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Seasonal variation and trade-off between frost resistance and photosynthetic performance in woody species

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Abstract

We tested the seasonal variability of frost resistance and photosynthesis characteristics, namely net photosynthetic rate at saturating irradiance and ambient atmospheric CO₂ concentrations, maximum carboxylation rate, and measurements based on the JIP-test, namely maximum quantum yield of PSII and the performance index. Additionally, we measured leaf functional traits, namely specific leaf area, leaf dry matter content, stomatal pore area index, and chlorophyll content. We assessed all traits weekly on two evergreen species Berberis vulgaris and Quercus × hispanica as well as the two summer green species Betula pendula and Quercus pubescens. We found that there was a high seasonal variability in all traits except stomatal pore area index. In a multivariate analysis, we could show trade-offs between growth rates and leaf thickness on the first axis, as well as resistance and performance on the second axis. The findings help understand ecophysiological processes and fine-tune biosphere models.

Introduction

Frost resistance (FR) and photosynthetic rates (Pₜₖ) are the most fundamental processes in plants, which enable them to exist and persist outside the tropics and drive species performance in a given habitat (Sakai and Larcher 1987, Larcher 2000, Körner 2003, Taschner and Neuner 2004, Bucher et al. 2018b). Both are adaptive traits which can strongly differ between species as well as between seasons, and are mostly influenced by photoperiod and temperature (Ulmer 1937, Schwarz 1970, Koski 1985, Sakai and Larcher 1987, Bucher et al. 2018b). An investment in FR as well as in Pₜₖ is energetically costly for plants, and we therefore expect a trade-off between frost resistance, as represented by FR, and performance, represented by Pₜₖ. There are two fundamental life history strategies for trees in temperate regions, namely being evergreen or being summer green and shedding the leaves in autumn. This has major implication for longevity of leaves, resource distribution, the necessity of investing in FR, and the overall canopy duration during which photosynthesis takes place (Kadereit et al. 2014). We therefore expect this to differ between life forms.

There are several methods for capturing frost resistance and photosynthetic performance. FR can be assessed easily via electrolyte leakage (Gurvich et al. 2002). Photosynthetic performance, however, can be captured via various techniques, such as measuring gas exchange (Farquhar et al. 1980, von Caemmerer and Farquhar 1981, Grassi et al. 2005, De Kauwe et al. 2016, Bucher et al. 2018a) or assessing chlorophyll a (Chl a) fluorescence via the JIP-test (Schreiber et al. 1995, Strasser and Srivastava 1995, Strasser et al. 2000, Bucher et al. 2018a). Photosynthetic rates, more specific the CO₂ uptake at saturating light conditions and current atmospheric CO₂ concentration as well as optimal humidity, Pₜₖ, can be assessed quickly. They can be used to calculate other ecophysiological parameters such as the maximum carboxylation rate, Vₘₐₓ (Farquhar et al. 1980, Wilson et al. 2000, Grassi et al. 2005, De Kauwe et al. 2016, Bucher et al. 2018a). Two
important parameters of Chl fluorescence, the maximum quantum yield of PSII, F/FR, and the absorption-based performance index, PI, are a quick method to assess the functionality of PSII and PSII. They are quicker and simpler to describe photosynthetic performance than measuring photosynthetic rates (Strasser and Srivastava 1995, Strasser et al. 2000, Bucher et al. 2018a). Chl fluorescence has been proven to indicate plant stress, such as ozone stress (Clark et al. 2000), water stress (Zivčák et al. 2008), temperature stress, such as frost or heat (Neuner et al. 1999, Maxwell and Johnson 2000, Neuner and Pramoskler 2006, Sharma et al. 2013, Bucher et al. 2018c), as well as the onset of autumn senescence (Holland et al. 2014).

