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Alpine forbs rely on different photoprotective strategies during spring snowmelt

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Abstract

Snowmelt in alpine ecosystems brings ample water, and together with above-freezing temperatures, initiates plant growth. In this scenario, rapid activation of photosynthesis is essential for a successful life-history strategy. But, strong solar radiation in late spring enhances the risk of photodamage, particularly before photosynthesis is fully functional. We compared the photoprotective strategy of five alpine forbs: one geophyte not particularly specialised in subnival life (*Crocus albiflorus*) and four wintergreens differing in their degree of adaptation to subnival life, from least to most specialised: *Gentiana acaulis*, *Geum montanum*, *Homogyne alpina* and *Soldanella alpina*. We used distance to the edge of snow patches as a proxy to study time-dependent changes after melting. We postulated that the photoprotective response of snowbed specialists would be stronger than of more-generalist alpine meadow species. F_v/F_m was relatively low across wintergreens and even lower in the geophyte *C. albiflorus*. This species also had the largest xanthophyll-cycle pool and lowest tocopherol and flavonoid glycoside contents. After snow melting, all the species progressively activated *ETR*, but particularly the intermediate snowbed species *G. acaulis* and *G. montanum*. The photoprotective responses after snowmelt were idiosyncratic: *G. montanum* rapidly accumulated xanthophyll-cycle pigments, tocopherol and flavonoid glycosides; while *S. alpina* showed the largest increase in plastochromanol-8 and chlorophyll contents and the greatest changes in optical properties. Climate warming scenarios might shift the snowmelt date and consequently alter the effectiveness of photoprotection mechanisms, potentially changing the fitness outcome of the different strategies adopted by alpine forbs.

1-Introduction

Alpine snowbeds can be considered extreme environments for plants. These habitats, covered by snow

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for most of the year, are located in places where snow accumulates for topographic or microclimatic reasons. Long-lasting snow cover has a dual effect on plants. On the one hand, it decreases freezing vulnerability because of the protective effect of snow, particularly during critical periods in spring (Körner 2003; Briceño et al. 2014). On the other hand, the heavy mass of snow imposes a series of physical and biological constraints on the plant life beneath, including a reduction in the vegetative period, anoxia and the facilitating spread of fungal infections (Körner 2003). As a consequence, these habitats foster a highly specialised flora (Körner 2003, Björk and Molau 2007, Leuschner and Ellenberg 2017), frequently dominated by hemicryptophytes (Ninot et al. 2013, Komac et al. 2015). Some of these species show unique characteristics such as the ability to re-start photosynthesis and growth weeks before snow melting is completed (Starr and Oberbauer 2003, Körner et al. 2019) or the high resistance to photoinhibition caused by the combination of high irradiance and low temperatures (Germino and Smith 2000). Overall, snowbed species show the highest degree of adaptation to subnival life.

Snowbeds are particularly vulnerable to climate warming (Matteodo 2016), both because this will bring an earlier date of snow melting and because a higher proportion of precipitation will occur as rain (Gobiet et al. 2014). Warmer conditions will change the micro-habitat in snowbeds as it is expected that early snowmelt will increase the risk of freezing damage to snowbed species that are intrinsically more freezing-sensitive than species inhabiting more-exposed sites (Bannister et al. 2005, Briceño et al. 2014, Wheeler et al. 2014). In addition, progressive warming of the climate will favour the replacement of highly specialised snowbed flora by more-competitive alpine meadow species (Schöb 2010, Tonin et al. 2019). In this sense, snowbed colonization by new species migrating from lower elevations, mainly driven by higher temperatures, is already evident in the Alps (Carbognari et al. 2014, Gristch et al. 2016) and likewise in the Snowy Mountains of Australia (Pickering et al. 2014). These reports describe changes in the plant community, while the ecophysiological drivers behind these changes have remained relatively poorly understood.

When plants (geophytes and hemicryptophytes) emerge from rapidly melting late-spring or early-summer snow, they are suddenly exposed to extremely high doses of shortwave visible and UV radiation while temperatures are still close to 0 °C. Additionally, both the high albedo of the receding snow pack and the relative enhancement of solar UV radiation at high elevations further increase the incident irradiance received by these plants (Blumthaler 2012). These conditions are potentially damaging to the photosynthetic apparatus because of an imbalance between light absorption and the capacity for photosynthetic utilisation (Huner et al. 1998, Germino and Smith 2000, Verhoeven 2013), and because of direct photodamage caused by the absorption of shortwave radiation (Takahashi and Badger 2011). Thereby, photoprotection is particularly critical to assure rapid re-establishment of photosynthesis and subsequent growth.

Some snowbed species regain photosynthetic function under snow, such as *Soldanella alpina* and *Eriophorum angustifolium*, others need some time to fully recover from their dormant state and a third

group starts to produce new photosynthetic tissues under the snow (Lütz 1996, Körner 2003). Despite the high photoprotective requirement when snow melts rapidly and the leaves suddenly become exposed, few studies have specifically addressed the role of different protective mechanisms in snowbed plants (Lütz 1996, Germino and Smith 2000). Some snowbed species such as *Soldanella alpina* have powerful antioxidant defences, with very high pools of ascorbate and xanthophyll cycle pigments (Streb et al. 2003), while photoprotection is assured by a greater use of energy in others. This is the case for *Geum montanum*, which attains high rates of the cyclic electron transport (Manuel et al. 1999), *Ranunculus glacialis* with high chlororespiratory activity (Streb et al. 2005, Laureau et al. 2013) and *Homogyne alpina* through enhanced photorespiration and catalase activity (Streb et al. 1997, Lütz 2010). The upregulation of these mechanisms acts as a sink for the electron transport chain, providing a mechanism for reducing overexcitation and preventing PSII damage. Some of these protective responses alter chloroplast ultrastructure, leading to the appearance of specific features such as bigger plastoglobules (Bascuñán-Godoy et al. 2010) and chloroplast protrusions (Moser et al. 2015).

These studies of the ecophysiology of photosynthesis upon plant emergence from snow have deeply described photoprotection in a small set of alpine plant species, most of which were also used in the present study. However, most, if not all, of these studies have been performed at mid-summer, while much less is known about how photoprotective mechanisms respond to the changing conditions prevalent in late spring when these plants are first exposed to full sunlight and temperature fluctuations during snowmelt (Streb and Cornic 2012). With increasingly earlier snowmelt, the relevance of responses of these species following exposure during spring will become even greater. Comparisons between co-occurring snowbed species adopting more generalist *vs* strongly specialised strategies are also scarce and serve to illustrate the relative effectiveness of these different approaches. We, therefore, have aimed to characterise the role of photoprotection mechanisms in alpine plants during the critical period of snow melting in late spring, when solar irradiance is maximal. We postulate that the photoprotective responses of snowbed specialists must be different to those of more-generalist alpine meadow species, with the former being constitutively stronger and the latter more plastic. To cover the range of biological strategies representative of this environment, we have studied the temporal responses after snow melting of four co-occurring evergreens whose leaves are retained over the whole winter season (including one “genuine” snowbed species, two “facultative” snowbed species, and one alpine meadow species) and of one geophyte that produces new leaves under the snow.

2-Materials and methods

2.1-Field site, meteorological conditions, studied species and experimental design.

Field measurements were performed in late spring 2019 (Julian days 148-151) on a south-facing slope in the vicinity of the Station Alpin du Lautaret of the University Joseph Fourier of Grenoble (45°02’N

6°24'E, 2100 m asl) in the French Alps (Fig. S1). In this site, both genuine snowbed species co-occur together with less specialised alpine meadow forbs.

