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ORIGINAL PAPERS

Freezing induces an increase in leaf spectral transmittance of forest understorey and alpine forbs

Twinkle Solanki[1] · José Ignacio García Plazaola[2] · T. Matthew Robson[1] · Beatriz Fernández Marín[3]

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Abstract

Evergreen plants growing at high latitudes or high elevations may experience freezing events in their photosynthetic tissues. Freezing events can have physical and physiological effects on the leaves which alter leaf optical properties affecting remote and proximal sensing parameters. We froze leaves of six alpine plant species (*Soldanella alpina*, *Ranunculus kuepferi*, *Luzula nutans*, *Gentiana acaulis*, *Geum montanum*, and *Centaurea uniflora*) and three evergreen forest understorey species (*Hepatica nobilis*, *Fragaria vesca* and *Oxalis acetosella*), and assessed their spectral transmittance and optically measured pigments, as well as photochemical efficiency of photosystem II (PS_{II}) as an indicator of freezing damage. Upon freezing, leaves of all the species transmitted more photosynthetically active radiation (PAR) and some species had increased ultraviolet-A (UV-A) transmittance. These differences were less pronounced in alpine than in understorey species, which may be related to higher chlorophyll degradation, visible as reduced leaf chlorophyll content upon freezing in the latter species. Among these understorey forbs, the thin leaves of *O. acetosella* displayed the largest reduction in chlorophyll (−79%). This study provides insights into how freezing changes the leaf optical properties of wild plants which could be used to set a baseline for upscaling optical reflectance data from remote sensing. Changes in leaf transmittance may also serve to indicate photosynthetic sufficiency and physiological tolerance of freezing events, but experimental research is required to establish this functional association.

T. Matthew Robson and Beatriz Fernández Marín shared senior authorship.

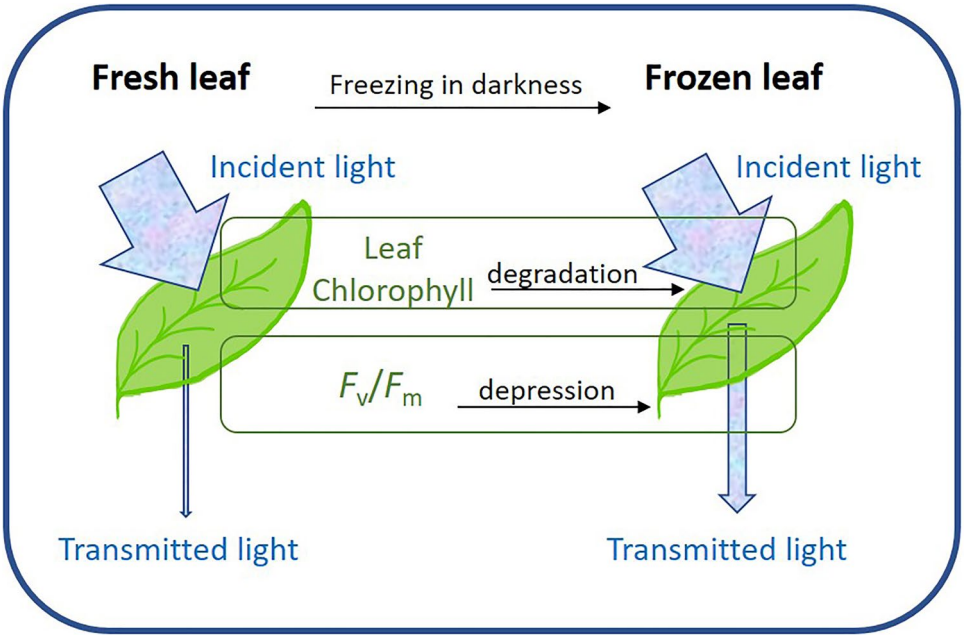
✉ T. Matthew Robson
matthew.robson@helsinki.fi

[1] Organismal and Evolutionary Biology (OEB), Viikki Plant Science Centre (ViPS), Faculty of Biological and Environmental Sciences, University of Helsinki, 00014 Helsinki, Finland

[2] Department of Plant Biology and Ecology, University of the Basque Country (UPV/EHU), Barrio Sarriena s/n, 48940 Leioa, Spain

[3] Department of Botany, Ecology and Plant Physiology, University of La Laguna (ULL), 38200 Tenerife, Spain

Graphical abstract



Keywords Frozen leaves · Extreme climatic events · Leaf optical properties · Spectral reflectance · Photoprotection · Leaf pigments

1 Introduction

Perennial plants in alpine environments and in temperate understory forests are at natural risk of freezing damage. This risk may be highest in early spring, when photosynthetic tissues emerge from under insulating snow cover, before attaining full frost hardiness [1, 2]. Exposure to such freezing events can damage buds and leaves, leading to reduced flowering or fruiting, and ultimately mortality in both alpine plants [3–6] and forest understorey species [4, 7, 8]. Plants have evolved various adaptations against freezing but nevertheless freezing resistance varies greatly across species, ranging from those that are very temperature sensitive to some alpine and boreal species that even survive extreme freezing temperatures [2, 9, 10]. Canopy cover partially buffers understorey plants from the sharp drops in temperature experienced in open environments of boreal and alpine regions [11], but alternately canopy interception and heterogeneity can reduce snow cover leaving ground vegetation exposed to freezing temperatures. Alpine plants are typically better adapted to freezing conditions than forest understorey species; however, even they can also suffer significant loss of biomass, fecundity and ultimately competitive ability following freezing events [12]. Freezing resistance can also vary through the seasons, among populations within a single species, and even among plant organs and developmental stages [2, 13–15]. Freezing definitely triggers physiological, biochemical and physical alterations in the leaves, regardless the capability of tissues to tolerate sub-zero temperatures.

