

Competitive effect of a native-invasive species on a threatened shrub in a Mediterranean dune system

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Abstract The canopy shade of the *Retama* species has been widely reported to ameliorate the environmental conditions in the understory, thus facilitating other species' establishment. The shading effect of the native-invasive leguminous shrub *Retama monosperma* (L.) Boiss on the endangered *Thymus carnosus* Boiss was analysed to determine a positive or negative net effect. Data was taken in all four seasons, representing contrasting light and water availability in a Mediterranean coastal dune ecosystem (SW Spain). The morphological and physiological status of sun-exposed *T. carnosus* plants growing in open areas versus shaded plants growing under *R. monosperma* were measured seasonally. Leaf mass area, leaf area index and pigment content showed typical sun–shade responses. In contrast, sun-exposed *T. carnosus* displayed higher stem water potential, transpiration rate and water use efficiency, both intrinsic and integrated, denoting low tolerance to the presence of *R. monosperma*. Five years after the measurements, canopy cover had decreased and mortality was higher in shaded plants, thus confirming the competitive effect of *R. monosperma* on *T. carnosus*. *R. monosperma* arises as a competitor for endangered *T. carnosus* communities,

consequently reinforcing its invasive behaviour. This species-specific shrub study demonstrates that eventual beneficial effects of *Retama* canopy may be overridden by competition in the understory, particularly in the case of species well-adapted to high light and low water levels.

Keywords Competition · Facilitation · *Retama monosperma* · *Thymus carnosus*

Introduction

Plant–plant interactions play a crucial role in plant community structure in dry conditions (Brooker et al. 2008; Armas and Pugnaire 2009). Both positive and negative effects are simultaneously implied in these interactions, the net balance resulting from unequal competitive and facilitative effects (Callaway et al. 1991; Callaway and Walker 1997). According to Holmgren et al. (2012), light and water are key resources for plant growth and survival, and their interacting effects can shape the nature of plant–plant interactions in a wide range of terrestrial ecosystems. Mediterranean environments are characterized by high light intensities combined with seasonal drought periods. Mediterranean plant species are well adapted to these factors, but in some cases, shading by neighbours' canopies has been shown to facilitate both survival and growth (Pugnaire et al. 1996; Maestre et al. 2003; Armas and Pugnaire 2005). Although, strictly speaking, shade only refers to low-light conditions, functionally and ecologically, shade involves a whole suite of effects on plants and environmental factors (Valladares et al. 2008). Some of these changes in the environment due to shading by the presence of a plant include higher nutrient and soil moisture availability, and lower evaporative demands due to increased humidity and/

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or reduced temperatures beneath plant canopies (Valladares and Pearcy 1998).

Under drought conditions, facilitation research suggests that the negative effects of light limitation in the shade can be offset by its benefits for plant water status (Callaway 1995; Holmgren 2000; Sánchez-Gómez et al. 2006 and references therein). But drought may also be critical in the shade, and then conflicting requirements can occur for biomass allocation, with drought conditions favouring the biomass investment towards roots, and shade favouring greater allocation to leaves, increasing their light interception at the expense of water capture (Smith and Huston 1989). As a consequence, plants may be able to persist under drought conditions in shaded habitats when the lesser evaporative demand below canopies reduces plants' water stress. Shading may therefore lessen the impact of drought by reducing water loss from soils and plants (Holmgren 2000; Prider and Facelli 2004). However, if environmental harshness is not ameliorated by the facilitator species, competitive interactions are likely to dominate (Tielbörger and Kadmon 2000; Maestre and Cortina 2004). Thus, shade in the understory of taller plant canopies may facilitate other plants' establishment as long as environmental conditions are better than in the open.

Retama species are green-stemmed, nitrogen-fixing leguminous shrubs. *R. monosperma* (L.) Boiss is native to coastal sandy areas SW of the Iberian Peninsula and NW of Africa (Talavera 1999). The influence of *R. monosperma* on its environment shows a predominance of positive effects regarding amelioration of temperature extremes, higher minimum relative humidity and enrichment of soil nutrients and organic matter (Muñoz Vallés et al. 2011). In relation to vegetation, the same authors found an increase in above-ground and below-ground plant biomass and species richness, mainly spring annuals. These findings agree with those from another widely studied *Retama* species, *R. sphaerocarpa* (L.) Boiss, which has been shown to have a facilitative effect on the environment and the understory vegetation in central and south-eastern Spain (Pugnaire et al. 1996; Moro et al. 1997; Rodríguez-Echevarría and Pérez-Fernández 2003; López-Pintor et al. 2006 and references therein), due to the attenuation of high radiation and temperature, increase in soil moisture and nutrient enrichment.

R. monosperma has been described as an exotic invasive in California, USA (Cal-IPC 2006; Randall 1997) and also in Australia (Randall 2007). In addition, the species was used in dune stabilization works in SW Spain in the early twentieth century (Kith y Tassara 1946) and has experienced a rapid recent expansion within its natural area of distribution (Valdés et al. 2007). Native species are often considered invasive when they increase in abundance or range following novel changes, especially human-caused

changes, in their natural habitats (Alpert et al. 2000). In this context, *R. monosperma* has been classified as a native-invasive species in Spain (Muñoz Vallés et al. 2011). Moreover, Valéry et al. (2009) also established that any species, whether native or exotic, that spreads in a region may be termed invasive, because in either case it is the same basic mechanism at work; namely, interspecific competition. For this reason, analysing the changes derived from *R. monosperma* invasion and its competition on other native species is crucial to assess its potential effects on coastal dune plant community dynamics. We selected the native endangered shrub *Thymus carnosus* Boiss to develop the interaction study because *R. monosperma* has colonized the last dune communities occupied by *T. carnosus* in the study area, and the results of the study may imply direct practical applications. Previous studies have found greater drought stress and a shift in *T. carnosus* water uptake patterns due to the presence of *R. monosperma* (Esquivias et al. 2014). Yet, a broader study aiming to evaluate the response of *T. carnosus* to different factors, including *Retama* canopy shade, is still lacking.

The objective of this study was to assess the net effect of the presence of *R. monosperma*, positive or negative, on morphological and physiological features (use of light and water) of *T. carnosus*, while also evaluating seasonal differences. For this purpose, we measured the performance of *T. carnosus* in its natural habitat, growing in full sunlight and in the shade of *R. monosperma* during each of the four seasons of the year, representing differing levels of water and light availability. The main hypotheses were: (1) *T. carnosus* would benefit from conditions of lower light and temperature during warmer seasons and greater soil nutrient content found under the *R. monosperma* canopy, and (2) the positive effect of the *R. monosperma* canopy may counteract drought stress for *T. carnosus*.

Materials and methods

Study area

The study was conducted in a coastal sandy dune system in El Rompido Spit, a natural protected area located in the Piedras River estuary, in Huelva, SW Spain (37°12'N, 7°07'W). The local climate is Mediterranean with an Atlantic influence, with a mean annual temperature of 18.1 °C. Monthly mean minimum temperatures range from 6.6 °C in January to 19.3 °C in August, while monthly mean maximum temperatures range from 16.3 °C in January to 31.8 °C in July. Average annual rainfall is 490 mm, with a pronounced drought period in summer (June–September) (30-year record, from 1971 to 2000; data from Huelva Meteorological Station, Agencia Estatal de Meteorología).

