


## Article

# Photoprotective Strategies in Mediterranean High-Mountain Grasslands

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**Abstract:** Albeit the remarkably high Ultraviolet B loads, high temperatures, and drought stress substantiate the need for efficient photoprotective strategies in Mediterranean high-mountain plants, these remain understudied. Considering the sensitivity of photosystems to extreme conditions, we evaluated an environmental gradient's weight on the photoprotection of five high-mountain specialists from Central Spain. Diurnal and seasonal variations in chlorophyll, chlorophyll fluorescence, carotenoids, and xanthophylls in consecutive and climatically contrasting years were taken to evaluate the effect of the impending climate coarsening at the photosystem level. Our results revealed significant differences among species in the xanthophyll cycle functioning, acting either as a continuous photoprotective strategy enhancing photochemistry-steadiness; or prompted only to counteract the cumulative effects of atypically adverse conditions. The lutein cycle's involvement is inferred from the high lutein content found in all species and elevations, acting as a sustained photoprotective strategy. These findings added to high de-epoxidation state (DEPS) and minor seasonal changes in the chlorophyll a/b ratio, infer the xanthophyll and Lutein cycles are crucial for upkeeping the photosystems' optimal functioning in these plants heightening their photoprotective capacity during periods of more unfavorable conditions. Nevertheless, an atypically dry growing season's detrimental effect infers the feasible surpassing of stress-thresholds and the precariousness of the communities' functional diversity under climate change.



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## 1. Introduction

Photoprotection is essential in high-mountain vegetation since these plants face the combined action of high irradiance with extreme temperatures during their brief growing period [1]. The latter is especially critical in plants from Mediterranean high-mountains, where the need for photoprotective strategies is undoubtedly exacerbated given the incidence of summer stress (i.e., the simultaneous effect of drought, elevated temperatures, and high irradiance) during the period when temperatures are sufficiently high to enable plant growth [2].

Light absorbing pigments, i.e., chlorophyll (Chl) and anthocyanins fulfill the first stage of photosynthesis, whose concentration and structure determine light absorption efficiency [3]. Stress conditions such as drought, extreme temperatures, and high light intensity impair plant physiological processes. They can directly or indirectly affect photosynthesis due to the high sensitivity of photosynthetic pigments to variations in environmental factors leading to the enhancement of photosynthetic production and accumulation of reactive oxygen species (ROS; [4–6]). The combined effect of adverse climatic factors can intensify this response, coupled with altering chlorophyll biosynthesis. Thus, resulting in

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changes in the light-harvesting complexes and the subsequent shift from efficient light- and energy-harvesting pigment-protein complexes towards those set for energy quenching [7]. To protect their tissues from the excess absorbed energy (i.e., avoid oxidative stress and resultant photoinhibition), plants can employ two main mechanisms: (1) modulate light capture through the development of thicker parenchyma and smaller chloroplasts to reduce the probability of light absorption, deriving in smaller antenna complexes and higher chlorophyll *a*/chlorophyll *b* ratio (Chl *a/b*) [8,9]; and (2) enhance the energy dissipation mechanisms and structures, achieved through the two components of non-photochemical quenching of chlorophyll (NPQ): the fast relaxing component (NPQ<sub>f</sub>) and the slow relaxing component (NPQ<sub>s</sub>) [4].

Thermal dissipation is a dynamic process associated with the NPQ<sub>f</sub> response where carotenoids, e.g., xanthophylls and lutein, are the main molecules involved in the excess energy dissipation captured by the chlorophyll molecules [4]. This thermal dissipation consists of the activation of the VAZ xanthophyll cycle (henceforth, VAZ) through the conversion of violaxanthin (V) into antheraxanthin (A) and, lastly, zeaxanthin (Z); strongly associated with stress-mediated processes that derive from an excess of energy absorption by chlorophyll molecules [10,11]. Similar to the relatively stable total chlorophyll content (Chl<sub>TOTAL</sub>) found in plants from temperate alpine environments (hereafter, alpine) throughout the day, the total pool of VAZ pigments in alpine species maintains constant diurnal values under favorable conditions [8,12,13]. However, the increment in the proportion of xanthophyll de-epoxidated compounds (i.e., A, Z) is acknowledged as a fast, protective response to diurnal variations in the environmental conditions (i.e., higher light intensity and temperatures at noon, opposite to lower early-day values); and to the incidence of abiotic stressors, such as heat and drought stress [12]. Therefore, a more critical requirement for photoprotection is expected in plants inhabiting stressful environments with co-occurring elevated temperatures, drought stress, and considerably high-UV intensities, such as Mediterranean high-mountains, in response to daytime increases in light intensity and temperatures [1,8,12,14].

Conversely, in all plants, changes in Chl<sub>TOTAL</sub> are expected as the growing season elapses, contrasting the diurnal changes in Chl<sub>TOTAL</sub> that represent <1% of the total content. In a growing season, high Chl<sub>TOTAL</sub> is expected during the periods of high vegetative activity followed by its decline as senescence approaches [15]. Nonetheless, variations in Chl<sub>TOTAL</sub> may occur in response to alterations in plant N status and scarce water availability. The latter is generally associated with the annual climate wavering in the study area [1,15]. Since changes in Chl in response to environmental wavering are relatively slow, it is essential to assess traits with a faster response to such constraints, such as chlorophyll fluorescence [16,17]. Under adverse conditions, chlorophyll fluorescence parameters such as the photosynthetic efficiency of the photosystem II ( $F_v/F_m$ ) provide a fast and sensitive indication of the physiological status of plants, added to allowing elucidating the degree of tolerance to high temperature and drought stress in several plant species across biomes [6,16,18,19].

In alpine species, photosynthesis has been validated as a well-adapted process to the interplay of challenging environmental conditions distinctive of high-mountain environments [4,20,21]. Several studies in alpine plants have established the decline in temperatures with increasing altitude as the main factor driving the buildup of antioxidants and ROS scavenging compounds, such as carotenoids [20,22–24]. The development of a functional system of hydrophilic and lipophilic antioxidants (e.g., carotenoids, VAZ), termed photoprotective strategies, has been acknowledged in alpine and lichen species as a proficient ROS scavenging mechanism in their photosystems, allowing efficient carbon assimilation and optimum use of light energy captured [20,21,25,26]. However, direct extrapolation of the strategies found in the alpine flora to the unique Mediterranean high-mountain plants does not appear straightforward. The latter is due to the prevalence of summer stress as the primary constraint for plant performance during their short growing season in these habitats [2]. The occurrence and the severity of summer stress underline the

more challenging conditions constraining plant life in Mediterranean high-mountains [2,27]. Furthermore, the marked interannual climate variability characteristic of these habitats, coupled with the higher frequency and severity of combined drought and heat episodes witnessed in recent years [28,29], adds further pressures to the establishment and survival of Mediterranean high-mountain plants [30,31].