Sharp drops in temperature can occur during spring in alpine zones and forest understories at high elevation or latitude [11]. These sudden fluctuations in temperature during periods when full physiological function is still returning to overwintering leaves often causes chlorophyll degradation [16] and stimulates accumulation of antioxidant pigments, such as anthocyanins, which ameliorate stress at low temperatures [17, 18], and other flavonoids which have additional roles including UV-screening, frost protection and herbivory defence [17, 19–21]. Freezing, also generates changes in the pigment composition of leaves: for instance, in frost-tolerant leaves freezing induces the de-epoxidation of xanthophyll cycle carotenoids involved in photoprotection [22], even in darkness [23–25].

In addition to the changes in pigments, freezing temperatures may cause structural changes to the leaf and leaf surface affecting their optical properties. On the one hand, physiological and anatomical responses to avoid leaf damage at cold temperatures, including acclimation of the cell membrane composition and cell structure, may modify leaf optical properties at time-scales of days to seasons [26]. On the other hand, ice accumulation in apoplastic spaces,

cellular cytorrhysis, or changes to the cuticle have associated effects on the scattering of radiation in the leaf [26–29]. In fact, ice optical properties greatly differ to those from liquid water, and its birefringence has recently been used to locate ice crystals within leaves and buds through reflected light microscopy [30].

Very few approaches are available to detect exotherms, to visualize ice formation and propagation thermally, or to evaluate ice allocation within plant tissues in order to address the mechanisms and consequences of plant freezing [27, 31–35]. However, by examining the transmittance of radiation in frozen leaves and comparing it with that of fresh leaves we can infer its functional significance, as well as providing information on reflectance that can be scaled-up to interpret remote sensing data [36] with broad applications in crop and ecosystem science.

All previous points considered, we aimed to determine the changes induced by freezing in the spectral reflectance and transmittance of leaves from alpine and understorey plants, spanning the ultraviolet (UV; 280–400 nm), PAR (400–700 nm) and far-red (700–900 nm wavelengths) regions of the spectrum. In order to provide insights into physiological processes potentially associated with changes in the spectra, we assessed the photosynthetic yield of photosystem II (F_v/F_m) and optically measured leaf pigments, before and upon freezing. To examine the consistency of these responses between alpine meadow species and boreal forest understorey forbs, we tested several plant species from each environment in spring soon after snowmelt. We expected alpine plant species, from a habitat where fluctuating temperatures and high irradiances are common, to be better acclimated to freezing, including having more photoprotective pigments. Because of this, we also expected alpine species to display a smaller increase in leaf spectral transmittance and smaller drop in photosynthetic yield following freezing compared to the forest understorey species.

2 Materials and methods

2.1 Collection sites, plant material and experimental design

Leaves of six of the most prominent springtime alpine species [37] were sampled from an alpine meadow in the subnival zone of the western French Alps adjacent to the *Station Alpin Joseph Fourier* (SAJF) botanical garden at the Col du Lautaret (45.0359° N, 6.4052° E) at 2150 m a.s.l, on 28th May 2019. These species were: *Soldanella alpina* (alpine snowbell), *Ranunculus kuepferi* (Pyrenean buttercup), *Luzula nutans* (wood rush), *Gentiana acaulis* (trumpet gentian), *Geum montanum* (alpine avens) and *Centaurea uniflora* (alpine knapweed). Likewise, leaves of three common forb species were sampled from the understorey of a mixed boreal forest [38], dominated by *Betula pendula* and *Betula pubescens* with some *Picea abies* trees, at Lammi Biological Station LBS, central southern Finland (61.05° N, E 25.04° E) at 130–135 m a.s.l on 15th June 2020. These understorey forbs were: *Hepatica nobilis* (liverwort), *Fragaria vesca* (wild strawberry) and *Oxalis acetosella* (wood sorrel). In the alpine meadow, only leaves from the previous year that had survived under snow cover through the winter were sampled, while in the understorey new leaves were also sampled. All leaves were collected close to solar noon between 12:00 and 14:00 to control for diurnal variation in leaf traits. A parallel set of leaves were sampled concurrently from which leaf area, fresh weight and dry mass (after drying to a constant weight at 60 °C) were recorded. For alpine species, 6 groups of 5 leaf disks were weighed, whereas among understorey species, 20–25 entire leaves were used, to calculate specific leaf area (SLA: leaf or disk area divided by dry mass) and leaf water content (LWC: one minus dry mass over fresh weight) [39] (Table 1). Mean daily temperature, precipitation and relative humidity in the alpine meadow during the experiment were 5.0° C, 0 mm and 73% (SAJF weather station, source: https://www.davis-meteo.com/Vantage-Pro2.php) and in the understorey they were 20.6° C, 0 mm and 80% (LBS, source: https://en.ilmatieteenlaitos.fi/) (Fig. 1).

2.2 Freezing treatments and chlorophyll fluorescence measurements

One mature leaf was harvested from three-to-four different plants of each species (and leaf-age class, where applicable) growing naturally in the alpine meadow and the forest understorey., Immediately after sampling leaves were taken to the lab for optical measurements. During the transfer period from field to lab, of about 10–15 min, the fresh leaves were kept in sealed plastic bags in a cool box. All three sets of measurements: optical properties, chlorophyll fluorescence and leaf pigments were made on every leaf. To freeze the leaves, they were placed horizontally flat between two sheets of paper towel, to ensure that they didn't crinkle on freezing, returned to sealed plastic bags and transferred to the freezer at − 18° C for 24 h in darkness, directly following room-temperature measurements on fresh leaves. This same set of leaves were remeasured when frozen, keeping the leaves in situ in the freezer to ensure that they remained frozen and at the same temperature throughout all the different measurements.