El Rompido Spit presents diverse geomorphologic formations, ranging from high beach and active dune communities to interior semi-stabilized and stabilized dunes, interdune wet depressions, salt marshes and brackish depressions (Gallego Fernández et al. 2006). The semi-stabilized and stabilized interior dunes represent the main habitat on the spit surface (57 %), and here the vegetation is dominated by *R. monosperma*, *T. carnosus*, *Helichrysum italicum* or *Artemisia campestris* subsp. *maritima*, spring annuals, and perennial herbaceous species (Muñoz Vallés et al. 2009).

The spit is 12 km long West to East, parallel to the coast, grows around 42 m per year (Muñoz Vallés 2009), and varies in width from 300 to 700 m. Field data were taken 6 km from the mainland, where livestock has no access. Wild rabbit (*Oryctolagus cuniculus*) was the only mammal herbivore present.

Study species

Thymus carnosus (Labiatae, *Thymus* hereafter) is an evergreen coastal shrub endemic to the SW of the Iberian Peninsula, classified as in danger of extinction (CR, IUCN) since 2005 in Spain (Cabezudo et al. 2005) and under regression in Portugal (ICN 2006). It is included in the catalogue of species of European Communities Council interest under the ‘Habitats’ Directive (Anonymous 1992). *Thymus* plants grow up to 45 cm high and present small, linear, fleshy leaves with rolled margins. Flowers appear from early May to July and seeds do not present any specialized dispersing mechanisms. *Thymus* plants develop a root system more than 1 m deep that can reach the water table in the study site (Esquivias et al. 2014). *Thymus* co-occurs with *R. monosperma* almost all over its range of distribution, and thus the assessment of the effect of *Retama* shading is extremely important regarding its conservation.

Retama monosperma (Leguminosae, *Retama* hereafter) is a woody shrub that reaches 4 m high and 9 m in diameter. With simple, linear leaves and evergreen photosynthetic stems, it remains leafless for most of the year, except in winter, although leaves fall rapidly after emergence. *Retama* has colonized the inland dunes of the coastal spit throughout the last eight decades and currently occupies almost the entire study area, having increased in surface by as much as 673.2 % from 1956 to 2001 (Gallego Fernández et al. 2006). In addition, it has also been observed to colonise the new areas formed in the spit. This rapid expansion capacity is mainly due to prolific fruit production, up to 2,800 fruits m⁻² (Muñoz Vallés 2009), and the dispersing activities of the wild rabbit, which improves seed germination rates after the passage of seeds through its gut (Del-lafiore et al. 2006).

The study was conducted in summer (29 August, end of dry period), autumn (30 November, recovery season), winter (27 February, minimum temperatures) and spring (31 May, growing season and beginning of dry period) of 2006–2007. We monitored adult *Thymus* plants in two light conditions: a sun-exposed habitat (referred to as open site hereafter, or T), and a shaded habitat beneath the *Retama* canopy (one *Thymus* plant per *Retama* plant; referred to as understory site hereafter, or T + R). We also measured the morphology of the *Retama* plants shading *Thymus* (referred to as R) to describe them ($n = 10$ per light condition and species, 30 plants in total, ten T, ten T + R and ten R). We randomly selected individuals with similar sizes in both light conditions; sun exposed *Thymus* were at least 1.5 m outside of *Retama* canopies to avoid shading during most of the day. The distribution of the studied *Retama* plants and *Thymus* in the open sites was not paired. Every individual was labelled, so that the same plants could be measured throughout the study.

Meteorological and microclimatic variables

Accumulated monthly precipitation, monthly mean temperature (T_{mean}) and solar radiation data from the closest meteorological station (Lepe, 12 km far from study site. Junta de Andalucía, Huelva, Spain) were used in this study. The atmospheric vapour pressure deficit (VPD) was calculated monthly for the whole study period and specifically for the sampling days. We recorded air temperature and relative humidity (RH) 5 cm above the soil every 30 min during sampling days from 1100 to 1900 h with microclimatic sensors (EasyLog USB, Lascar Electronics, Salisbury, UK), with the exception of the summer sampling date, on which humidity was not measured due to a failure in the RH sensor. Sensors were placed above bare soil and under *Retama* canopies ($n = 1$ per site). Photosynthetic active radiation (PAR) was measured by means of a PAR meter integrated into an IRGA (LCi-Portable Photosynthesis, ADC System, UK) ($n = 30$ per site).

Morphology and leaf area index

Plant height, canopy minor and major diameters, and leaf area index (LAI) were measured both in *Thymus* and *Retama* before the study started, in July 2006. We used diameters to calculate projected canopy area (cover) as an ellipse. LAI was estimated by means of a LAI-2000 Plant Canopy Analyser (Li-Cor, NE, USA).

We also measured height, diameters, and LAI in 2011 in the same individuals in order to compare with the initial measurements, and to evaluate plant survival.

Chlorophyll *a* fluorescence and photoinhibition

In situ chlorophyll *a* fluorescence signals were monitored on attached, fully expanded leaves with a pulse-amplitude modulation technique using a portable fluorometer (mini-PAM, Walz, Effeltrich, Germany). Fluorescence was excited by a pulse of modulated red light from a light-emitting diode (LED, type H-3000; Stanley) connected to a fibre optic. Calculations were performed following Genty et al. (1989). Leaves were dark-adapted with leaf clips for over 20 min, a period determined to be sufficient to allow complete re-oxidation of photosystem II (PSII) reaction centres. Maximum photochemical efficiency of PSII (F_v/F_m) was determined from the ratio of variable to maximal fluorescence as $F_v/F_m = (F_m - F_0)/F_m$, where F_0 = initial and F_m = maximal fluorescence of dark-adapted leaves. Leaf samples were exposed to a weak modulated measuring beam from a LED at a 600 Hz frequency to assess F_0 , and then a saturating light pulse to determine F_m .

Three measurements per individual were carried out at complete darkness before dawn and at midday for F_v/F_m , and the average mean values per individual and site were calculated.

Chronic and dynamic photoinhibition (PI_{chr} and PI_{dyn} , respectively) were calculated following Werner et al. (2002). PI_{chr} was calculated as the percentage reduction in predawn F_v/F_m relative to the annual maximum F_v/F_m of both sites [$(F_v/F_m)_{max}$] (Eq. 1), and PI_{dyn} was calculated from midday F_v/F_m as the additional decrease relative to pre-dawn F_v/F_m (Eq. 2). Total photoinhibition (PI_{tot}) was calculated as the addition of PI_{chr} and PI_{dyn} (Eq. 3).

$$PI_{chr} = [(F_v/F_m)_{max} - (F_v/F_m)_{pd}] / (F_v/F_m)_{max} \times 100 \quad (1)$$

$$PI_{dyn} = [(F_v/F_m)_{pd} - (F_v/F_m)_{md}] / (F_v/F_m)_{max} \times 100 \quad (2)$$

$$PI_{tot} = (PI_{chr} + PI_{dyn}) \quad (3)$$

where $(F_v/F_m)_{pd}$ and $(F_v/F_m)_{md}$ are pre-dawn and midday F_v/F_m values, respectively. $(F_v/F_m)_{max}$ is F_v/F_m annual maximum, calculated as the average of the maximum recorded values from the 2 months with highest F_v/F_m . Photoinhibition values were expressed as a percentage of reduction in $(F_v/F_m)_{max}$ values. Any photoinhibitory reduction in F_v/F_m at any time of the day may be composed of both chronic photoinhibition, such as in the case of the depressed predawn F_v/F_m values, and dynamic photoinhibition resulting in a further (fully reversible) decrease during the day (Werner et al. 2002).

