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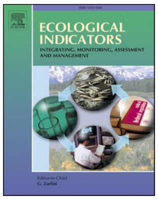

Original Articles

Spectral indices to evaluate the physiological response of Mediterranean shrubs to browsing

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ABSTRACT

Herbivory by large ungulates may cause important physiological and spectral changes in plants, which may serve as ecological indicators of herbivore overabundance. This study examines the physiological and spectral response of plants to recent and prolonged browsing through remote sensing techniques. We selected 30 plants from two Mediterranean shrub species with contrasting palatability: *Cistus ladanifer* (non-palatable) and *Phillyrea angustifolia* (highly palatable). Plants were subjected to three treatments: 1) no browsing (deer exclosure), 2) simulated recent browsing (clipping 30% of branchlets), and 3) intense, long-life natural browsing by deer (*Cervus elaphus*). Leaf physiological variables (chlorophyll, flavonols, anthocyanins, Nitrogen Balance Index (NBI), chlorophyll fluorescence, and stomatal conductance) were measured, and 27 spectral indices were calculated from reflectance data. The less herbivore-adapted *Cistus* showed lower chlorophyll (−27.7%), nitrogen (−26.8%), and stomatal conductance (−19.24%) when browsed compared to non-browsed plants, while no differences were found in the more palatable *Phillyrea*. Reflectance indices *R750/R800* and *R740/R800* related with chlorophyll fluorescence, were significantly lower for the intense, long-life browsing treatment in both species. Other indices such as *R690/R600* only showed significant differences between species. NDPI and SRPI indices, related to carotenoid/chlorophyll ratio, showed seasonally variable responses to browsing, with higher levels in spring for both herbivory treatments. We conclude that some spectral indices related to chlorophyll/carotenoids were able to detect physiological stress from deer herbivory, with key seasonal and species-specific variations for both, palatable and non-palatable shrubs. This demonstrates the potential and context-dependent use of spectral indices for the ecology and management of herbivore-dominated environments.

1. Introduction

Plant-animal interactions are primary drivers of ecosystem dynamics. Herbivory, the consumption of plant material by animals, is a key interaction that significantly modifies vegetation structure, community composition and productivity in all ecosystems (Huntly, 1991). Wild ungulates, such as deer (Cervidae) are among the most important herbivores (Burbaitė & Csányi, 2010), and represent fundamental actors in shaping ecological communities (García-Herrera & Botín, 2005; Zamora et al., 2004). Over the last decades, wild ungulate populations have increased significantly in many areas of the Northern hemisphere (Carpio et al., 2021; Côté et al., 2004). The causes of the current overabundance are diverse, including factors such as lack of native predators, abandonment of agricultural areas, and increase of forest land (Valente et al., 2020). This overabundance of wild ungulates typically triggers cascading effects that disrupt ecosystem dynamics, and have negative socioeconomic consequences (Carpio et al., 2017). For instance, wild ungulates affect vegetation regeneration and recruitment by feeding directly on seeds or saplings (Lecomte et al., 2017; Perea et al., 2020), or indirectly by reducing shrub cover and altering optimal

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microsites for seed germination and seedling survival (Perea & Gil, 2014; Smit et al., 2008). Moreover, wild ungulates shape species composition by preferring some woody plants over others, primarily based on plant palatability and seasonal availability (Fernández-Olalla et al., 2006; Miranda et al., 2011). Thus, ungulate diet selection, causes a reduction of highly preferred species, which may become less abundant or even disappear in areas with high deer densities (Perea et al., 2014; Rooney, 2009). Selective feeding pressure also leads to decreased plant species diversity and the long-term homogenization of vegetation (Perea et al., 2014; Rooney, 2009).

Continuous and intense browsing by ungulates induces stress in plants, affecting their growth, proper physiological functioning, and reproductive success and fitness (Perea et al., 2015b). Plant responses to herbivory are complex and often involve significant changes in chemical and morphological traits (Karban & Myers, 1989), with some of these responses affecting ungulate browsing preferences (Massei et al., 2000). Physiological processes such as photosynthesis is affected by direct ungulate herbivory. One notable example is "photosynthetic overcompensation", where some plant species increase their photosynthetic rates in response to herbivory (Caldwell et al., 1981; Capó et al., 2024; Gordaliza et al., 2025; Lemoine & Budny, 2022; Tiffin, 2000). Chlorophyll *a* fluorescence, which reflects the photochemical efficiency of light transformations into chemical energy, has been utilized to assess tolerance to herbivory damage (Madriaza et al., 2019). Variations in other physiological parameters, such as leaf content of chlorophyll, anthocyanins and flavonoids, have also been analyzed as early physiological responses to ungulate herbivory due to their roles in light absorption, photoprotection and defense (Capó et al., 2024; Pellissier, 2013). Additionally, some studies have found specific chemical responses in woody plants triggered by elicitors present in the saliva of large herbivorous mammals. For example, Ohse et al. (2017) observed an increase in growth-related phytohormones and secondary defense compounds, such as flavonols, after applying roe deer saliva to clipped apical buds and leaves. Similarly, another research found that simulated browsing, involving tearing and the application of moose (Alces alces) saliva, affected the growth of Salix caprea (Bergman, 2002).

Remote sensing, mainly through hyperspectral sensors, has emerged as a key tool for detecting stress in vegetation (Zhang et al., 2021). Hyperspectral sensors capture vegetation reflectance across numerous bands in the electromagnetic spectrum, producing what is known as the spectral signature. The spectral signature varies depending on vegetation traits, such as species phenology, and stress (Behmann et al., 2014). By combining the reflectance intensity of multiple bands across the spectrum, vegetation indices (VIs) have been developed to correlate with structural, chemical, or functional characteristics of vegetation (Peñuelas & Filella, 1998). The use of spectral imagery in plant-animal interactions is receiving a growing interest. Spectral sensors can classify taxa in heterogeneous environments and create maps of wild ungulate food resources, termed "foodscapes" (Alonso-Martínez et al., 2020). Moreover, the use of VIs for detecting woody plants stress, productivity and phenology changes caused by ungulate browsing has been successfully applied in recent studies (Balata et al., 2022; Comas, 2020). This methodology is highly valuable in studying and characterizing vegetation, facilitating landscape diagnostics and land management. However, no studies have compared the hyperspectral responses of plant species with contrasting palatability (i.e., preferred vs. non-preferred plants) or analyzed the use of spectral VIs to evaluate deer herbivory depending on the species' susceptibility consumption.