Based on the series of photoprotective mechanisms found in alpine and their Andean counterparts [19], the present study aims to assess a) the role of thermal dissipation in alleviating the potential photoinhibitory effects of summer stress in herbaceous Mediterranean high-mountain specialists. Specifically, due to the possible broad differences in environmental conditions along local elevation gradients in the study area, we expect to find (1) an increased level of de-epoxidation of VAZ pigments in individuals from higher altitudes in response to high photo-oxidative pressures (i.e., greater thermal amplitude and light intensity; [23]); coupled with (2) an increased Chl*a/b* ratio and lower  $F_v/F_m$  in populations from the dry-lower end of the elevational gradient as a local advantage outcome of their adaptation to more intense drought stress conditions compared to their high-elevation counterparts [32–35]. Contrary to prostrate growth forms, herbaceous species do not possess an atmosphere-decoupling capacity that grants them substantial advantages to the diurnal environmental variations in these summits. The latter is particularly critical on clear summer days when the incidence of excessive high-solar radiation and the absence of wind may increase diurnal ambient and leaf temperatures, leading to the surpassing of critical plant thresholds [1]. In this sense, (3) we expect that the low-elevation individuals will accumulate a higher content of xanthophyll de-epoxidated compounds at midday and late afternoon than during the morning to prevent the imperilment of the repair mechanisms of PSII [1,36,37].

Mediterranean high-mountains have been classed as “islands of biodiversity” since these summits have served as a refuge for specialized Euro-Siberian species that cohabit with xerophilous species, well-adapted to summer stress and occupying areas from the highest peaks to the tree limit [38,39]. Contrasting the multiple studies illustrating the wide variety in photoprotective strategies in different plant species and habitats [13,40,41], information is scarce on their specific response to climate variation or the extent of their role in the primary functional type comprising the plant communities from Mediterranean high-mountains, hemicryptophytes. However, some research efforts are available on rosettes and cushion-form species from Mediterranean high-mountains [42,43], advocating for their photosystem’s robustness relative to unfavorable conditions. Therefore, this study aims to determine the diurnal variations in the xanthophyll pigment cycle and assess the photochemical efficiency of the PSII and the seasonal variations in chlorophyll content in these Mediterranean high-mountain hemicryptophytes to elucidate their role in photoprotection as both the growing season and summer stress advance. This information is of great ecological significance since most of these species are narrow endemics isolated in these mountains’ highest summits, facing a highly precarious future (i.e., *Erysimum penyalarens*). Therefore, understanding their response to changing climatic conditions, added to the latent similarities in these responses among species, is a priority for the adequate management of these highly vulnerable habitats.

## 2. Materials and Methods

### 2.1. Study Site and Plant Material

Our study was conducted throughout the 2017–2019 growing seasons in the Bola del Mundo summit’s surroundings, located within the Sierra de Guadarrama National Park, an E-W mountainous range dividing the Iberian plateau. These mountains experience a Mediterranean-type climate with an intense dry season during summer from June to September (<10% of total annual rainfall), which coincides with the short active growing season of vegetation [2]. Four sites were established along the elevation gradient at 2244, 2172, 2052, and 1980 m, on NW and W facing slopes, placing one plot (20 × 20 m) per site on homogeneous grasslands. The selected species provided a complete phylogenetic

representation of the herbaceous specialists comprising these grassland communities [44]. In turn, we chose the altitudinally widespread species in this summit: *Erysimum penyalarens* (Pau) Polatschek, *Festuca curvifolia* Lag. ex Lange and *Senecio pyrenaicus* subsp. *carpetanus* (Willk.) Rivas-Mart., and two species circumscribed to elevations above 2000 m, i.e., *Hieracium vahlia* Froelich. and *Jurinea humilis* (Desf.) DC. We ensured all the selected individuals of the same species were of similar size and at the same phenological stage (i.e., vegetative growth) in every sampling event to minimize the variations due to ontogeny.

The sampling occurred in two phases, given the limited biomass available in 2017 in all species (dry winter; Figure S1) and the lack of reference information and available expertise of the seasonal or diurnal variation in both photosynthetic parameters and photoprotective pigments in these specialists. Samples were collected for an initial assessment of seasonal variation in photosynthetic pigments during the first phase and carried over similarly in the 2018 growing season. Samples consisted of five fully developed leaves from ten individuals per species collected at each site throughout the entire growing season (June–September) in climatically contrasting years (2017 and 2018). The second sampling phase (2018 and 2019) consisted of the sample collection to quantify photoprotective pigments. For these determinations, fully developed leaves of five mature individuals were collected per species on three consecutive, sunny days in July 2018 and 2019 in the morning, midday, and afternoon. In this second stage, sampling in two successive years allowed counting in the annual climate variability in Mediterranean areas. After excision, samples were immediately frozen in liquid nitrogen to avoid pigment degradation. Samples were taken at the two most contrasting elevations of the selected elevation gradient for a preliminary assessment of xanthophyll pigments' content between populations from both edges of the local distribution in response to the environmental gradient.

Topsoil water content (SWC) was measured in all plots at the time of plant sampling using a hand-held, time-domain reflectometry sensor (FieldScout TDR 300; Spectrum Technologies, Plainfield, IL, USA) to a depth of 10 cm and taking ca. 5 measurements per plot in randomly located sites endowed with plant cover. Daily patterns of irradiance and temperature, monthly temperature, and rainfall values were obtained from the meteorological station Puerto de Navacerrada, Madrid, run by the Spanish Institute of Meteorology, AEMET. Diurnal temperature variation and mean SWC, the latter up to the days of sample collection, for 2018 and 2019 are in Table 1. These denote a similar ambient temperature during the sampling for xanthophyll and lutein pigments required for valid inter-annual comparisons.

**Table 1.** Environmental conditions during sample collection for the xanthophyll cycle experiment (July 2018 and 2019). Average topsoil water content (SWC) values are shown only for the highest and lowest sites of the studied elevational gradient from up to the time of sampling for the xanthophyll pigment analyses—Climate Info Source: Spanish Institute of Meteorology, AEMET.

