-treatment methods available for minimising the impact of particle size (scatter). The effects of overlapping absorption bands can be eliminated, and the spectral resolution can be improved by calculating the first and second derivatives of the spectral data (Savitzky & Golay, 1964; Cen & He, 2007).

After pre-treatment, the spectra can be used in model development. The calibration process establishes a correlation between the chemical information contained in a substance's spectral properties and the chemical information obtained through more conventional wetchemistry techniques. The latter is applied only at the beginning, while calibration is intended to create a predictive regression equation so that the constituents of interest may be assessed using NIRS alone, eliminating the need for extensive application of expensive and timeconsuming wet-chemistry procedures (Stuth et al., 2003). The primary goal of any calibration technique is to ensure that the initial sample subset chosen for calibration accurately represents the overall spectral variance present in all samples (Foley et al., 1998). The calibration involves any of Multivariate Regression Procedures, e.g., Multiple Linear Regression (MLR), Principal Component Regression (PCR) or Partial Least Squares Regression (PLSR, Martens and Næs, 1991; Shenk & Westerhaus, 1991; Foley et al., 1998; Cen & He, 2007). PCR and PLSR are the most popular approaches for calibrating NIRS data because they reduce massive, multivariate, and frequently highly correlated datasets to focus on relevant variables (Wold, 1975; Shenk & Wasterhaus, 1992; Næs et al., 2002). Both techniques allow models with more variables than samples and integrate independent variables into factors to minimise data dimensionality and eliminate co-linearity. Nevertheless, both procedures yield comparable results, although PLSR requires fewer principal components (PCs). A model may be overfitting if it can predict training data but not independent data. A high number of PCs decreases variability not accounted for by the model (Lawler et al., 2006). For this reason, PLSR is the preferred method for determining the linear combination of wavebands to explain the response variable. However, overfitting can still occur with any of these models. Discriminate analysis

can be performed with PC and partial least squares techniques for categorical response variables. Machine learning methods, including Artificial Neural Networks (ANN), Random Forests (RF), and Cubists are becoming more popular for constructing non-linear models, although they are still new for NIRS calibrations (Pérez-Marín et al., 2006).

Cross-validation, also known as Standard Error of Cross Validation (SECV) or the "leave one out approach", is the most often used method for verifying the calibration model. It is used to estimate the error of prediction for unknown samples by simulating the prediction process by leaving a portion of the data set out (the validation samples), developing the calibration model on the remainder of the data matrix, and making predictions for the samples left out using previously developed calibration models (Foley et al., 1998). This procedure is often performed numerous times, such that each sample is excluded once. Although cross validation is often employed in developing NIRS models, the ability of the model to fit new data depends on whether the training data are spectrally representative of the total data population. Cross-validation alone is sufficient for feasibility studies, but independent data should be used to validate model performance before applying it to a specific real-world application (Næs et al., 2017). Cross-validation can also hint at a model's performance on future datasets by comparing the similar standard errors of training and cross-validation testing. If they are dissimilar, it suggests that the amount of spectral diversity in the population may exceed the capacity of the training dataset. After this penultimate step in the mentioned procedure, the spectrometer is calibrated, and it is possible to analyse around 100 samples daily for various components/constituents simultaneously (Rothman et al., 2009).

All the samples used in this thesis were scanned with the NIRST<sup>TM</sup> DS 2500 FOSS analyser under the ISIScan<sup>TM</sup> Routine Analysis Software (Foss, Hillerød Denmark), and desirable constituents were calculated with WinISI 4 Calibration Software (Foss, Hillerød Denmark) according to different calibration sets. The analyser automatically calculates the average of 8 successive scans at a resolution of 0.5 nm, which gives the spectrum of each sample, recorded as the logarithm of the reciprocal of reflectance (amount of radiation reflected from the sample – Dryden, 2003). The software displays the curve of the reciprocal logarithm of reflectance and a curve of absorbing components in a close-to-linear relationship. The peak values of the two curves occur at wavelengths that correspond to absorption bands in the sample (i.e., lower reflectance – Norris et al., 1976; Hruschka, 1990).