In this study, we examine the response of two abundant Mediterranean shrub species to the herbivory stress caused by deer browsing. We analyze whether changes in leaf physiology and VIs caused by two levels of herbivory vary depending on the season and the palatability of the plant species. For this purpose, we evaluated various physiological variables and the leaf spectral signature of two species with contrasting palatability: *Cistus ladanifer* (non-palatable) and *Phillyrea angustifolia* (highly palatable). We compared the responses of both woody species across two seasons and explored the potential relationship between leaf physiology and spectral vegetation indices.

We hypothesize that browsing affects both physiological and spectral variables depending on the season, plant species and browsing intensity. We expect that leaf chlorophyll and nitrogen content, as well as light use in photochemical processes decrease under intense, lifelong browsing. In contrast, we anticipate that the concentration of defensive compounds (anthocyanins and flavonols) increases with herbivory stress. We predict that the most palatable species will adjust its physiology to better adapt to ungulate herbivory stress. We propose that specific spectral indices may exhibit associations with plant physiology and serve as reliable indicators for detecting and evaluating deer herbivory.

2. Material and methods

2.1. Study area

This study was conducted in the protected area of Cabañeros National Park, situated within the Montes de Toledo mountain range, northwest of Ciudad Real (Castilla-La Mancha, central Spain). The elevation ranges from 700 to 1100 m above sea level. The climate is Mediterranean, characterized by a pronounced summer drought period (3–5 months, from June to September), with rainfall predominantly occurring in spring and autumn. The mean annual precipitation is approximately 550 mm, and the mean annual temperature is about 12.4 °C. Summer temperatures can reach 40 °C, while winters are cold, with potential frosts occurring from November to March (García-Herrera & Botín, 2005). Soils in the region are acidic (pH = 5.2) and shallow, primarily composed of clay with varying amounts of quartzite pebbles and slates (Perea & Gil, 2014).

The vegetation is dominated by Mediterranean shrublands and oak forests, featuring evergreen and semi-deciduous species such as *Quercus ilex, Quercus suber, Quercus faginea,* and *Quercus pyrenaica*. The canopy cover of these formations varies, with extensive areas of open woodland. The understory is characterized by evergreen shrubs (including *Cistus ladanifer*, *Erica australis*, *Erica scoparia*, *Rosmarinus officinalis*, *Lavandula pedunculata*, *Thymus mastichina*, *Phillyrea angustifolia* and *Rubus ulmifolius*) along with annual herbaceous plants, primarily grasses (García-Herrera & Botín, 2005; Perea et al., 2015a). The National Park hosts a high density of red deer (*Cervus elaphus*), with an average density exceeding 20 individuals per km^2. Other wild ungulates, such as wild boar (*Sus scrofa*, 2.4 ind/km^2) and roe deer (*Capreolus capreolus;* < 2.6 ind/km^2) are scarce in the area compared to red deer (Carpio et al., 2024; Linares & Urivelarrea, 2020). Red deer is by far the main large herbivore in the area, browsing more intensely in winter (December–March) and summer (July–September) when no alternative high-quality food is available (Perea & Gil, 2014).

2.2. Studied species

The selected Mediterranean shrub species were *Phillyrea angustifolia* and *Cistus ladanifer* (see Supplementary Fig. S1 for images). Both species are dominant in the shrubland of the study area. *Phillyrea angustifolia* is a sclerophyllous evergreen shrub belonging to the Oleaceae family, widely distributed in the western Mediterranean region (Puértolas Simón et al., 2013). In contrast, *Cistus ladanifer* is a resinous perennial shrub from the Cistaceae family, endemic to the western Mediterranean (Castroviejo, 1986). Its leaves release a resinous exudation primarily composed of flavonoids, which protect against fungal infections, shield from UV radiation (Chaves et al., 1997) and deter herbivores (Sosa et al., 2004). Both species can grow up to 4 m tall but are usually smaller due to multiple limiting environmental conditions.

The physiology of these two species undergoes significant seasonal changes, as they adapt to the extremely pronounced variations of the Mediterranean climate (Chaves et al., 1997; Nunez-Olivera et al., 1996; Valares Masa et al., 2016). These woody plants exhibit different levels of palatability; *Phillyrea angustifolia* is considered a highly palatable shrub while *C. ladanifer* is identified as much less palatable (Fernández-Olalla et al., 2006; Miranda et al., 2011; Perea et al., 2014).

2.3. Data collection

We selected a fenced area (46.37 ha) that has been enclosed for 20 years (since February 15, 2004), to assess variations in physiology and spectral signatures of the target species. We compared three levels of deer browsing: 1) no browsing (within the fence), 2) simulated recent browsing by clipping with shears 30 % of the plant branchlets twice within one year (within the fence), and 3) intense, natural browsing throughout the plant's life (outside the fence). For simulated recent herbivory, we clipped twigs in early spring (April) and late summer (September) 2023, attempting to emulate red deer foraging habits by removing branchlets from the upper crown of plants. Although clipping does not fully replicate the effects of herbivory (Ohse et al., 2017), it remains a widely used method to simulate herbivory in both herbaceous (Jarque-Bascuñana et al., 2022) and woody plants (Peláez et al., 2025).

We followed a six-rank (0–5) approach to assess browsing intensity on vegetation, as outlined by Perea et al. (2015b):

- 0 = No browsing evidence
- 1 = Few twigs browsed (<10 %)
- 2 = Many twigs browsed (10–30 %)
- 3 = Intense but sustainable browsing (30–60 %)
- 4 = High browsing (>60 %), with no reproductive structures
- 5 = Maximum browsing intensity, with only a few leaves remaining.

For the intense natural browsing treatment outside the fence, we specifically selected plants with browsing ranks of 3, 4, or 5, representing the general status of the species in the area. For the simulated recent browsing treatment, we assigned a browsing rank of 2 (30 % of twigs clipped), while plants in the no browsing treatment were ranked as 0. In total, we selected and labelled 30 plants for the experiment, comparing 15 individuals of each species and 5 plants per treatment and species (see Supplementary Fig. S1).

Data were collected during two contrasting phenological periods: late spring (June 6, 2023) and winter (February 19, 2024). All measurements were conducted on clear, sunny days, during peak sunlight hours around midday.

We assessed six physiological variables at the leaf scale for each plant. Using an optical sensor (Dualex™ Scientific+, FORCE-A, Orsay, France), we quantified chlorophyll, flavonol and anthocyanin leaf content. Nitrogen balance index (NBI) was also measured with the Dualex device. NBI is a chlorophyll/flavonols ratio (related to nitrogen/carbon allocation) that can be used as a proxy of nitrogen status in plants (Agati et al., 2016; Cartelat et al., 2005). These measurements were taken for two or three fully expanded leaves from each individual plant. Leaf chlorophyll fluorescence was measured on two leaves per plant using a pulse-modulated fluorometer (FMS2, Hansatech Instruments Ltd, Norfolk, UK) to estimate the quantum yield of photosystem II (Φ_{PSII}) under ambient light from steady-state fluorescence (F_s) and maximum fluorescence (F'_m) as: $(F'_m - F_s) / F'_m$). Leaf stomatal conductance to water vapour (g_s) was measured with a leaf porometer (SC1, Decagon Devices, Inc., NE, USA) on one or two leaves per plant.