Leaf functional traits capture long-term adaptations of plants to their environment rather than short-term physiological responses as captured by FR and PI. They are used more and more in ecological research as they can provide further insights into the fundamental mechanisms of a species' adaptation to its environment (Violle et al. 2007, de Bello et al. 2010, Pérez-Harguindeguy et al. 2013, Römermann et al. 2016). Traits, such as specific leaf area (SLA), leaf dry matter content (LDMC), and stomatal pore area index (SPI), have been shown to be proxies of photosynthetic performance as well as competitive strength (Rosenzweig 1987, de Bello et al. 2010, Pérez-Harguindeguy et al. 2013, Bucher et al. 2016). SLA, for example, is an indicator of growth rate, indicates competitive strength and stress tolerance (Garnier 1992, Reich et al. 1997, Poorter et al. 2009, Pérez-Harguindeguy et al. 2013). LDMC is a proxy of the investment in structural compounds indicating tougher, long-lived leaves and good defence and resistance especially to herbivory (Pérez-Harguindeguy et al. 2013). Stomata are the mediators of gas exchange (Chaerle et al. 2005, Roelfsema and Hedrich 2005). The stomatal pore area index (SPI) encompasses both stomatal density and size and thus relates to transpiration and photosynthetic rates (Sack et al. 2003, Bucher et al. 2016). Chlorophyll content (Chl) can be measured in situ with spectroscopic techniques and is closely related to photosynthetic rates (Evans 1989, Zhu et al. 2012).

Seasonal variation in FR, PI, and leaf functional traits are of major importance for the economy of the plants, as the balance of the trade-off between the maintenance of FR vs. the investment in photosynthesis-related processes is important to minimise energy investments and maximise performance (Polgar and Primack 2011, Römermann et al. 2016, Bucher et al. 2018b). Plants harden in autumn and gradually deharden in spring, reducing the investment in FR during the summer (Aulitzky 1961, Sakai and Larcher 1987, Körner 2003, Neuner and Pramoskler 2006, Bucher et al. 2018b). Photosynthetic rates usually show higher values in summer and lower in winter (Larcher 1994, Öquist and Huner 2003, Adams et al. 2004, Neuner and Pramoskler 2006, Holland et al. 2014, Bucher et al. 2018b). Chloroplasts break down very early during leaf senescence, so metabolically, carbon assimilation is replaced by catabolism of the Chl and other macromolecules (Lim et al. 2007). For the summer green western larch (Larix occidentalis), Chl content declines by 11% before photosynthetic rates decline, whereas F/FR does not decline until late during senescence (Rosenthal and Camm 1997). For evergreen species, both leaves, which are newly formed and leaves, which overwintered and were formed the previous year, contribute to the overall functionality of the organism. Analysing and quantifying the differences between leaf types is crucial to capture physiological processes on an organism scale and also to help establish sampling procedures and avoid systematic errors when only sampling one leaf type.

To study the trade-off between frost resistance and photosynthetic performance as well as the seasonal variation in these parameters, we monitored FR and PI (measured via gas exchange and Chl a fluorescence) as well as leaf functional traits, namely SLA, LDMC, SPI, and Chl, on two evergreen and two summer green species in the Botanical Garden of the Friedrich Schiller University Jena, Germany. To investigate the seasonal variation in these parameters as well as the trade-off between FR and PI, we measured all parameters on a weekly basis. More specifically, we asked the following research questions: (1) Is there a seasonal variation in FR, PI, and leaf functional traits in summer green and evergreen species? (2) Do leaves, which are newly formed, differ from leaves, which overwintered? (3) Is there a trade-off between frost resistance and photosynthetic performance and how do leaf functional traits help understand the functional mechanisms behind it?

Understanding these processes and seasonal variations help us predict species behaviour under changing climate conditions and broaden our knowledge of the use of traits to capture trade-offs between resistance and performance.

**Materials and methods**

**Plant material:** We selected four woody species, namely two evergreen species Berberis vulgaris and Quercus × hispanica as well as two summer green species Betula pendula and Quercus pubescens. There was only one individual per species in the Botanical Garden, so we chose to measure multiple leaves per individual and week to capture the variability within the individuals (Fig. 1). To avoid midday depression of photosynthesis, we did not perform measurements (neither gas exchange nor Chl a fluorescence measurements) after 12:00 h (Demmg-Adams et al. 1989, Lichtenthaler et al. 2005). We selected sun leaves for all analyses. Plants in the Botanical Garden did not suffer from nutrient deficiencies as fertilizer was applied. Plants were irrigated during the extremely dry summer of 2018. For each species, we selected and studied ten leaves per week (Fig. 1). On five leaves, we assessed FR, PI, Vcmax, Chl a fluorescence, SPI, and Chl. On the remaining five leaves, we recorded Chl a fluorescence, SLA, and LDMC as well as Chl. The measurements could not be carried out on the same leaves, as some of the measurements were destructive (FR) or left traces on the leaves (SPI). The measurements were carried out from 9 April till 19 June 2018 and again from 3 September till 28 November 2018 as we wanted to capture the phases
Frost resistance was assessed with a device (LI-COR Bioscience, Piscataway, New Jersey, USA). From these values, the stomatal pore area index, and chlorophyll content were assessed. On leaf two, chlorophyll fluorescence, specific leaf area, dry matter content as well as chlorophyll content were recorded. On each sampling event, there were five replicates of leaf one and leaf two per species, so ten leaves were analysed per species.