	Year	1980 m.a.s.l.		
		Morning	Midday	Afternoon
Temperature (°C)	2018	19.1	21.03	22.6
	2019	19.0	24.4	21.6
		2244 m.a.s.l.		
	2018	19.86	20.02	19.86
SWC (% by vol.)	2019	18.9	22.3	19.8
		1980 m.a.s.l.		
	2018		5.10	
	2019		1.4	
		2244 m.a.s.l.		
	2018		2.37	
	2019		1.68	

## 2.2. Xanthophyll Cycle Pigments Analysis

Fully developed leaf samples were collected at the time of optimal plant activity from fully developed individuals of each species on three clear days at 9, 13, and 16 h in July 2018 and 2019. Leaf samples were collected from individuals growing in opposite ends of the selected elevation gradient (1980 and 2244 m) and immediately frozen in liquid nitrogen, subsequently stored at  $-20^{\circ}\text{C}$  until analysis. For extraction, ca. 100 mg<sub>FW</sub> of each leaf sample was powdered with liquid nitrogen in a cool mortar and further extracted with 2 mL of acetone. Next, the homogenate was centrifuged at  $0^{\circ}\text{C}$  for 10 min at  $13,000\times g$ . Supernatants were collected and deposited in new tubes, following each pellet's resuspension in 2 mL of acetone for re-extraction as previously described. Combined supernatants were adjusted to a final volume of 5 mL, filtered through a  $0.2\text{ }\mu\text{m}$  syringe filter, and stored at  $-20^{\circ}\text{C}$  until analysis.

Samples were analyzed by reverse-phase HPLC to determine the content of antheraxanthin (A), violaxanthin (V), zeaxanthin (Z), and lutein (Lut) according to the method by García-Plazaola and Becerril [45]. Chromatographic analyses were performed with an Agilent 1260 instrument (Agilent Technologies, Santa Clara, CA, USA), using a Mediterranea Sea18 reverse-phase column ( $150\times 4.6\text{ mm}$ ;  $3\text{-}\mu\text{m}$  particle size) (Teknokroma Analitica S.A., Barcelona, Spain). Two mobile phases were implemented, with the following compositions: (A) acetonitrile: methanol: water (84:9:7); and (B) methanol: ethyl acetate (68:32). Photosynthetic pigments were eluted using a linear gradient from 100% A to 100% B for 12 min, followed by isocratic elution with 100% B for 6 min. Next, a linear gradient from 100% B to 100% A was applied for 1 min. Finally, an isocratic elution of 100% A was used for 6 min to allow the column to re-equilibrate before the next injection. Injection volume was  $20\text{ }\mu\text{L}$ , with  $1.2\text{ mL/min}$  for solvent flow rate and a working pressure of approx. 82.7 bar. Methanol was flushed into the column for overnight storage. Samples were scanned with a photodiode array detector within the 250–450 nm range, with eventual scanning from 250–700 nm to detect impurities. Peak integration and areas were calculated using Chemstation software (Agilent Technologies, Santa Clara, CA, USA). Lutein and xanthophyll-cycle pigments were quantified by comparison with standards. The de-epoxidation state of the xanthophyll pigment pool was estimated as follows:

$$\frac{(Z + 0.5 * A)}{(V + A + Z)}$$

## 2.3. Chlorophyll Quantification

Chlorophyll *a* (Chl<sub>a</sub>), Chlorophyll *b* (Chl<sub>b</sub>), and total carotenoid content were determined according to Lichtenthaler [41]. For extraction, ca. 200 mg<sub>FW</sub> of plant material was used per replicate, following its freeze-drying and subsequent dry weight (DW) measurement. In a new test tube, approximately 5 mg of the freeze-dried tissue was transferred, followed by the addition of 100  $\mu\text{L}$ -distilled water for rehydration of the sample. After 10 min, 8 mL of 96% ethanol was added to the rehydrated samples, followed by 20 s on the vortex. The test tubes were wrapped in aluminum foil and incubated over-night at room temperature in an exhaustion hood. The following morning, samples were vortexed to homogenate the sample and left 10–20 min to sediment particulates. The absorbance of the extracts was measured using a UV-Vis spectrophotometer at 470.0, 648.6, and 664.2 nm, using 96% ethanol as blank. Extracts were recurrently measured at 750 nm to correct for impurities. According to the protocol, photosynthetic pigment content was determined on a DW basis with the following formulas.

$$C_a = \frac{(13.36 A_{664.2} - 5.19 A_{648.6}) * 8.1}{\text{DW}} \quad \left[ \text{mg g}^{-1}\text{DW} \right]$$

$$C_b = \frac{(27.43 A_{648.6} - 8.12 A_{664.2}) * 8.1}{\text{DW}} \quad \left[ \text{mg g}^{-1}\text{DW} \right]$$

$$C_{x+c} = \frac{(4.785 A_{470} + 3.657 A_{664.2} - 12.76 A_{648.6}) * 8.1}{DW} \quad \left[ \text{mg g}^{-1} \text{DW} \right]$$

## 2.4. Chlorophyll Fluorescence Parameters

Chlorophyll fluorescence parameters were assessed using a portable pulse-modulated fluorometer (Handy PEA+ Chlorophyll Fluorimeter; Hansatech Instruments Ltd., Norfolk, UK) on three fully expanded, dark-adapted leaves per individual from plants growing at 1980 and 2244 m. Leaf samples were dark-adapted for 20 min before the measurement to allow for complete reoxidation of the PSII reaction centers and ensure that all energy-dependent quenching is relaxed to obtain a reliable measure of the minimal fluorescence ( $F_0$ ). Following

Measurements were taken throughout the day, expressly matching the intervals of sample collection for the VAZ pigment analyses in 2018 and 2019: in the morning (9:00 to 11:00 GMT), during the periods of maximum solar radiation (12:00 to 14:00 GMT), and in the afternoon (16:00–18:00 GMT). Maximal fluorescence ( $F_m$ ) and minimal fluorescence ( $F_0$ ) were used to compute the maximal photochemical efficiency of PSII ( $F_v/F_m$ ) for dark-adapted leaves [6].

## 2.5. Statistical Analysis

Differences in Lut and VAZ's diurnal variations were assessed per species between two climatically contrasting years (2018, 2019) in samples from contrasting elevations. Data were analyzed using MANOVAs to determine every single factor's effect over all the responses (i.e., V, A, Z, and lutein content). Additionally, nested ANOVAs were performed to identify significant differences per photoprotective pigment within each year and per species. Linear Mixed Models (LMMs) were implemented with elevation and year as fixed factors, with temperature and SWC as covariates, and individual as the random factor. Individual LMMs for each species were also performed, with elevation and date of sampling as fixed factors and individual as a random factor.