Meteorological data were obtained from a nearby (< 100 m distance) meteorological station in the Botanical Garden of the Station Alpin Joseph Fourier. Microclimatic data were additionally obtained with EL-USB-2-LCD sensors and data loggers (Lascar Electronics) placed 10 cm above ground (Fig S1A). Spectral irradiance was measured with a Maya 2000 Pro array spectroradiometer (Ocean Optics; D7-H-SMA cosine diffuser, Bentham Instruments Ltd.) recently calibrated to give faithful readings in the UV-visible regions of the solar spectrum. Measurements included dark and UV-filter calibrations to account for stray light in the UV region and noise in the baseline readings. To obtain maximum resolution in the UV regions, bracketing at ×10 integration time was used for these spectral regions, following the protocols Aphalo et al. (2016) and Aphalo et al. (2017).

Five plant species were studied (Fig. 1); four of them are hemicryptophytes with perennial leaves (*Soldanella alpina* L., *Homogyne alpina* L., *Geum montanum* L. and *Gentiana acaulis* L.) and one, *Crocus albiflorus* Kit. ex Schult, is a geophyte producing new leaves in spring (Aeschimann et al. 2004). Among them, *S. alpina* belongs to the snowbed flora, *H. alpina* and *G. montanum* can be occasionally found in snowbeds but are typical alpine meadow species and *G. acaulis* and *C. albiflorus* are alpine meadow plants rarely found in snowbeds (Aeschimann et al. 2004). Thus, a gradation of strategies was evaluated during the study.

Physiological responses after snow melting were characterised across five spatial transects per species in small snow patches (mean and SD patch area 191±41 m^2). Around the snowbed, in the already melted area, samples were collected at three different distances from the edge (each transect represents a biological replicate): the first one in the melting edge, the second at 20 cm from the edge and the third 1-m away from the edge (Fig S1). Given the observed melting rate (approximately 15 cm per day), these relative distances from the edge are equivalent to approximately 1 day and 5-7 days without snow cover, respectively. Consequently, distances to the snow patch can be also interpreted in terms of "time after snowmelt". One to three leaves from each species were studied at each sampling point. Physiological measurements were carried out first (chlorophyll fluorescence, optically estimated flavonoid glycoside content and leaf transmittance spectra) and sampling for biochemical analyses were performed immediately after under a uniformly cloudy sky (PAR 288-707 µmol photons m^{-2} s^{-1}) and air temperatures ranging between +6.6 and +11 °C. Samples for biochemical analyses (approximately 40 mm^2 leaf area) were collected per replicate, immediately frozen in liquid nitrogen and stored at -80 °C until extraction.

2.2-Chlorophyll fluorescence

Chlorophyll *a* (Chl *a*) fluorescence was measured in the same five plants per species and measurement point as those used for biochemical analyses. Measurements were done between 11:00 and 15:00 using a portable modulated Plant Stress Kit fluorometer (Opti-Sciences). The operating quantum efficiency of PSII was measured by using the Opti-Sciences' Y(II) meter. In intact illuminated leaves

(i.e. directly exposed to natural solar radiation) the operating quantum efficiency of PSII was estimated as $\Delta F/F_m' = (F_m' - F_s)/ F_m'$, where F_m' is the maximum Chl *a* fluorescence induced with a saturating pulse and F_s is the actual Chl *a* fluorescence under illumination. Saturating pulses to calculate F_m' values were applied according to Loriaux et al. (2013) protocol. The irradiance (PAR) was measured with the Leaf-Clip built-in quantum sensor and the electron transport rate (*ETR*) was estimated using the absorptance coefficient calculated for each leaf with the two RGB sensors placed in the measuring head. To allow inter-species comparisons, fluorescence measurements were performed during a uniformly cloudy afternoon under a narrow range of non-saturating irradiances: 382-473 µmol photons m^{-2} s^{-1} (Table S2). Immediately after $\Delta F/F_m'$ determinations, leaves were dark-adapted with leaf clips (30 min) (Fig. S1b), the maximum Chl *a* fluorescence (F_m) was induced with a saturating pulse (0.8 s duration, 7000 µmol photons m^{-2} s^{-1}) while the initial fluorescence (F_o) was recorded with red modulated measuring light (ML<1 µmol photons m^{-2} s^{-1}). The F_v/F_m was then estimated by the ratio $F_v/F_m = (F_m - F_o)/F_m$.

2.3-Flavonoid measurements

Flavonoid glycoside (flavonols in dicots. and flavones in monocots.) and anthocyanin measurements of each leaf were made with a Dualex Scientific + (Force-A, Paris-Sud, France) immediately on sampling, directly following the Chl *a* fluorescence measurements and prior to placing these leaves in liquid N. Both sides of each leaf were measured in the same region of the leaf lamina to obtain optical indices of flavonoid glycosides and anthocyanins from both the upper (adaxial) and lower (abaxial) epidermis. These optical indices are given by the Dualex from the abs. at 375 nm and 530 nm, respectively, by comparison of Chl *a* fluorescence at each of these wavelengths based on the epidermal absorbance 375 nm (flavonoid glycosides) and 530 nm (anthocyanins) wavelengths, calculated by comparison of Chl *a* fluorescence excited by these wavelengths and by red light. An index of leaf chlorophyll content per unit area is obtained concomitantly by the device using the light transmitted in the red and near-infrared regions. Taking measurements from both epidermises allowed us to calculate the upper/lower ratios of flavonoid glycoside and anthocyanin.

2.4-Spectral measurements

A leaf from each plant was placed in the Jaz Spectro-Clip (Ocean Optics), and reflectance was measured from the adaxial (upper) epidermis and transmittance through the leaves was measured beneath the abaxial (lower) epidermis using a coordinated dual spectrometer system. A xenon light source provides a pulse of light at a trigger rate of 10 ms. Each leaf measurement comprised ten consecutive spectra recorded over a 4-min period, during which time there was no noticeable trend to suggest any short-term time-dependent variation in optical properties. The flash rate was set at 200 Hz with an intensity of 400 V. Each leaf transmittance and reflectance spectra measured on a sample (S), were matched with a dark (D) and reference (Ref.) measurement using black and white Spectralon diffuse-reflectance reference targets (WS-1-SL, Ocean Optics). Spectral transmittance (Tλ) and reflectance (Rλ) were calculated according to Eq.1.

$$T\lambda \; or \; R\lambda = \frac{S - D}{Ref. - D} * 100 \qquad \text{(Eq.1)}$$

Spectral absorptance (Aλ) was calculated as the proportion of emitted light remaining after subtracting that of Tλ and (Rλ). Post-processing of the raw spectra was done using the Photobiology suite of packages in R (Aphalo, 2015).

2.5-Biochemical analyses

Plant material was ground under liquid N_2 and extracted with acetone (buffered with 0.5 g $CaCO_3$ L^{-1}). The extracts were then centrifuged at 16100 *g* and 4°C and pigment composition was analysed by HPLC using a reverse phase C18 column (Waters) as described previously (García-Plazaola et al. 1999) using a photodiode array detector (Waters model 996) for chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*), lutein (Lut), neoxanthin (Neo), β-carotene (β-Car), violaxanthin (V), antheraxanthin (A) and zeaxanthin (Z) and a fluorescence detector (Waters model 474) for α-tocopherol (α-toc) and plastochromanol-8 (PC8). Considering that carotenoid and lipophilic antioxidants are typically located in plastids, their concentrations were expressed as chlorophyll ratios.

