

SECONDARY METABOLITES AND NUTRIENTS IN WILD OLIVE IN RESPONSE TO MEDITERRANEAN SEASONALITY AND BROWSING INTENSITY

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Abstract

Secondary metabolites are related to the defensive needs of plants and could act as indicators of the degree of stress experienced by vegetation. Understanding how individual woody plants respond chemically to different degrees of browsing would provide information applied to herbivore management in the face of environmental changes. However, very few studies compare more than two levels of browsing intensity. In addition there is very little information on joint variations in leaf nutrient and polyphenol content in response to various browsing intensities in woody plants of Mediterranean ecosystems across the different seasons.

We developed an experimental procedure under natural conditions in order to explore the relationships between browsing intensity and secondary metabolites and nutrients in wild olive leaves from southern Spain. We studied total polyphenolic compounds (TPC) and condensed tannins (CT) at increasing browsing intensities from 0 to 3 ranges, throughout the year (January, June and September) according to seasonal climatic variations and the trees' biological cycle. Variations of nutritional quality were assessed through nitrogen (N) and carbon (C) content, and their ratios (C/N), (N/TPC) and (N/CT) as palatability indices.

We found stress due to Mediterranean abiotic factors driving the main variation of secondary metabolite contents, its relationship with nitrogen and carbon content, and its seasonal variations. An increase in secondary metabolite content with increasing browsing intensity was detected, but seasonal climatic variations lead to an increase in secondary metabolites to a greater extent than browsing, with the interactions also important. According to our results the efficacy of secondary compounds in preventing browsing is limited, perhaps efficient at moderate browsing levels but at more intense browsing levels the effect may be the opposite, compromising plant growth and maintenance, especially in the more restrictive seasons of the Mediterranean climate and coinciding with factors such as drought. Our findings on seasonal variations in the nutritional quality-palatability of leaves are important for understanding differential feeding behavior and plant selection of ruminants in a broad context of plant-herbivore interaction useful for herbivory management in Mediterranean ecosystems.

Keywords: Animal-plant interactions, Chemical plants defense, Factorial experiment in a completely randomized design, Mediterranean environmental constraints, Palatability nutritional quality, Selective browsing

Abbreviations: *CT: Condensed Tannins; TPC: Total Polyphenolic Compounds*

Introduction

Plants' carbon-based secondary metabolites constitute the world of chemical signals through which plants relate to their environment (Chen et al. 2022, Mostafa et al. 2022). Among these compounds the total polyphenolic compounds (TPC), a heterogeneous group of carbon-based aromatic substances, play an important role in practically all the interactions that a plant establishes with its environment (Harborne 1966, Waterman & Mole 1994). The condensed tannins (CT) are a type of polyphenol that produce harder leaves and reduce palatability (Waterman & Mole 1994). Polyphenols show great antioxidant activity and can be used by plants as protection mechanisms against abiotic and biotic stresses (Ksouri et al. 2008, Anu et al. 2018). Thus the total polyphenol contents, an important biological and ecological significance, is related to the plants' defensive needs and could act as an indicator of the degree of stress experienced by the plants (Gil & Tuteja 2010, Ashraf et al. 2018).

In the Mediterranean area the environmental and climatic conditions that change with stationarity pose important stress for the plant. Mediterranean summers are characterized by the simultaneous occurrence of high light intensities, high temperatures and low water availability, three factors that can control, individually and in combination, the emissions of phenolic compounds (Loreto et al. 2014). In addition, Mediterranean ecosystems, especially in the south of the Iberian Peninsula, have evolved under high browsing pressure (Azorit et al. 2005, 2006), and in recent times a decrease in tolerance to ungulate pressure has been documented in general in all habitats from woodlands to grasslands, including those called *dehesas* and *montados* (Velamazán et al. 2020). Therefore, plants would have to develop a greater structural or chemical response effort when continuous and prolonged browsing by ruminants is added to environmental factors.

Understanding how individual woody plants respond chemically to different degrees of browsing in Mediterranean ecosystems would allow monitoring plant status through secondary metabolite contents and could provide information applied to minimizing the negative effects of herbivory; and would help us in understanding how plant assemblages respond to changing environments (Hester et al. 2009). However, chemical response may also vary with seasons, the plant life cycle, organ studied (Brahmi et al. 2013), as well as leaf age (Rugna et al. 2008), and intensity of damage caused by herbivores (Scogings et al. 2011). Moreover, very few studies have compared more than two levels of browsing intensity (Scogings et al. 2011, Nosko et al. 2020) and there is very little information on joint variations in leaf nutrient and polyphenols content in response to different browsing intensities in woody plants from Mediterranean ecosystems across the seasons.

In this study we explore the relationships between browsing intensity and secondary metabolites and nutrients in mature leaves of wild olive (*Olea europaea L. subsp. europaea var. sylvestris*), an

abundant Mediterranean woody species that is among the most selected by both domestic and wild ruminants. We developed an experimental procedure under natural conditions to test the effects of different degrees of browsing in a Mediterranean ecosystem of southern Spain on the variation of total polyphenolic compounds (TPC), condensed tannins (CT) and nutrients such as nitrogen (N), as well as carbon content (C) at different browsing intensities (increasing from 0 to 3 ranges), in different seasons of the year (January, June, September) according to the biological cycle of the wild olive tree. First, we explored how stress due to Mediterranean abiotic factors drives the variation of secondary metabolites, its relationship with nitrogen and carbon content, and its seasonal variations. Next, given that browsing is a factor that drives the total production of polyphenolic compounds and influences the nutritional and structural status of woody plants (Scogings et al. 2011), we hypothesized a significant increase in the content of polyphenolic compounds in browsed compared to non-browsed trees subjected to the same climatic and environmental conditions. We expect an increase in the content of secondary metabolites as browsing intensity increases, and that browsing causes an increase in polyphenols and tannins to a greater extent than seasonal climatic variations and plant life cycle events.

Variation in wild olive nutritional attributes as a function of seasonality and browsing level were also assessed to help explain differential ruminant feeding behavior and seasonal plant selection (Azorit et al. 2012). Both phenols and tannins work together as chemical defenses of plants to prevent or reduce the attacks of herbivores on woody plants (Croteau et al. 2000). However, chemical defense appears at times to be ineffective in deterring ruminants browsing (Nosko et al. 2020), and it has even been reported that intensive and severe browsing has the opposite effect, causing an increase in N concentrations or a reduction of TPC and CT in individual plants (Person et al. 2005). Heavily browsed plants may be characterized by C-poor/N-rich tissues (Scogings et al. 2011, Morrison et al. 2022), and leaves richer in nitrogen than in secondary metabolites could make these parts more palatable and more vulnerable to consumption by ruminants. Therefore, N as an indicator of total protein was related to phenols and tannins content in a ratio N/TPC and N/CT, as well as leaf carbon nitrogen ratio C/N to assess the variations in nutritional quality and palatability (Palo et al. 1993). We expect a differential response to four levels of browsing and seasonal variation in nutritional quality, which may help explaining differential feeding behavior and plant selection in a broad context of plant-herbivore interaction in a Mediterranean ecosystem.