The assessment of significant differences in the chlorophyll fluorescence parameters and the de-epoxidation state (DEPS, %) of the VAZ cycle between years and elevations was performed using ANOVAs. Preliminary tests for normal distribution and homogeneous variance were performed using the Shapiro-Wilk test and Levene test with the functions "Shapiro.test" and "leveneTest" from packages "dplyr" and "car" respectively [46,47]. Significant differences between means were determined with the least significant difference test calculated at the 5% level.

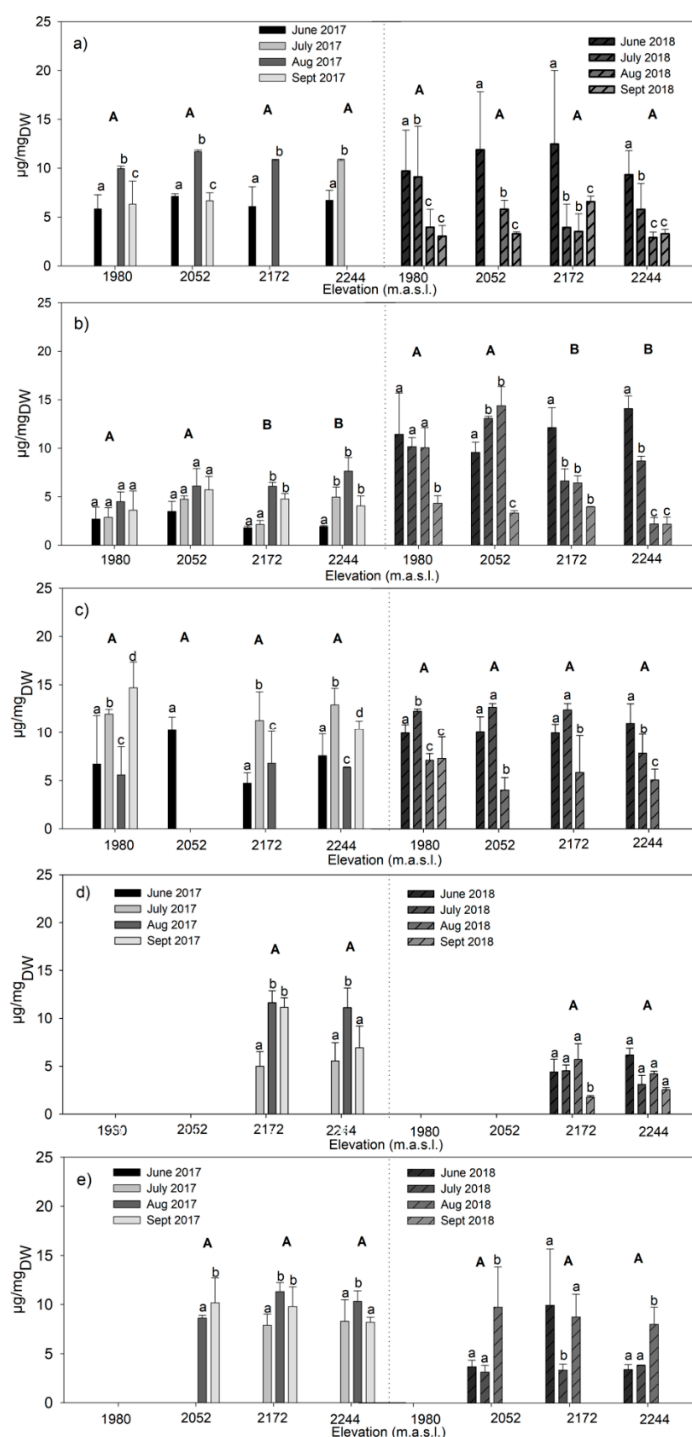
Variations in chlorophyll content and related ratios (Chl  $a/b$ ,  $Cab/x_c$ ) throughout the elevation gradient were analyzed in two consecutive and climatically contrasting years (2017, 2018) using LMMs, via restricted maximum likelihood [48]. Given the registered decrease in SWC as the growing season elapsed within each year (Table S3), we performed an analysis for each species considering the additive effect of elevation and SWC at each date of sampling (within a year) as fixed factors, and individual as a random factor since several measurements were collected through time. All models were created using the function "lme" from package "nlme" [49].

All statistical analyses were performed using the R statistical [50].

## 3. Results

### 3.1. Chlorophyll Content, Chl $a/b$ , and $Cab/x_c$ Ratio

Significant differences between years and among species were found in  $\text{Chl}_{\text{TOTAL}}$ , while the significant effect of elevation was specific to each species (Figure 1). In the analysis with species as a random factor, significant differences in  $\text{Chl}_{\text{TOTAL}}$  were found between climatically contrasting years, with a significantly higher  $\text{Chl}_{\text{TOTAL}}$  under milder weather (i.e., 2018) than during an atypically dry year ( $p < 0.01$ ).



**Figure 1.** Seasonal changes in Chl<sub>TOTAL</sub> in (a) *Erysimum penyalarens*, (b) *Festuca curvifolia*, (c) *Senecio carpatanus*, (d) *Hieracium vahlia*, and (e) *Jurinea humilis* throughout the elevational gradient. Data from two consecutive years of study (2017, 2018). Different lower-case letters indicate significant differences ( $p < 0.05$ ) between dates at a given elevation, and upper-case letters indicate significant differences between elevations ( $p < 0.05$ ).

During an atypically dry year (i.e., 2017), there was a significant temporal increase in Chl<sub>TOTAL</sub> through the first half of the growing season in *E. penyalarens* and *H. vahlia* ( $p < 0.05$ ; Figure 1a,d) at all elevations. In contrast, Chl<sub>TOTAL</sub> declined significantly through the season in *E. penyalarens* during the mild growing season of 2018 ( $p < 0.001$ ), while in *H. vahlia*, Chl<sub>TOTAL</sub> showed no significant seasonal changes ( $p > 0.05$ ; Figure 1d). In both years,

the  $\text{Chl}_{\text{TOTAL}}$  in *S. carpetanus* (Figure 1c) generally showed a significant increase through the period of high plant activity (June–July;  $p < 0.05$ ), followed by its subsequent decline at the end of the growing season, without significant differences among elevations. As for *F. curvifolia*, a significantly higher  $\text{Chl}_{\text{TOTAL}}$  was found in 2018 than in the 2017 individuals. Moreover,  $\text{Chl}_{\text{TOTAL}}$  significantly declined with increasing elevation during the growing season of 2018 ( $p < 0.05$ ), contrasting its significant seasonal increase at higher elevations ( $p < 0.05$ ) when the SWC suffered rapid declines.  $\text{Chl}_{\text{TOTAL}}$  was significantly higher in *J. humilis* in 2017 than in 2018, without significant differences among elevations.