with major change, i.e., the dehardening and hardening of the plants after and just before the winter. Evergreen species could be measured more frequently, as summer green species formed their leaves later in the season (Table 1). In sampling weeks 6–9, evergreen species started to form new leaves as well and it was possible to differentiate visually between newly formed leaves and the leaves formed in previous years. After week 9, no difference could be visually detected anymore. We therefore collected data on both new and old leaves during that time to get an estimate about the overall resistance and performance of the plant and to analyse the functional differences between the leaf types.

Electrolyte leakage: Frost resistance was assessed via electrolyte leakage as described by Gurvich et al. (2002). For each leaf, six discs with a diameter of 0.5 cm were cut out and partitioned into two treatments (with three replicates per treatment): a frost treatment at -18°C and a control at room temperature. Plant tissues were exposed to these temperature treatments for 12 h and then gradually warmed up again to room temperature. Conductivity was measured after warming up with a LAQUAtwin B-771 (HORIBA Instruments, Piscataway, New Jersey, USA) and again after boiling the samples for 15 min, where approximately 100% of the cells in a tissue died and the membranes leaked, which lead to maximum conductivity. The percentage of electrolyte leakage (PEL) was then calculated for each treatment as the quotient between the conductivity before and after the boiling of the samples × 100. The effective PEL (PEL_{eff}) was then calculated as the difference between the PEL of the samples stored in the freezer and the PEL of the control treatment. High values of PEL_{eff} thus indicate low FR.

The net photosynthetic rate at saturating irradiance and at ambient atmospheric CO₂ concentrations (P_{NAT}) was measured using the Li-6400XT (LI-COR Bioscience, Lincoln, Nebraska, USA). Irradiance was kept constant at 1,500 µmol(photon) m⁻² s⁻¹ and CO₂ concentration at 400 ppm. Block temperature was set to 20°C to make the measurements comparable throughout the season.

We used the ‘one-point method’ to calculate V_{max} from P_{NAT} measurements (De Kauwe et al. 2016, Bucher et al. 2018a).

Chl a fluorescence was determined using a Plant Efficiency Analyser, a portable continuous excitation time resolved Chl fluorimeter (PocketPEA, Hansatech Instruments, King’s Lynn, UK). Leaves were dark-adapted for 30 min using black leaf clips prior to measurements. The maximum PSII quantum yield (F_{m}/F_{v}) and the Chl a fluorescence performance index (PI_{abs}; Strasser and Srivastava 1995, Clark et al. 2000) were recorded.

Specific leaf area and leaf dry matter content were determined via assessing the dry and fresh mass using a fine scale (ABJ, Kern & Sohn GmbH, Balingen, Germany), leaf area was recorded via scanning the fresh leaves (CanoScan LiDE110, Canon, Tokyo, Japan). Both parameters were then calculated in R using the package LeafTraits (Bernhardt-Römermann, unpublished).

Stomatal pore area index: Stomatal imprints from the adaxial and abaxial leaf side were taken using the clear nail polish method as described in Hilu and Randall (1984). Stomata were counted and guard cell length was measured with a light microscope (Olympus CH20, Olympus, Shinjuku, Japan). From these values, the stomatal pore area index, SPI, was calculated as proposed by Sack et al. (2003). None of the species studied showed stomata on the upper leaf side, which was checked on every sample.

The relative Chl concentration was measured using an atLeaf PLUS device (atLeaf, Wilmington, Delaware, USA) and then converted to total Chl concentration using the conversions on the homepage (https://www.atleaf.com/SPAD).

Statistical analysis: To test differences of the parameters between species, we performed analysis of variance (ANOVA) followed by Tukey’s multiple comparisons of means tests. To assess the seasonal variation in the parameters recorded (PEL_{eff}, P_{NAT}, V_{max}, F_{m}/F_{v}, PI_{abs}, SLA,
LDMC, SPI, and Chl), we performed linear models using the parameters measured as dependent variables and day of the year the measurements (doy) as well as doy² as the explanatory variable to account for nonlinear relationships. To account for species-specific responses, species identity was included in the model as a covariate as well as the twofold interactions with doy:species and doy²:species. These full models were then simplified backwards, until the minimum adequate model was found as described in Crawley (2013).