Gas exchange and leaf mass area

Net photosynthetic (A), transpiration (E) and stomatal conductance (g_s) rates were measured on mature leaves of three terminal shoots per plant (several leaves per shoot) during clear sunny days, with an open system, compact infra-red gas exchange analyser (LCi-Portable Photosynthesis, ADC, UK). The average mean values per plant and site were calculated. Intrinsic water use efficiency (WUE_i) was calculated as A/g_s . The WUE of plants is a key aspect of their water economy and of their performance and survival in arid sites (Damesin et al. 1997; Valladares et al. 2008).

Results were expressed on a projected leaf area basis, calculated using an image area analyser (Midebmp, R. Ordiales, Almería, Spain, 2000) from scanned images of all the leaves from each measured shoot, collected following the gas exchange measurements. After drying for 48 h at 70 °C, leaf mass area (LMA) was calculated as the ratio of dry mass to leaf area. LMA can be understood as the leaf-level cost of light interception (Gutschick and Wiegell 1988; Poorter et al. 2009).

Due to technical problems, gas exchange measurements could not be carried out on the winter sampling date.

Leaf pigments content

Two samples of leaves from each *Thymus* plant were collected. One sample (about 1 g) was immediately stored in liquid nitrogen and kept in the laboratory at -24 °C until the pigments were extracted. The other sample was kept refrigerated in individual hermetic plastic bags, and fresh mass (fm) was registered in the lab within 3 h. Leaf samples were dried at 70 °C for 48 h and dry mass (dm) was measured in order to obtain the fm/dm relationship.

Chlorophyll *a* and *b* and carotenoid pigments were extracted with 100 % acetone, determined spectrophotometrically, and calculated on a dry mass basis following Lichtenthaler (1987).

Leaf C and N content, stable isotopes analyses

On the winter and spring sampling days, leaves of sampled *Thymus* ($n = 5-10$ individuals per site) were collected for isotopic ($\delta^{13}C$ and $\delta^{15}N$) and elemental (carbon and nitrogen concentrations) analyses. Leaves were oven dried at 70 °C until a constant weight was reached and ball-milled to a fine powder (Retsch MM 400, Haan, Germany). Samples were transferred into tin capsules and injected into an elemental analyser coupled in continuous flow to an isotope ratio mass spectrometer (EA-CF-IRMS, Thermo Delta V Advantage, Bremen, Germany) at the Universidad Autónoma de Madrid Stable Isotopes Laboratory (Spain).

The accuracy of the measurements was $\pm 0.1\text{--}0.2\text{‰}$ for $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$. The isotopic abundance was expressed in delta notation (δ) in parts per thousand (‰) as

$$\delta_{\text{sample}} (\text{‰}) = (R_{\text{sample}} / R_{\text{standard}} - 1) \times 1,000 \quad (4)$$

where R_{sample} and R_{standard} are the molar ratios of heavy to light isotopes of the sample and the international standard (NBS18 for $\delta^{13}\text{C}$, atmospheric N for $\delta^{15}\text{N}$).

According to Farquhar et al. (1989), $^{13}\text{C}/^{12}\text{C}$ discrimination ($\Delta^{13}\text{C}$) was calculated as:

$$\Delta^{13}\text{C} (\text{‰}) = (\delta^{13}\text{C}_{\text{air}} - \delta^{13}\text{C}_{\text{sample}}) / (1 + \delta^{13}\text{C}_{\text{sample}}) \quad (5)$$

where $\delta^{13}\text{C}_{\text{air}}$ is that of the source CO_2 , considered as a constant value of -8‰ in open air conditions, and $\delta^{13}\text{C}_{\text{sample}}$ is obtained from plant leaf material, calculated in Eq. 4. Because of the integrative response of $\Delta^{13}\text{C}$ to multiple eco-physiological constraints through time, C isotopes can be used to assess traits that co-vary with gas exchange, C gain, and water relations, including water use efficiency (WUE) (Farquhar and Richards 1984; Dawson et al. 2002). Thus, $\Delta^{13}\text{C}$ is a time-integrated measure of WUE. As $\Delta^{13}\text{C}$ and A/E are negatively related (Farquhar et al. 1989), values of $\Delta^{13}\text{C}$ generally decrease with an increased WUE.

Stem water potential

Stem water potential was measured before dawn (Ψ_{pd}) and at midday (Ψ_{md}) in terminal shoots of every marked plant (one shoot \times ten plants \times two sites). Measurements were performed immediately after the shoot was cut. Water potential was determined using a Scholander pressure chamber (Scholander et al. 1965; Manofrigido, Portugal).

Statistical analyses

Repeated-measures analysis of variance (RM-ANOVA) were carried out, with season (summer, autumn, winter, and spring) as the within-subjects effect to test for seasonal differences, and site (open vs. understory) as the between-subjects effect. RM-ANOVA was used in the following variables: LMA, $(F_v/F_m)_{\text{pd}}$, $(F_v/F_m)_{\text{md}}$, PI_{chro} , PI_{dyn} , PI_{tot} , A , WUE_i , total chlorophyll ($a + b$), chlorophyll a/b , carotenoids, carotenoids/total chlorophyll, Ψ_{pd} and Ψ_{md} . Pairwise comparisons (between sites and among seasons) were run after a conservative Bonferroni correction (Rice 1989). Differences between years in morphology and LAI measurements for *Thymus* and *Retama* were tested by means of one-way ANOVAs. All values conformed to the normality assumption. In the cases where Mauchly's sphericity was not assumed, we applied Greenhouse–Geisser correction. Homogeneity of variances was checked using Levene's test. When variables were heterocedastic (transpiration rate,

E) we applied the alternative non-parametric test for one-way ANOVA and RM-ANOVA (Mann–Whitney and Friedman tests, respectively). Differences between sites in air temperature and relative humidity (RH) variables were analysed by Wilcoxon matched-pair signed ranks test. Standard score values were calculated for temperature and RH in open and understory sites every 30 min in each of the four seasons, using the following equation:

$$z = (x - \mu) / \sigma \quad (6)$$

where z is the standard score value, x is every 30 min' value, μ the mean value and σ is the standard deviation from all measurements in open and understory sites ($n = 30$). Differences between years in *Thymus* canopy cover and mortality were tested by means of a Chi square test. Significance level was set at 0.05. The relationship between A and g_s were curve-fitted by linear and quadratic regressions. Data analyses were performed with the SPSS v.18.0.0 software (SPSS Inc., Chicago, IL, USA).