Within a year, significant seasonal changes in the  $\text{Chl } a/b$  ratio occurring alongside the aggravation of seasonal drought were almost unanimous in all models per species, with few exceptions. Similarly, the analysis revealed the lack of significant effect of elevation on the  $\text{Chl } a/b$  ratio in all species ( $p > 0.05$ ; Figure S2). However, the  $\text{Chl } a/b$  ratio increased significantly in *H. vahlii* towards the end of the 2017 growing season ( $p = 0.034$ ; Figure S2d), accompanied by the decline in SWC, opposite to the lack of seasonal changes the following year. In *J. humilis*, the  $\text{Chl } a/b$  ratio showed a significant increase only in individuals from the highest elevation in both years. As for the  $\text{Chl } a/b$  ratio in *E. penyalarensis*, it increased significantly both seasonally ( $p < 0.05$ ) and with elevation in 2018, being significantly higher in low-elevation individuals compared to their high-elevation counterparts ( $p = 0.041$ ). Similarly, in *F. curvifolia*, a significant increase in the  $\text{Chl}_{\text{TOTAL}}$  was observed towards the end of the 2018 growing season and rising elevation (Figure S2a,b).

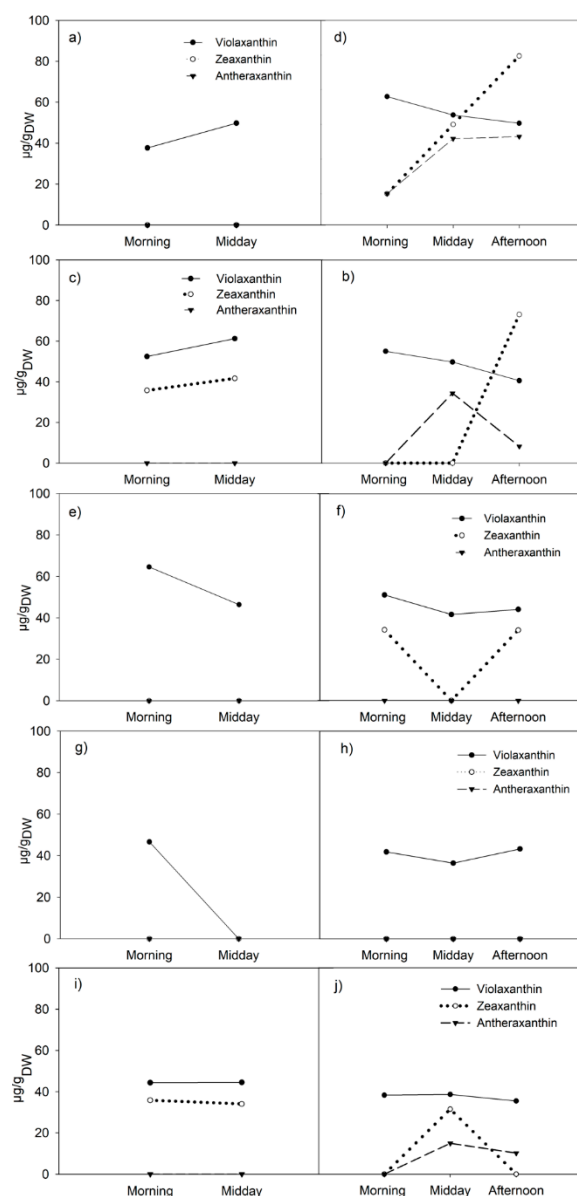
The total chlorophyll vs. carotenoids ratio ( $\text{Cab}/\text{xc}$ ) varied between years ( $p = 0.021$ ), and the effect of elevation diverged among species (Figure S3). In 2017, the  $\text{Cab}/\text{xc}$  ratio in *E. penyalarensis* declined substantially during the growing season. However, the disappearance of the above-ground portion of this species during the growing season prevented spotting any seasonal trends in 2017. Nonetheless, a significant elevational decrease was observed in the  $\text{Cab}/\text{xc}$  ratio in this species. Conversely, in 2018 the  $\text{Cab}/\text{xc}$  ratio was maintained through the season, with slightly higher values towards vegetative activity optimum and followed by its gradual decline as the growing season elapsed (Figure S3a).

Contrasting the significantly higher values maintained throughout the entire 2017 growing season ( $p < 0.01$ ), the  $\text{Cab}/\text{xc}$  ratio in 2018 in *H. vahlii* showed lower seasonal values coupled with no significant variations through the season nor among elevations (Figure S3d). In 2018, a significant seasonal decline was observed in the  $\text{Cab}/\text{xc}$  ratio in *J. humilis* in the extremes of its elevational distribution in this summit (i.e., 2052 and 2244), contrary to the lack of significant seasonal changes observed in 2017 (Figure S3e). As for *F. curvifolia* and *S. carpetanus*, no significant seasonal or elevational changes in the  $\text{Cab}/\text{xc}$  ratio were registered in these species in both years of study (Figure S3b,c).