2.6-Statistical analyses

One-way ANOVA with Tukey HSD test as *post-hoc* was conducted to evaluate significant differences among the positions 0, 20 and 100 cm from the snow edge for each species. Pearson correlation was used to evaluate the relationships among measured variables. All statistical analyses were conducted with SPSS 2019 (IBM Corp.) at an alpha level of 0.05.

3-Results

In 2019, the study period "Julian days 148-151" corresponded to snow retreat at the study site. During this period and the preceding week, the average temperature in the experimental field site was +5.9°C with a maximum of +13.6°C and a minimum of -0.7°C. The irradiance peak at solar noon during sunny days was over 2000 µmol photons m^{-2} s^{-1} of photosynthetically active radiation (PAR), while the maximum unweighted ultraviolet-B radiation (UV-B: 280-315 nm) was more than 3.4 µmol photons m^{-2} s^{-1}. There was a large difference in irradiance between the snowpatch surface, which was c 10-15% higher due to reflected radiation from the surrounding snow, and that 100 cm away from the snowpatch (Fig. S2, Table S1). This is apparent in the different UV-B:PAR ratios between the irradiance on snow (2.06) and on the ground (1.71). The UV-B:PAR ratio is even higher in radiation reflected from the snowpatch (2.11 UV-B:PAR ratio), adding as much as 48% to the solar radiation (Table S1).

Apart from the physical effect on plants of the release from snow cover, environmental conditions also differed between the edge of the snowpatch and the points situated at 1-m distance (notice that distance to the snow is used as a proxy of time after melting) during the three days prior to the physiological measurements (Fig. S1d). In particular, at the snow-edge, the air temperature was up-to 5°C lower during daylight hours (particularly on sunny days) and the minimum temperature was

slightly buffered compared to the 1-m point. Consequently, the extremes of the recorded temperatures during the study period were -3.1°C (edge) and -3.4°C (1 m) for the minimum, and +25.3°C (edge) and +28.6°C (1 m) for the maximum. No significant differences were found for PAR or T_{leaf} across species or sites (Table 1, Table S1).

The five species studied here represent a diversity of strategies but some general patterns were apparent. Thus, irrespective of strategy, maximal photochemical efficiency (F_v/F_m) was relatively low in all species at the time of snow melting and did not rise significantly over the following days. On average, F_v/F_m was 0.69-0.70 in all species except for the sprouting leaves of *C. albiflorus*, which had values significantly lower than the rest of the species (Fig. 1, Table 1). In contrast to the lack of response of F_v/F_m, the actual photochemical efficiency ($\Delta F/F_m'$) under ambient conditions increased rapidly in all species, except in *H. alpina* upon snow melting (Fig. 1). After one day, this increase was small in *G. montanum*, *C. albiflorus* and *S. alpina* but it sustained and was statistically significant after one week, while in *H. alpina* $\Delta F/F_m'$ increased after one day but decreased thereafter. The highest $\Delta F/F_m'$ values were obtained in *G. montanum*. Considering that $\Delta F/F_m'$ was measured at a relatively constant PAR, the electron transport rate (*ETR*) increased from 2 to 3-fold in all species except *H. alpina* (Fig. 1). The *ETR* was highest in *G. montanum*, but photochemistry was activated fastest following snowmelt in *G. acaulis* (3.5-fold rise from 0 to 100 cm from the snowpatch). It should be noted that leaf temperature during the measurements was slightly lower (on average 1.7-2.0°C) at the snow edge (Table S2), except for *C. albiflorus* whose erect leaves were out of the snow influence.

In parallel with photochemical changes, pigment composition also varied in some of the studied species. Thus, Chl content increased by 56% in *S. alpina*, while the ratio of Chl *a*/*b* decreased significantly in *C. albiflorus*, indicating a reorganisation of the photosynthetic apparatus (Fig. 2). The ratios of the main carotenoids (Lut, Neo and β-Car) to chlorophyll did not change significantly in any of the studied species, with the exception of a marked decrease in lutein (Fig. S3). Xanthophyll cycle (VAZ) ratio to Chl was much higher (3 to 4-fold) in expanding leaves of *C. albiflorus* than in the other species (Fig. 3). However, due to the rapid Chl synthesis, the VAZ/Chl ratio decreased markedly with leaf maturation, reaching values close to 2-fold higher in *C. albiflorus* than in the mature leaves of the other species. VAZ/Chl did not vary with the time after snowmelt in most other species, except for *G. montanum*, in which it increased by 80% (Fig. 3). Irrespective of VAZ pool size, the epoxidation state of the cycle (A+Z)/(V+A+Z) did not change with time in any of the species studied (Fig. 3).

The pools of the main lipophilic antioxidants, α-toc and PC8, varied greatly among species, being the lowest in *C. albiflorus* and the highest in *G. acaulis* (Fig. 4). When there were time-dependent variations after snowmelt, both antioxidants had a marked decline in *C. albiflorus* and *G. acaulis*. In *G. montanum*, the pattern of α-toc was the opposite; it became enriched along the transect. The flavonoid glycoside content tended to increase with the time after snow melting, but this trend was only statistically significant for *G. montanum* (Fig. 4). Conversely, there was a non-significant trend towards a decrease in the anthocyanin content of all species but *H. alpina*.

Generally, at the snowpack edge, leaf reflectance was higher and absorptance lower than at 1-m distance in both the UV and green parts of the spectrum, whereas reflectance was lower in the far-red (Fig. 5). Leaves of *S. alpina* showed the strongest trend for a change in leaf optical properties along the transect, although similar trends were also visible in *G. montanum* and *G. aucalis* (Fig. 5). The optical properties of *H. alpina*, in general, failed to differ along the transects. Between 320-800 nm, the spectral properties of leaves of each species were similar, although *G. aucalis* was notably more reflective than the others. At the extremes of the spectrum in the UV-B and near-infrared, species diverged from each other. Three of the species, apart from *G. montanum*, were more reflective in the UV-B region on initial emergence from under the snowpack than at 1-m distance (Fig. 5). These data can also be considered by comparing spectral regions. In Fig. S4 the differences in the red, far-red, green and in the UV regions can be easily visualised.

When all photoprotective parameters were considered together, a coordinated light acclimation response among photosynthetic pigments was apparent, with positive correlations between Lut/Chl, β-Car/Chl, VAZ/Chl and AZ/VAZ, and Chl *a*/*b* ratio (Fig. S5). On the other hand, flavonoid glycosides correlated negatively with other photoprotective parameters, including VAZ/Chl, AZ/VAZ, β-Car/Chl or Lut/Chl and also with Chl *a*/*b* ratio (Fig. S5). A spider plot was used to illustrate a unified view of the suite of responses of all species (Fig. 6). It shows that the progressive activation of photochemistry and *ETR* represents the most prominent trend after the snowmelt along the transects (Fig. 6).

Apart from the time-dependent activation of protective attributes, there were significant differences amongst species (Table 1); the most pronounced being between *C. albiflorus* and the other species. In particular, the contents of lutein and VAZ were 2 to 3-fold higher in *C. albiflorus* (Table 1, Figs. 3 and S3). Conversely, its α-toc and, to a lesser extent, PC8 were much lower than in the other species (Fig. 4). Despite having a similar Chl content to the other species, the ratio Chl *a*/*b* was higher in *C. albiflorus* (Table 1). Carotenoid contents differed among most species, in particular in *C. albiflorus* (Table 1) that showed higher Lut and lower Neo than the others. The lipophilic antioxidants α-toc and PC8 were higher in *G. acaulis* and *S. alpina*. Interestingly the rate of photoprotective response to snow melting was also species-dependent. *G. montanum* actively induced a rise in its xanthophyll cycle pool, tocopherol and adaxial flavonoid glycosides, while *C. albiflorus* followed the opposite pattern. Interestingly, PC8 content decreased in the less-specialised *C. albiflorus*, but was relatively constant in the intermediate species and accumulated in the most-specialised species, *S. alpina* (Figs. 4 and 6B). Overall, the photochemical activity of the facultative snowbed species *G. acaulis* and *G. montanum* recovered fastest under the field conditions in our study (Fig. 6B).