To analyze the differences between newly formed leaves and leaves which overwintered in the two evergreen species, we performed Welch’s two sample t-test or Wilcoxon rank sum test, depending on data distribution tested with the Shapiro-Wilk normality test in all parameters (PELₐ, Pₑₐ, Vₑₐ₃₉₉, Fₑ/Fₐ, PLₑₐ, SLA, LDMC, SPI, and Chl).

To analyze trade-offs between frost resistance and photosynthetic performance and the association with leaf functional traits, we performed linear models with species and the interaction of species and traits as explanatory variables for each trait separately. If possible, these were then simplified as described above. This was done using leaf-level data of the replicate measurements each week, or mean values per species and sampling date, depending whether the traits had been measured on the same leaf or on a different one (Fig. 1). In a second step, we analyzed the relationships in the multivariate trait space and performed a principal component analysis (PCA) with the species-wise mean values per sampling date, depending on whether the traits had been measured on the same leaf or on a different one (Fig. 1). In a second step, we analyzed the relationships in the multivariate trait space and performed a principal component analysis (PCA) with the species-wise mean values per sampling date, depending on whether the traits had been measured on the same leaf or on a different one (Fig. 1). Furthermore, the mean values in between old and new leaves within the first weeks of the measurement period represent the total resistance and performance better than the individual values. Confidence ellipses are drawn in the plot for each species.

All statistical analyses were performed using R (R Core Team 2017), the PCA was computed using the ‘vegan’ package (Oksanen et al. 2007) and displayed graphically using ‘ggplot2’ (Wickham 2009), ‘devtools’ (Wickham and Chang 2017) and ‘ggbiplot’ (Vu 2011).

### Results

**Seasonal variation:** An overview of all parameters measured including mean values and ranges is presented in Table 1. All parameters measured showed a species-specific seasonal variation. FR showed a strong species-specific seasonal variation with lower values, i.e., higher resistance in early spring and late autumn (R² = 0.55, F₁₂, ᵃ₀ = 40.3, p < 0.001; Fig. 2A). *Quercus pubescens* showed almost a linear decrease in PELₐ, thus an increase in FR during the course of the year. The relationship was defined by doy, doy² and species as well as the two-fold interactions thereof. Net photosynthetic rates also showed a pronounced seasonal variation with a strong species-specific pattern. Again, the relationship was defined by doy, doy² and species as well as the two-fold interactions thereof (R² = 0.61, F₁₂, ᵃ₀ = 50.8, p < 0.001; Fig. 2B). The two summer green species showed overall higher values in

### Table 1: Characterisation of four woody species with respect to their mean and range in measured trait values. Given is the mean value for each species as well as the range, i.e., the minimum and maximum values measured, of frost resistance measured as effective percentage of electrolyte leakage (PELₐ), net photosynthetic performance index (SPI), specific leaf area (SLA), leaf dry matter content (LDMC), stomatal pore area index (SPI), as well as chlorophyll content (Chl). Differences between species were assessed with ANOVA, Tukey HSD post hoc test, depending on data distribution tested with the Shapiro-Wilk normality test in all parameters (PELₐ, Pₑₐ, Vₑₐ₃₉₉, Fₑ/Fₐ, PLₑₐ, SLA, LDMC, SPI, and Chl). Results are reported, the statistical results are given as n.s. – not significant, ‘·’ – result is not significant, ‘*’ – p < 0.05, ‘**’ – p < 0.01, and ‘***’ – p < 0.001. Letters denote significant differences at the 0.05 level. The canopy duration indicates, during which period leaves were displayed on summer green trees.