Results

Meteorological variables showed the characteristic Mediterranean weather pattern with a marked seasonality in temperatures and rainfall distribution. Nevertheless, precipitation was especially pronounced in November and February and scarce during December and January compared with the 30-year historical data (Fig. 1). VPD was maximal in August and minimal in February, although on the summer sampling day, 29 August, VPD was double than the mean of the month (2.6 kPa compared to 1.35 kPa). This day was also the hottest date of the whole study period,

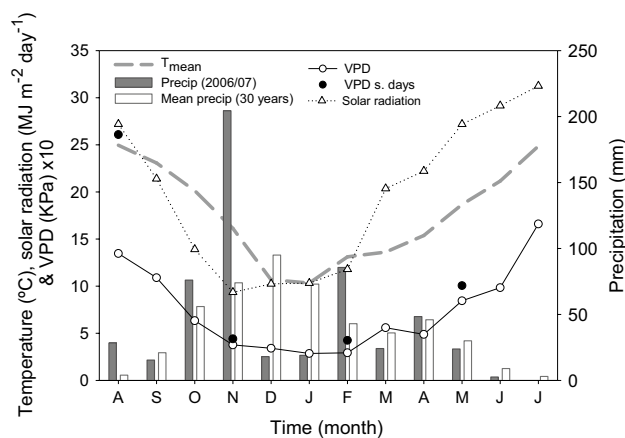


Fig. 1 Monthly mean temperature (T_{mean}), monthly precipitation during the study period (Precip 2006/2007), mean monthly precipitation over 30 years (Mean precip), monthly mean vapour pressure deficit (VPD), sampling day VPD (VPD s. days), and monthly solar radiation throughout the study period (August 2006–July 2007)

Table 1 Maximum PAR at midday (Max. PAR md), % PAR in the understory with respect to open site, absolute maximum (T_{\max}), absolute minimum (T_{\min}) and mean temperatures (T_{mean}), and rela-

tive humidity (RH) microclimatic measurements, taken in the open (T) and the understory sites (T + R) during sampling days (11 a.m.–19 p.m.)

	Max. PAR md ($\mu\text{mol m}^{-2} \text{s}^{-1}$)	% PAR under- story	T_{\max} (°C)		T_{\min} (°C)		T_{mean} (°C)		RH (%)	
			T	T + R	T	T + R	T	T + R	T	T + R
Summer	1,787	18.3	43.0	35.5	28.0	26.5	35.7	31.3***	–	–
Autumn	1,480	21.9	29.5	18.5	9.0	10.0	19.2	15.2**	46.6	53.4*
Winter	1,580	13.6	32.5	25.0	15.5	18.0	22.5	22.2	56.0	58.7
Spring	1,720	23.1	41.5	35.5	23.0	22.5	35.0	26.0***	32.3	46.8**

Data are means for T_{mean} and RH measurements ($n = 15\text{--}17$)

Asterisks indicate significant differences between sites in each season (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; Wilcoxon matched-pair signed-rank test)

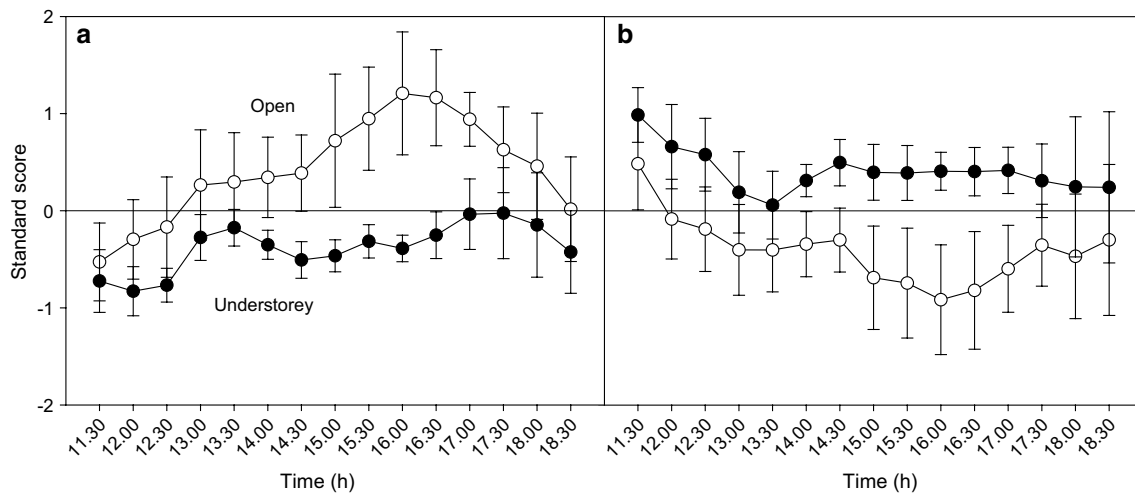


Fig. 2 Standard score values ($z = (x - \mu)/\sigma$) for **a** air temperature and **b** relative humidity in the open site (open symbols) and the understory site (filled symbols), represented at 30 min intervals throughout

the sampling days (11 a.m.–19 p.m.). Values are mean \pm SE from all seasons ($n = 4$ per time interval)

with air temperature reaching 38 °C during the day in the meteorological station.

The percentage of PAR in the understory compared to in the open sun ranged between the minimum in winter (13.6 %) and the maximum in spring (23.1 %), always being lower than 400 $\mu\text{mol m}^{-2} \text{s}^{-1}$ in any season (Table 1). The reduction in midday maximum temperature in the understory compared to the open sites attained 7.5 °C in summer and winter, 11 °C in autumn and 6 °C in spring. Mean temperature was also lower under the *Retama* canopy in all seasons except winter, whereas RH was significantly higher in autumn and spring (Table 1). The mean standard score values from all seasons, plotted at 30-min intervals throughout the day, showed that the temperature was above the mean in the open site, and RH values were below the mean (Fig. 2).

Table 2 Morphology measures (height and cover) and leaf area index (LAI) in open-site *Thymus* (T), understory *Thymus* (T + R) and *Retama* plants (R)

		2006	2011	
Height (m)	T	0.36 \pm 0.02	0.39 \pm 0.02	*
	T+R	0.39 \pm 0.05	0.29 \pm 0.05	
	R	2.77 \pm 0.19	2.76 \pm 0.14	
Cover (m ²)	T	0.25 \pm 0.04	0.49 \pm 0.10	b
	T+R	0.39 \pm 0.13	0.18 \pm 0.12	
	R	17.84 \pm 2.71	23.82 \pm 3.41	
LAI	T	3.03 \pm 0.17	2.82 \pm 0.28	*
	T+R	1.95 \pm 0.18	1.61 \pm 0.25	
	R	1.57 \pm 0.24	2.01 \pm 0.15	

Data are mean \pm SE ($n = 10$ in 2006; $n = 6\text{--}10$ in 2011)

Asterisks indicate significant differences between open-understory sites per year (* $P < 0.05$; *** $P < 0.001$). Different letters indicate significant differences between years per species and site

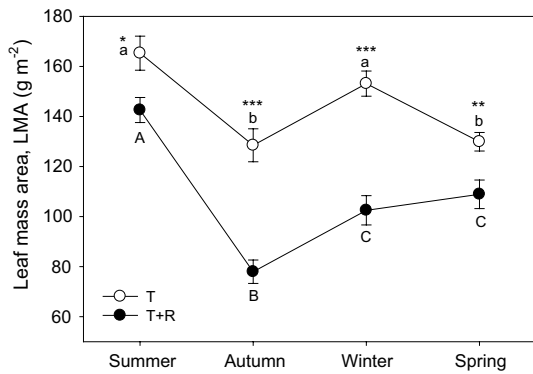


Fig. 3 Leaf mass area (LMA) in open-site *Thymus* (T) and understory *Thymus* (T + R). Values are mean ± SE ($n = 10$ per site). Asterisks indicate significant differences between sites in each season ($*p < 0.05$; $**p < 0.01$; $***p < 0.001$). Different letters indicate significant differences among seasons within sites ($p < 0.05$; lower case letters for T, upper case letters for T + R)

In 2006, all *Thymus* individuals were of similar size in terms of height and canopy cover in both sites, whereas LAI was higher in the open-site plants (Table 2). Five years later, open-site *Thymus* increased in canopy cover (0.24 m^2), and open-site plants were higher than understory plants. LAI was similar in 2011 in both sites, showing higher values in open-site *Thymus*. The height and LAI of *Retama* plants were similar after the 5-year period, whereas canopy cover increased significantly ($p < 0.001$, Table 2).