Materials and Methods

Study area

Field studies were conducted in the *Dehesa del Santo Cristo* (JA-30029-AY), of 495 ha in the municipality of Baños de la Encina (Jaén) in the south of Spain (38°10'N 3°47'W). The area is within a Special Area of Conservation of the Natura 2000 Network in the basin of the Rumblar river (Figure 1). The altitude ranges between 200 m and 450 m above sea level. The area has a Mediterranean-type macroclimate with an irregular distribution of rainfall characterized by an almost total absence of rainfall together with high temperatures during the summer. Throughout the year, the temperature generally varies from 2°C to 36°C and rarely falls below -2°C or rises

above 45°C. The mean annual rainfall during the study period (2020-21) was 563 mm with more than 40% of rain falling during spring and ~50% during autumn-winter (Figure 2).

Figure 1. Study area and experimental studio design in the "Dehesa del Santo Cristo" (38°10'N 3°47'W) in Baños de la Encina (Jaén) South of Spain.

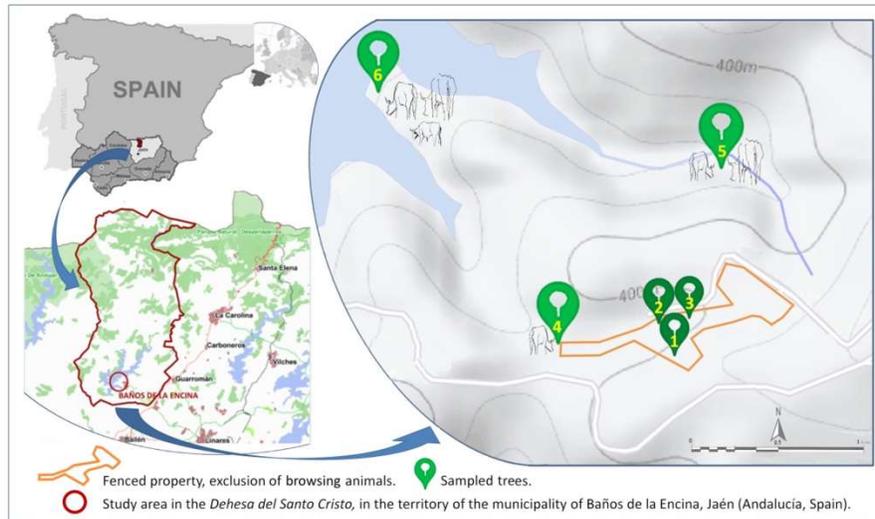
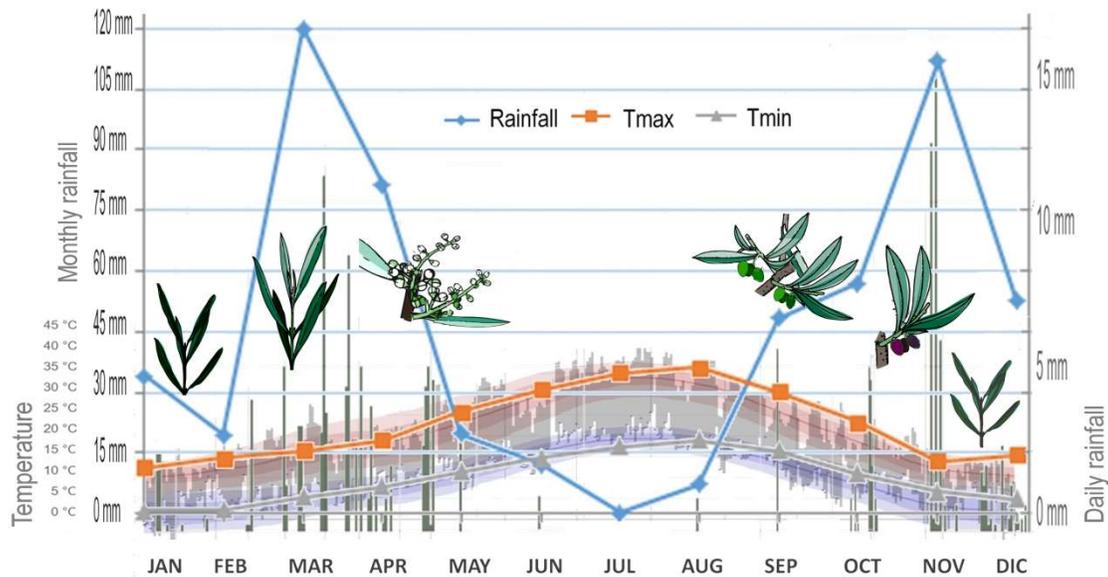


Figure 2. Rainfall and maximum and minimum temperature values for the study period 2020-21, and main events of the biological cycle of the olive tree throughout the year.



The plant communities are typical of siliceous soils and the savannah-like landscapes of the Iberian Peninsula, known as *dehesas* or *montados*, have been a dominant part of this agroforestry system. Currently, the study area is a degraded Mediterranean dehesa predominate with large areas of *Pinus pinea* reforestation. The dominant shrub cover is of rockroses (*Cistus* spp.), yellow broom (*Retama sphaerocarpa* L.), rosemary (*Salvia rosmarinus*), thyme (*Thymus vulgaris*), lavender

(*Lavandula stoechas*), and marjoram (*Origanum majorana*). Shrubs also include oleander (*Nerium oleander*) and other woody tree species such as mastic (*Pistacia lentiscus*), kermes oak (*Quercus coccifera*), and wild olive (*Olea europaea* L. subsp. *europaea* var. *sylvestris*). The wild olive, an evergreen woody species, is the ancestor of the cultivated olive tree (*Olea europaea* L. subsp. *europaea* var. *europaea*) present throughout the Mediterranean basin.

Most of the territory is public property where pastures are used mainly for cattle with a density varying from 10 to 20 ruminants km⁻². There are several small private properties dedicated to the cultivation of orchards and olive groves. One of these farms, with an area of 4 hectares and fenced to prevent the entry of livestock, was used in this study as an experimental exclusion zone for the sampling of wild olive trees and olive trees (as a control of possible genetic variations) without browsing (Figure 1).

Experimental design, browsing intensity rating and trees selected for sampling.

We conducted our research on the wild olive, which is among the most selected woody plants by domestic and wild ruminants, both by cervids (Rodríguez Berrocal 1977) and bovids (Martínez-Martínez 1992). In Mediterranean ecosystems of southern Spain the wild olive coexists with high densities of ruminants, with ruminants' browsing selection on wild olive reaching proportions close to 12% of the total biomass consumed, depending on the species and habitat (Martínez-Martínez 1992), especially in autumn and winter (Azorit et al. 2012). This species is an abundant woody plant, thermophilic and highly adapted to the harsh environmental conditions of the Mediterranean climate. It can tolerate drought stress and different temperature regimes, although it is sensitive to cold and excessive humidity (Arenas-Castro et al. 2020, Fanelli et al. 2022).

Four browsing intensities were established and six different tree treatments were selected. Three non-browsed trees were sampled: one olive tree and two wild olive trees, excluded from herbivory on a fenced property (the olive and a wild olive tree were irrigated, and one wild olive without irrigation to evaluate the differential effect of drought). Three wild olive trees browsed naturally over a long-term use by large herbivores (mainly cows) were also selected and sampled (Figure 1). In the study area, ruminant densities average 15 cows/km², and the variation in browsing intensity was mainly due to the existence of an environmental gradient of browsing drivers such as distance to water, altitude, land slope and proximity to inhabited fenced areas, which favored differential access of cows to trees and consequently the existence of more browsed trees than others.