We recorded the spectral signatures of the leaves using a GER 1500 spectroradiometer, which captures 512 bands covering wavelengths from 285 nm to 1095 nm (UV, Visible, and Near Infrared). The field of view for the spectroradiometer lens is 4 degrees. Measurements were taken at a distance of 10–20 cm from the plant, resulting in a scanned area of approximately 2 cm^2, with the lens directed towards the centre of the plant from above, ensuring it was as perpendicular as possible while avoiding any obstructions. White standard measurements were conducted immediately before the leaves spectral measurement.

2.4. Data analysis

2.4.1. Spectral indices

The data captured by the field spectroradiometer were processed by calculating 27 spectral indices for subsequent statistical analysis

Table 1
Spectral indices selected for assessing herbivory stress by deer browsing in Mediterranean shrubs.

Spectral index	Formula	Related to	Reference
Ant (Leaf area index)	*R800/R675*	Structural index: Green biomass	Jordan, 1969
clPRI (Photochemical Reflectance Index)	*(R531-R645)/(R531 + R645)*	Photosynthetic light-use efficiency	Drolet et al., 2005
EVI (Enhanced Vegetation Index)	*2.5*((R900-R680) /(R900+ (6*R680) −(7.5*R680) + 1))*	Structural index: Green Biomass with a background and atmosphere correction	Huete et al., 2002
NDPI (Normalized Difference Pigment Index)	*(R430-R680-)/(R430 + R680)*	Pigment index: Carotenoids/Chlorophyll ratio	Peñuelas et al., 1995a
NDVI (Normalized Difference Vegetation Index)	*(R900-R680)/(R900 + R680)*	Structural Index: Green biomass	Gamon et al., 1995Huete et al., 2002
NDVIchl (Normalized Difference Vegetation Index Chlorophyll)	*(R750-R705)/(R750 + R705)*	Structural Index: Green biomass (high sensitive to pigment changes)	Gitelson & Merzlyak, 1994
SIPI (Structure Insensitive Pigment Index)	*(R800-R445)/(R800-R680)*	Pigment index: Carotenoids/Chlorophyll ratio	Peñuelas et al., 1995a
SR (Simple Ratio)	*R900/R680*	Structural index: Green Biomass	Gamon et al., 1995
SRPI (Simple Ratio Pigment Index)	*R430/R680*	Pigment index: Carotenoids/Chlorophyll ratio	Peñuelas et al., 1995aPeñuelas et al., 1995c
PRI (Photochemical Reflectance Index)	*(R531-R570)/(R531 + R570)*	Photosynthetic light-use efficiency	Drolet et al. 2005Peñuelas et al., 1995bGarbulsky et al., 2011Zarco-Tejada et al., 2003
PRI13 (Photochemical Reflectance Index)	*(R531-R670)/(R531 + R670)*	Photosynthetic light-use efficiency	Drolet et al., 2005
WI (Water Index)	*R900/R970*	Water content	Peñuelas et al., 1997
	R685/R655 *R690/R655* *R690/R600* *R740/R800* *R750/R800*	Chlorophyll fluorescence	Dobrowski et al., 2005Zarco-Tejada et al., 2000aZarco-Tejada et al., 2000bZarco-Tejada et al., 2003
Spectral curvature optical indices	$R683^2/(R675/R690)$ $(R675*R690)/(R683^2)$ $R685^2/(R675*R690)$	Chlorophyll fluorescence	Zarco-Tejada et al., 2003Zarco-Tejada et al., 2000aZarco-Tejada et al., 2000b
Canopy reflectance optical indices in the 680–690 nm region	*R680/R630* *R685/R630* *R687/R630* *R690/R630*	Chlorophyll fluorescence	Zarco-Tejada et al., 2000b
	R735/R645 *R735/R660* *R735/R680*	Chlorophyll fluorescence	Zarco-Tejada et al., 2000a

(Table 1). These indices are related to structural characteristics of the plant (green biomass) and physiological properties (pigment ratios, water content, photosynthetic capacity or chlorophyll fluorescence).

2.4.2. Statistical analysis

Initially, we identified potential outliers within the dataset and eliminated values that were 1.5 times greater than the interquartile range (IQR), which is the difference between the third quartile (Q3) and the first quartile (Q1, Schwertman et al., 2004). As a result, four outliers were removed from measurements of chlorophyll, flavonols, anthocyanins and NBI for one *P. angustifolia* plant.

We fitted 33 linear models using a Gaussian error distribution and an identity link function (LM) to evaluate the effects of season (i.e., winter and spring), species (i.e., *Cistus ladanifer* and *Phillyrea angustifolia*), and treatment (i.e., intense browsing, recent browsing, and no browsing) on 6 physiological variables and 27 spectral indices recorded for both study species. We used *lm* function from the package *stats* for R 4.2.2 software (R Core Team, 2022). Model selection was conducted using the *dredge* function from the *MuMIn* package (Bartoń, 2023) to generate models based on subsets of predictors from a global model and evaluate their performance using the Akaike Information Criterion (AIC). Then we calculated the relative importance of all predictors for each model averaging approach. This parameter is the cumulative sum of the AIC weights across all models where the predictor is present (0 = minimum importance to 1 = maximum importance). We also checked the assumptions of the full models. The normality assumption was checked using the Shapiro-Wilk test on the model residuals with *shapiro.test()* and *residuals()* functions of the package *stats*. Homoscedasticity was assessed using Levene's test, which is implemented in the *leveneTest()* function of the car package. We evaluated the linear model (LM) assumptions of the retained models following the guidelines of Zuur et al. (2007). We applied logarithmic, square root or Box-Cox transformations to the response variable when necessary to ensure compliance with homoscedasticity and normality requirements. We performed a Tukey post-hoc test at $\alpha = 0.05$ to examine significant differences between treatment levels.

Finally, we analysed multiple correlations between all pairs of physiological variables and vegetation spectral indices (N = 528 combinations) using the Spearman method. Since we conducted multiple tests on the same dataset, we applied the Holm-Bonferroni correction method (Holm, 1979) to identify statistically significant relationships.