4-Discussion

4.1-Trends in photoprotection with time after snowmelt

In the present study, responses to snow melting were studied in five alpine plants representing a range of ecological strategies. Among these species, *C. albiflorus* was the only species producing new leaves

from resting bulbs (Fig S1), while the others reactivated their photosynthesis in over-wintering leaves. The trade-offs between maintaining leaves through winter or remaining leafless under the snow have been well characterised in terms of carbon economy (Polgar and Primack 2011). Irrespective of the foliar strategy, the rapid activation of photosynthesis after snow melting is an essential attribute to assure fitness in highly competitive alpine environments (Hülber et al. 2011). Using the distance to the melting edge of the snow patch as a temporal marker, we have studied the photoprotective component of photosynthesis reactivation in several species with different degrees of adaptation to subnival life. Among them, *S. alpina* is considered to be a genuine snowbed species, with the capacity to maintain metabolic activities and growth at temperatures close to 0°C (Körner et al. 2019), while the others represent examples of alpine meadow species with different levels of adaptation to the stresses associated to long-lasting snow cover (Streb and Cornic 2012).

Irrespective of the time after snowmelt, all the studied species showed some degree of photoinhibition during daytime (e.g. F_v/F_m values <0.8), although the extent of such a down-regulation in the PSII photochemical efficiency differed amongst species (Fig. 1), with the strongest down-regulation being found in the new leaves of *C. albiflorus*. Interestingly similar low values of F_v/F_m were reported in mid-summer (not after emergence from snow) for *S. alpina*, *H. alpina* and *G. montanum* (Wildi and Lütz 1996; Streb et al. 1997), and for other montane species such as *R. myconi* (Fernández-Marín et al. 2020b), suggesting that intrinsically low values of F_v/F_m could be a frequent trait of alpine plants. This low efficiency could be interpreted as a conservative strategy to reduce the risk of excess energy flow towards reactive oxygen species when carbon assimilation is restricted. The correlation between F_v/F_m and VAZ/Chl or AZ/VAZ ratios was very weak (Fig S5), suggesting that this relationship is a constitutive characteristic not dynamically regulated by the operation of the xanthophyll cycle (Fig. 3) or by leaf chilling temperatures during the measurements (e.g. between 5 and 7ºC, Table 1) that occur in most cold-adapted plants (Míguez et al 2015).

One of the most consistent trends with the increasing time after melting was the activation of photochemical processes ($\Delta F/F_m$' and *ETR*) (Fig 1). Interestingly, this activation was neither related to changes in F_v/F_m nor with enhanced de-epoxidation of the xanthophyll cycle (Figs 3 and S5). This indicates that the initial activation of photosynthesis (first week after snow melting) is independent of the complete recovery from winter photoinhibition or the operation of the xanthophyll cycle. The lower air temperature in the vicinity of the snow mass (Table S2) could be one of the factors limiting the photochemical activity in all species except *C. albiflorus*, whose leaves emerge vertically from the snow (Fig. S1C).

The activation of photochemistry was in general not accompanied by substantial changes in photoprotective traits, except in the expanding leaves of *C. albiflorus* where several compounds decreased in concentration (VAZ, α-toc, PC8) (Figs. 3 and 4), and in the overwintering leaves of *G. montanum* where VAZ and α-toc increased in parallel with *ETR*. It is interesting that VAZ and α-toc pools correlated negatively with each other (Pearson correlation coefficient=0.971, P<0.05, Fig. S5),

suggesting a functional redundancy within the photosynthetic tissue, as has been described in *Arabidopsis thaliana* mutants (Havaux et al. 2005). In contrast to the pattern shown by antioxidant molecules in the present study (Fig. 4), it has been reported that the antioxidant enzymes of *S. alpina* increase their activity rapidly after snow melting (Streb et al. 1997) and that its foliar carotenoids and UV-absorbent flavonoids increase after the shade to sun transfer (Bidel et al. 2020). With the exception of *H. alpina*, passive filters such as anthocyanins and flavonoid glycosides in overwintering leaves also decreased after emerging from snow (Fig. 4) and this may be associated with the small drop in UV absorptance once the snow had melted. Arctic and alpine plants can start to accumulate flavonoid glycosides while still emerging from under the snowpack (Solanki et al., 2018), presumably driven by the combination of blue and UV radiation transmitted through the snow (Robson and Aphalo 2019) together with cold-temperature induction (Schultze and Bilger 2019). Consequently, virtually all the UV radiation that was not reflected was absorbed when the leaf was above the snow. However, this flavonoid glycoside accumulation may be reversed as warmth and irradiance temporarily decline with the reduced albedo associated with the transition from snow to vegetation cover, leading to less scattered and reflected radiation with distance from the snowpack (Fig. S2). These compounds could also be retained in overwintering leaves, as has been shown for flavonoids in leaves of *S. alpina* (Laureau et al 2015), enhancing photoprotection after snowmelt or playing other roles in the adaptation to subnival life. In particular, anthocyanins have a plethora of protective functions (Gould et al 2004; Sudheeran et al 2020), including antifungal and antimicrobial properties (Schaefer et al 2008), which could be of great value during the long period of snow cover.

4.2-Species segregate in photoprotection according to over-wintering strategy

The composition of photosynthetic pigments and tocochromanols in alpine forbs has scarcely been studied in detail in a couple of species. A study of plants growing at 2000 m a.s.l. in the Austrian Alps in late summer, found *H. alpina* to have slightly higher Chl *a*/*b* and VAZ/Chl ratios that than those we report here (Wildi and Lütz 1996). These slight differences could be explained by the high temperatures typical of mid-summer (July and August) (Wildi and Lütz 1996), compared to those at our study site in late spring. In a different study, it was reported that the antioxidant pool of *H. alpina* was half of the co-occurring *S. alpina* (Streb et al. 1997, 1998). The latter species, the only genuine snowbed plant in the present study (Körner et al. 2019), is characterised by a high degree of constitutive photoprotection (Streb et al. 2003) when compared to other alpine species. In this species, the pools of tocopherol reported in the literature (Streb et al. 1997, 1998, 2003, Wildi and Lütz 1996) are slightly lower than those measured in the present study, while VAZ pools were slightly higher. Again, the fact that those studies were performed at mid-summer, as opposed to late spring, could be indicative of seasonal trends in photoprotection. Besides, *S. alpina* was the only species to show significant changes in absorptance of visible light after its release from snow cover (Fig. S4), likely caused by structural rearrangements (Davis et al. 2011). Chlorophyll biosynthesis was also noticeable,

with a two-fold increase in just one week. Interestingly, a sister species (*S. pusilla*) is known to be metabolically active at 0°C and to initiate the development of new tissues under such conditions (Körner et al. 2019).