With respect to survival and canopy status in 2011 compared with 2006, the situation was as follows: in the open site, 10 % of *Thymus* plants died, 10 % decreased and 80 % increased in canopy cover; whereas in the understory, 44.4 % died, 44.4 % decreased and 11.11 % increased in canopy cover (Chi square test, $p < 0.05$ for site effect). One *Retama* individual dried up and, despite resprouting, the *Thymus* plant beneath its canopy was discarded for survival control purposes.

LMA was significantly higher in the open-site plants in all seasons (Fig. 3). The highest LMA (165.3 ± 6.8 and $142.6 \pm 5 \text{ g m}^{-2}$ in T and T + R, respectively) was found in summer for both sites. RM-ANOVA showed significant differences for site, season, and site × season effects (Table S1).

PSII predawn maximum photochemical efficiency (F_v/F_m)_{pd} showed higher values for open-site *Thymus* (site effect, $p = 0.007$; Table S1; Fig. 4a). At midday, F_v/F_m was significantly higher for understory plants (site effect, $p = 0.016$, Table S1), especially in spring (Fig. 4b).

Seasonal differences per site were found for both sites at midday following the expected seasonal pattern: the lowest values were in summer and spring, the periods with higher temperature and radiation input. The theoretical optimum (0.7–0.8 for woody species, Maxwell and Johnson 2000) was reached in all cases.

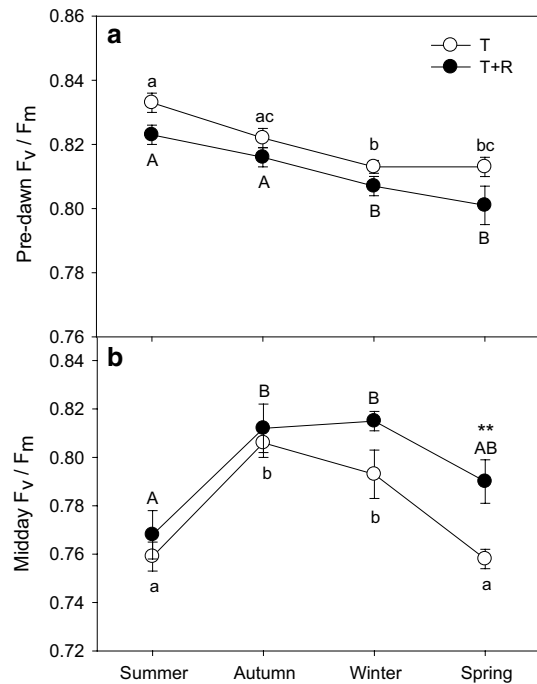


Fig. 4 a Predawn and b midday maximum photochemical efficiency of PSII (F_v/F_m) throughout the study periods in open-site *Thymus* (T) and understory *Thymus* (T + R) (mean ± SE, $n = 10$ per site). Asterisks show significant differences between sites in each season ($**p < 0.01$). Different letters show significant differences among seasons within sites ($p < 0.05$; lower case letters for T, upper case letters for T + R)

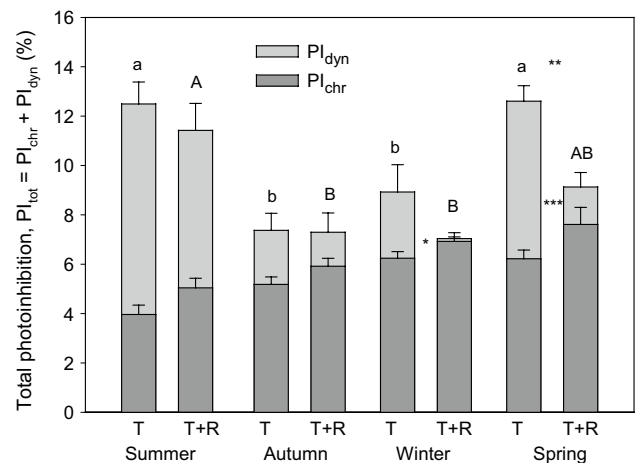


Fig. 5 Chronic (PI_{chr}), dynamic (PI_{dyn}) and total photoinhibition (PI_{tot}) throughout the study periods in open-site *Thymus* (T) and understory *Thymus* (T + R) (mean ± SE). Asterisks show significant differences between sites in each season ($*p < 0.05$; $**p < 0.01$; $***p < 0.001$; asterisks at bar level refer to PI_{dyn} , and upper asterisk refer to PI_{tot}). No significant differences were found for PI_{chr} in each season separately). Different letters show significant differences among seasons within sites ($p < 0.05$; lower case letters for T, upper case letters for T + R)

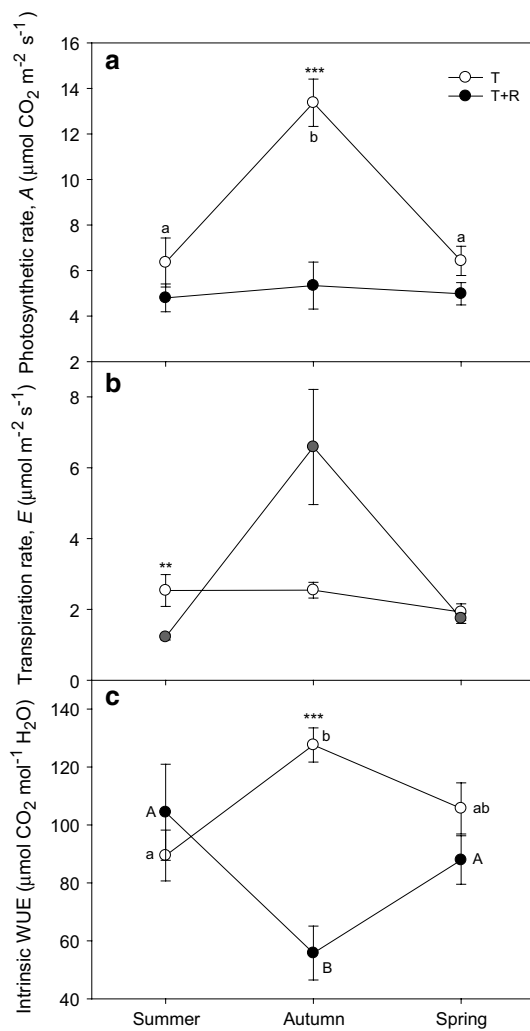


Fig. 6 **a** Photosynthetic rate (A), **b** transpiration rate (E) and **c** intrinsic water use efficiency (WUE_i) throughout the study periods except winter in open-site *Thymus* (T) and understory *Thymus* (T + R) (mean \pm SE, $n = 10$ per site). Asterisks show significant differences between sites in each season (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). Different letters show significant differences among seasons within sites ($p < 0.05$; lower case letters for T, upper case letters for T + R)

PI_{tot} ($PI_{chr} + PI_{dyn}$) was similar in both sites for all studied seasons except for spring, the growth period, when it was significantly higher in open-site *Thymus* (Fig. 5). PI_{dyn} showed differences between sites in winter and spring, open-site plants displaying higher values. However, PI_{chr} was higher in understory *Thymus* throughout the seasons (site effect, $p = 0.007$, Table S1).