3. Results

3.1. Plant physiology

Except for anthocyanins, our model selection identified shrub species as the most important factor (relative importance > 0.9) in explaining the observed variability in plant phisiology (Table 2). The second main effect was the season, as it was a significant predictor for chlorophyll, flavonols, anthocyanins and NBI. We obtained the maximum relative importance (value of 1) for chlorophyll, flavonols, anthocyanins and NBI (Table 2). Moreover, the interaction between season and plant species was statistically significant for chlorophyll and NBI, with a relative importance of 0.98 (Table 2). The interaction between species and the treatment was significant for chlorophyll, anthocyanins, NBI and g_s, and their relative importance ranged from 0.68 (anthocyanins) to 0.96 (NBI). The interaction between treatment and season, and the triple interaction among season, species and treatment, was not significant in any model (Table 2).

Chlorophyll content in *C. ladanifer* showed lower values under intense browsing treatment than under simulated recent browsing (p-value = 0.005) and no browsing (p-value < 0.001). Chlorophyll levels were higher in winter than in spring for both species (Fig. 1) (p-value < 0.001). Notably, *C. ladanifer* exhibited significantly greater chlorophyll values than *P. angustifolia* (p-value = 0.02), with the difference being more pronounced in winter than in spring (Fig. 1) (p-value < 0.01).

Cistus ladanifer exhibited higher anthocyanin values when browsed (primarily in winter), whereas no differences were observed for *P. angustifolia* across treatments (Fig. 1). Anthocyanin (Anth) levels were higher in spring than in winter for both species (p-value < 0.001).

For *C. ladanifer,* NBI values from the intense browsing treatment were significantly lower than those from the no-browsing treatment (p-value < 0.001). NBI increased in winter (p-value = 0.001), primarily for *C. ladanifer* (p-value < 0.001), and it had higher NBI values compared to *P. angustifolia,* particularly in winter (with significant differences between species only in winter, p-value < 0.001). NBI values differed significantly between species only under the treatment with no browsing (p-value < 0.001).

Overall, the flavonol level increased in winter (p-value < 0.001) with *P. angustifolia* exhibiting higher values than *C. ladanifer* (Fig. 1) (p-value < 0.001).

Although there were no significant differences in stomatal conductance (g_s) across treatments when species were pooled, *C. ladanifer* exhibited lower g_s under intense browsing. Conversely, *C. ladanifer* had higher Φ_{PSII} (p-value < 0.001) and g_s (p-value < 0.001) values than *P. angustifolia* in both seasons.

3.2. Hyperspectral indices

The herbivory treatment (or some of the interactions with the other predictors) had a significant effect on 8 out of the 27 spectral indices (Table 3). Season or some of its interactions were significant predictors for all spectral indices (Table 3), and the most important predictor for all

Table 2

Summary of the linear models (P-values and relative importance) to assess the effect of season (Ssn), species (Sp) and treatment (Trm) and their interactions on six plant physiological variables [chlorophyll, flavonols, anthocyanins, NBI leaf content, quantum yield of photosystme (Φ_{PSII}) and stomatal conductance (g_s)] recorded in two Mediterranean shrubs with contrasting palatability: *Cistus ladanifer,* non-palatable, and *Phillyrea angustifolia,* highly palatable, browsed by red deer in the Cabañeros National Park in central Spain. Season (Spring and Winter), Species (*C. ladanifer* and *P. angustifolia*), treatment (intense –longlife- browsing, recent browsing, and no browsing).

Variable response	Predictors													
	Season (Ssn)		**Species (Sp)**		**Treatment (Trm)**		**Ssn:Sp**		**Ssn:Trm**		**Sp:Trm**		**Ssn:Sp:Trm**	
	p	RI	*p*	RI	*p*	RI	*p*	RI	*p*	RI	*p*	RI	*p*	RI
Chlorophyll	**<0.001**	1	**0.002**	1	0.363	0.97	**0.005**	0.98	0.159	0.46	**0.002**	0.96	0.726	0.12
Flavonols	**<0.001**	1	**<0.001**	1	0.696	0.32	0.501	0.32	0.210	0.15	0.902	0.04	0.982	0
Anthocyanins	**<0.001**	1	0.097	0.91	0.637	0.75	0.368	0.34	0.758	0.13	**0.023**	0.68	0.885	0.01
NBI	**0.002**	1	**<0.001**	1	0.245	0.99	**0.005**	0.98	0.416	0.34	**0.004**	0.98	0.49	0.08
Φ_{PSII}	0.334	0.56	**<0.001**	1	0.997	0.17	0.238	0.26	0.306	0.04	0.855	0.02	0.838	0
g_s	0.663	0.55	**<0.001**	1	0.279	0.82	0.151	0.31	0.379	0.16	**0.032**	0.72	0.440	0.03

p = *p-value* for the full model. Bold type highlights those variables that had a significant effect on the response (*p*-value < 0.05).

RI = Relative Importance is the cumulative sum of the AIC weights across all models where the predictor is present on the model averaging approach (0 = minimum importance to 1 = maximum importance).