### 3.2. Diurnal Variations in Xanthophyll-Cycle Pigments

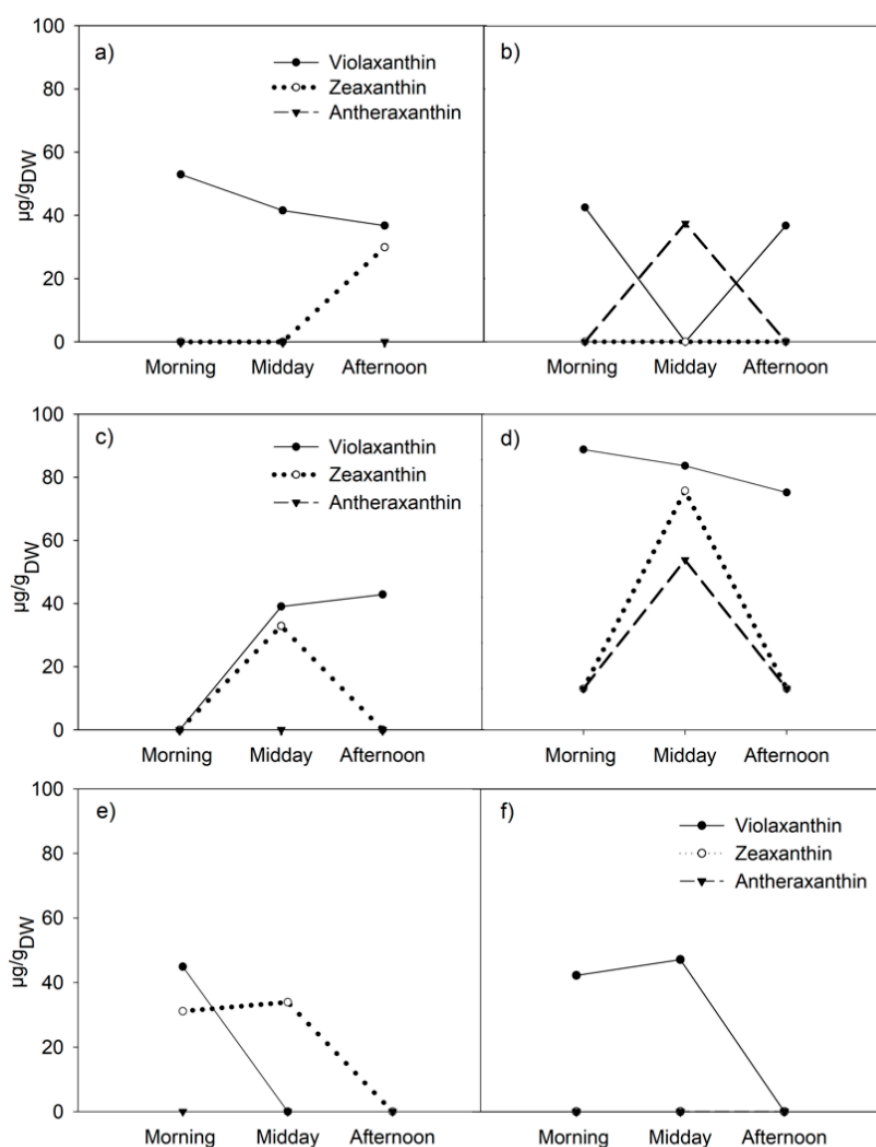
Xanthophyll composition and lutein showed a marked diurnal variation among species and at both elevations. Statistical analyses revealed Z, and A levels differed significantly among species and years, while only V showed a significant variation with elevation.

Overall, the highest levels for the xanthophyll cycle pool and the Lut content were found in the species growing at the high-elevation (i.e., 2244 m; Figure 2, Table S1). No significant differences were found in the VAZ pool between elevations ( $p > 0.05$ ). Component analysis of the xanthophyll cycle (V, A, Z) differed between years and was significantly higher at the highest site ( $p < 0.001$ ) compared to the low-elevation counterparts. In 2019, samples from the uppermost site exhibited the highest content of de-epoxidated compounds A at midday (i.e., *F. curvifolia*, *H. vahlii*, *J. humilis*), and of Z in late afternoon samples (i.e., *F. curvifolia* and *J. humilis*).



**Figure 2.** Diurnal variations in xanthophyll cycle pigments in *Festuca curvifolia* (a,b), *Jurinea humilis* (c,d), *Erysimum penyalarens* (e,f), *Senecio carpetanus* (g,h), and *Hieracium vahlia* (i,j) collected at the highest site (2244 m) at the optimum of plant activity in 2018 (left panel) and 2019 (right panel), respectively.

Concerning 2018, the highest Z content was found in low elevation samples of *E. penyalarens* and *S. carpetanus* at midday and the end of the day in *F. curvifolia* (Figure 3). Lut content showed maximum levels in midday samples of *F. curvifolia* and *E. penyalarens* from low-elevation individuals (Table S1), contrasting the observed in their high-elevation counterparts with minor diurnal variations (Table S1). As for *S. carpetanus*, Lut levels decreased throughout the day at the lowest site while reaching maximum levels at the start and end of the day in samples from the highest elevation. No significant variation in Lut levels was found throughout the day or between years in *H. vahlia* and *J. humilis* ( $p = 0.35$  and  $p = 0.62$ , respectively).