<table>
<thead>
<tr>
<th>Species</th>
<th>Life form</th>
<th>Canopy</th>
<th>Pₑₐ (%)</th>
<th>Vₑₐ₃₉₉ [μmol(CO₂) m⁻² s⁻¹]</th>
<th>Fₑ/Fₐ</th>
<th>PLₑₐ [μmol(CO₂) m⁻² s⁻¹]</th>
<th>SLA [m² g⁻¹]</th>
<th>LDMC [g kg⁻¹]</th>
<th>SPI</th>
<th>Chl [mg cm⁻²]</th>
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</thead>
<tbody>
<tr>
<td><em>Betula pendula</em></td>
<td>evergreen</td>
<td>winter</td>
<td>31.6</td>
<td>18.6</td>
<td>0.71</td>
<td>0.82</td>
<td>11.5</td>
<td>22.9</td>
<td>0.231</td>
<td>455.9</td>
</tr>
<tr>
<td><em>Quercus pubescens</em></td>
<td>evergreen</td>
<td>winter</td>
<td>31.8</td>
<td>27.6</td>
<td>0.71</td>
<td>0.82</td>
<td>11.5</td>
<td>22.9</td>
<td>0.231</td>
<td>455.9</td>
</tr>
<tr>
<td><em>Quercus ilex</em></td>
<td>evergreen</td>
<td>winter</td>
<td>31.6</td>
<td>27.6</td>
<td>0.71</td>
<td>0.82</td>
<td>11.5</td>
<td>22.9</td>
<td>0.231</td>
<td>455.9</td>
</tr>
<tr>
<td><em>Quercus pyrenaica</em></td>
<td>evergreen</td>
<td>winter</td>
<td>31.6</td>
<td>27.6</td>
<td>0.71</td>
<td>0.82</td>
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Table 1: Characterisation of four woody species with respect to their mean and range in measured trait values. Given is the mean value for each species as well as the range, i.e., the minimum and maximum values measured, of frost resistance measured as effective percentage of electrolyte leakage (PELₐ), net photosynthetic performance index (SPI), specific leaf area (SLA), leaf dry matter content (LDMC), stomatal pore area index (SPI), as well as chlorophyll content (Chl). Differences between species were assessed with ANOVA, Tukey HSD post hoc test, depending on data distribution tested with the Shapiro-Wilk normality test in all parameters (PELₐ, Pₑₐ, Vₑₐ₃₉₉, Fₑ/Fₐ, PLₑₐ, SLA, LDMC, SPI, and Chl). Results are reported, the statistical results are given as n.s. – not significant, ‘·’ – result is not significant, ‘*’ – p < 0.05, ‘**’ – p < 0.01, and ‘***’ – p < 0.001. Letters denote significant differences at the 0.05 level. The canopy duration indicates, during which period leaves were displayed on summer green trees.

### Notes

- ANOVA tests were used to determine significant differences among species, depending on data distribution tested with the Shapiro-Wilk normality test in all parameters (PELₐ, Pₑₐ, Vₑₐ₃₉₉, Fₑ/Fₐ, PLₑₐ, SLA, LDMC, SPI, and Chl).
- Tukey HSD post hoc tests were used to determine significant differences between species.
- The canopy duration indicates, during which period leaves were displayed on summer green trees.

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<th>LDMC [g kg⁻¹]</th>
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<td>evergreen</td>
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<td>31.6</td>
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<td>0.71</td>
<td>0.82</td>
<td>11.5</td>
<td>22.9</td>
<td>0.231</td>
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<td><em>Quercus pubescens</em></td>
<td>evergreen</td>
<td>winter</td>
<td>31.6</td>
<td>27.6</td>
<td>0.71</td>
<td>0.82</td>
<td>11.5</td>
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<tr>
<td><em>Quercus ilex</em></td>
<td>evergreen</td>
<td>winter</td>
<td>31.6</td>
<td>27.6</td>
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<td><em>Quercus pyrenaica</em></td>
<td>evergreen</td>
<td>winter</td>
<td>31.6</td>
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<td>11.5</td>
<td>22.9</td>
<td>0.231</td>
<td>455.9</td>
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Fig. 2. Seasonal variation of parameters recorded, namely (A) frost resistance as effective percentage of electrolyte leakage (PEL_{eff}), (B) photosynthetic rates (P_{net}), (C) maximum carboxylation capacity (V_{max}), (D) efficiency of PSII (F_{v}/F_{m}), (E) performance index (PI_{abs}), (F) specific leaf area (SLA), (G) leaf dry matter content (LDMC), (H) stomatal pore area index (SPI), and (I) chlorophyll content (Chl). Species are represented by colour, the evergreen species *Berberis vulgaris* and *Quercus × hispanica* are displayed in black circles and dark grey squares, respectively. The two summer green species are *Betula pendula* (pale grey triangles) and *Q. pubescens* (light grey diamonds). Leaves, which were visually newly formed and not fully grown, are indicated by open symbols.
The seasonal variation was more pronounced in evergreen species than in summer green species. The interaction between doy² and season was significant in the seasonal variation in SLA, whereas summer green species showed slightly higher values in mid-season than early and late (R² = 0.74, Fig. 2F). The seasonal variation in LDMC, however, was more pronounced than in SLA, with evergreen species showing higher values in early spring and summer green species showing lower values in spring and again a pronounced decrease in autumn (R² = 0.52, Fig. 2E). The seasonal variation in SPI was very low. The interaction between doy² and season was not significant and could be removed from the full model. Only Q. pubescens showed a noticeable difference in SPI values throughout the season with higher values in spring and autumn than in summer (t = –7.6, p < 0.001; Fig. 2G).