Indices of leaf chlorophyll content, and epidermal flavonoid glycosides (flavonols in dicots., flavones in monocots.) and epidermal anthocyanins were measured once from the adaxial side of each leaf with a Dualex Scientific + (Force-A, Paris Sud, France). Maximum quantum yield of PS_{II}

Table 1 Leaf morphological traits from the alpine and understorey species used in the experiment

	Leaf production	Leaf anatomy	Leaf shape	Trichomes	Specific leaf area ($mm^2\ mg^{-1}$) ¥	Leaf area (cm^2) ¥	Leaf water content (%)
Alpine species							
Centaurea uniflora	Perennial	Dorsiventral	Narrow lanceolate	Dense short rough hair	15.647 ± 1.532	2.69 ± 0.75	76.78 ± 1.01
Gentiana acualis	Annual/perennial	Dorsiventral	Elliptical to lanceolate	Hairless	11.914 ± 1.405	10.16 ± 3.52	70.05 ± 2.10
Geum montanum	Perennial	Dorsiventral	Pinnate	Densely Hairy	11.305 ± 0.805	5.87 ± 0.56	64.54 ± 0.64
Luzula nutans	Perennial	Isobilateral	Narrow lanceolate	Hairy	18.752 ± 1.375	1.76 ± 0.17	81.07 ± 1.36
Ranunculus kuepferi	Perennial	Dorsiventral	Narrow lanceolate		13.291 ± 1.372	3.82 ± 0.29	79.32 ± 0.06
Soldanella alpina	Perennial	Dorsiventral	Kidney	Hairless	12.407 ± 0.77	3.59 ± 1.01	70.57 ± 1.14
Understorey species							
Hepatica nobilis	Once annually	Dorsiventral	Lobate 3 leaflets	Few hairs	25.64 ± 4.33	9.28 ± 3.23	71.49 ± 0.25
Oxalis acetosella	Sequentially growing season	Isobilateral	Palmate trifoliate	Few hairs	81.97 ± 13.16	5.53 ± 1.44	82.86 ± 2.59
Fragaria vesca	Dimorphic spring/autumn	Dorsiventral	Palmate 3 leaflets	Hairy	48.09 ± 4.54	14.82 ± 2.16	67.08 ± 2.52

Leaves of plants growing in the experimental sites at the time of measurement were used to calculate specific leaf area (SLA), leaf area (LA) and leaf water content (LWC), as the mean (± 1 standard deviation) of 20–25 leaves of understorey plants, and of six groups of five leaf disks from alpine species

(F_v/F_m), as indicator of photosynthetic capacity, was measured with a FluorPen (PSI, Drásov, Czechia) following 30 min of dark acclimation using leaf clips. We made a set of measurements of leaf spectral transmittance and reflectance of radiation for each of the three leaves per alpine species or four leaves per understorey species and leaf-age class. These measurements were repeated on the same leaves once frozen, except for reflectance of alpine species which was not logistically possible. Following these measurements and 30-min dark-adaptation, F_v/F_m (mini-PAM, Heinz-Walz, Effeltrich, Germany) was recorded as a means of indirectly assessing true freezing within the tissues of the same set of frozen leaves. The frozen leaves of alpine species were too fragile to allow Dualex and reflectance measurements before leaves defrosted.

2.3 Measuring and processing of leaf optical properties

Leaf spectral transmittance and reflectance were measured across the spectral region (250–892 nm), encompassing all the wavelengths of ambient UV-B and UV-A radiation, and visible and far-red light that plants naturally receive in sunlight. Reflectance of radiation from the adaxial (upper) side and transmittance to the abaxial (lower) side of one leaf from three-to-four replicate plants per species and age class were measured with a dual integrating-sphere system, Jaz Spectro-Clip (Ocean Optics, Dundane, FL, USA). These measurements were all taken from the same part of the fresh and frozen leaves selected for all other optical measurements, avoiding the midrib where possible. The Jaz Spectro-clip comprises several modules including a dual spectrometer, a pulse xenon light source, data processing unit and battery. The two integrating spheres in the system collect light transmitted though the leaf as well as reflected light. The Jaz modular spectrometer was used as a standalone for optical measurements, the trigger rate was set to 10 ms with a hold-off time and trigger delay of 1 ms, and the spectrometer integration time was set to 1000 ms. Each leaf's transmittance/reflectance spectrum was an average of six consecutive individual recordings of spectra without both boxcar-smoothing and non-linearity detection. Measurements of each leaf took *c* 4 min., 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 (or one flash every 5 ms) with an intensity of 400 Volts. Each leaf transmittance and reflectance spectrum measured on a sample (*S*), was matched with a dark (*D*) and reference (*R*) measurement, using black and white Spectralon diffuse-reflectance reference targets (WS-1-SL, Ocean Optics).