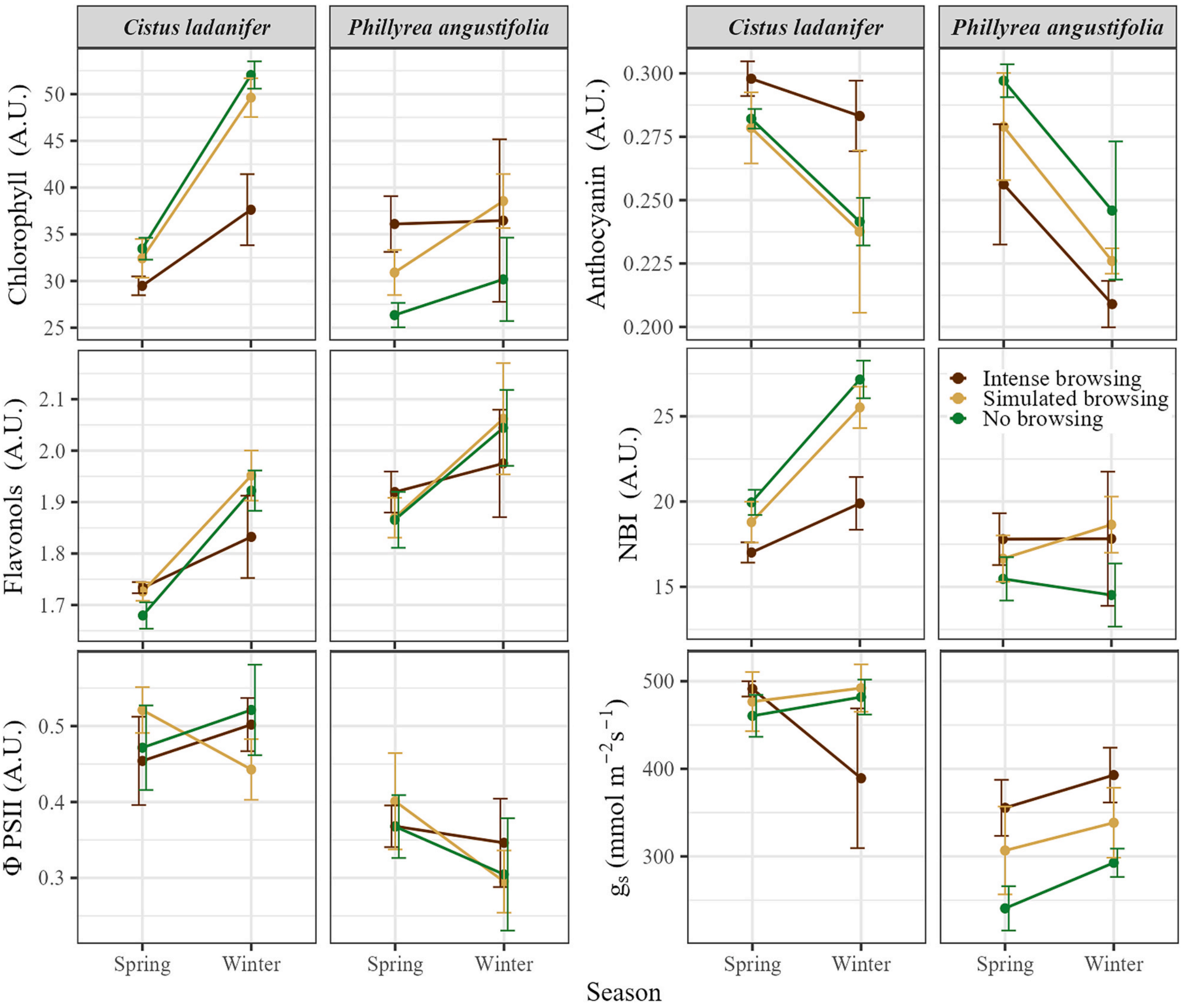

Fig. 1. Physiological variables (chlorophyll, anthocyanins, flavonols, NBI, chlorophyll fluorescence and stomatal conductance) of *C. ladanifer* and *P. angustifolia* in two seasons (spring vs winter), and three treatments of red deer browsing (Green = no browsing; Yellow = simulated recent browsing; dark brown = intense long-life browsing) in the Cabañeros National Park, Central Spain. Error bars indicate standard error. A. U. = Adimensional units. (*) = Treatment and species interaction had a significant effect on the response (*p*-value < 0.05). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

models (Table 3). Species was a significant factor for eight indices, with a relative importance > 0.9 (Table 3).

We identified some indices affected by the herbivory treatment, which are related to chlorophyll fluorescence (Fig. 2) or the carotenoid/chlorophyll ratio (Fig. 3).

Intense browsing had significantly lower values than the other two treatments for the *R740/R800*, and *R750/R800* indices (Fig. 2; Tukey test p-value < 0.05). These indices were lower under intense browsing treatment for both species in both seasons. The indices exhibiting a three-way interaction between season, species, and treatment ((*R685ˆ2)/(R675*R690)*; *R735/R645*; *R735/R660*; *R735/R680*) showed significant differences among browsing treatments depending on species and season. The indices *R735/R645*, *R735/R660*, and *R735/R680* were highly similar, producing nearly identical values due to the close proximity of their reference wavelengths in the spectrum. Similarly, the indices *R740/R800* and *R750/R800* were also closely related to each other (see Supplementary Fig. S2).

The indices *R735/R645, R735/R660* and *R735/R680* showed significantly lower values in winter than in spring for *P. angustifolia* (Tukey test p-value < 0.05) under intense browsing. Moreover, *(R685ˆ2)/(R675*R690)* showed significantly lower values in winter than in spring for *C. ladanifer* (Tukey test p-value < 0.02) for treatments with no browsing and simulated recent browsing. This interaction had a very low relative importance (RI < 0.05).

When we analyzed the data for each season separately, we found that the NDPI and SRPI indices in spring were lower in the no-browsing compared to intense browsing treatment (p-value < 0.05) for both species (Fig. 3). In winter, this relationship was reversed exhibiting higher index values for plants with no browsing, although this difference was not statistically significant (Tukey test p-value > 0.05).

There was no model where the interaction between species and season or species and treatment was significant. The interaction between season and treatment was a main factor in three indices. The three-way interaction between season, species and treatment was a significant predictor for 4 indices, but this predictor had a very low relative importance (<0.05).

It was observed that indices related to photosynthetic light use efficiency and water content exhibited higher values during the spring season. In contrast, indices that exhibited higher levels during the winter season were those related to the carotenoids/chlorophyll ratio (see

Table 3

Relative importance (RI), from 0 to 1, of each predictor (Ssn= Season; Sp=Species; Trm = Treatment) on the model averaging approach with 27 hyperspectral indices as response variables. The predictors in bold are statistically significant for the spectral index model (p-value < 0.05).

Index	Predictors						
	Ssn	**Sp**	**Trm**	**Ssn: Sp**	**Ssn: Trm**	**Sp: Trm**	**Ssn: Sp: Trm**
_(R675*R690)/(R683ˆ2)_	**1**	0.56	0.24	0.16	0.03	0.02	0
_(R683ˆ2)/(R675*R690)_	**1**	0.63	0.21	0.2	0.02	0.03	0
_(R685ˆ2)/(R675*R690)_	**1**	0.56	0.39	0.19	0.06	0.06	**0.02**
Ant	**1**	**0.92**	0.17	0.37	0.03	0.04	0
clPRI	**1**	**0.9**	0.23	0.33	0.05	0.03	0
EVI	**1**	0.44	061	0.13	0.18	0.04	0
NDPI	0.77	0.53	0.7	0.33	**0.64**	0.07	0.01
NDVI	**1**	0.79	0.28	0.48	0.07	0.11	0.03
NDVIchl	**1**	0.65	0.3	0.44	0.08	0.13	0.03
PRI	**1**	0.44	0.19	0.16	0.03	0.03	0
PRI13	**1**	**0.91**	0.19	0.27	0.03	0.02	0
_R680/R630_	**1**	0.81	0.17	0.22	0.03	0.02	0
_R685/R630_	**1**	**0.86**	0.2	0.23	0.04	0.03	0
_R685/R655_	**1**	0.4	0.32	0.12	0.1	0.06	0
_R687/R630_	**0.97**	**0.93**	0.28	0.25	0.14	0.05	0
_R690/R600_	**1**	**0.98**	0.29	0.3	0.1	0.04	0
_R690/R630_	**1**	0.47	0.41	0.12	0.11	0.03	0
_R690/R655_	**1**	0.56	0.23	0.17	0.03	0.02	0
_R735/R645_	**1**	0.58	0.5	0.26	0.2	0.11	**0.05**
_R735/R660_	**1**	0.59	0.48	0.21	0.17	0.08	**0.04**
_R735/R680_	**1**	0.58	0.5	0.13	0.17	0.08	**0.04**
_R740/R800_	**1**	**0.96**	**1**	0.3	**0.85**	0.24	0.01
_R750/R800_	**1**	**0.95**	**0.99**	0.28	0.76	0.17	0
SIPI	**1**	0.56	0.33	0.25	0.13	0.03	0
SR	**1**	0.8	0.24	0.5	0.05	0.09	0.02
SRPI	0.83	0.5	0.78	0.27	**0.74**	0.07	0.01
WI	**1**	0.5	0.33	0.14	0.14	0.09	0.01

Fig. S3). Additionally, while some indices related to the amount of green biomass and chlorophyll fluorescence exhibited higher values in winter, others, such as NDVI, showed higher values during the spring season.