### Trade-off between resistance and performance:

The pairwise trait-trait relationships are summarised in Table 2 and graphically represented in Fig. 2S (supplement). There are positive relationships between PEL-eff and all parameters related to photosynthesis (Pₙₙₙₙ, Vₐₐₐₐₐ, Fₚₚₚₚ, PIₐₐₐₐ), indicating that the higher the performance, the lower the investment in FR. For some trait-trait relationships, there was a stronger difference in Chl content between evergreen and summer green species. While evergreen species had higher contents in early spring and in late autumn, the summer green species had the opposite tendency with lower values in spring and autumn (R² = 0.70, F₁₇, 7₄₄ = 145.1, p < 0.001; Fig. 2f).

### Old and newly formed leaves in evergreen species:

When looking at the differences between leaves formed in the previous year(s) and leaves from the same growth period, we found that some traits differed significantly between leaves of different age whereas others did not (Fig. 1S, supplement).

### Table 2. Pairwise relationships between all functional traits measured.

<table>
<thead>
<tr>
<th>Pₑₑₑₑₑₑ</th>
<th>Pₛₛₛₛₛₛ</th>
<th>Vₐₐₐₐₐₐ</th>
<th>Fₚₚₚₚ</th>
<th>PIₐₐₐₐ</th>
<th>SLA</th>
<th>LDMC</th>
<th>SPI</th>
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<tr>
<td>Pₛₛₛₛₛₛ</td>
<td>R² = 0.17</td>
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<td>Fₚₚₚₚ</td>
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<td>R = 0.17</td>
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<td>Fₚₚₚₚ</td>
<td>Fₚₚₚₚ, Fₚₚₚₚ</td>
<td>R = 0.38</td>
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<td>Fₚₚₚₚ</td>
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<td>R = 0.60</td>
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<td>Fₚₚₚₚ</td>
<td>Fₚₚₚₚ</td>
<td>R = 161.9***</td>
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<td>Fₚₚₚₚ</td>
<td>Fₚₚₚₚ</td>
<td>R = 59.8***</td>
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<td>Fₚₚₚₚ</td>
<td>Fₚₚₚₚ</td>
<td>R = 42.6***</td>
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<td>Fₚₚₚₚ</td>
<td>Fₚₚₚₚ</td>
<td>R = 16.6***</td>
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<td>Fₚₚₚₚ</td>
<td>Fₚₚₚₚ</td>
<td>R = 20.9***</td>
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<tr>
<td>Fₚₚₚₚ</td>
<td>Fₚₚₚₚ</td>
<td>R = 34.8***</td>
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<td>Fₚₚₚₚ</td>
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<td>R = 540.6***</td>
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<tr>
<td>Fₚₚₚₚ</td>
<td>Fₚₚₚₚ</td>
<td>R = 21.1***</td>
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was a clear divide in between evergreen and summer green species, such as in the relationships between \( P_{\text{Nat}} \) and \( V_{\text{max}} \), respectively, and \( \Pi_{\text{abs}} \) and Chl, respectively, where summer green species showed a stronger relationship with a much steeper slope. There was a strong positive relationship between Chl and \( \Pi_{\text{abs}} \) and LDMC, respectively. The PCA showed a clear separation between the species and the two life forms indicated by ellipses mainly on the first axis (Fig. 3). The first axis represented the trade-off between SLA on one side and LDMC, SPI, Chl, and \( \Pi_{\text{abs}} \) on the other, whereas the second axis represented the trade-off between \( F_{\text{el}} \) (PEL\textsubscript{eff}) and \( P_{\text{Nat}} \), \( V_{\text{max}} \), and \( F_{\text{i}}/F_{\text{m}} \). Summer green species tended to show higher growth rates and gas-exchange rates, whereas evergreen species invested more in leaf thickness and resistance as indicated by high LDMC, Chl, LDMC, and \( \Pi_{\text{abs}} \) and experienced less stress as indicated by \( F_{\text{i}}/F_{\text{m}} \).