The application of a completely randomized design implies using homogeneous experimental units, that is, we only select trees of the same size, age and similar controlled maintenance conditions, but with a sufficiently differentiated browsing status. The browsing status of each selected tree was described visually as the fraction (%) of productive canopy volume (including branches with leaves or buds) considered to be missing due to browsing and considering whether the tree was browsed only laterally or also showed apical browsing (Nosko et al. 2020). We used a semi-quantitative scoring scale like that considered as an objective measure in forest regeneration and browsing impact inventories (Scogings et al. 2011, Kupferschmid 2018). As summarized in

Table 1, four degrees of browsing intensity were described as: *not browsed* (0), *lightly browsed* (1), *moderately browsed* (2) and *heavily browsed* (3).

Trees Sampled	Browsing intensity	Rating	browsing intensity descriptions	Trees Location
Protected into fenced-exclusion of ruminants				
1	0	Not browsed	with irrigation (<i>O. europaea</i> var. <i>europaea</i>)	38°10'41.6"N 3°47'11.5"W
2	0	Not browsed	with irrigation (<i>O. europaea</i> var. <i>sylvestris</i>)	38°10'42.2"N 3°47'11.5"W
3	0	Not browsed	without irrigation (<i>O. europaea</i> var. <i>sylvestris</i>)	38°10'42.2"N 3°47'11.0"W
Browsed naturally by ruminants				
4	1	Lightly browsed	< 15% affected (leaves and bud removal) tree browsed laterally	38°10'40.2"N 3°47'18.8"W
5	2	Moderately browsed	~ 25% affected (leaves, bud, shoot) tree were browsed laterally	38°10'52.9"N 3°47'05.5"W
6	3	Heavily browsed	> 50% loss including leader shoot missing lateral and apical browsing as well	38°10'57.3"N 3°47'36.2"W

Table 1. Browsing intensity rating in the 6 experimental tree samples at three times of the year (January, June and September) according to the seasonal biological cycle of olive (*Olea europaea* var. *europaea*) and wild olive (*Olea europaea* var. *sylvestris*) in the Mediterranean ecosystem

The leaves sampled were taken from below 1.5 m on trees, and fresh leaves were collected in 2020-21 at three times of the year following biological cycle states: (1) vegetative rest state in January after the fruits' ripening completion in autumn; (2) leaves grown and flowers blooming in June, and (3) fruits ripening in September (Figure 2). Fruits begin to change coloration in October and continue with darkening until December. The quantity of leaves grown and flowers blooming in June depends on spring rainfall, which influences the growth of new shoots and inflorescences (Consolo 1997).

Laboratory procedures and methanol extracts

The leaves were manually separated from the branches to obtain a weight of at least 10 g. Leaves were stored in paper bags and thus transported to the research laboratory. These leaves were washed in the shade to remove any soil or micro-organisms, were measured both in length and in width to assess size differences among trees, and then dried and stored in an oven at 65°C until

analysis. The extracts were prepared according to the methodology of Casas-Sánchez et al. (2007), with minor modifications. 10 g of leaves were soaked in 50 ml of methanol, placing them in an orbital shaker (Optic ivymen system Cod. 5312001 COMECTA-IVYMEN 100-240 V/50Hz Sevidel #:1145, Made in Spain) for 24 h. at room temperature ($25 \pm 1^\circ\text{C}$). The extracts were filtered through Whatman n° 1 filter paper (Sigma Aldrich, France). These extracts were stored, in small topaz bottle, at 4°C until used for determinations a few days later.

Determination of total polyphenolic compounds (TPC)

Polyphenol content was determined using the Folin-Ciocalteu procedure as described in Edziri et al. (2019). The samples were previously diluted with methanol (1:5) due to the large amounts of phenolic components in the samples. Total phenols were determined by mixing 250 μl of the methanolic extract of each sample with 1 ml of distilled water and 250 μl of Folin-Ciocalteu reagent. After 3 min. incubation, 250 μl of Na_2CO_3 (7%) was added to the mixture. The final volume was adjusted to 5 mL with distilled water and mixed thoroughly, then incubated for 90 min at room temperature (25°C) in the dark. The absorbance of the resulting blue complex was measured at 765 nm in a VARIAN Cary 4000 UV-vis spectrophotometric (USA). Gallic acid was used to make the standard curve. The range of the calibration curve for gallic acid was 0-400 $\mu\text{g/ml}$ ($y = 0.0979x + 0.018$; $r^2 = 0.9983$). The TPC was expressed as mg gallic acid equivalents per gram of dry weight (mg GAE/g DW).

Determination of condensed tannins (CT)

Condensed tannins (CT) were determined according to the method of Julkunen-Titto (1985) with minor modifications. An aliquot of 200 μl of each extract and standard solution was mixed with 6 ml. of 4% vanillin dissolved in methanol, and then was added 3 ml of HCl concentrate. The mixture was incubated in the dark, at ambient temperature, for 20 min. The absorbance against blank was read at 500 nm in a VARIAN Cary 4000 UV-vis spectrophotometric (USA). (+)-Catechin was used to make the standard curve (0.05-1 mg/ml; $y = 0.801x + 0.0555$; $r^2 = 0.9992$; where y is the absorbance; x is the solution concentration). The results were expressed as mg catechin equivalents (mg CEQ/g DW).

Determination of carbon (C) and nitrogen (N)

Ground dry leaves (0.1 g DW) were also used for the determination of nutritional and structural indices. An elemental Analyzer (Thermo Finnigan Flash EA1112) by Micro Dumas Combustion was used for total nitrogen (N) and carbon (C) elemental analysis (Farina et al. 1991, Tellado et al. 2015). Then N as an indicator of total protein was related to tannins content in a ratio N/CT in order to assess the variations in nutritional quality and palatability (Palo et al. 1993). Leaf C/N ratio and N/TPC ratio were also assessed.

Data analysis

A two-factor factorial experiment in a completely randomized design was conducted in order to investigate the effects of two explanatory variables (or factors), the browsing intensity and month of the year, on each of the four response variables, total polyphenolic compounds (TPC), condensed tannins (CT), carbon (C) and nitrogen (N) and the three ratios C/N, N/CT and N/TPC. This type of design offers an efficient way to obtain the maximum information from a small

number of experiments and the replication is necessary to estimate the interaction effects (Montgomery 2017). In our study three replicates of each experiment were taken for each of the six levels of factor 'browsing intensity', represented by six different trees, in each of the three months of the year considered.

First, in the preliminary data exploration we provided a descriptive statistical analysis of the response variables of interest for each factor in each month (January, June and September) and we carried out an analysis of the correlation between the response variables considered. Also, we plotted the interaction effects between the two factors, browsing intensity and month, together with the standard errors of the means, for each of the response variables. Secondly, we analyzed the significance of the effects of browsing intensity and month on the variation of each of the response variables studied. To do so, we fitted factorial analysis of variance models including the main effects of the indicated factors (browsing intensity and month) and their interaction effect. In those cases in which we were faced with non-normal and heteroscedastic data a previous data transformation was realized in order to satisfy the underlying assumptions of the model (log-transformed in the TPC, N, C/N and N/TPC models).

Once significant effects were detected for each fitted factorial model, they were further compared through a post hoc comparison analysis based on the Holm method to control the total error rate (Bretz et al. 2010).