Regardless of the treatment, we found 6 indices that were significantly different between the two species (Fig. 4).

For *C. ladanifer*, indices such as *R685/R630*, *R687/R630*, and *R690/R600* were significantly higher compared to *P. angustifolia*. These indices are related to green biomass and chlorophyll fluorescence. Conversely, indices clPRI and PRI13, associated with photosynthetic efficiency, were higher for *P. angustifolia* than for *C. ladanifer*.

3.3. Correlation between physiology and spectral indices

No strong correlations were identified between physiological variables and spectral indices (only for those that represented statistically significant relationships). The strongest correlations observed were between chlorophyll and the indices _(R683ˆ2)/(R675*R690)_ (correlation coefficient = 40), _(R675*R690)/(R683ˆ2)_ (correlation coefficient = -40), but they were not significant (p-values > 0.05) (see Supplementary Fig. S2).

4. Discussion

Both physiological and spectral responses of plants showed significant differences across herbivory treatments. These differences were noted between plants with no browsing or in simulated recent browsing and plants subjected to intense, long-term browsing. Interestingly, no significant differences were observed between simulated recent browsing (1 year) and no browsing at all. The loss of 30 % of twigs on two occasions, separated by several months, does not appear to induce significant stress for the plant, at least within the first year after damage, consistent with previous studies showing a slow response of plants to light herbivory damage (Comas, 2020; Holland et al., 2013). Another important limitation of the simulated browsing treatment was the absence of deer saliva during plant clipping. While the mechanical damage was simulated through cutting, the chemical component of deer saliva was not incorporated. Therefore, the absence of this chemical cue may explain why no significant changes were observed between the

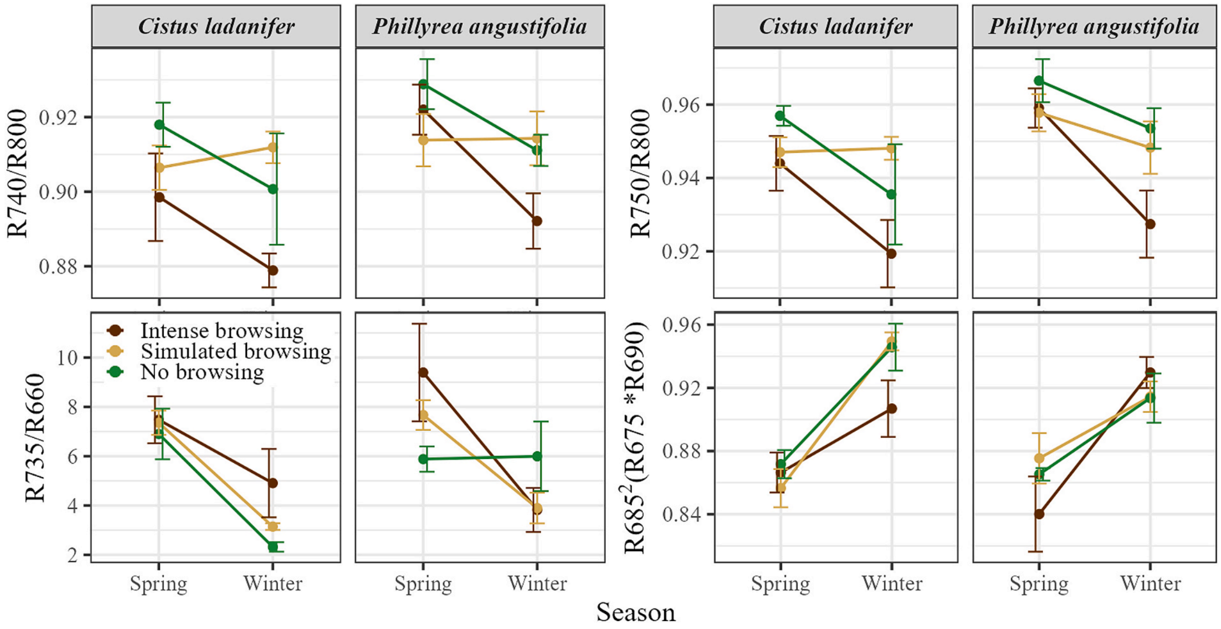

Fig. 2. Spectral indices related to chlorophyll fluorescence for which the browsing treatment or some interaction with treatment were significant (ANOVA p-value < 0.05). Error bars indicate standard error. (Spring = June 2023; Winter = February 2024; Green = no browsing; Yellow = Simulated recent browsing; Red = Intense, long-life browsing). See Table 1 for information on spectral indices. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

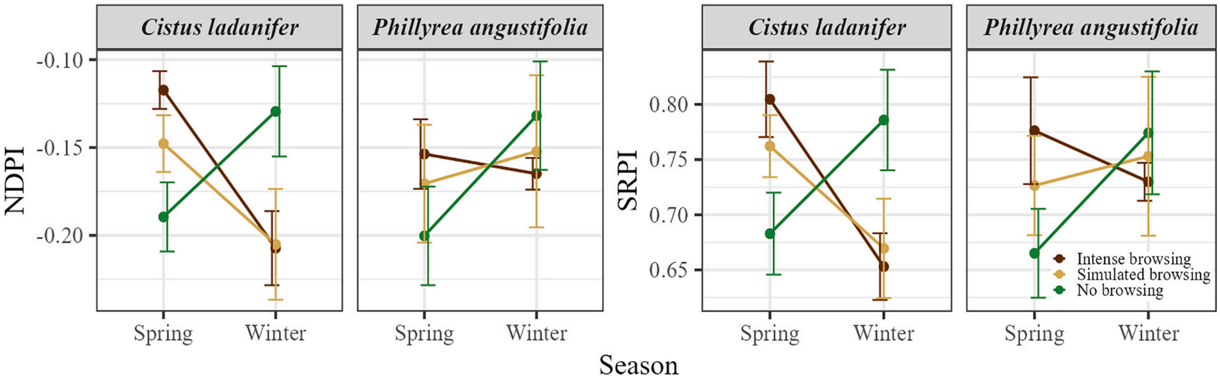

Fig. 3. Spectral indices related to the carotenoid/chlorophyll ratio for which the interaction between browsing treatment and season was significant (ANOVA p-value < 0.05). Error bars indicate standard error. Spring = June 2023; Winter = February 2024; Green = no browsing; Yellow = Simulated recent browsing; Red = Intense, long-life browsing). See Table 1 for information on spectral indices. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