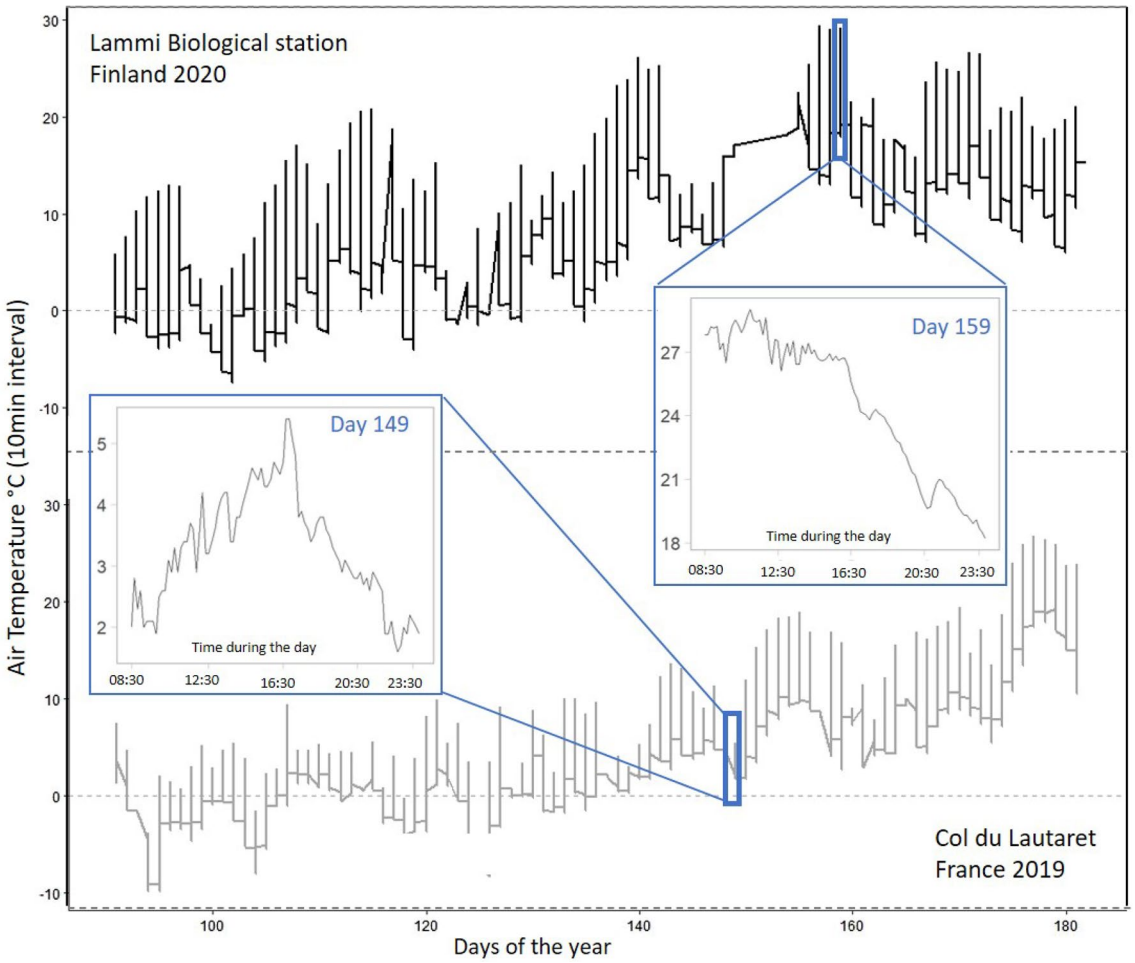


Fig. 1 Time series of mean air temperature measured at 10 min interval at both sampling locations; Lammi Biological Station, Finland (understorey species) and the Col du Lautaret, France (alpine species). Inset plots in blue boxes zoom in on the temperature on the day when plant leaves were sampled from both the locations

The instrument calculates the spectral transmittance ($T\lambda$) and reflectance($R\lambda$) according to Eq. 1.

$$T\lambda/R\lambda = \frac{S-D}{R-D} \times 100, \quad (1)$$

Post-processing of the raw spectra was done using the Photobiology suite of packages in R [40], to detect out-of-range values. A "lowess" function was selected to smooth the spectrum, after testing and comparing those smooth functions available, reducing the effect of bad pixels without overfitting the data.

2.4 Statistical analyses

Differences in the transmittance, reflectance and absorptance of leaves were compared in two ways, treated either as continuous spectra or as discrete spectral regions calculated from these spectra. The mean of spectra (from 3 to 4 leaves) for a species, or freezing treatment, were considered to differ significantly when their 95% confidence bands were non-overlapping, plotted in the same figure. Whereas, species or freezing-related differences for discrete spectral regions were tested through ANOVA.

Two-way ANOVA was used to test whether the spectral transmittance in blue, green, red and far-red (and likewise F_v/F_m) differed among species, before vs upon freezing. Differences in leaf epidermal pigments (flavonols, anthocyanin and chlorophyll content) due to leaf age, among understorey species, and before vs upon freezing, were tested by a three-way ANOVA, and among alpine species leaf epidermal pigments were test using a single factor ANOVA. Function glht from 'multicomp' package was used to estimate within-species pair-wise comparisons and multiple comparisons between fresh and frozen leaves.

3 Results and discussion

Freezing caused changes in the optical properties (reflectance and transmittance across the UV, PAR and far-red spectrum), as well as pigments (optically measured flavonols, anthocyanins and chlorophylls) of frozen leaves compared with fresh leaves from species growing in alpine and forest understorey environments. While a few studies have addressed spectral characterization of leaf-injury induced after freezing in crop plants [36, 41] and in trees [42], to the best of our knowledge, this is the first study examining the spectral transmittance and reflectance of frozen leaves in wild species. The results of this series of measurements of leaf optical properties, and associated leaf pigments in the same leaves, could provide insights into variation in the amount of light reaching the mesophyll upon freezing and set a baseline for remote sensing where such data on leaf transmittance and reflectance of frozen leaves in winter are required as inputs for modeling radiative transfer.

3.1 Freezing increased transmittance of leaves of both alpine and understorey species

The transmittance spectra of fresh leaves from our studied species followed characteristic patterns similar to those previously reported for leaves from a variety of species [43, 44] (Fig. 2). A typical leaf appears green to the human eye, due to the strong absorption by photosynthetic pigments