**Discussion**

In this study, we found that PEL\textsubscript{eff}, \( P_{\text{Nat}} \), \( V_{\text{max}} \), \( F_{\text{i}}/F_{\text{m}} \), \( \Pi_{\text{abs}} \), SLA, LDMC, SPI, and Chl varied during the growth period in a species-specific manner and that there was a trade-off between performance and growth rates on one hand and leaf thickness and resistance on the other hand. There was a profound difference between evergreen and summer green species in many parameters.

Frost resistance showed clear seasonal variations with a profound dehardening in spring and hardening in autumn. In woody plants, the main driving forces of frost hardening are typically photoperiod and temperature (Schwarz 1970) which led to the observed increase in FR starting from the end of summer, i.e., the end of August. This effect was much more pronounced in evergreen species, which rely on the reuse of leaves in the following years to come, than in summer green species. Especially in *Q. pubescens*, hardly any hump shaped relationship could be detected, but rather a linear increase of FR over time was found. This response in seasonal variability of *Q. pubescens* confirms findings of Taschler et al. (2004), who showed that woody species are more frost susceptible during shoot expansion and regrowth in spring. In *Q. × hispanica*, young leaves were also less frost resistant than older leaves, whereas there was no difference in *B. vulgaris* between leaves of different age. Performance, as captured via gas exchange and Chl fluorescence, also showed remarkable seasonal variations. In our study, \( P_{\text{Nat}} \) and \( V_{\text{max}} \) showed similar patterns in their seasonal variation. Overall, summer green species showed higher \( P_{\text{a}} \) than that of evergreen species, yet a strong decline with senescence, which was almost contrary to our findings in evergreen species, which showed low seasonal variation and higher \( P_{\text{a}} \) in early spring. *Q. pubescens* showed low \( P_{\text{a}} \) in early spring, whereas *B. pendula* started with high values in young leaves. A similar pattern could be observed in \( \Pi_{\text{abs}} \), which has been proven to correlate well with \( P_{\text{Nat}} \) and \( V_{\text{max}} \) in previous research (Bucher et al. 2018a). However, the seasonal variation in \( \Pi_{\text{abs}} \) was much more pronounced than in gas-exchange measurements and evergreen species showed higher values in \( \Pi_{\text{abs}} \) than that of summer green species. Differences between gas exchange and Chl \( a \) fluorescence measurements could be due to the fact, that only the uppermost layers of plant tissue are studied in Chl \( a \) fluorescence technique. When measuring the adaxial side only, we merely probed the chloroplasts of the palisade parenchyma cells but not the entire leaf (Vogelmann and Evans 2002, Lichtenthaler et al. 2005), whereas gas exchange engulfs both sides of the leaves. \( F_{\text{i}}/F_{\text{m}} \) was a good indicator of autumn senescence as previously described by Holland et al. (2014) in summer green species, but showed little seasonal variation in evergreen species. *Q. pubescens* showed low values in spring, as did *Q. × hispanica* in younger leaves, probably hinting to a not fully developed photosynthetic apparatus. The low seasonal variation and the high early-spring values in SLA confirms findings of Römermann et al. (2016), who found that SLA peaks in early spring for most of the 16 summer green woody species studied. This can be attributed to the initial period of leaf growth and laminar expansion during which SLA is high (Steer 1971). Once cell expansion has finished, dry matter, especially in the form of nonstructural carbohydrates, accumulates in the cells (Palacio et al. 2008, Römermann