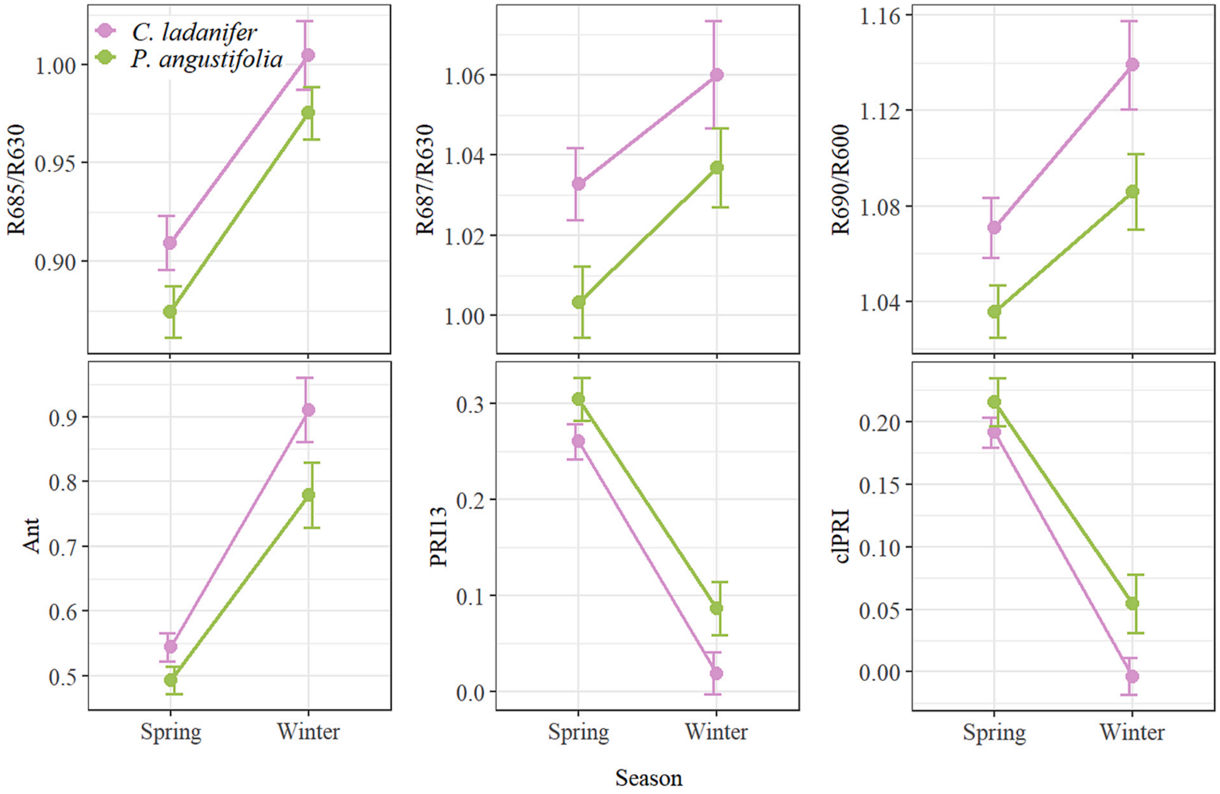

Fig. 4. Spectral indices for which only season and species were significant predictors (p-value < 0.05). Error bars indicate standard error. (Spring = June 2023; Winter = February 2024; Purple = *Cistus ladanifer*; Green = *Phillyrea angustifolia*). See Table 1 for information on vegetation indices. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

natural browsing and the 30 % clipping treatment in either species (Ohse et al., 2017; Bergman, 2002). Whereas many studies have shown an effect of insect herbivory on the vegetation spectral response (Fuenzalida et al., 2019; Mirik et al., 2012; Stone et al., 2001) very few have addressed the effect of increasing herbivory intensity and none of them by large herbivores such as deer. According to preliminary results by Comas (2020), spectral responses of vegetation did not change significantly at low levels of deer herbivory, whereas vegetation indices exhibited sharper increases or decreases when browsing intensity was high (degree > 3). This study only found differences for intensively browsed plants, which persisted throughout the plant's lifespan. Such findings suggest the existence of a threshold for herbivory damage beyond which plants exhibit more distinct responses.

The response to browsing varied by species. Thus, the intense lifetime browsing of *P. angustifolia* resulted in higher chlorophyll and nitrogen levels than those found in unbrowsed or lightly browsed plants, while *C. ladanifer* showed a decrease in these two variables under intense browsing conditions. In response to stress, chlorophyll concentration has been shown to vary with stress levels, increasing under low stress and decreasing under very high levels (Agathokleous et al., 2020). Intensively browsed plants had at least 50 % of their leaf and twig biomass consumed during the year of sampling, reflecting high red deer abundance. Consequently, the increase in chlorophyll and nitrogen in *P. angustifolia* and the decrease in these variables in *C. ladanifer* may reveal differences in stress intensity and varying fitness in response to ungulate herbivory. Based on our observations, *C. ladanifer* appears to spread more within the fenced area than outside, while *P. angustifolia* is more abundant in areas with high deer density. In a scenario of prolonged and intense herbivory, it seems that *C. ladanifer* suffers more damage than *P. angustifolia*. The physiological responses of *P. angustifolia* may indicate an adaptive strategy, whereas those of *C. ladanifer* may reflect symptoms of stress.

Differences in the responses of these two species to intense browsing were likely due to variations in palatability. *Cistus ladanifer* defends itself through lower palatability, thereby reducing the likelihood of consumption, while *P. angustifolia* is better adapted to browsing. Other studies have documented a trade-off between defense and tolerance to herbivory (Rosenthal & Kotanen, 1994; Salgado-Luarte et al., 2023). Less palatable plants invest more in physical and chemical defenses to avert herbivory. In contrast, more palatable plants are consumed more frequently but demonstrate enhanced tolerance to stress (Bilbrough & Richards, 1993; Salgado-Luarte et al., 2023). Chlorophyll and nitrogen are essential compounds for plant health (Leghari et al., 2016; Pavlovic et al., 2014). The "dilution effect" suggests that heavily browsed plants may retain higher nutrient concentrations in the remaining leaves compared to unbrowsed ones, an effect not observed in heavily browsed *C. ladanifer*. Sustained defoliation reduces carbon and nitrogen photoassimilation, potentially leading to nutritional deficiencies over time. Unexplored aspects, such as photosynthetic overcompensation or root growth following browsing (Drexhage & Colin, 2003), could also underpin the different sensitivities of nitrogen and chlorophyll in response to herbivory stress in both species. While our study does not evaluate plant survival, we have observed that browsed *C. ladanifer* plants resprout poorly and eventually die when browsing is severe. Further studies are needed to identify factors beyond palatability that influence the survival of both species under high herbivory pressure.