C. albiflorus had the most distinct strategy. Amongst the five species, it had the highest photoinhibition (Fig. 1), the highest Chl *a*/*b*, Lut/Chl, VAZ/Chl and AZ/VAZ ratios (Fig. 2, 3 and S3), and the lowest α-toc content (Fig. 4). The distinct response of *C. albiflorus* may derive from the new and vertical leaves that emerge from the melting snow (Fig. S1C), whereas all other species already had mature perennial leaves under the snow. Young greening tissues are particularly sensitive to harsh alpine conditions until they reach maturity, as has been shown in *Eriophorum angustifolium* (Lütz 1996). In our study, *C. albiflorus* had a higher Chl *a*/*b* ratio than all the other species. The lower Chl *a*/*b* ratio of these species could indicate that they have more antennae proteins per unit of reaction centre, which would favour light harvesting under the snow or on cloudy days (Esteban et al. 2015). Interestingly, *C. albiflorus* was apparently able to compensate for the low α-toc values typical of young leaves (Hormaetxe et al. 2004, Lizarazo et al. 2010) by maintaining a high VAZ pool. Its leaf VAZ/Chl values, particularly close to the edge of the snow patch (around 250 μmol mol^{-1}Chl), were clearly above what is typical for boreoalpine species (100 mmol mol^{-1}Chl on average) (Fernández-Marín et al. 2018, 2019, 2020a). On the other hand, wintergreen species compensated for the risk of overexcitation of photosynthetic apparatus with higher α-toc, PC-8 and flavonoid glycoside contents than *C. albiflorus* (Fig. 4). Together, this suite of responses suggests a functional trade-off or a switch in strategies between responses that may occur in other time frames.

5-Concluding remarks

In alpine ecosystems, snowmelt marks the onset of growth and the race for plants to complete their annual life cycle within the short growing season. When considering long-lasting snow patches, the inception of growth is postponed to late spring or early summer when plants potentially receive very high intensities of solar radiation. Consequently, when overwintering or new expanding leaves are starting to re-activate photosynthesis, they may also suddenly be exposed to the strong UV radiation and PAR that are amongst the highest on Earth, in combination with temperatures that, because of the proximity of the snowpack, often remain relatively low. This cocktail of environmental stress factors represents an extreme environment for plants, meaning photoprotection mechanisms play a pivotal role in guaranteeing that photosynthesis can be readily activated after snowmelt. While the results obtained here do not indicate a distinctive photoprotective strategy for species adapted to subnival life, we have found important interspecific differences. Whatever the photoprotection strategy, alterations in the date of spring snow melting caused by climate warming will undoubtedly affect the importance of such mechanisms and their contribution to the overall plant success. It is likely that this will alter the ecological relationships among species, with the more specialized snowbed species being displaced by more plastic alpine meadow forbs.

Author contributions

J.I.G-P., B.F-M, and T.M.R. designed the study. J.I.G-P., B.F-M, T.S. and T.M.R. performed the field measurements. J.I.G-P. and A.S. performed the laboratory measurements. J.I.G-P., B.F-M, and T.M.R. discussed the results and wrote the manuscript.

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Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request

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Supporting information

Additional supporting information may be found online in the supporting information section at the end of the article:

Fig. S1. Experimental site and environmental conditions during the field campaign.

Fig. S2. Spectral photon irradiance at the experimental site.

Fig. S3. Carotenoid (β-carotene, lutein and neoxanthin) ratios to chlorophyll.

Fig. S4. Mean optical properties of leaves (reflectance and absorptance) of each of the four species studied in several spectral regions.

Fig. S5. Pearson correlation matrix of the main parameters measured across all species and sampling sites.

Table S1. Spectral photon irradiances in two locations and its supplementation by radiation reflected by snow were calculated from the spectra reproduced in Fig S2.

Table S2. Leaf temperature (T_{leaf}, in °C), PAR (µmol m^{-2} s^{-1}) and electron transport rate (µmol e^- m^{-2} s^{-1}) during measurements of chlorophyll fluorescence.

Figure legends:

Fig. 1. Photochemical parameters (F_v/F_m, $\Delta F_v/F_m$' and Electron Transport Rate) in leaves of the five species studied along transects at three distances (0, 20 and 100 cm) from the edge of the snow patches. Boxes represent the interquartile range of the data, the central line represents the median and whiskers the minimum and maximum range excluding outliers. Letters denote significant differences (P<0.05, one-way ANOVA and Tukey's posthoc test, n=5) among positions along the transect. Upper panels show images of the flowers of the respective species. The colour code for each species is based on flower colours.

Fig. 2. Chlorophyll content (in µmol m^{-2} leaf area) in leaves of the five species studied at three distances (0, 20 and 100 cm) from the edge of the snow patches. Boxes represent the interquartile range of the data, the central line represents the median and whiskers the minimum and maximum range excluding outliers. Letters denote significant differences (P<0.05, one-way ANOVA and Tukey's posthoc test, n=5) among positions in the transect from the snow patches.

Fig. 3. VAZ ratio to chlorophyll (in mmol mol^{-1} Chl) and de-epoxidation index (A+Z)/(V+A+Z) in

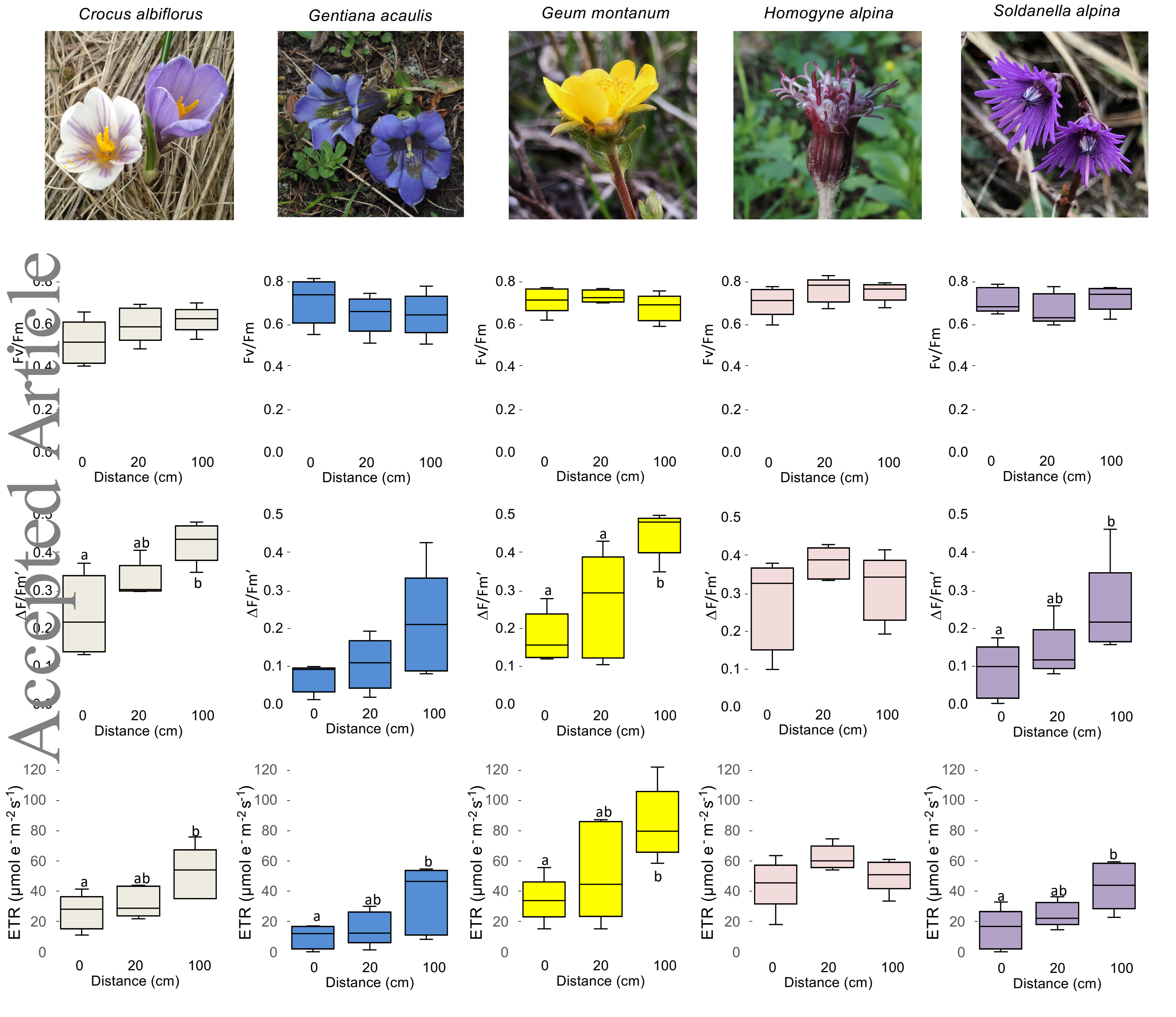
leaves of the five species studied at three distances (0, 20 and 100 cm) from the edge of the snow patches. Boxes represent the interquartile range of the data, the central line represents the median and whiskers the minimum and maximum range excluding outliers. Letters denote significant differences (P<0.05, one-way ANOVA and Tukey's posthoc test, n=5) among positions in the transect from the snow patches.