The first type of comparisons consisted of analyzing the effect of browsing intensity (0-3) by pairwise comparisons of the trees with levels 3, 4, 5 and 6, for each month. Second, the effect of browsing was quantified by pairwise comparing the months, first for trees without browsing (0), i.e. with tree levels 1, 2 and 3, and then for trees with levels 4, 5 and 6 which experienced browsing intensity from 1 to 3. Simultaneous confidence intervals were computed (with a 95% family-wise confidence level) to estimate the difference between the effects of the two months compared in each case on the mean response variable. Note that the simultaneous confidence intervals obtained from the factorial models corresponding to the response variables TPC, N, C/N and N/TPC were backtransformed from log. Finally, in order to assess the differential effect of drought on non-browsing trees in relation to the response variables comparisons were made between irrigated trees (levels 1 and 2) and non-irrigated trees (levels 3 and 4) for each month. For this purpose, the difference in the average effect on each variable between the irrigated trees and the non-irrigated ones, for each month, was estimated by means of simultaneous confidence intervals. Statistical analysis was carried out using R software v.4.2.1 (R Core Team 2020).

Results

Phenols, tannins, nutritional quality and palatability for each of the six trees levels (1-6) representing four browsing intensity levels (0-3) in three months (January, June and September) are shown in the Table 2, which summarizes the descriptive statistics, means and standard deviations. Figure 3 shows the correlation analysis between variables, highlighting a strong and positive correlation between N - TPC and N - TC in January but not in June or September, while a strong and positive correlation between TPC and CT is observed in January but negatively in

September. In Figure 4 we show the interaction effects plots between tree treatments and month, together with the standard errors of the means for total polyphenolic compounds (TPC), condensed tannins (CT), carbon (C) and nitrogen (N), whereas Figure 5 shows the three ratios C/N, N/CT and N/TPC. The significance of these interaction effects is contrasted in Table 3. Indeed, this table shows the F-statistics and the corresponding p-values for the main effects, browsing intensity and month, as well as for their interaction, and the percentages of variability explained by the fitted factorial models which are considerably high as shown by the values of the adjusted R-squared coefficient (from 88% to 99.4%).

Variable	Month	With irrigation		Without irrigation			
		1	2	3	4	5	6
		Sampled trees		Browsing intensity			
		0	0	0	1	2	3
TPC (mg g ⁻¹ DW)	January	0.351 ± 0.053	0.366 ± 0.026	0.732 ± 0.035 ^a	0.748 ± 0.010 ^a	0.980 ± 0.040 ^b	2.228 ± 0.100 ^c
	June	1.071 ± 0.042	4.485 ± 0.392	4.146 ± 0.418 ^a	4.133 ± 0.085 ^a	4.558 ± 0.533 ^a	6.969 ± 0.379 ^b
	September	0.942 ± 0.026	1.333 ± 0.075	0.972 ± 0.046 ^a	0.835 ± 0.071 ^b	1.223 ± 0.000 ^c	3.247 ± 0.093 ^d
CT (mg g ⁻¹ DW)	January	0.130 ± 0.013	0.139 ± 0.016	0.125 ± 0.019 ^a	0.224 ± 0.014 ^b	0.222 ± 0.011 ^b	0.265 ± 0.040 ^c
	June	0.221 ± 0.041	0.329 ± 0.023	0.353 ± 0.039 ^a	0.337 ± 0.009 ^a	0.334 ± 0.042 ^a	0.331 ± 0.045 ^a
	September	0.139 ± 0.021	0.226 ± 0.019	0.179 ± 0.016 ^{ab}	0.207 ± 0.016 ^a	0.158 ± 0.009 ^{ab}	0.127 ± 0.018 ^b
C (%) DW)	January	48.609 ± 0.082	46.614 ± 0.089	49.385 ± 0.066 ^a	50.239 ± 0.247 ^b	49.728 ± 0.325 ^{ab}	49.284 ± 0.099 ^a
	June	47.907 ± 0.316	46.684 ± 0.081	49.832 ± 0.577 ^a	50.715 ± 1.010 ^b	48.980 ± 0.211 ^c	48.541 ± 0.099 ^c
	September	47.699 ± 0.572	48.227 ± 0.103	49.469 ± 0.231 ^a	48.887 ± 0.125 ^a	48.836 ± 0.179 ^a	49.064 ± 0.547 ^a
N (%) DW)	January	1.551 ± 0.018	1.683 ± 0.010	1.139 ± 0.030 ^a	1.864 ± 0.031 ^b	1.966 ± 0.011 ^c	2.599 ± 0.007 ^d
	June	1.729 ± 0.062	1.705 ± 0.026	1.507 ± 0.004 ^a	1.220 ± 0.069 ^b	1.139 ± 0.020 ^c	1.592 ± 0.007 ^d

	September	1.416 ± 0.026	1.778 ± 0.001	1.279 ± 0.006 ^a	1.388 ± 0.007 ^b	1.100 ± 0.004 ^c	1.564 ± 0.027 ^d
	January	31.350 ± 0.406	27.696 ± 0.106	43.378 ± 1.187 ^a	26.960 ± 0.322 ^b	25.292 ± 0.022 ^c	18.960 ± 0.093 ^d
C/N	June	27.732 ± 1.183	27.383 ± 0.374	33.073 ± 0.477 ^a	41.621 ± 1.529 ^b	43.031 ± 0.949 ^c	30.487 ± 0.201 ^d
	September	33.706 ± 1.030	27.122 ± 0.037	38.682 ± 0.363 ^a	35.226 ± 0.087 ^b	44.386 ± 0.013 ^c	31.374 ± 0.193 ^d
	January	12.057 ± 1.308	12.247 ± 1.333	9.299 ± 1.627 ^a	8.346 ± 0.446 ^a	8.876 ± 0.468 ^a	7.186 ± 0.802 ^a
N/CT	June	8.010 ± 1.584	5.197 ± 0.314	4.307 ± 0.441 ^a	3.622 ± 0.262 ^a	3.445 ± 0.402 ^a	4.869 ± 0.725 ^a
	September	10.358 ± 1.675	7.914 ± 0.690	7.182 ± 0.682 ^a	6.718 ± 0.534 ^a	6.988 ± 0.403 ^a	12.508 ± 1.833 ^b
	January	4.474 ± 0.584	4.611 ± 0.345	1.558 ± 0.080 ^a	2.491 ± 0.028 ^b	2.009 ± 0.074 ^c	1.168 ± 0.054 ^d
N/TPC	June	1.617 ± 0.107	0.383 ± 0.041	0.366 ± 0.039 ^a	0.295 ± 0.012 ^{ab}	0.252 ± 0.026 ^b	0.229 ± 0.013 ^b
	September	1.502 ± 0.022	1.337 ± 0.078	1.318 ± 0.069 ^a	1.670 ± 0.154 ^b	0.900 ± 0.003 ^c	0.482 ± 0.018 ^d

Table 2. Descriptive statistical summary and mean (± SD) concentration of total polyphenolic compounds (TPC), condensed tannins (CT), nitrogen (N), carbon (C) and their ratios: (C/N), (N/TPC), (N/CT) as palatability indices in Olive (*Olea europaea* var. *europaea*-tree 1-) and wild olive (*Olea europaea* var. *sylvestris*- trees from 2 to 6) in a Mediterranean ecosystem from southern Spain; at 6 trees treatments for increasing browsing intensities from 0 to 3 ranges, in January, June and September. Significant pairwise comparisons between treatment trees without irrigation are indicated in each month by different letters in superscript (p < 0.05).