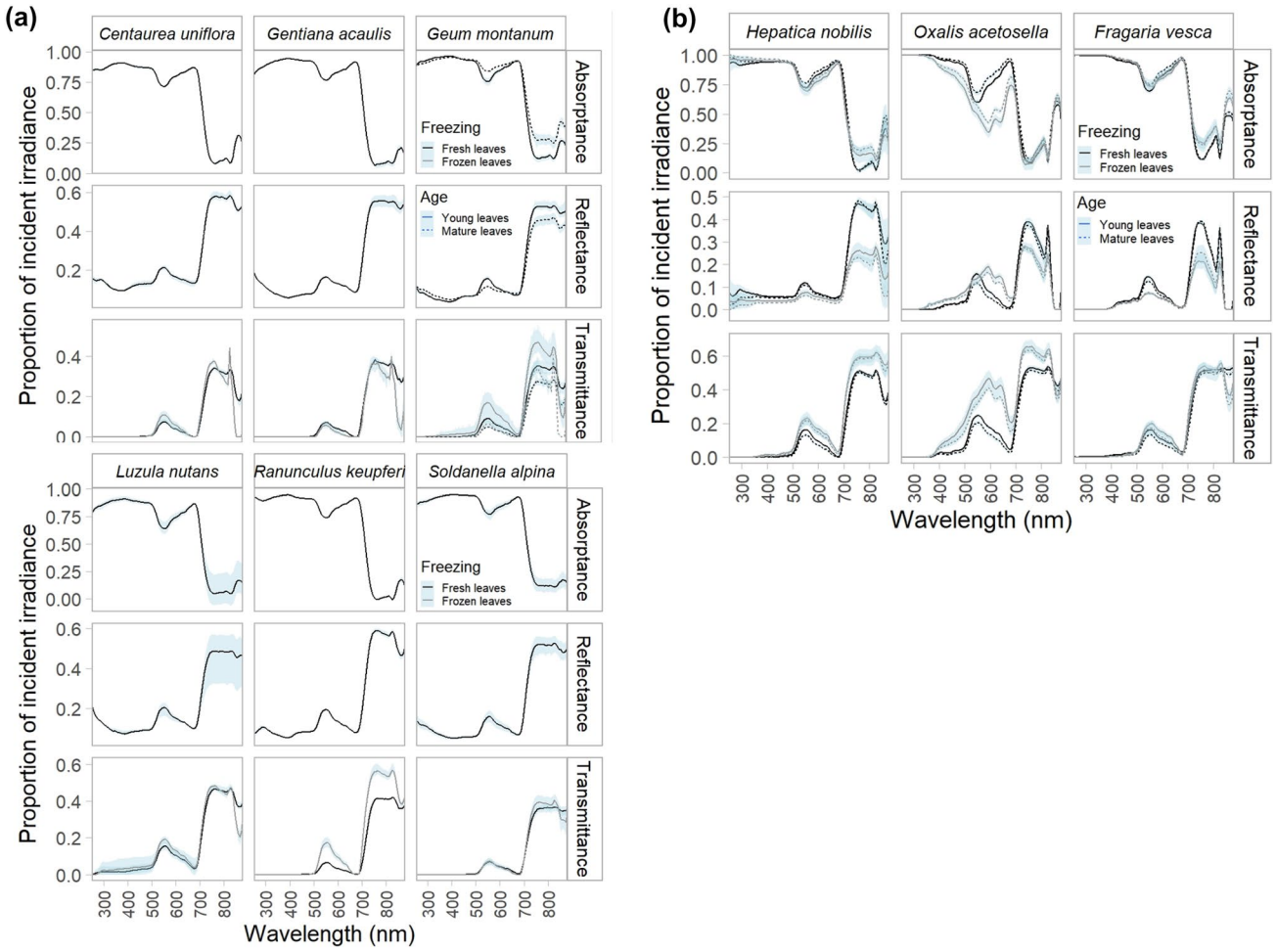


Fig. 2 Measured spectral reflectance and transmittance and calculated absorptance (280–892 nm) for fresh leaves prior to freezing (black line) and for frozen leaves (gray line). The blue band indicates the 95% confidence interval around each spectrum, for mature leaves (solid line) and young leaves (dashed line). Where the bands around spectra are non-overlapping, the difference between them is considered statistically significant. **a** Alpine species: Each spectrum is the mean of three leaves, with each leaf's spectrum composed of six consecutive spectra from a single position on the leaf. Note that reflectance spectra were not recorded from frozen leaves except for *G. montanum*. **b** Understorey species: Each spectrum is the mean spectra from four leaves collected from different locations in the understorey of the stand. Each leaf's spectrum is composed of six consecutive spectra from a single position on the leaf

of light at other wavelengths in the visible spectrum (Photosynthetically Active Radiation—PAR, 400–700 nm). At longer wavelengths, in the near and short-wave infrared (700–2500 nm) green leaves typically have prominently higher reflectance and transmittance, with very little absorptance; properties linked to their water content and structural features [45–49]. In our experiment, leaf spectral transmittance increased in response to freezing but the size of this effect differed across the spectrum (Fig. 3). Overall, the increase in the percentage of radiation transmitted was largest in the far-red (increasing by 4.1% in alpine sp and 7.6% in understorey sp) and in the PAR (1.4% alpine sp and 4.5% understorey sp); where specifically the difference in transmittance between fresh and frozen leaves was greater in the green (500–600 nm; 1.84% alpine sp and 7.74% understorey sp) than the red (600–700 nm; 0.14% alpine sp and 5.45% understorey sp) and blue (400–500 nm; 0.40% alpine sp and 1.85% understorey sp) spectral regions (Figs. 2 and 4, Table S5). Overall, frozen leaves also transmitted 0.60% (alpine sp) and 0.24% (understorey sp) more UV-A radiation (315–400 nm) than fresh leaves of both the understorey and alpine species, but this effect was minimal in the UV-B (280–315 nm) because all leaves transmitted negligible radiation (< 0.1%) in this spectral region, decreasing at shorter wavelengths (Fig. 2, Table S5).

The enhancement of transmittance induced by freezing was generally greater in the leaves of understorey than alpine species (Fig. 2a, b). However, in both environments there were some species which deviated from this general pattern of response. Even once frozen, the leaves of most