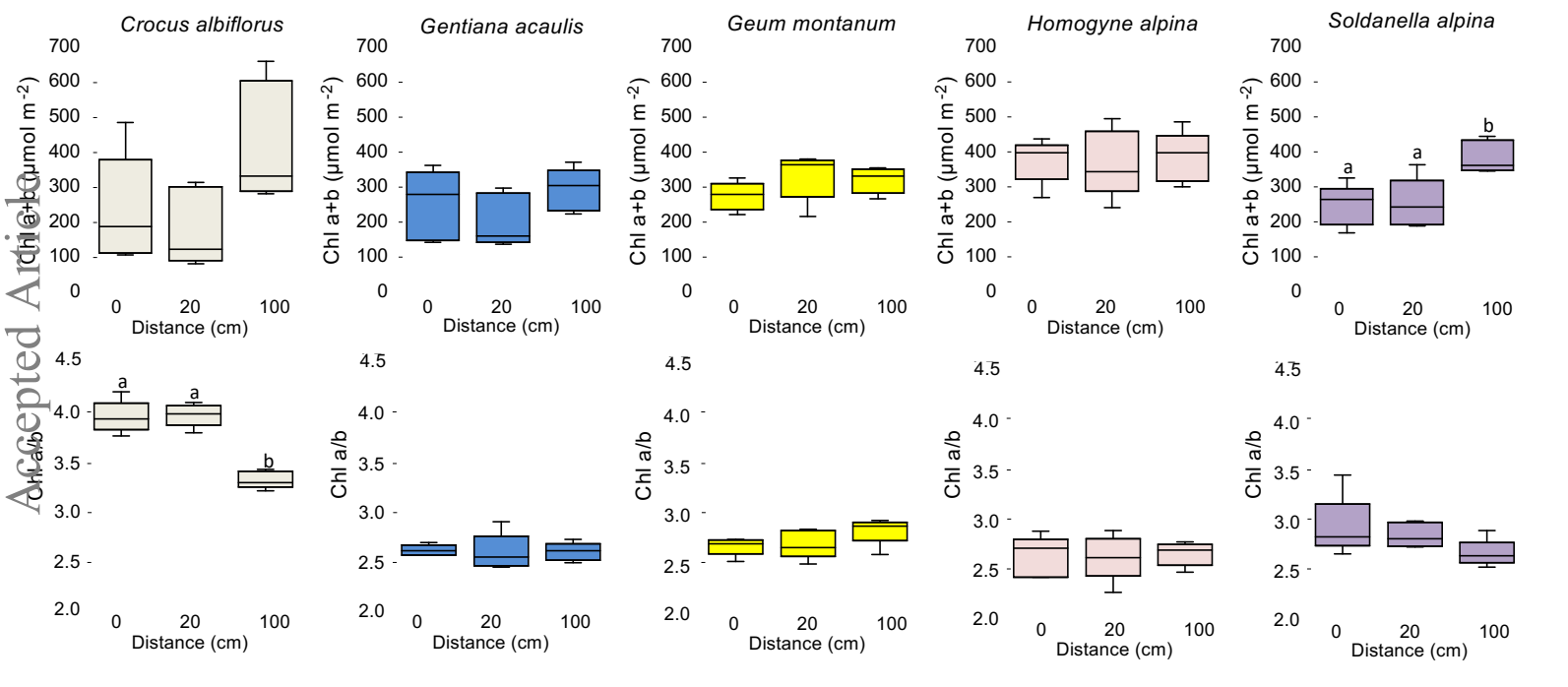
Fig. 4. α-tocopherol and plastochromanol-8 ratios to chlorophyll (in mmol mol^{-1} Chl) and flavonoid glycoside and anthocyanin contents (in arbitrary units) in leaves of the five species studied at three distances (0, 20 and 100 cm) from the edge of the snow patches. Due to the morphological structure of *C. albiflorus* leaves accurate measurements of anthocyanins were not possible. Boxes represent the interquartile range of the data, the central line represents the median and whiskers the minimum and maximum range excluding outliers. Letters denote significant differences (P<0.05, one-way ANOVA and Tukey's posthoc test, n=5) among positions in the transect from the snow patches.