Source of variation	TPC		CT		C		N		C/N		N/CT		N/TPC	
	F	p-value	F	p-value	F	p-value	F	p-value	F	p-value	F	p-value	F	p-value
Tree (1-6)	548.84	0.00	19.58	0.00	69.38	0.00	453.16	0.00	599.27	0.00	19.68	0.00	472.12	0.00

Mont h (J- J-S)	2607	0.0	158.	0.0	2.7	0.0	621.	0.0	656.	0.0	110.	0.0	3062	0.0
	.76	00	80	00	5	77	57	00	77	00	59	00	.75	00
Tree × Mont h	83.3	0.0	17.7	0.0	10.	0.0	290.	0.0	310.	0.0	8.41	0.0	88.1	0.0
	3	00	7	00	00	00	69	00	90	00	00	00	6	00
Adju sted R ²	99.4%		91.6%		89.2%		99.2%		99.3%		88%		99.4%	

Table 3. Results of the models fitted for the two-factor designs considered for each of the seven response variables (F ratios, p-values and adjusted R-squared are shown for each model).

Figure 3. Correlation matrix among all the response variables for January, June and September.

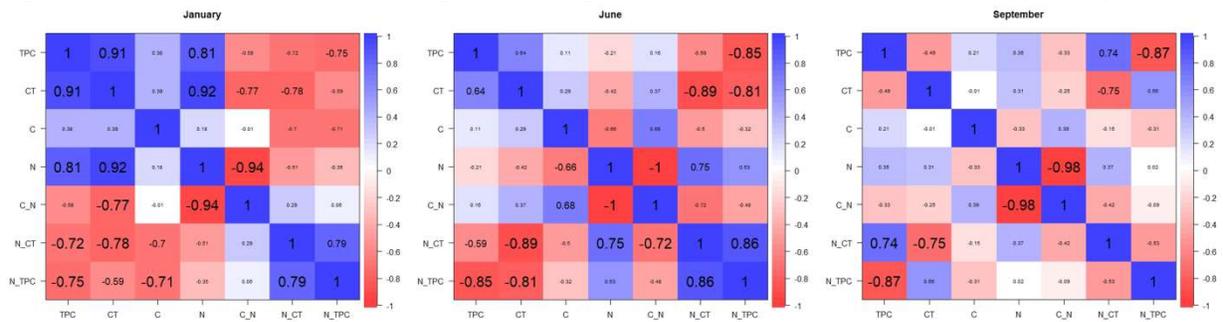


Figure 4. Interaction effects plots between tree treatments and months with standard errors of the means for TPC, CT, N, and C.

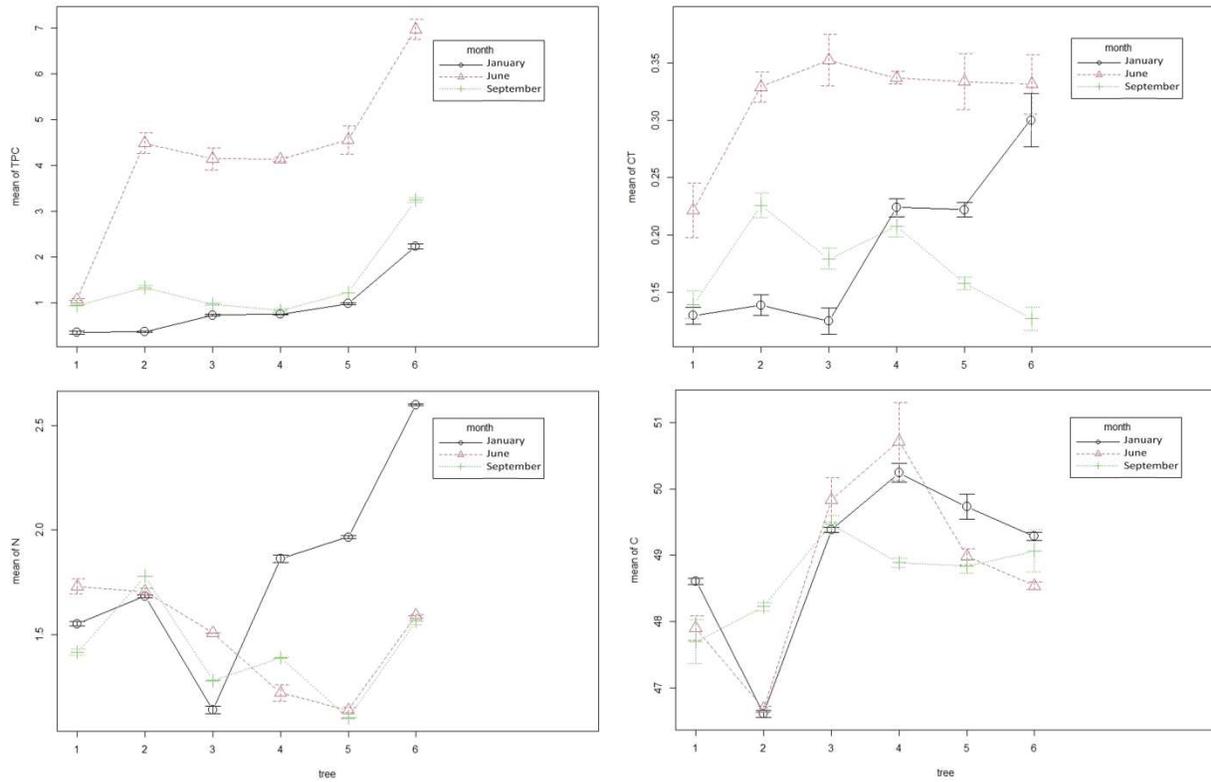
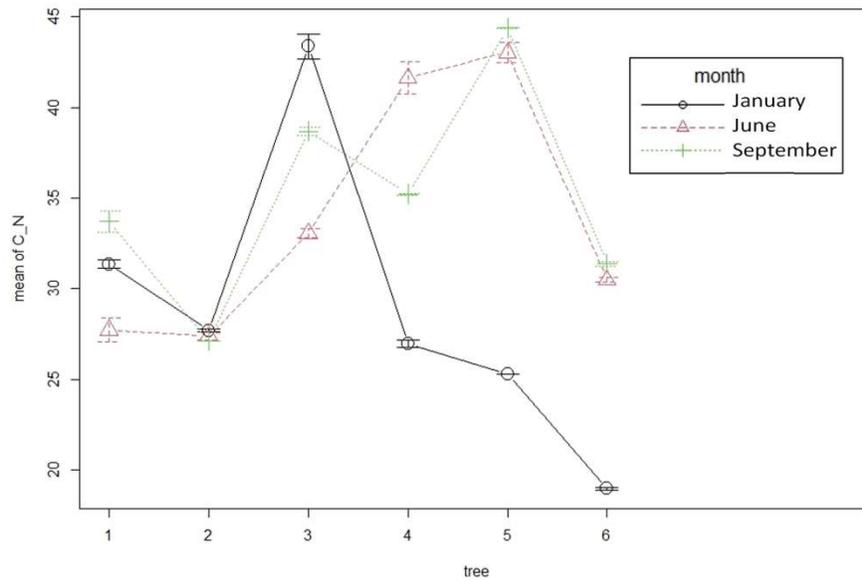
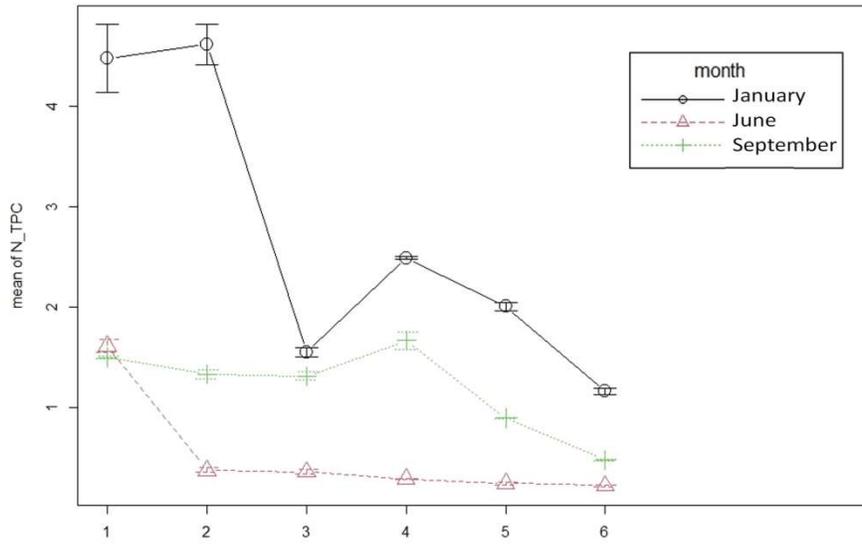
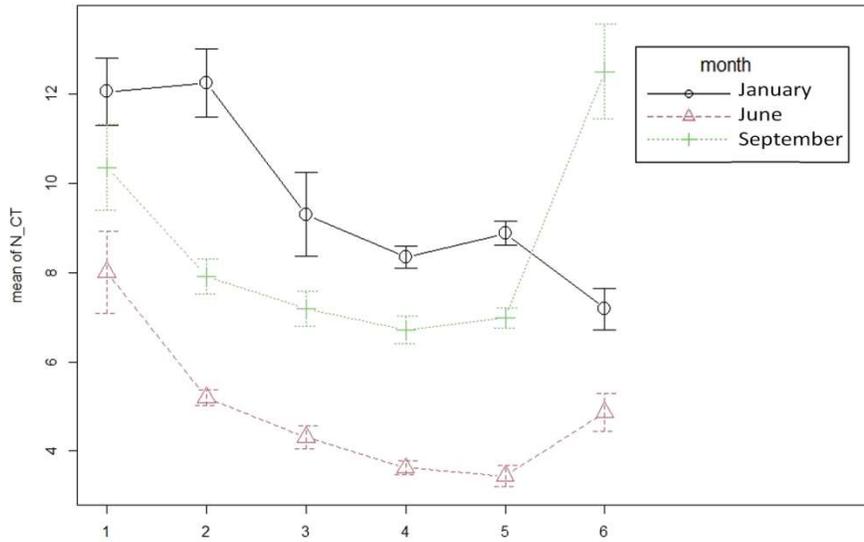