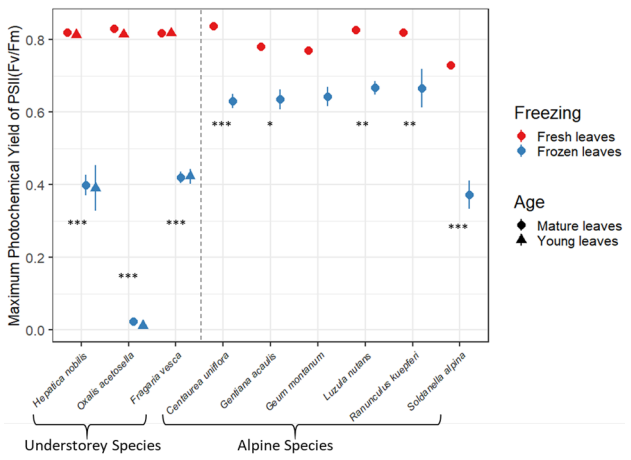


Fig. 3 Maximum quantum yield of photosystem II (PS_{II}) photochemistry (F_v/F_m) of six alpine species for fresh leaves prior to freezing and for frozen leaves. Each point is the mean ± 1 SE of four leaves. Significant differences between fresh and frozen leaves for each species are indicated as follow: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. *S. alpina* among alpine species, and *O. acetosella* among all understorey species, had significantly lower value of F_v/F_m than the rest ($p < 0.001$)

alpine species (five of the six species) were opaque to UV-A radiation and blue light (Figs. 2a, and 4), and *L. nutans* only transmitted less than 0.02% UV-A radiation (Figs. 2a and 4). *Luzula nutans* was the only monocot among the species that we tested, and had high SLA and LWC compared with the other alpine species (Table 1), as well as a very different leaf structure; which may explain this difference in transmittance [50, 51]. A typical monocot leaf has a compact palisade mesophyll often referred to as chlorenchyma and lacks spongy mesophyll; this causes light interception, scattering and transmittance to be very different from species with both spongy and palisade mesophyll cells [52]. Among alpine species, only leaves of *G. montanum* and *R. keupferi* transmitted significantly more green light (increasing by 2.7% and 4.8%) and far-red light (increasing by 6.5% and 11.3%) when frozen than fresh (Fig. 2a, Table S4); indicating that freezing had a greater impact on leaf optical properties in these species than in the other alpine forbs. Among the understorey species, fresh leaves of *O. acetosella* had the highest spectral transmittance among the species we measured; and likewise, once frozen, its leaves transmitted the most spectral irradiance (Figs. 2b, 4 and Table S4). Irrespective of damage, the fact that *O. acetosella* has very thin leaves [53–56] likely explains why their transmittance was highest, this contrasts with *H. nobilis* which has thick leaves through which very little radiation is transmitted (Figs. 2b and 4, Table 1) [54]. As well as leaf structure, specific leaf area and leaf anatomy, difference in transmittance among species may change due to acclimation to the physical environment, such as temperature fluctuations and high incident irradiance, e.g., inducing phototropin-mediated chloroplast stacking, whereby chloroplasts align against the anticlinal cell walls increasing light transmittance through the leaf [55, 57]. The processes leading to apoplastic ice allocation, organelle reorganization and other ultrastructural changes upon freezing are as yet poorly understood in plant leaves. However, recent methodological advances have enabled ice accommodation within leaf cross-sections to be visualized [30], and for ultrastructural evaluation of frozen leaves by transmission electron microscopy [58]. These techniques appear to be promising new tools to complement the interpretation of changes in spectral signatures of frozen leaves in the near future.

Notably, the reflectance of *O. acetosella* leaves in the PAR increased when frozen, with the green peak seemingly shifting towards longer wavelengths (Fig. 2b). Leaf absorbance in this spectral region is mainly attributable to chlorophyll, hence this change may be the result of cell lysis affecting the pH, degrading chlorophyll and producing pheophytin [42]. It is also possible that disruption of the membrane and cell structure of the leaf during freezing contributed to the changes recorded in transmittance and reflectance, reducing the scattering of radiation by internal structures,