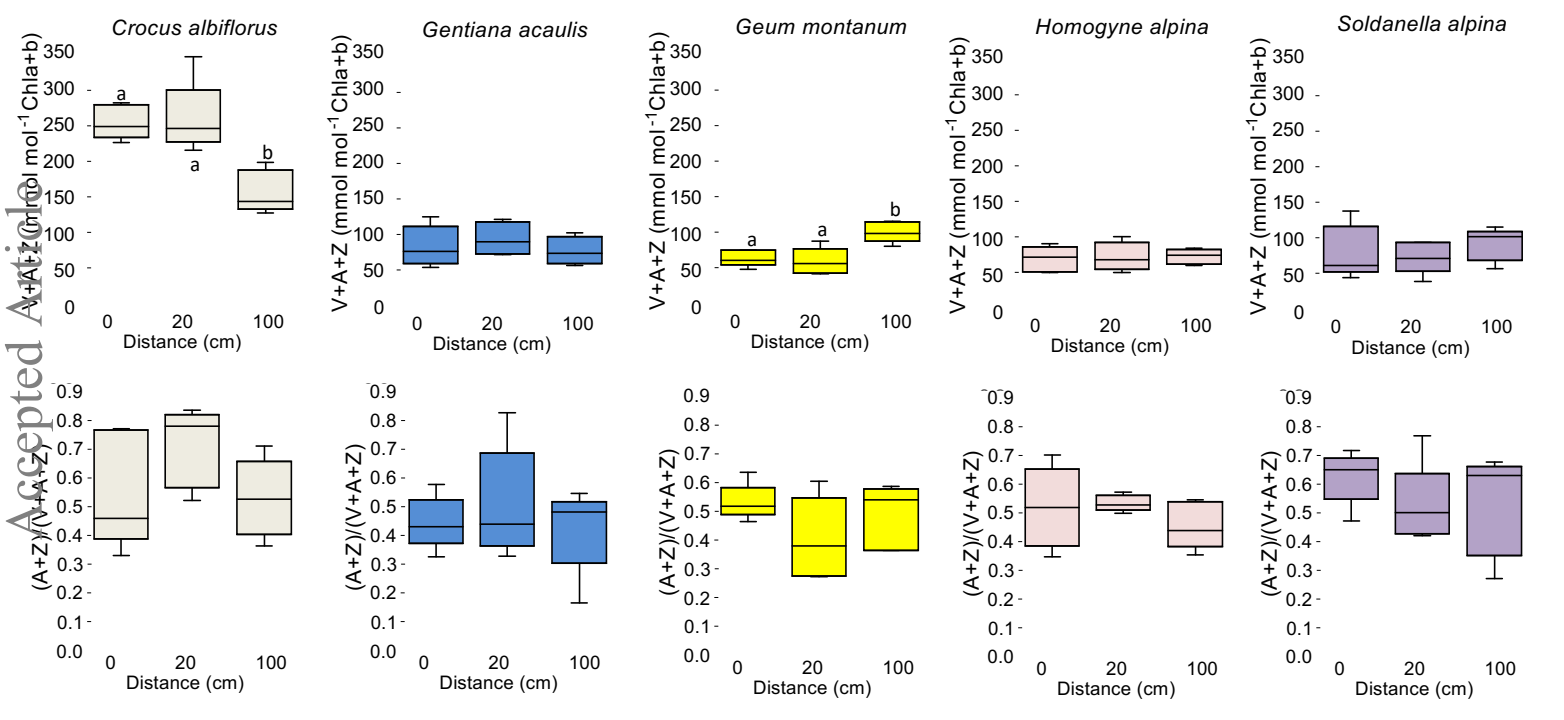
Fig. 5. The optical properties of leaves of four of the five species studied: *C. albiflorus* leaves were to narrow and ribbed to be measured. Each spectrum was plotted at 0.5 nm resolution between 290-880-nm wavelength, as bands representing +/- 1 SE centred around the mean from five replicate transects. Values are the proportion of incident irradiance reflected and absorbed by leaves as measured by the Jaz Spectro-Clip.

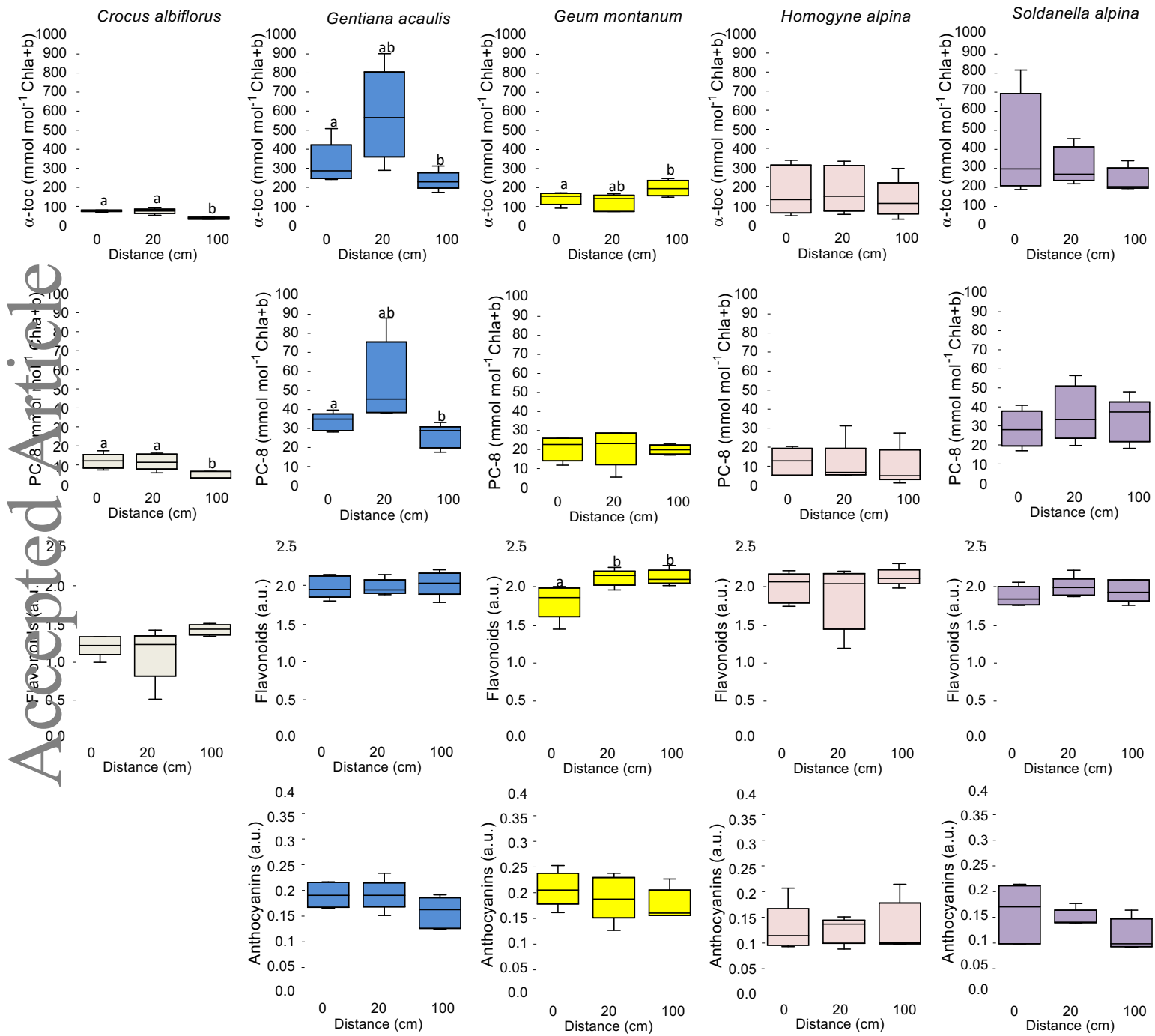
Fig. 6. Spider plot providing a unified view of trends in photochemistry, photoprotection and leaf environment (A) along the transect and (B) across species. Panel (A) depicts the fold-change for every parameter when compared to the edge of the snow patch (0 cm), thus values around 1 denote no change, <1 denote decrease and >1 denote rise upon snow melting. All species were considered together. Panel (B) depicts the fold-change for every parameter when comparing values at 100 cm with values at 0 cm for each species individually. Thus, values <1 denote decrease and > 1 rise upon snow melting.