PI_{tot} was low in general for *Thymus*, reaching values lower than 15 % in all study seasons. Nevertheless, in summer and spring PI_{tot} was significantly higher than in autumn and winter, the less stressful seasons ($p < 0.005$). Although this tendency was not reflected in PI_{chr} for any light condition, it was reflected in PI_{dyn} for open-site plants, with the lowest values also in autumn and winter (Fig. 5).

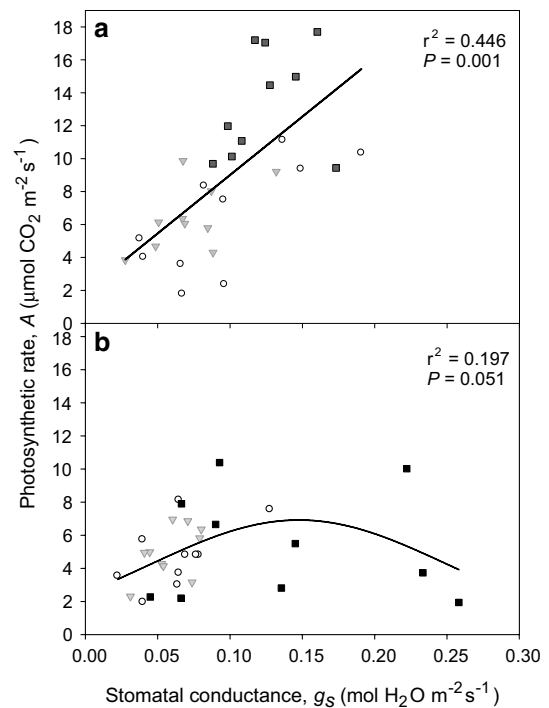


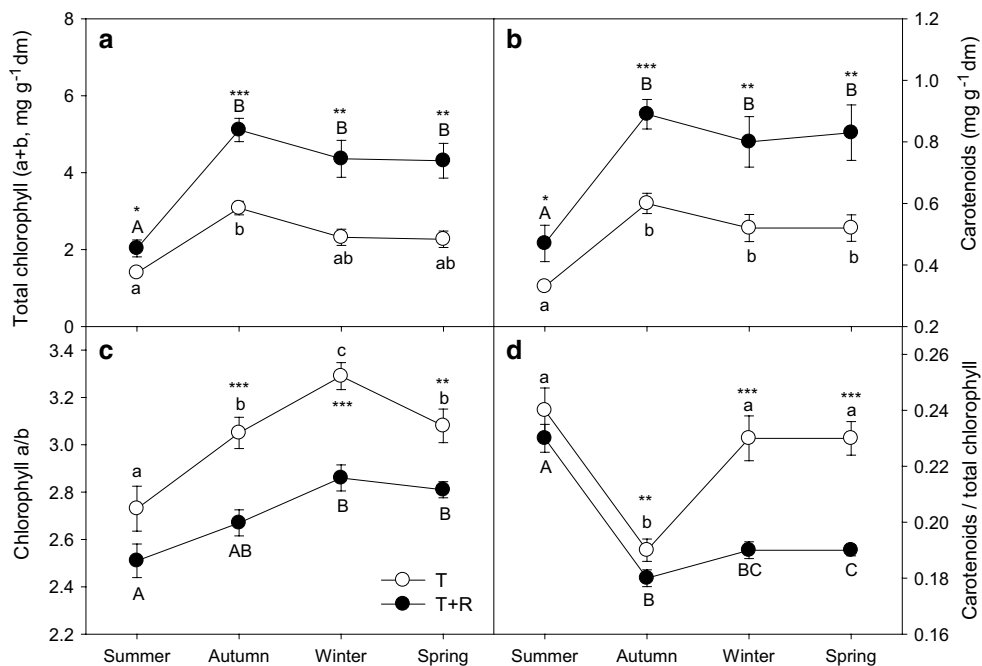
Fig. 7 Relationship between photosynthetic rate (A) and stomatal conductance (g_s) in **a** open-site *Thymus* (linear regression) and **b** understory *Thymus* (quadratic regression), in summer (open circles), autumn (closed squares) and spring (grey triangles) ($n = 30$ per site)

Net photosynthetic rate (A) showed higher values in open-site *Thymus* in autumn (Fig. 6a). Transpiration rate (E) was significantly different between sites in summer (Fig. 6b), with open-site plants displaying higher values. WUE_i was significantly lower in understory plants in autumn (Fig. 6c).

Open-site plants displayed the highest A values in autumn, the season with the lowest VPD, whereas understory *Thymus* did not show any difference among seasons. Results for E showed no seasonal differences neither in the open nor in the understory plants ($p > 0.05$, Friedman test, Fig. 6b). Open-site *Thymus* WUE_i increased in autumn with respect to summer, while understory WUE_i was the lowest in autumn. RM-ANOVA indicated site, season, and site \times season effects in A ($p < 0.001$), and site and site \times season effects in WUE_i ($p = 0.009$ and $p < 0.001$, respectively, Table S1).

We examined the relationship between A and g_s after segregating the data according to site and season (Fig. 7). *Thymus* in the open-site showed a linear relationship ($p = 0.001$), with similar g_s and A values in summer and spring, and maximum values in autumn (Fig. 7a). In understory plants, a higher increase in g_s in autumn yielded lower A values (Fig. 7b), which caused the low WUE_i in the same season.

Fig. 8 **a** Total chlorophyll content (*a + b*), **b** chlorophyll *alb*, **c** carotenoids content and **d** carotenoids/total chlorophyll content throughout the study periods in open-site *Thymus* (T) and understory *Thymus* (T + R) (mean ± SE, *n* = 10 per site). Asterisks indicate significant differences between sites in each season (**p* < 0.05; ****p* < 0.01; *****p* < 0.001). Different letters indicate significant differences among seasons within sites (*p* < 0.05; lower case letters for T, upper case letters for T + R)



Total leaf chlorophyll (*a + b*) and carotenoid content was lower in open-site *Thymus* in all seasons (69–53 % regarding T + R from summer to spring in total chlorophyll, and 70–63 % in carotenoids, Fig. 8a, b), although the relationships chlorophyll *alb* and carotenoids/total chlorophyll were revealed to be higher for open-site plants in all seasons except summer (Fig. 8c, d).

The seasonal trend was similar in general for both sites, showing significantly lower values in summer for chlorophyll *a + b*, carotenoids and chlorophyll *alb* ratio. Chlorophyll *a + b* in summer compared to the autumn, winter and spring rates was reduced by 55, 40, and 38 %, respectively, in open-site plants, and 60, 54, and 53 % in understory *Thymus*. RM-ANOVA revealed site and season effects for all the variables, and site × season interaction in the case of carotenoids/total chlorophylls (Table S1).

Leaf nitrogen content (%N) was higher in understory plants in all measured seasons, and the C/N ratio was higher in open-site *Thymus* in spring (Table 3). Carbon isotope discrimination ($\Delta^{13}\text{C}$) varied with canopy exposure and seasons, being lower (higher integrated WUE) in the open site with respect to the understory site, and in spring with respect to winter. $\delta^{15}\text{N}$ did not show significant differences between sites, but it was significantly closer to zero in winter for both light conditions (Table 3).

