

Inter- and intraspecific variation in stomatal pore area index along elevational gradients and its relation to leaf functional traits

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Received: 19 June 2015 / Accepted: 9 January 2016 / Published online: 16 January 2016
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Abstract Stomata are mediators of gas exchange and thus important for photosynthesis and plant performance. The aim of this study was to analyze the ecological explanatory power of the stomatal pore area index (SPI) calculated via stomatal size and density. We studied the SPI on sun leaves of 22 herbaceous species on 22 study sites being distributed along two elevational gradients in the northern Alps ranging from 700 to 1800 m a.s.l.. We analyzed its correlation with other functional traits related to plant performance namely specific leaf area (SLA), area-based leaf nitrogen and carbon (N_{area} and C_{area} , respectively) as well as carbon discrimination $\Delta^{13}\text{C}$

within as well as between species. On a subset of four species we also measured light-saturated net photosynthetic rate at ambient CO_2 concentration (A_{sat}) and stomatal conductance on all sites. We found that SPI was positively correlated with A_{sat} , yet the relation was weaker than expected. The reaction of SPI along the elevational gradients was highly species-specific and related to variations in other investigated leaf traits. The relationship with functional traits, however, differed between the inter- and intraspecific level in strength and direction. SPI was positively related to N_{area} and C_{area} and negatively with SLA and $\Delta^{13}\text{C}$ for most species. However, we found no significant relation considering species mean values for $\Delta^{13}\text{C}$ and N_{area} . The relation of SPI to SLA was the most consistent displaying no difference when comparing the relation between and within species. This research shows that different processes may act on different

Communicated by T.L. Pons.

Electronic supplementary material The online version of this article (doi:[10.1007/s11258-016-0564-2](https://doi.org/10.1007/s11258-016-0564-2)) contains supplementary material, which is available to authorized users.

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organizational levels leading to the detected differences in trait–trait correlations on the inter- and intraspecific levels. It may have important consequences also for macroecological and modelling studies.

Keywords Stomatal pore area index (SPI) · Potential conductance index (PCI) · Plant functional traits · Stomata · $\Delta^{13}\text{C}$ · Altitude

Introduction

Stomata are one of the most fundamental novelties in the evolution of higher plant species enabling them to conquer terrestrial land surface. They mediate the gas exchange between the plants photosynthetic layer and its environment and keep the balance between uptake of CO_2 , which is essential for photosynthesis, and water loss via transpiration (Chaerle et al. 2005; Roelfsema and Hedrich 2005). Regulation of gas exchange depends on both, density and aperture of stomata and is mediated via changes in turgor pressure of guard cells. The change in aperture is triggered by external as well as internal factors though signalling pathways are not entirely understood yet (Beerling and Chaloner 1993; Jones 1998; Roelfsema and Hedrich 2005; Kim and Maik 2010).

Since both, size and density, have an impact on gas exchange, Sack et al. (2003) proposed the stomatal pore area index (SPI). This index was later introduced again by Holland and Richardson (2009) as potential conductance index (PCI), both taking size (as squared guard cell length²) as well as density into consideration. Other authors also proposed similar indices such as the maximum leaf conductance which uses among others stomatal pore area instead of guard cell dimensions (Franks and Farquhar 2001) following early work by Brown and Escombe (1900), or the stomatal area index (SAI) which is the product of guard cell length and density (Ashton and Berlyn 1994). Few but large stomata might be as effective for stomatal conductance as many but small stomata, a fact neglected when looking either at stomatal density or stomatal size. SPI was found to be related to leaf hydraulic conductance (Sack et al. 2003) which was correlated with photosynthesis rates across species (Brodribb et al. 2005). However, it is not clear, how

SPI responds to changing abiotic conditions on both, the inter- and especially the intraspecific level, and how this relates to functional traits associated with photosynthetic performance and growth.

The density of stomata and their diffusive conductance are positively correlated with net-photosynthesis rates and biomass production (Wong et al. 1979; Kundu and Tigerstedt 1999; Woodward et al. 2002). Sun leaves were found to have higher stomatal densities than shade leaves within the same species (Hanson 1917; Gay and Hurd 1975) and xeric species tend to have higher stomatal densities than mesic species (Gindel 1969; Carpenter and Smith 1975). The formation of stomata is continuous throughout leaf development enabling plants to respond to changing environments (Nadeau and Sack 2002). So far it is known that stomatal development is controlled by environmental conditions e.g. by CO_2 concentration, light intensity, temperature, nutrient availability and water supply, and also by gene expression, being itself controlled by the environment (Woodward 1987; Woodward and Bazzaz 1988; Nadeau and Sack 2002; Woodward et al. 2002; Qiang et al. 2003; Chaerle et al. 2005; Casson and Gray 2008; Lau and Bergmann 2012). Stomatal length remains rather constant within species but is highly species-specific unlike stomatal densities, which vary strongly within species depending on external conditions (Ashton and Berlyn 1994; Richardson et al. 2001).