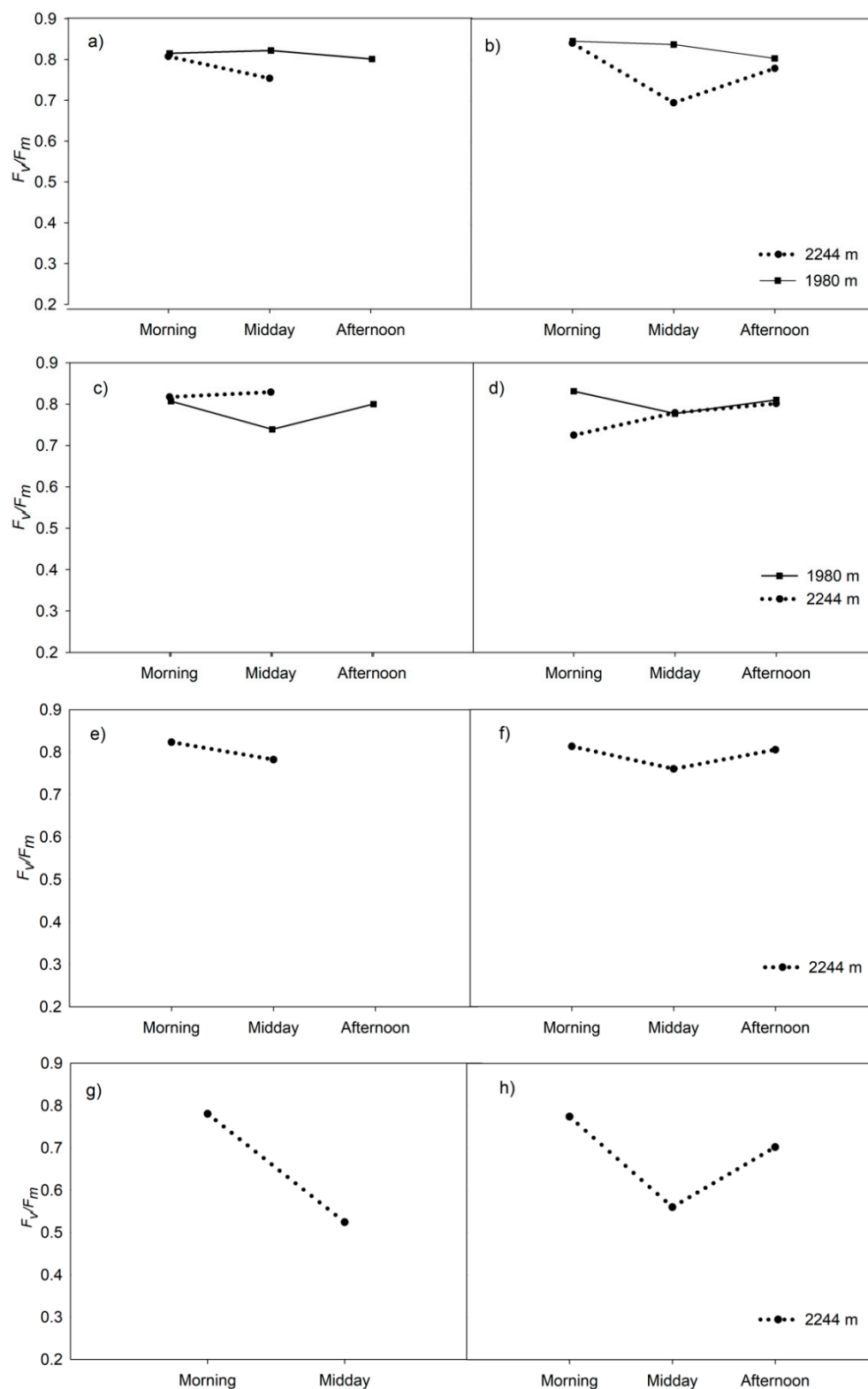


**Figure 3.** Diurnal variations in xanthophyll cycle pigments in *Festuca curvifolia* (a,b), *Erysimum penyalarens* (c,d), and *Senecio carpetanus* (e,f) collected at 1980 m in two climatically contrasting years. Figures for each species correspond to the 2018 growing season (left panel) and the 2019 growing season (right panel).

No significant differences were found in DEPS (%) between years nor elevations ( $p = 0.5$  and  $p = 0.08$ , respectively). Nonetheless, the majority of species from the highest elevation (except *S. carpetanus*) showed a greater conversion rate of the xanthophyll cycle pool into A-Z (43–60%) in the drier growing season of 2019, contrasting the conversion ratio (40–50%) in the milder conditions of 2018. Conversely, we found few variations in the DEPS in low-elevation species between climatically contrasting years (approx. 50%, Table S1).

### 3.3. Chlorophyll Fluorescence

Diurnal variations in the  $F_v/F_m$  were evident in most of the species assessed, denoting a substantial decrease at the time of highest light intensity and temperatures (midday), particularly in high-elevation individuals (Figure 4; Table S2). Nevertheless, all species achieved a complete recovery of the  $F_v/F_m$  at the end of the day.



**Figure 4.** Changes in the photochemical efficiency of PSII,  $F_v/F_m$ , throughout the day in *Erysimum penyalarens* (a,b) and *Senecio carpetanus* (c,d) from contrasting elevations (1980 and 2244 m); as well as in *Hieracium vahlii* (e,f), and *Jurinea humilis* (g,h) growing at 2244 m. Measurements were taken at the time of sample collection for the VAZ analysis in the 2018 (left panel) and 2019 (right panel) growing seasons.

Furthermore, the  $F_v/F_m$  displayed no significant differences between elevations in those species found throughout the entire elevation gradient ( $p = 0.07$ ), maintaining values within the optimum designated for this parameter ( $F_v/F_m \geq 0.75$ ).

#### 4. Discussion

Beyond their function in the light-harvesting process, carotenoids (e.g., V, A, Z, Lut) play a fundamental role in photoprotection, avoiding ROS formation in the photosystems of plants subject to unfavorable environmental conditions [4,51].

Under field conditions, alpine herbs display a pronounced variation in the content of phenolic compounds and the  $Cab/x_c$  ratio with elevation changes, resembling our findings in Mediterranean high-mountain herbs and denoting the high antioxidant capacity of high-altitude plants in response to combined abiotic stressors, compared to lowland species (Figure S3; [1,13,24]). Our results showed significant differences in the xanthophyll cycle's functioning among species at high elevations, having the VAZ cycle acting either as a continuous photoprotective strategy or elicited only under more adverse environmental conditions. On the one hand, a repeated, substantial increase in the xanthophyll cycle's de-epoxidation was observed in herbs circumscribed to elevations above 2100 m (i.e., *H. vahlii*, *J. humilis*) compared to the other species and elevations. In accordance, the decreased  $F_v/F_m$  measured in *H. vahlii* and *J. humilis* at the periods of higher temperatures and light intensity indicates more energy is dissipated as heat, remaining in line with studies inferring the combination of ecological factors, as is the case with summer stress, can eventually lead to increased NPQ [52,53]. These findings suggest the xanthophyll cycle functions as an inbuilt continued photoprotective strategy in these two specialists to enhance photosynthesis' steadiness via NPQ<sub>f</sub> and stabilize PSII reaction centers [14]. On the other hand, the remaining species displayed significant differences in the behavior of the xanthophyll cycle between climatically contrasting years, such as the significant diurnal increase of de-epoxidated compounds A and Z observed in *E. penyalarens* and *F. curvifolia* subject to the drier, warmer conditions in 2019, differing from their response under milder weather during the same period (i.e., 2018). These results resemble those in *Phacelia secunda* [42], suggesting the activation of the xanthophyll cycle in high-mountain specialists essentially to counteract the negative, cumulative effects of more adverse summer stress on their photosystems.

The observed higher DEPS found in most of these specialists at contrasting elevations (2244 m: 43–60%, 1980 m: 50%) at the periods of maximum plant activity and higher summer stress could suggest their increase to allow higher thermal dissipation under adverse conditions. These findings, coupled with the observed maintenance of optimal  $F_v/F_m$  values, suggest a potential acclimation strategy to avoid photoinhibition, mediated by the severity of the conditions occurring in the growing season and not local microclimate [8]. On another note, since the conversion of V into either abscisic acid or Z can be influenced by environmental factors, the high DEPS achieved in low-elevation individuals of *S. carpetanus* during the colder, wetter conditions of 2018 could be a strategy to avoid low-temperature photoinhibition when experiencing temperatures below the ideal for its growth, as was the case in the days before the sampling [8].