**Fig. 3.** Principal component analysis of all traits selected. Colours represent species. *Berberis vulgaris* is displayed in dark green, *Quercus × hispanica* in light green, both being evergreen species. The two summer green species are *Betula pendula* (yellow) and *Q. pubescens* (orange). Given is the frost resistance measured as effective percentage of electrolyte leakage (PEL\textsubscript{eff}), the net photosynthetic rate at saturating irradiance and ambient atmospheric CO\(_2\) concentrations (\( P_{\text{m}} \)), the maximum carboxylation rate (\( V_{\text{max}} \)), the maximum quantum yield of PSII (\( F_{\text{i}}/F_{\text{m}} \)), the absorption based performance index (\( \Pi_{\text{abs}} \)), the specific leaf area (SLA), the leaf dry matter content (LDMC), the stomatal pore area index (SPI), as well as the chlorophyll content (Chl).
et al. 2016). This is also reflected in LDMC values, which increased over time especially in evergreen species. The leaves of the evergreen species, which overwintered, however, showed an initial decrease in LDMC and later on an increase contrasting summer green and newly formed leaves. Previous studies showed an initial increase of LDMC and a subsequent decrease during the growth period in woody species (Jurik 1986, Palacio et al. 2008, Dubey et al. 2011, Römermann et al. 2016) and in some herbaceous taxa (Bucher et al. 2018b) which has been attributed to the accumulation of nonstructural carbohydrates and retranslocation of valuable compounds during senescence. Evergreen species thus seem to accumulate compounds in their overwintering leaves, which could relate to FR during cold periods. We could not observe a strong variation in SPI during the season similar to Römermann et al. (2016) and Bucher et al. (2018b). In previous research, we found a strong link between SPI and FR (Bucher et al. 2018b), which could not be confirmed by this study. However, SPI scaled very well with Chl, which in turn scaled well with LDMC and Plines. Thus, this relationship could have been masked by leaf thickness. Chl showed strong differences between evergreen and summer green species. Whereas evergreen species showed the highest values in the beginning and end of the measurement period, summer green species showed a mid-season peak. This might explain similar patterns in gas-exchange rates. In Platanus occidentalis, leaf nitrogen, which is partially located in Chl, showed a decline during senescence (Adams et al. 1990). In this study, the decrease in PSII fluorescence during senescence was attributed to a decreased reabsorption of PSII fluorescence.

There are few studies, which include the difference between newly formed and old leaves. In general, there were stronger differences between new and old leaves in Q. × hispanica than that in B. vulgaris. This might be due to the fact, that Q. × hispanica, a hybrid between Q. cerris and Q. suber, is evergreen in our garden, yet there have been reports that this species is only partially wintergreen and senescences its leaves if the winter is too severe. Notably, there were stronger differences in traits associated with leaf morphology, such as SLA, LDMC, and Chl, whereas differences in FR, Pn, and Vc were less pronounced. In most of these traits, the newly formed leaves of evergreen species were more similar to the newly formed leaves of the summer green species than to the old leaves of the same species. Thus, sampling protocols should take this difference into account, as sampling only one leaf type early in the year would strongly bias the results and not capture the processes on an organism scale.

In our study, there was a profound trade-off between resistance and performance as could be demonstrated with the trade-off of PElmax and the two gas-exchange parameters Pn and Vc. However, this pattern was much clearer in the multivariate trait space than in the univariate analysis on the intraspecific scale. Again we could demonstrate that Pn is well described by Plines (see Bucher et al. 2018a), yet this link was much stronger in summer green than that in evergreen species. This could be due to differences in morphology and leaf thickness, as summer green species form thinner leaves and less Chl. Additionally, only the uppermost layers of a leaf are captured with the PocketPEA (Vogelmann and Evans 2002, Lichtenenthaler et al. 2005), which might have caused some fundamental differences in between evergreen and summer green plants. Chl seemed to capture resistance and performances very well showing a negative relationship with FR and a positive one with the parameters assessing photosynthetic performance, especially Plines. We could demonstrate with our multivariat analysis that the trade-off is rather in between photosynthetic rates and FR than between growth rates and FR, which confirmed work by Larcher (1994), who found that overwintering leaves stayed photosynthetically active but invested their assimilates rather in the accumulation of more carbohydrates and thus higher FR than in growth. In previous studies, FR was linked negatively to SLA and growth rates and positively to SPI, indicating high photosynthetic rates (Sakai and Larcher 1987, Bucher et al. 2018b) due to a conflict of resource investment (Larcher 1994, Körner 2003). We could not confirm a relationship between stomatal traits and FR found by Bucher et al. (2018b) in six herbaceous plant species. This might have been confounded as mentioned above by high variations in leaf thickness. In our study, there were distinct differences between evergreen and summer green species, yet the two Quercus species were more similar to each other than the other two other species, which hints to an effect of phylogeny.

This study provides valuable insights into the differences between evergreen and summer green species, differences between old and newly formed leaves in evergreen species, and the trade-off between resistance and performance. It also shows how easy to measure plant functional traits can expand our knowledge about ecophysiological processes. Understanding seasonal variations and incorporating them in e.g., terrestrial biosphere models (TBMs) will help to fine-tune the models and increase their reliability. In future studies, the differences in this trade-off as inflicted by changing abiotic conditions and different leaf thicknesses should be tested and the phylogenetic signal as might be seen in the similar patterns of the two oak species should be analysed using higher species numbers.

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