There are several leaf traits related to plant performance which could theoretically go along with changes in SPI. Li et al. (2015) found that leaf economics traits (e.g. specific leaf area (SLA), leaf nitrogen content) are relatively independent from hydraulic traits (e.g. stomatal traits) in a subtropical forest when comparing different species, yet little is known on their relations on an intraspecific scale. SLA is often positively related to relative growth rates across species and scales positively with mass-based light-saturated photosynthesis rates (Garnier 1992; Pérez-Harguindeguy et al. 2013). Since it is a function of leaf dry matter content and leaf thickness (Pérez-Harguindeguy et al. 2013), SLA should relate negatively to area-based light-saturated photosynthesis rates and SPI. A relationship of SPI with area-based leaf nitrogen (N_{area}) can be expected from the often found relationship of N_{area} and maximum photosynthesis rates (e.g. Wright et al. 2004). N_{area} is a good proxy for plant photosynthetic capacity, since most of

the nitrogen is located in enzymes of the Calvin cycle. There are strong positive species-specific linear relationships of leaf nitrogen and RubisCO, leaf nitrogen and chlorophyll concentration, leaf nitrogen and maximum leaf conductance as well as photosynthesis rates (Evans 1989; Bond et al. 1999; Wright et al. 2004) so we expect also a positive relationship of N_{area} to SPI. Area-based leaf carbon (C_{area}) is also related to leaf thickness which is known to increase with elevation and thus we expect a positive relation with SPI. The majority of carbon is located in structural compounds within a cell and mainly located in lipids, lignin, cellulose and proteins, whereas other components such as organic acids are low in carbon content or carbon-free (e.g. minerals; Poorter et al. 1992). The enzyme RubisCO discriminates against ^{13}C and favours ^{12}C as reactant. Photosynthetic carbon isotope discrimination ($\Delta^{13}\text{C}$) is low if either stomata are closed causing a strong gradient between internal (c_i) and external CO_2 concentration (c_a) of the leaves (i.e. it is a proxy for a plant's intrinsic water use efficiency) or if there is a high demand of CO_2 (Farquhar et al. 1989; Pérez-Harguindeguy et al. 2013). We expect a high SPI or high “potential conductance” to cause on average also a higher “realized conductance” and thus high c_i/c_a which should be reflected in high $\Delta^{13}\text{C}$ values.

With our study we aim at quantifying changes in the SPI with changing environmental conditions on different organizational levels (i.e. on the inter- and intraspecific level) and at unravelling correlations with hydraulic as well as leaf economic traits. We hypothesize that intraspecific patterns should follow the interspecific patterns, i.e. that SPI should decrease with increasing elevation in response to a decrease in temperature and partial pressure in CO_2 , irrespective of the organizational level considered. Accordingly, also trait–trait correlations should be consistent when analyzing them on the inter- and intraspecific level. The results of this research will lead to a better understanding of the factors that are captured by stomatal parameters (here: SPI) and they will further provide a more profound background on its use as a functional trait in further (macroecological) approaches. To approach our questions, we measured stomata density, stomatal length, SLA, area-based nitrogen and carbon concentration of the leaves as well as the carbon isotope discrimination on 22 herbaceous species along two elevational gradients. On a subset of

four species, light-saturated net photosynthetic rate at ambient CO_2 concentration (A_{sat}) and stomatal conductance (g_s) were measured to test our hypothesis that A_{sat} is related to SPI. Elevational gradients offer the possibility to explore adaptations to changes in abiotic environments over short spatial distances and represent important “natural experiments” to study ecological and evolutionary responses of plants since both mean annual temperatures as well as air pressure decline with increasing elevation (Körner 2007). There are studies comparing the changes in functional traits among species interspecifically and/or in closely related plant genera (e.g. Körner et al. 1986; Wright et al. 2004; Li et al. 2015) and on intraspecific variations in single traits (e.g. Rosbakh et al. 2015 for SLA variations) but little is known on changes in trait–trait correlations on inter- versus intraspecific levels along elevational gradients. More specifically, we addressed the following issues:

1. To see whether SPI changes with changing environmental conditions on an inter- and intraspecific scale, we analyzed its response along two elevational gradients for 22 herbaceous species.
2. In order to test the relation of SPI to other leaf functional traits, we analyzed its relation with SLA, N_{area} and C_{area} and $\Delta^{13}\text{C}$ both on an interspecific as well as intraspecific level.
3. To test whether SPI is related to photosynthesis rates, we measured A_{sat} to analyze its relation to SPI on a subset of four species.