Figure 5. Interaction effects plots between tree treatments and months with standard errors of the means for N/CT, N/TPC, and C/N.



We can observe the variation experienced by each response variable for each tree sampled being clearly dependent on the month. They are significant at 5% in all models, with the exception of the month effect which is not significant for the model corresponding to the carbon content (C); however, the interaction with factor tree is significant. Trees 3, 4, 5 and 6 representing browsing levels 0, 1, 2 and 3, all without irrigation, are pairwise compared for each month with the results also shown in Table 2. In Table 4, results of the pairwise comparisons between months for trees without browsing (0), i.e. with levels 1, 2 and 3, and then for trees with browsing (1-3 considered together), i.e. with tree levels 4, 5 and 6, are shown. In trees without browsing, the highest values of TPC, CT and N occur in June and the lowest in January. However, for C there are no significant differences between months. On the other hand, the effect of browsing does not influence the differences observed between months for TPC. It does influence the averages of CT and C which are now significantly lower in September and the average N, higher in January.

Comparisons between months		TPC	CT	C	N	C/N	N/CT	N/TPC
For Trees (1, 2, 3) without browsing (0)	Jan-Jun	(-0.638, -0.601)*	(-0.200, -0.140)*	(-0.363, 0.487)	(-0.087, -0.063)*	(0.117, 0.164)*	(4.201, 6.525)*	(1.401, 1.626)*
	Jan-Sep	(-0.324, -0.255)*	(-0.080, -0.020)*	(-0.687, 0.163)	(-0.027, -0.002)**	(-0.000, 0.042)***	(1.554, 3.878)*	(0.715, 0.876)*
	Jun-Sep	(0.780, 0.961)*	(0.090, 0.150)*	(-0.749, 0.101)	(0.052, 0.080)*	(-0.123, -0.086)*	(-3.809, -1.485)*	(-0.317, -0.253)*
For Trees (4, 5, 6) with browsing (1, 2, 3)	Jan-Jun	(-0.651, -0.615)*	(-0.094, -0.034)*	(-0.087, 0.764)	(0.337, 0.372)*	(-0.384, -0.358)*	(2.996, 5.320)*	(1.154, 1.356)*
	Jan-Sep	(-0.177, -0.093)*	(0.076, 0.136)*	(0.396, 1.246)*	(0.317, 0.352)*	(-0.363, -0.335)*	(-1.764, 0.560)	(0.384, 0.514)*
	Jun-Sep	(1.245, 1.473)*	(0.140, 0.200)*	(0.057, 0.908)*	(-0.027, -0.002)**	(0.014, 0.057)*	(-5.921, -3.597)*	(-0.386, -0.328)*
Comparisons between trees with irrigation vs without irrigation plus lighth browsing (1)				TPC	CT	C	N	
Trees 1+2 vs 3+4		January		(-0.508, -0.350)*	(-0.078, 0.003)***	(-2.733, -1.669)*	(0.040, 0.075)*	
Trees 1+2 vs 3+4		June		(-0.671, -0.565)*	(-0.107, 0.032)*	(-3.510, -2.446)*	(0.133, 0.171)*	

Trees 1+2 vs 3+4	September	(0.127, 0.490)*	(-0.048, 0.026)	(-1.747, -0.684)*	(0.092, 0.129)*
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Trees treatments are: (1): (*Olea europaea* var. *europaea*) not browsed (level 0) and with irrigation; (2): (*Olea europaea* var. *sylvestris*) not browsed (level 0) and with irrigation; (3): (*Olea europaea* var. *sylvestris*) not browsed (level 0) and without irrigation; (4): (*Olea europaea* var. *sylvestris*) browsed (level 1) and without irrigation, (5): (*Olea europaea* var. *sylvestris*) browsed (level 2) and without irrigation, (6): (*Olea europaea* var. *sylvestris*) browsed (level 3) and without irrigation.

Table 4. Results of the post-hoc comparisons and 95% simultaneous confidence interval. For the cases in which the p-values were statistically significant, this is indicated by (*) p-value < 0.001, (**) p-value < 0.01, (***) p-value < 0.05.

Simultaneous confidence intervals are also shown in Table 4 to estimate the differences found in the average effect on TPC, CT, C and N reached in the irrigated trees (trees 1 and 2) and in the non-irrigated trees (trees 3 and 4) for each month, to quantify the effect of drought. In the case of C, the average effect is greater for non-irrigated trees and in the case of N the average effect is greater for irrigated trees. Lack of irrigation or drought leads to lower nitrogen production. For TPC and CT variables the average mean is greater for non-irrigated trees in January and June. On the other hand, in September the effect on TPC is greater for irrigated trees while in CT irrigation has no significant effect.