Differences in plant Ψ_{pd} were significant for site effect (*p* = 0.005, Table S1), understory plants displaying more negative values than open-site ones in summer and spring (Fig. 9a). Ψ_{md} also showed differences between sites in winter and spring, but with opposite responses in both seasons: open-site plants had lower Ψ_{md} in winter and

Table 3 Leaf total carbon and nitrogen contents (%C and %N), C/N ratio, carbon isotope discrimination ($\Delta^{13}\text{C}$) and stable nitrogen isotopes ratio ($\delta^{15}\text{N}$) in winter and spring for open-site (T) and understory (T + R) *Thymus* plants

		Winter	Spring		
%C	T	48.65 ± 0.28	49.13 ± 0.32		
	T+R	48.44 ± 0.19	48.66 ± 0.10		
%N	T	1.25 ± 0.09	1.09 ± 0.07	*	***
	T+R	1.62 ± 0.14	1.84 ± 0.13		
C/N	T	40.69 ± 3.02	46.56 ± 2.78		**
	T+R	30.71 ± 2.51	27.32 ± 1.90		
$\Delta^{13}\text{C}$	T	22.36 ± 0.21	20.44 ± 0.27	a	b
	T+R	25.19 ± 0.26	22.14 ± 0.76	*** a	** b
$\delta^{15}\text{N}$	T	-0.27 ± 0.37	-1.78 ± 0.33	a	b
	T+R	-0.80 ± 0.43	-2.49 ± 0.08	a	b

Data are mean ± SE (*n* = 5–10)

Asterisks indicate significant differences between open-understory sites in each season (* *P* < 0.05; ** *P* < 0.01; *** *P* < 0.001). Different letters indicate significant differences between seasons per site

higher values in spring than understory *Thymus* (Fig. 9b). Ψ showed the expected general seasonal pattern in understory *Thymus*, reaching maximum values during autumn and winter and falling after spring to minimum values at the end of summer (Fig. 9a, b). Open-site *Thymus* did not show exactly this tendency, as Ψ_{pd} values in summer were

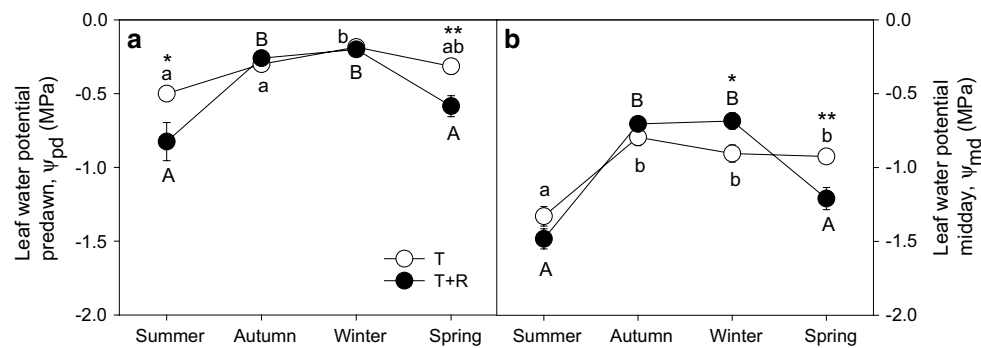


Fig. 9 **a** Predawn (Ψ_{pd}) and **b** midday (Ψ_{md}) stem water potential in open-site *Thymus* (T) and understory *Thymus* (T + R) (mean \pm SE, $n = 10$ per site). Asterisks indicate significant differences between

sites in each season ($*p < 0.05$; $**p < 0.01$). Different letters indicate significant differences among seasons within sites ($p < 0.05$; lower case letters for T, upper case letters for T + R)

similar to those in autumn and spring and only higher in winter. Ψ_{md} values were the lowest only in summer in the open-site plants. RM-ANOVA detected significant differences for seasons and site \times season effects in both Ψ_{pd} and Ψ_{md} (Table S1).

Discussion

Despite the attenuation of high radiation and temperature and the increase in relative humidity and nutrient enrichment below the *Retama* canopy, results showed that the physiological performance, growth and survival of *Thymus* plants was negatively affected by the presence of *Retama*. *Thymus* appears to be a very well-adapted species to this semi-arid environment, as evident in the more pronounced net competitive effect of *Retama* in spring and summer.

Increased drought stress under the *Retama* canopy

The possibility of positive plant–plant interactions due to attenuation of extreme microclimatic conditions beneath the *Retama* canopy was overridden by reduced water availability for *Thymus* during summer and spring, as demonstrated by the lower Ψ_{pd} , Ψ_{md} and E in understory *Thymus*. In addition, understory plants displayed a higher PI_{chr} , directly related to the lower F_v/F_{mpd} . Photoinhibition will be produced not only due to light excess, but also enhanced by drought stress (Björkman and Powles 1984; Valladares and Pearcy 1997) because water scarcity may limit the capacity of plants to use the incident light for photosynthesis. Indeed, a previous study demonstrated that the presence of *Retama* produces greater soil moisture depletion in the understory in comparison with the open site, and a shift in water exploitation pattern from permanent water table in open-site *Thymus* to soil and rain water in understory plants (Esquivias et al. 2014). In the same study, leaf

proline content (a water stress indicator) was found to be higher in understory *Thymus* in spring and summer. These results support the conclusion of higher water deficit under *Retama* canopies in the warmer seasons.

Open-site *Thymus* showed a more conservative water-use pattern or WUE_i (A/g_s), being able to fix more carbon with the same stomatal closure. Both integrated (Δ) and intrinsic *Thymus* WUE were higher in the sun than in the shade, similar to results found in *Arctostaphylos uva-ursi* (Valladares et al. 2008), a Mediterranean species showing low shade-tolerance. In addition, the higher WUE_i found in autumn than in summer for open-site plants is in line with other studies on Mediterranean species that also reported a decrease in WUE as water deficit increased (Moriani et al. 2002; Llorens et al. 2003; Álvarez-Cansino et al. 2010), in contrast to other studies showing the opposite pattern (Farquhar and Richards 1984; Ehleringer and Cooper 1988). In the Mediterranean climate, on autumn days, it is not surprising that shrub species display high WUE_i , as photosynthetic rates can be higher due to the high radiation, rainfall recovery and decreased VPD. Moreover, it has been suggested that efficient use of water is a conservative eco-physiological strategy that can be detrimental in a competitive, water-limited environment (DeLucia and Heckathorn 1989; Read and Farquhar 1991; Llorens et al. 2003), as we observed both in the seasonal and site patterns (Fig. 6c).

In cases of facilitation, photosynthetic rates and stomatal conductance are usually lower in open sites than in the understory due to photoprotection in dry areas (Callaway and Pugnaire 1999; Valladares and Pearcy 2002). This contrasts with results found in our study in autumn. Understory plants showed A values that were constant in all seasons independent of rain events. In fact, even when understory *Thymus* opened the stomata in autumn with rainfall recovery, plants could not attain high carbon assimilation rates. It is likely that this was because plants did not achieve the minimum radiation required for photosynthesis, as there

was no stomatal limitation. The maximum photosynthetic rate was found in autumn in open-site *Thymus* due to recovery from summer drought after the first rains, which were especially abundant the year in which the study was carried out. Nonetheless, the sampling days in spring were long after the last precipitations (29 days) and temperatures were rising again, as well as the VPD, which was likely to have caused lower carbon assimilation due to a reduced stomatal conductance. In addition, high radiation in summer and spring could have also been affecting open-site *Thymus* and causing the low photosynthetic rates found in those periods. The higher A values found in sun-exposed plants in autumn, as well as more pronounced seasonal changes in open sites, are in accordance with findings by Zaragoza-Castells et al. (2008) in a study with another Mediterranean species, *Quercus ilex* subsp. *ballota*, and with findings by Valladares et al. (2008) with *A. uva-ursi*.