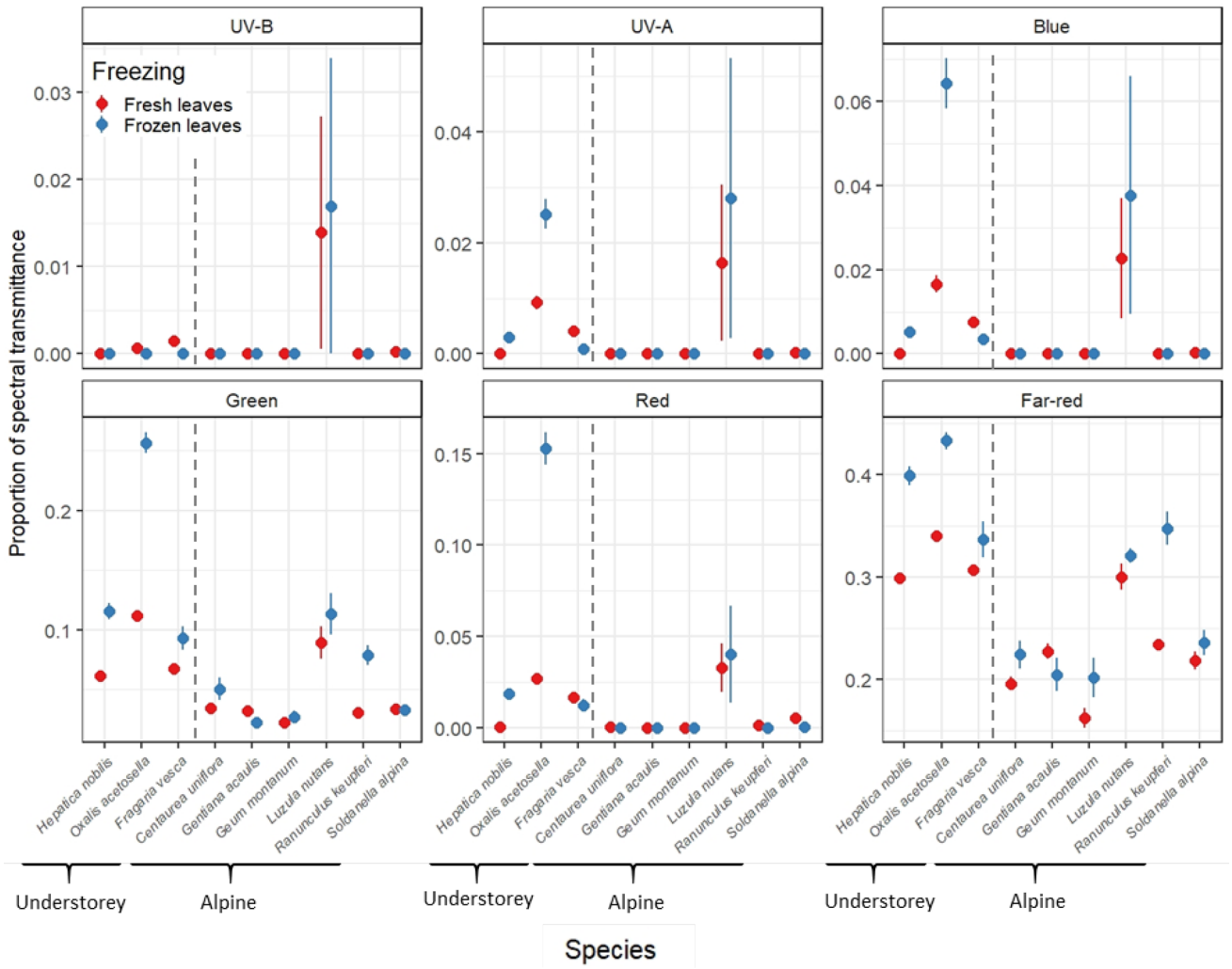


Fig. 4 Spectral reflectance, absorptance and transmittance plotted for different spectral regions: UV-B, UV-A, blue, green, red and far-red for all understorey and alpine plant species, comparing fresh leaves prior to freezing against frozen leaves. Spectral regions were calculated from the spectra plotted in Fig. 2A, B. Only mature leaves were used in this plot (Fig. 2A, B)

which would also result in reduced back-scattered radiation escaping the leaf. In all three understorey species, freezing reduced leaf reflectance in the far-red region. The amount of radiation reflected in the far-red is thought to be controlled by surface properties, air spaces and the internal structure of the leaf [52]. Additionally, the phase transition from water to ice has been found to displace reflectance spectra to longer wavelengths in the green and far-red regions during freezing of oil-seed rape (*Brassica napus*) leaves [36].

When exposed to freezing temperatures and high irradiance, leaves can suffer severe photo-inhibitory stress and damage to the photosynthetic apparatus. In freezing-tolerant species, this risk is effectively counteracted with photoprotective mechanisms [59–61]. Our experiment, however, was conducted in darkness to avoid photoinhibition. Fresh leaves from the understorey species showed high F_v/F_m values of 0.82, and similarly, fresh leaves of the alpine species had high F_v/F_m at 0.78 on average. Values of F_v/F_m slightly below 0.8, such as these, are common under optimal conditions for alpine species generally [24, 25, 62]. Acclimation of photosynthetic apparatus from freezing-tolerant species to low temperatures is usually accompanied by a downregulation of predawn F_v/F_m values [23, 24]. Interestingly, in our study, the F_v/F_m depression due to freezing was higher in the understorey than the alpine species. The greater susceptibility of understorey species to freezing damage may have been exacerbated because they were sampled later into the growing season than the alpine species, meaning that they are likely to have completely dehardened prior to our freezing treatment [14, 63]. Amongst the understorey species, F_v/F_m was lowered most by freezing in *O. acetosella* (Fig. 3) which was consistent with the largest increase in transmittance occurring in this species, along with the greatest loss of chlorophyll and lowest flavonoid indices