Anthocyanin is a pigment that plays important roles in protection against herbivores (Lev-Yadun & Gould, 2008) and ultraviolet (UV) radiation (Hatier & Gould, 2008), among other stressors (Moustaka et al., 2020). The anthocyanin content of *C. ladanifer* was higher in plants exposed to intense browsing, indicating higher stress under a similar browsing pressure compared to *P. angustifolia*. This result aligns with the findings of Capó et al. (2024), who found that *Quercus ilex* seedlings excluded from herbivory exhibited lower anthocyanin levels than those exposed to herbivory.

Stomatal conductance (g_s) is crucial for regulating the exchange of carbon dioxide and water vapour between the atmosphere and the plant and is essential for optimal plant physiological functions, including leaf thermoregulation and photosynthesis (Farquhar & Sharkey, 1982). *Cistus ladanifer* exhibited lower g_s in cases of intense browsing, while *P. angustifolia* maintained higher g_s values across both seasons. The closure of stomata is a common response to varying stress factors, including biotic ones (Lin et al., 2022). Lower nitrogen concentrations can be directly and indirectly associated with diminished g_s in response to abiotic stresses (Quero et al., 2006), which may similarly relate to severe browsing.

We identified two spectral indices, *R740/R800* and *R750/R800*, for which herbivory treatment was significant in the model. These indices displayed significant differences between the intense browsing treatment and the other two herbivory treatments for both species. Both indices indicated lower values under intense browsing in both seasons. Chlorophyll fluorescence has been shown to correlate with the reflectance ratio at wavelengths 750 and 800 (or 740 and 800) (Zarco-Tejada et al., 2000a) Additionally, these indices may reflect the increase in oxidized compounds, such as quinones, commonly known as brown pigments, which are typically formed through the activity of secondary metabolites that emerge in response to environmental stress (Ashraf et al., 2018; Edreva et al., 2008). Zarco-Tejada et al., (2000b) noted that significant differences in reflectance between stressed and non-stressed plants (due to high temperature and lack of irrigation) occurred in the wavelengths ranging from 690 to 700 nm and from 740 to 750 nm. This suggests that these indices may be used to identify individuals under high levels of browsing stress and could be highly applicable in diagnostic management. It should be noted that spectral measurements were taken at the leaf scale; therefore, at the whole-plant scale, other indices such as NDVI or those related to green biomass might prove more effective in distinguishing heavily browsed individuals. Still, our results indicate that even in the remaining leaves, differences in reflectance can be detected through these indices.

Two additional indices, NDPI and SRPI, reflected an opposite impact of intense browsing on plants during spring and winter (Fig. 3). In winter, indices were higher for unbrowsed plants, whereas in spring, those subjected to intense and simulated recent browsing showed elevated values. These indices are related to the carotenoid/chlorophyll ratio (Peñuelas et al., 1995a) indicating that the treatment significantly influences changes in the carotenoid/chlorophyll ratio between seasons. According to (Peñuelas et al., (1995c), the SRPI index positively correlated with termite attacks on apple trees due to reductions in leaf chlorophyll content. The absence of correlations between these indices and leaf chlorophyll content in our study (see Supplementary Fig. S2) suggest variations may rely more on carotenoid content or the carotenoid/chlorophyll ratio. Additionally, browsing can affect leaf phenology, potentially anticipating or delaying leaf emergence (Post et al., 2024) which in turn impacts the carotenoid/chlorophyll ratio and its seasonality. Higher NDPI and SRPI values in intensely browsed plants during spring may reflect delayed leaf phenology (i.e., younger leaves) compared to unbrowsed plants. Similarly, new shoots produced by clipped plants could lead to higher NDPI and SRPI values compared to unbrowsed plants. Seasonal fluctuations in physiological variables and spectral signatures support earlier studies observing strong phenological differences in Mediterranean environments (Capó et al., 2024; Comas, 2020; Gori et al., 2019; Nunez-Olivera et al., 1996; Valares Masa et al., 2016; Zunzunegui et al., 2011).

Plant species was identified as a main factor in several spectral indices, independent of herbivory treatment. This finding indicates that these indices can be used to identify both taxa regardless of herbivory pressure. The classification of species based on vegetation indices or spectral signatures has previously been successfully employed in Mediterranean environments (Alonso-Martínez et al., 2020). Future studies should evaluate prolonged responses, particularly to the simulated recent herbivory (beyond 1 year), and involve more species and larger sample size to enhance our understanding of the potential spectral responses of plants to herbivory.

5. Conclusion

Plants subjected to intense browsing throughout their lives exhibit physiological changes. No significant differences were observed between plants experiencing light, recent browsing and those with no browsing.

The physiological response to deer herbivory differed significantly between species. The more palatable species, *Phillyrea angustifolia*, exhibited more adaptive physiological changes in response to intense browsing compared to *Cistus ladanifer,* which displayed signs of physiological stress.

The different spectral signatures of *P. angustifolia* and *C. ladanifer* regardless of browsing treatment reveals a strong potential for using these indices for species identification. R750/R800 and R740/R800 are effective spectral indices in identifying plants under high levels of deer herbivory stress, demonstrating their potential utility for diagnosing and managing vegetation in environments with high ungulate densities.

Seasonal variations were notable within the physiology and spectral signatures of both, palatable and non-palatable shrubs. Chlorophyll, flavonols and NBI increased in winter, while anthocyanins decreased. Spectral indices such as SRPI and NDPI were significantly affected by browsing treatments, presenting opposing trends based on treatment conditions across seasons.

CRediT authorship contribution statement

Carmen Rello: Writing – original draft, Investigation, Formal analysis, Data curation. **Emmanuel Serrano:** Writing – review & editing, Supervision, Methodology, Funding acquisition, Data curation, Conceptualization. **Jesús Rodríguez-Calcerrada:** Writing – review & editing, Supervision, Methodology, Data curation, Conceptualization. **Iolanda Filella:** Writing – review & editing, Supervision. **Josep Peñuelas:** Writing – review & editing, Supervision. **Ramón Perea:** Writing – review & editing, Supervision, Methodology, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ecolind.2025.114367.

Data availability

Data will be made available on request.

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