Light adaptation

The response of *Thymus* plants to light availability was as expected for sun–shade conditions in the case of LMA, LAI, pigments content or midday chlorophyll fluorescence. The result for LMA is ecologically adaptive: by increasing the area of a given unit of leaf biomass, the interception of light is increased under low-light conditions, while more photosynthetic biomass per unit leaf area enhances photosynthetic capacity in high light (Poorter et al. 2009). Moreover, as shown by LAI, stronger foliage aggregation reduces energy absorption and water loss in sun-exposed habitats, and thus is expected to enhance tolerance to drought, while the opposite is needed for improved light capture under shade conditions (Hallik et al. 2009). Thus, higher LAI and LMA of *Thymus* in the open site conferred greater morphological capacity for photoprotection and water loss avoidance.

The decrease in leaf chlorophyll is common during the summer in Mediterranean plants, and constitutes a reversible process that allows a decrease in light harvesting and reduces risks of over-excitation and photodamage (Balaguer et al. 2002). This reduction of the antenna size of up to 60 % in *Thymus* allows a sustained functionality of PSII, as indicated by summer midday maximal F_v/F_m values no lower than 94 % of the annual maximal values. When compared with other Mediterranean Labiatae species such as *Lavandula stoechas* or *Rosmarinus officinalis* (Munné-Bosch et al. 1999), we found similar results, showing the great level of adaptation of *Thymus* to its environment. Even greater results are found comparing both *Thymus* sites: there was 30 and 47 % less chlorophyll in open-site plants than in understory plants in August and May, respectively. This led to <5 %

F_v/F_{mmd} reduction in all seasons, but the differences were only significant in spring. This, accompanied by the increase in the chlorophyll a/b and carotenoids/chlorophyll ratios, typical under high light conditions (Niinemets et al. 1998), partly explains the absence of damage to PSII in sun-exposed plants (Demmig-Adams and Adams 1996). These results show how chlorophyll loss in open-site *Thymus* is an adaptation against photodamage, but not an irreversible event. At the same time, exposure to the sun of open-site plants was reflected in higher PI_{dyn} , but neither in an increase in PI_{chr} nor in lower carbon assimilation.

Sun plants exhibited efficient photoprotection against strong irradiance due to both the architecture of the crown and the physiology of leaves, similar to other Mediterranean species as *Heteromeles arbutifolia* (Valladares and Pearcy 2002). However, in understory plants, the increased investment in aerial resources to cope with low light conditions probably restricted investment in the capture of below-ground resources, which further reduced the competitive ability for water acquisition.

Nutrients and light availability

For *Thymus*, growing in a nutrient-poor environment such as sand dunes, the fact of being shaded by an N-fixing leguminous shrub could have been an advantage over open-site plants. Nevertheless our results show that, in spite of values that indicate a higher leaf N accumulation, there was not an associated increase in A or WUE_i in understory *Thymus*. In fact, individuals in the open sites showed similar or even higher carbon assimilation rates with less N content. It is possible that shaded *Thymus* invested N in chlorophyll production that was not enough to fulfill photosynthetic requirements (as they showed lower A), whereas open-site plants invested more in structural components (higher C/N).

The higher leaf N content in understory plants was not related to a change in $\delta^{15}N$ values, which were similar between sites in winter and spring (Table 3). Our results indicated a lack of relationship between understory *Thymus* and fixed atmospheric N via direct transfer from *Retama* roots. These results support those found by Rodríguez-Echevarría and Pérez-Fernández (2003) in a study on *R. sphaerocarpa*. The presence of multiple N-sources with distinct isotopic values, mycorrhizal associations, temporal and spatial variation in N availability, and changes in plant demand can all influence plant $\delta^{15}N$ (Dawson et al. 2002). In fact, the pattern of decreasing $\delta^{15}N$ from winter to spring in both sites could be indicating N depletion in the ecosystem due to higher N use during the growing season (Högberg 1997).

Net *Retama* effect

The overall effect of *Retama* on its surrounding environment and the soil under its canopy may include other features such as allelopathic chemicals, mycorrhizas, soil resources or physical factors. Although we cannot discard the potential effects of these factors, in this study we have focused on the response of *Thymus* to water and light, because these are the most important drivers of stress in Mediterranean ecosystems. However, we consider that it is the cumulative effect of several factors that explain why *Thymus* plants performed better physiologically in open areas than in the understory. The measured variables show drought stress in the understory and adaptation to low light availability, but not facilitation by the presence of *Retama*. As argued by Smith and Huston (1989), physiological trade-offs may affect the capacity of a plant to simultaneously tolerate shade and drought. *Thymus* plants would be even less capable of investing in the aerial and the underground fractions when their productivity was low due to shading, which probably restricted *Thymus* growth.

An analysis of changes over time can help to elucidate the net outcome of species interaction more definitely. After 5 years, the morphological measurements showed a cover increase in open-site plants. In addition, understory *Thymus* presented a lower height, as well as a higher mortality. These results are supported by analyses of spatial patterns of the distribution of the species in the same study area, which showed a repulsion pattern of *Retama* over *Thymus* plants (Zunzunegui et al. 2012), thus reinforcing the competitive net effect of *Retama* in the long term.

The effect of wild rabbit on the decrease in size and survival of understory *Thymus* could be another factor to take into consideration, as rabbits have an important role as dispersers of *Retama* seeds (Dellafiore et al. 2006). Nevertheless, we discard rabbit disturbance, firstly because *Thymus* plants are not consumed by any herbivore (Zunzunegui et al. 2012). Secondly, although warrens always appear associated with high *Retama* cover, the areas with *Thymus* are less used than expected, with only seven warrens observed in more than 50 ha of total surface (Dellafiore et al. 2008). And no warrens were detected in our understory sites. Finally, although rabbits would be present in the area, we consider that any possible effect produced by their activity could be similar on open-site *Thymus* plants and understory ones.

Despite the negative net effect of *Retama* on *Thymus* plants assessed in this study, a hypothetical positive effect of adult *Retama* plants on *Thymus* seedlings must not be discarded, as other studies have demonstrated early facilitation turning into competition when plants reach the adult state (Armas and Pugnaire 2009). On the other hand, the widely studied facilitative effect of *Retama* species over the

understory has been done mainly with herbaceous species; interaction results with woody shrubs are dependent on the species (Padilla and Pugnaire 2009). This statement is in agreement with Valladares et al. (2008) when suggesting facilitation as species-specific. Further research is needed to assess *Retama*'s potential competitive effect on other Mediterranean dune shrub species, both in its native range of distribution and where it has become an exotic invasive species, which could have serious consequences for plant community dynamics.

Concluding remarks

Despite the potential for *Retama* to provide improved growing conditions through enhanced nutrient availability and lower temperatures, this study shows that the presence of *Retama* was detrimental to the size and overall physiological response of *Thymus*. We conclude that water scarcity in interaction with low light availability under the *Retama* canopy probably overrode facilitation of *Thymus* by *Retama*. *Thymus* has demonstrated to be well-adapted to drought at high light intensities, and even though it showed some degree of morphological plasticity in the shade, *Thymus* was negatively affected by the presence of *Retama*. As long as *Retama* is present throughout the area of distribution of *Thymus*, the same mechanisms could be reproduced, with the consequent displacement of *Thymus*. Therefore, *R. monosperma* arises as a new threatening factor for the endangered *T. carnosus*. In addition and following Valéry et al. (2009), *R. monosperma* interspecific competition would confirm the invasive behaviour of this native species.

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