The less-studied Lutein epoxide cycle (Lx), occurring parallel to the more studied VAZ cycle, involves the interconversion of lutein and lutein 5–6 epoxide [51]. The correlation of Lut conversion with NPQ and the subsequent restoration of the Lut pool several days after light stress support the Lx cycle's role in photoprotection and its contribution to long-term light acclimation in plants [51]. The maintenance of high diurnal Lut levels found in all species from the highest elevation and its marked enhancement in individuals from the lower-end (*E. penyalarens*, *F. curvifolia*, and *S. carpetanus*), particularly under more unfavorable conditions, suggests the involvement of the Lx cycle as an additional photoprotective strategy in the leaves from these specialists, acting as a sustained energy-dissipation mechanism [51,54].

In the context of foreseen summer stress aggravation in Mediterranean high-mountains [37], reduced water availability can severely affect the  $\text{Chl}_b$  content in plants while unaltering  $\text{Chl}_a$ . Resembling the findings of Shi et al. [55] in alpine species subject to an autumn-warming treatment, the high  $\text{Chl}_{\text{TOTAL}}$  found in the studied herbs towards the end of the 2018 growing season allows inferring an alteration in the chlorophyll biosynthesis. However, contrary to the suggested enhancement of photosynthetic capacity under elevated temperatures for alpine species, temperature increases in Mediterranean high-mountains could result in greater constraints than benefits to plant life. The observed increase in the  $\text{Chl } a/b$  ratios at the end of the growing season in *H. vahlia* and *S. carpetanus* (2017) and *J. humilis* (2017, 2018) could be associated with the breakdown of chlorophyll during plant senescence [56,57].  $\text{Chl}$  breakdown via the conversion of  $\text{Chl}_b$  into  $\text{Chl}_a$  before its entrance to the catabolic pathway could explain the increment of more than 100% observed in the species mentioned above.

Moreover, the minor variations in the  $\text{Chl } a/b$  with the seasonal escalation of summer drought and with altitude contrasted our initial assumptions, inferring a greater endurance of the photosynthetic apparatus in the studied high-mountain specialists under summer stress conditions. In line with the latter, the increased  $\text{Chl}_{\text{TOTAL}}$  in all species at the time of maximum plant activity (July), coupled with the activation of photoprotective strategies and decreased  $F_0$ , could suggest an acclimation strategy for improved PSII efficiency to endure the incidence of summer stress at critical developmental stages [58]. These findings, coupled to the higher DEPS found in these species, suggest the activation of photoprotective mechanisms (i.e., xanthophyll cycles, both VAZ and lutein) as indispensable to guarantee the maintenance of optimal functioning of the photosystem in these species growing under challenging conditions.

The significant differences found per species between climatically contrasting years allow suggesting the optimum climate for each studied species in these mountains in the context of shifting climate. For instance, drier and warmer growing seasons could hinder the growth of *E. penyalarensis*, as suggested by the substantial enhancement in the VAZ pool in samples from the uppermost site, low  $\text{Chl}_{\text{TOTAL}}$ , and scarcity of individuals at all elevations subject to an atypically dry growing season. These findings contrast the smaller VAZ pool, higher  $\text{Chl}_{\text{TOTAL}}$ , and species abundance observed under milder conditions the following year in the same period, pointing out the constraint that graver summer stress could pose over this endemic species, which could potentially derive into costly investments on enhanced photoprotection to avoid oxidative damage and photoinhibition under aggravated conditions [12]. Furthermore, our findings highlight the potential vulnerability of *E. penyalarensis* to the surpassing of its higher stress-threshold for summer stress with the expected lengthening and exacerbation of summer stress in these mountains as an outcome of climate change [37]. Conversely, and akin to the findings by Buchner et al. [12] in *Senecio incanus*, our results in *S. carpetanus* and *F. curvifolia* indicate the xanthophyll cycle operates in response to the daily thermal and light intensity variations in these species, rather than in response to year-to-year climate wavering (Figures 2 and 3). The latter is associated with superior life strategies and functional trait differentiation in *S. carpetanus* and *F. curvifolia*, resulting from the selection over time of functional traits granting greater tolerance to the coarsening climate in this mountain range and the firm competitiveness and dominance in these xerophytic pastures, respectively [28,44].

**Supplementary Materials:** The following are available online at <https://www.mdpi.com/1424-2818/13/3/137/s1>, Figure S1: Number of days per month with a temperature above the normal monthly values ( $>T_m$ ), and the number of days with no rainfall per month (0 rain) for the 2017–2019 growing seasons in Puerto de Navacerrada (1894 m). Data from meteorological station Puerto de Navacerrada, Madrid run by the Spanish Institute of Meteorology (AEMET), Figure S2: Variation in  $\text{Chl } a/b$  ratio in two consecutive years in (a) *Erysimum penyalarensis*, (b) *Festuca curvifolia*, (c) *Senecio carpetanus*, (d) *Hieracium vahlia*, and (e) *Jurinea humilis*. Data are given in means per species at all elevations. Different lower-case letters indicate significant differences among dates in the growing season ( $p < 0.05$ ) and different upper-case letters indicate significant differences among elevations ( $p < 0.05$ ), Figure S3:

Seasonal variation in the Chl<sub>TOTAL</sub> vs. carotenoids ratio ( $Cab/x_c$ ) in all studied species, throughout the altitudinal gradient (1980–2244 m) in (a) *Erysimum penyalarens*, (b) *Senecio carpetanus*, (c) *Festuca curvifolia*, (d) *Hieracium vahlii*, and (e) *Jurinea humilis*. Data corresponds to the years 2017 and 2018. Different lower-case letters indicate significant differences ( $p < 0.05$ ) between dates at a given elevation and different upper-case letters indicate significant differences between elevations ( $p < 0.05$ ), Table S1: Xanthophyll component analysis and lutein levels, de-epoxidation state of xanthophyll cycle pigments during the day in the studied species. ND, no data, Table S2. Diurnal changes in the photochemical efficiency of PSII,  $F_v/F_m$ , and the related parameters of minimal fluorescence ( $F_0$ ) and maximal fluorescence ( $F_m$ ) measured in *E. penyalarens* and *S. carpetanus* growing at the extremes of the elevation gradient (1980 and 2244 m) and in *H. vahlii*, and *J. humilis* from 2244 m. Measurements were taken at the time of sample collection for the VAZ analysis in 2018 and 2019. ND, no data, Table S3. Seasonal variation in SWC along the studied elevation gradient and through the active growing season of vegetation in the study site. Data corresponds to in-site measurements taken on each year of study (2017, 2018 and 2019). ND, no data.

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