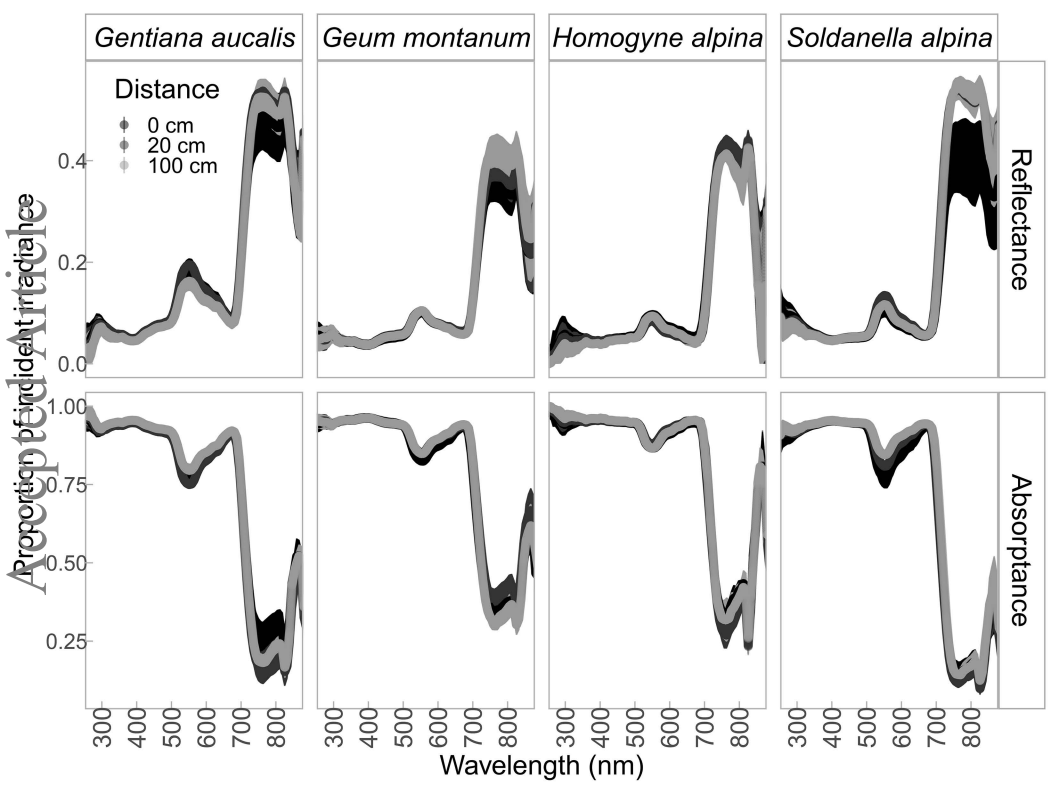
Table 1. Species average values (all measurements pooled) for pigment and tocochromanols, adaxial flavonoid glycosides and leaf temperature (°C) during the measurements. Total chlorophyll content is expressed in μmol g^{-1} DW. Carotenoids and tocochromanols are expressed in mmol mol^{-1} Chl. Flavonoid glycosides are relative units. Data are average ± SE. When significant, differences amongst species are depicted with lowercase letters (P<0.05, one-way ANOVA and Tukey's posthoc test, n=15).

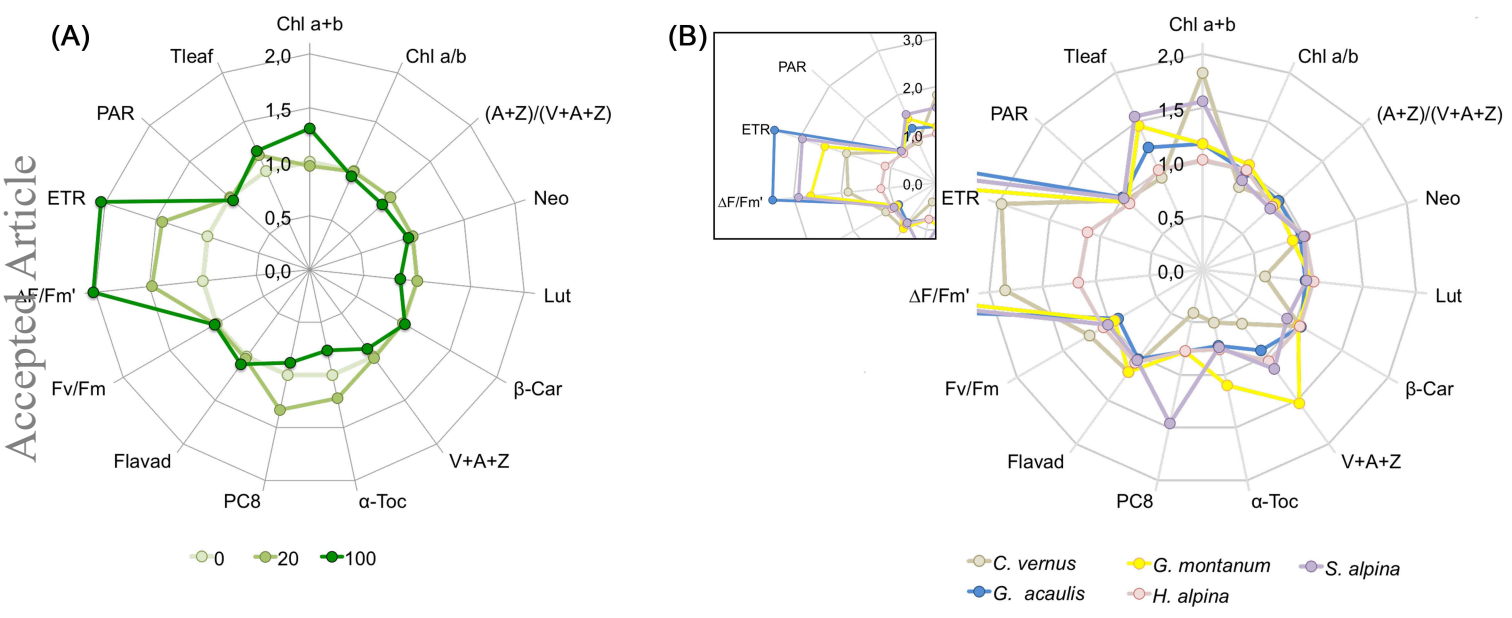












Species	Chl a+b	Chl a/b	Neo	Lut	β-Car	VAZ	AZ/VAZ	α-Toc	PC8	$_{Ad}$Flav	FvFm	ΔF/Fm'	T_{leaf}
Crocus albiflorus	274 ± 45 ab	3.7 ± 0.1 a	38 ± 1 d	323 ± 23 a	103 ± 2 a	223 ± 15 a	0.588 ± 0.044 a	63 ± 5 c	10 ± 1 b	1.22 ± 0.06 b	0.57 ± 0.02 a	0.32 ± 0.03 a	6.5 ± 0.4
Gentiana acaulis	244 ± 22 b	2.6 ± 0.0 b	44 ± 1 c	184 ± 8 b	93 ± 2 c	84 ± 6 b	0.447 ± 0.038 b	377 ± 53 a	38 ± 4 a	1.97 ± 0.03 a	0.65 ± 0.02 b	0.12 ± 0.03 b	7.1 ± 0.6
Geum montanum	304 ± 14 ab	2.7 ± 0.0 b	50 ± 1 ab	152 ± 3 b	98 ± 1 abc	77 ± 6 b	0.466 ± 0.030 ab	149 ± 12 c	20 ± 2 b	1.97 ± 0.05 a	0.70 ± 0.01 b	0.29 ± 0.04 a	6.6 ± 0.6
Homogyne alpina	371 ± 19 a	2.6 ± 0.0 b	46 ± 2 bc	149 ± 6 b	94 ± 2 bc	75 ± 4 b	0.490 ± 0.025 ab	167 ± 29 bc	11 ± 2 b	1.95 ± 0.07 a	0.73 ± 0.02 b	0.31 ± 0.02 a	7.3 ± 0.3
Soldanella aplina	291 ± 22 ab	2.8 ± 0.1 b	52 ± 1 a	166 ± 4 b	102 ± 3 ab	83 ± 7 b	0.549 ± 0.036 ab	299 ± 42 ab	33 ± 3 a	1.90 ± 0.03 a	0.69 ± 0.02 b	0.16 ± 0.03 b	6.5 ± 0.7