TPC content also increased significantly with the level of browsing, with the response to browsing intensity 3 being two points higher than that of intensity level 2, in all months of the year. As can be seen in Table 2, the level of browsing intensity 3 differs significantly from the lower ones for TPC. As for tannins (CT), no significant differences were observed in June in the concentration of tannins in the leaves of the olive trees browsed differently, while in September a different pattern of decrease was observed depending on the level of browsing and an increase in January. In relation to palatability and nutritional quality ratios we found significant differences between trees 3 to 6, indicating a differential response to browsing intensity and season of the year. In September the N/CT ratio was significantly higher for browsing level 3, indicating higher palatability than in trees with light and moderate browsing where palatability was lower. Meanwhile, in relation to N/TPC the variations are statistically significant in January and September with an opposite response of decreasing values as browsing intensity increases (decreasing palatability). The C/N ratio shows a statistically significant decrease from level 0 to level 3 of browsing, increasing the nutritional quality of leaves in January, while in June and September trees with light and moderate browsing show higher C/N values than heavily browsed trees.

Discussion

Responses due to abiotic stressor in combination with four browsing intensity levels

In general large seasonal variations were observed, with higher phenolic and tannin contents in June in both browsed and non-branched trees. Since the Mediterranean environment is characterized by prolonged hot and dry seasons and recurrent heat waves, polyphenols are particularly widespread and play an important physiological role in woody plants due to abiotic stress stimuli (e.g. drought, high temperature, light intensity) particularly in the most constraining season, which is summer. In fact, a high seasonal variability of the content of polyphenolic compounds in Mediterranean vegetation was also reported, with concentrations increasing by an order of magnitude in summer compared to winter (Seco *et al.*, 2011). In the present study we found increases in concentrations of polyphenols (TPC) four times greater in June than in the rest of the seasons studied (Table 2, Figure 4).

In a Mediterranean climate, the dryness of the soil also increases the production of chemical defenses with high antioxidant capacity (Bueno *et al.*, 2020). But when quantifying the effect of drought, significant differences were only obtained in nutritional variables such as nitrogen (N) and carbon (C). In the case of C, the average effect is greater for trees without irrigation and in the case of N, the average effect is greater for trees with irrigation. Drought, in our study, along with lack of irrigation leads to lower nitrogen production, while rainfall of ~50% of the annual total fallen during the autumn and winter of the study period may explain the positive correlation between N - TPC and N-CT found in January. For TPC and CT variables, the average mean is greater when lacking irrigation also in January.

Both phenols and tannins act as nutritional deterrents when concentrated in large quantities in the leaf, due to their bitter taste. These secondary metabolites play a key role as plant defense compounds against environmental stress; but in their relationship with other species, the role they play as mediators of plant-herbivore interactions stands out (Winkel-Shirley 2002, Nosko *et al.* 2020). In the study area the wild olive tree faces abiotic stressors along with the ungulate herbivores, so we expect that browsing causes an increase in polyphenols and tannins to a greater extent than seasonal climatic variations and plant life-cycle events. Specially, in some months such as June we have observed the greatest chemical response when both types of stresses are combined. However, abiotic factors were much more influential in all cases (see Table 3). We expected a significant increase in the content of secondary metabolites in browsed trees compared to non-branched trees subjected to the same climatic and environmental conditions. So significant differences were detected between the browsing intensity levels also in January and September, whereas in June no significant differences were detected between browsing (1) and browsing (2) treatments, but only for browsing (3) which is the experienced by the tree *heavily browsed* (see Table 1). We also expected an increase in the content of secondary metabolites as browsing intensity increased. But this was not exactly the case and, although we did find significant increases in TPC with browsing, the same did not occur with tannins. There are no differences in tannins between treatments without browsing and with browsing in June, but there are in January and September (Table 3, Figure 4). Trees respond differently to browsing by producing tannins or not depending on the month. In January the increase in CT with browsing is significant and in June it remains constant, while in September CT decreases in the most browsed trees. The actual

difference in tannins was very small between browsed and unbrowsed trees. Tannins have a more costly synthesis for the plant and may only be activated when a critical level of biomass removal in the plant is exceeded; on the other hand, their concentrations vary with the growing season, decreasing when the growth of the plant is more active (Nosko et al. 2020).

Seasonal leaf nutritional quality-palatability variations and ruminants feeding behavior

In general, browsing is an important food resource in late winter and late summer in Mediterranean ecosystems and browsing on Mediterranean woody species is very strong in September because after a very dry summer there is no pasture and ruminants feed on the vegetation available to them. But previous studies have shown differences in diet selection among deer species (and both sexes) that are not always explained by the abundance of the resource, or by predictions derived from body size or physiological states of ruminants (Azorit et al., 2012). The study of the relationship between the nutritional attributes of plants and the selection of plants according to different metabolic demands could better explain the feeding behavior of ruminants (Azorit et al., 2012).

In fact, it has been observed that especially in autumn and winter the browse selection of ruminants on wild olive reaches higher proportions, close to 12% of the total biomass consumed (Martínez-Martínez, 1992). This coincides with the higher N/CT and N/TPC ratios (higher palatability) in January and September, both in browsed and non-branched trees, and coincides with the low C/N values (higher proportional amount of protein) detected in January on wild olive trees, decreasing significantly and progressively at all browsing levels (Figure 5). At this time of the year, we could say that wild olive is more nutritious and palatable, which would explain the high consumption of this woody plant by ruminants at this time of the year.

Chemical defense appears at times to be ineffective in deterring ruminants' browsing (Nosko et al., 2020), and it has even been reported that intensive and severe browsing has the opposite effect, causing an increase in N concentrations or a reduction of TPC and CT in individual plants (Persson *et al.*, 2005). Browsing reduces the number of leaves and thus the concentrations of these secondary metabolites and increases N concentrations in individual plants, so heavily browsed plants may be characterized by C-poor/N-rich tissues (Scogings *et al.*, 2011; Morrison *et al.*, 2022), but leaves richer in nitrogen than in secondary metabolites such as tannins or phenols make these parts more palatable and more vulnerable to consumption by ruminants. Our results agree with these statements since we observed high C/N values in the trees with light and moderate browsing levels in June and September but very low values in January, especially in the trees with the most intense browsing levels. This makes it more nutritious than at other times of the year, and its leaves more nutritious than those of the less browsed trees. As for the N/TPC ratio, a progressive decrease (less nitrogen in relation to phenolic compounds) was observed with increasing browsing intensity, with the lowest values (less palatability) appearing in the most browsed trees in all the months studied (Figure 5, Table 4). As for the N/CT ratio, it did not vary in January and June but in September it was significantly higher in tree 6 (the most intensively browsed tree), which also makes it more palatable and attractive than the other trees (4 and 5) subjected to light and moderate browsing.