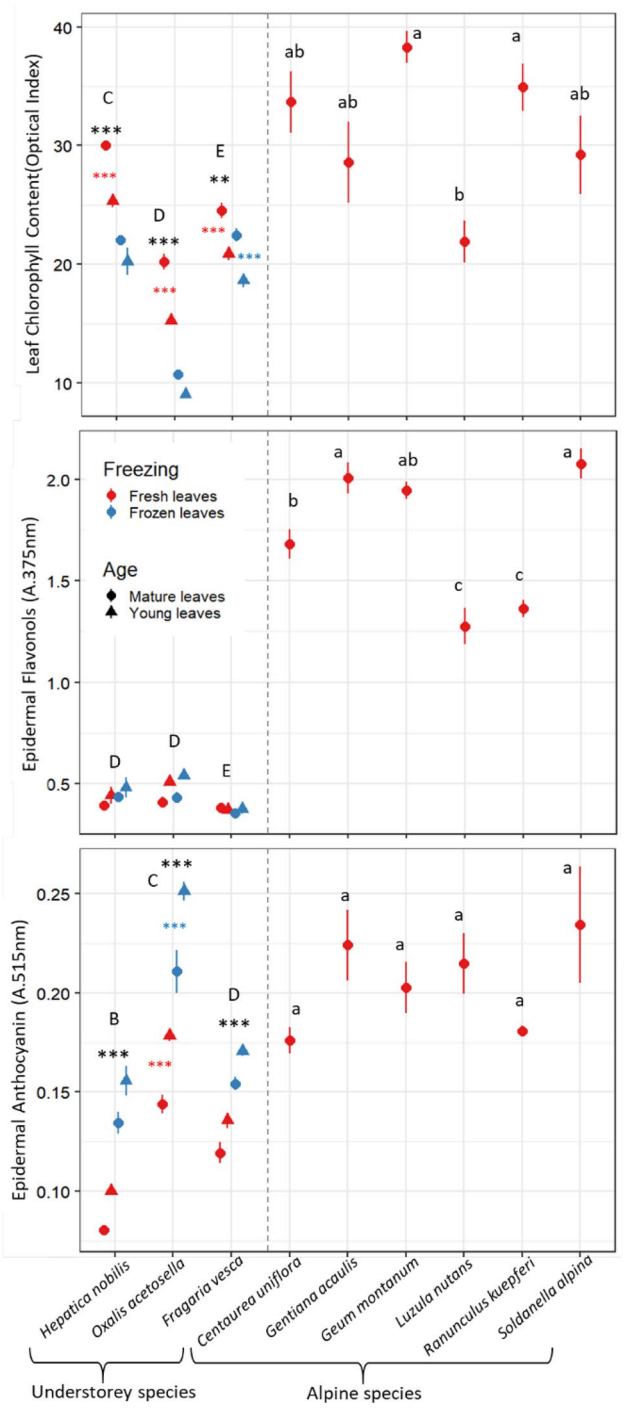


Fig. 5 Leaf chlorophyll (**a**), and epidermal flavonol (b) and anthocyanin (**c**) contents of fresh leaves of six alpine species (Lautaret) before freezing, and three understorey forbs (Lammi) for fresh leaves prior to freezing (red points) and frozen leaves (blue points). Significant differences between fresh and frozen leaves for each species are indicated as black asterisks, and differences between mature and young leaves as blue and red asterisks: $*p<0.05$, $**p<0.01$, $***p<0.001$. Mean values with different letters indicate significant differences among understorey species (upper case) and alpine species (lower case). Each point is the mean ± 1 SE of 3 and 4 leaves for alpine and understorey species, respectively. Where Dualex data are absent, leaves were too delicate for this measurement when frozen

(Figs. 2b and 5). Amongst alpine species, *S. alpina* had both the lowest F_v/F_m in fresh leaves (0.72 ± 0.01), and the biggest decrease in frozen leaves (to 0.37 ± 0.04), compared with F_v/F_m in other alpine species which only declined to about 0.60 (Fig. 3). While we did not find a difference in the extent to which F_v/F_m in mature leaves, that had overwintered, was depressed by freezing compared with current year's leaves, previous studies [64, 65] have reported young leaves of alpine species to be more freezing sensitive, since ice formation can more easily damage their photosynthetic apparatus than in mature leaves.

3.2 Changes upon freezing in optically measured leaf pigments

Leaf chlorophyll, epidermal flavonol and anthocyanin contents were measured optically on the leaves of understorey species before and upon freezing, whereas these optical traits were only measured in fresh leaves of alpine species. The fresh leaves of alpine species contained higher epidermal flavonoids as well as higher chlorophyll content than the fresh leaves of understorey species (Fig. 5a and b). This presumably is a consequence of the accumulation of these pigments in response to the higher solar radiation, in particular UV-B radiation, received in the alpine environment than in the boreal understorey, and its colder springtime temperatures with large diurnal fluctuations [38, 66], which stimulate increased photoprotection and chlorophyll accumulation [60, 66]. It is conceivable that excision of the fresh leaves prior to measurement could affect their physiology and pigment values prior to freezing, but comparison with other attached leaves of these species did not provide evidence for such an effect (unpublished data).

For all the understorey species, the index of epidermal flavonols was higher than that of anthocyanins, but these indices differed in response to freezing (Fig. 5b and c). In understorey species, the epidermal anthocyanin index significantly increased upon freezing in darkness ($p<0.001$) (Fig. 5c). However, there was no parallel increase in the optical index of UV-screening epidermal flavonols upon freezing in *O. acetosella* or *F. vesca*, it only increased in *H. nobilis* (Fig. 5b). Without a quantitative comparison of the metabolites of fresh and frozen leaves, we are not able to attribute these increases in absorbance to changes in the concentration or location of these classes of flavonoid. Even well-acclimated alpine species such as *Ramonda myconi* cease enzymatic activity at − 15 °C [24], so at most these leaves would have been metabolically active only as their temperature dropped prior to freezing, meaning there would have been little opportunity for anthocyanin synthesis.

In contrast to flavonols, the drop in optically measured leaf chlorophyll content upon freezing differed significantly among species. Chlorophyll content was

substantially reduced in frozen leaves of *H. nobilis* (− 39%) and *O. acetosella* (− 79%) compared with fresh leaves, but only reduced to a lesser extent in leaves of *F. vesca* (− 11%) (Fig. 5a, Table S2). These results suggest that there was degradation of leaf chlorophyll during freezing [67], which is consistent with the larger increase in PAR transmittance in *H. nobilis* and *O. acetosella* than in *F. vesca* upon freezing (4.1). Transmittance of frozen leaves also increased at longer wavelengths, but a study of the leaf ultrastructure would be required to distinguish the relative contributions to this effect from leaf structural changes and pigment degradation. The two obligate understorey species were presumably less well adapted to tolerate sudden freezing to − 18 °C or less well acclimated to cold conditions at the time of sampling than *F. vesca* which grows across a broader environmental niche [68, 69].

Interpretation of the changes in optically measured leaf pigments and leaf optical properties that we report could be improved by identifying whether these differences are underpinned by biochemical changes in the leaf or structural changes and cellular damage during freezing. These effects can be related to the freezing resistance strategy and acclimation of the different species that we studied. Upscaling the changes in leaf optical properties, measured for the alpine and understorey species in our study, provides possibilities to interpret remotely sensed canopy-level changes in absorbed PAR on freezing [36, 70]. Leaf reflectance spectra provide reflectance indices useful in understanding the physiological status of plants, e.g., chlorophyll indices and the photochemical reflectance index (PRI), which allow scaling from the leaf to the canopy. The species-level information we provide could be incorporated in ecosystem-scale models to improve estimates of their contribution to photosynthesis [48, 71, 72].

4 Conclusions

We found that across alpine and understorey species, freezing increases the spectral transmittance of mature leaves in spring. The largest reductions in transmittance were in the PAR, particularly the red and green, but there was also a small species-specific increase in transmittance of UV-A radiation. Transmittance of far-red radiation increased and its reflectance decreased upon freezing. In understorey species, the spectral peak characteristic of in vivo absorbance by chlorophyll of the leaf shifted towards longer wavelengths on freezing, possibly as a symptom of membrane damage and a change in its molecular configuration or pH, though this suggestion requires specific examination. Among obligate species understorey, optically assessed leaf chlorophyll content was particularly decreased by freezing.

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Declarations

Conflict of interest The authors declare no conflict of interests.

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