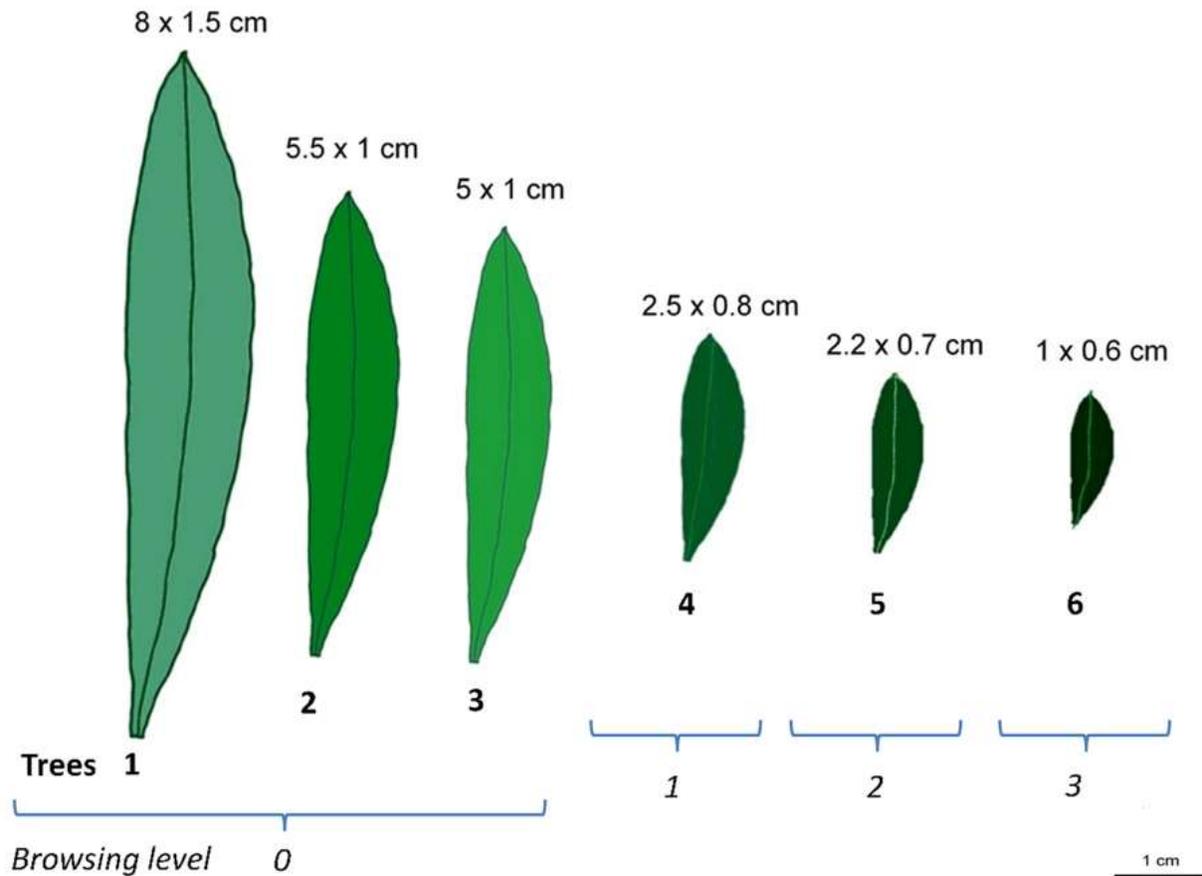
In theory, levels of these chemicals can also increase after browsing, leaving the plant ready to defend itself against future herbivore attack (Morrison *et al.*, 2022). But according to our results, the efficacy of secondary compounds in preventing browsing is limited, perhaps efficient at moderate browsing levels but at more intense browsing levels the effect may be the opposite, compromising plant growth and maintenance. In addition, ruminants are also adapted to these secondary metabolites and can counteract the effects of tannins.

Low intake of tannin-rich foods is attributed to low palatability due to the sensation of astringency that tannins confer to food by binding to salivary proteins which subsequently do not exert their lubricating function in the mouth, giving an unpleasant sensation of dryness and hardness. However salivary proteins have been identified as one of the animal's defense mechanisms against tannins. In ruminants it has been suggested that tannin-binding salivary proteins (TBSPs) are present in browsers which thrive on tannin-rich diets, such as deer (Shimada, 2006). Therefore, aspects related to oral cavity physiology and saliva production/composition, which is different in different ruminant species, is in relation to intraspecific differences in acceptance/avoidance of and tolerance to these secondary compounds (Jerónimo *et al.*, 2016). They would also have to be taken into consideration in future studies of plant-herbivore interaction in contexts such as the ones we are dealing with.

A preliminary approach to leaf size reduction as structural response to biotic stressors

Woody evergreen species growing in the Mediterranean area have evolved under high browsing pressure, producing small leaves and dense shoots which can decrease the foraging efficiency of herbivores and therefore reduce the damage produced on plants (Massei *et al.*, 2000; Scogings *et al.*, 2011). This structural defense together with the chemical defenses would reinforce the strategies of the wild olive tree to defend itself against herbivores. Stress generally stimulates carbon fluxes from primary to secondary metabolic pathways, including a shift of available resources in favor of secondary product synthesis. Plants can show certain plasticity in their leaf morphology before adverse situations, changing the length and size of leaves compared to non-branched plants. In fact, we found 3 different leaf sizes depending on the type of olive tree sampled and whether they were exposed to browsing. We found larger leaves in olive trees (1), medium leaves in non-branched olive trees (2 and 3), and small leaves in browsed olive trees (4, 5, and 6). As can be seen in Figure 6, wild olive trees show increasingly smaller leaves the higher the intensity of browsing. This could hinder foliage browsing, as the density of vegetation hinders easy access to leaves and prevents ungulates from taking large bites, i.e., small leaves decrease ungulate reward per unit time (Morrison *et al.*, 2022).

Figure 6. Leaf size of the 6 trees sampled and average of the length and width in cm.



As we have seen in our experimentation browsing on woody plants can lead to the induction of these defense chemicals. On the other hand, the plant may also slow down growth in order to adapt to different stressful situations, due to compensatory mechanisms between growth and plant defense (Morrison et al., 2022). However, competition for the allocation of resources for growth maintenance or plant defense could be responsible for reduced leaf size and decreased secondary metabolites in heavily browsed trees, compromising their survival under climate change and prolonged drought.

Conclusion

Stress due to Mediterranean abiotic factors causes seasonal variations in the content of secondary metabolites and their relationship with nitrogen and carbon content. We also found an increase in secondary metabolite content with increasing browsing intensity, but seasonal climatic variations cause an increase in secondary metabolites to a greater extent than browsing. Although a significant increase in the content of polyphenolic compounds was detected in browsed trees compared to non-branched trees under the same climatic and environmental conditions, the most influential factors in the responses detected by the wild olive trees were those due to climatic seasonality.

According to our results, the efficacy of secondary compounds in preventing browsing is limited, perhaps efficient at moderate levels of browsing but at more intense levels of browsing the effect may be the opposite, compromising plant growth and maintenance, especially in the more restrictive seasons of the Mediterranean climate due to factors such as drought.

Our findings on seasonal variations in the nutritional quality-palatability of leaves are important for understanding the dietary choices of ruminants. They may also help to explain differential feeding behaviors and plant selection of ruminants in a broad context of plant-herbivore interaction useful for the management of herbivore herds in Mediterranean ecosystems.

These variations in secondary metabolite content in differential response to browsing levels as an indicator of herbivore abundance and/or access to trees could also provide information applied to management in Mediterranean ecosystems.

Credit authorship contribution statement

Milagros Bueno: Methodology and Laboratory analysis, Writing and editing original draft; **Antonia Oya:** Experimental design, Formal analysis, Writing and editing original draft; **Rafael Carrasco:** Sample collection, Field work, Discussion and Project management; **Concepción Azorit:** Conceptualization, Experimental design, Project management, Resources, Writing and editing original draft, Supervision, Corresponding author. All authors reviewed the article for final approval.

Declaration of Competing Interest

The authors declare no conflict of interest.

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Data availability

The current study data sets are available in supplementary information files. More detailed data and sampling may be available upon reasonable request to the corresponding author and head of Research Group PAI-RNM-175.

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