Materials and methods

Study site and sample collection

Leaf samples were collected in the growing season of 2012 (end of April to mid-September) along two south facing elevational gradients near Garmisch-Partenkirchen in the Northern Limestone Alps (Mount Kramer and Mount Kreuzeck, hereafter referred to as “Kramer” and “Kreuzeck”) ranging from 700 to 1800 and from 800 to 1700 m a.s.l., respectively. We selected 22 herbaceous plant species growing on meadows or on forest edges and covering a wide elevational range (based on BERGWALD database, GIVD-ID EU-DE-002; Ewald 2012), all of them

displaying C_3 metabolism. Samples were collected every 100 m increase in elevation on altogether 22 sites as far as individuals were present at the given elevational belt [Online Resource 1 gives an overview of the species, nomenclature follows Wisskirchen and Haeupler (1998)].

For each species, three sun leaves of five replicate mature individuals per elevational belt were examined. One of them was used for stomatal imprints, the other two to determine SLA, N_{area} , C_{area} and $\Delta^{13}\text{C}$. Except for the differences in elevation, we always selected similar sites for a species (i.e. mid-meadow or forest edge) and we selected leaves from the same position within the individuals (constant within species) to minimize sampling bias. Plant functional traits (stomatal traits, SLA, N_{area} and C_{area} as well as $\Delta^{13}\text{C}$) were measured at the time of full flowering of the individual plants following standardized protocols (Pérez-Harguindeguy et al. 2013). In Online Resource 2, the changes of the plant functional traits with elevation are given.

Leaf trait measurements

Stomatal traits

Two imprints from the abaxial surface of sun leaves and one from the adaxial surface were taken using the clear nail polish method as described in Hilu and Randall (1984). For the species used in gas exchange measurements, exactly the same area as the cuvette was covering was taken for measurements. For the remaining species one of the leaves as described above was used for stomatal analysis. Samples were analyzed using a light microscope (Axiostar Plus, Carl Zeiss, Jena, Germany) equipped with a scale grid at 400-fold magnification. Stomata were counted in two fields of view per stomata imprint; guard cell length was measured on two closed stomata per field of view. Veins were omitted since stomata are absent there (Martin and Glover 2007). SPI (dimensionless) was calculated as proposed by Sack et al. (2003) as follows:

$$\text{SPI} = (\text{guard cell length})^2 \cdot \text{stomatal density} \quad (1)$$

Since stomatal densities and size (i.e. guard cell length) differ on abaxial and adaxial sides (compare also Online Resource 1, 3 and 4) both sides were

recorded separately and summed up afterwards to get a total value for SPI of the plant. SPI represents a relative measure of the ratio of total stomatal pore area to leaf area.

Specific leaf area

SLA is defined as the ratio of fresh leaf area to dry mass (Pérez-Harguindeguy et al. 2013). To determine SLA, two leaves of the same individuals (including petioles if present) were scanned (CanoScan LiDE110, Cannon, Tokyo, Japan) and subsequently dried to constant weight (96 h, 50 °C in a drying oven). Dry weight was recorded afterwards. Leaf area was calculated in R using the R-Package LeafTraits (Bernhardt-Römermann, unpublished).

Leaf nitrogen and carbon and $\Delta^{13}\text{C}$

All leaves previously used for determining SLA were pooled per site and species and ground using a ball mill. An amount of 0.2 mg of the milled material was weighed into tin capsules and combusted in an elemental analyzer (NA 1110, Carlo Erba, Milan, Italy) coupled to an isotope ratio mass spectrometer via a Conflow interface. Each sample was measured against CO_2 -working standard reference gas previously calibrated against secondary stable isotope reference material. After every 20th sample, a solid laboratory standard (wheat flour) was run to check for instrument drift and to assess the measurement precision ($\text{SD} \pm 0.06 \text{ ‰}$). Leaf nitrogen and carbon concentration as well as $\delta^{13}\text{C}$ were determined. Leaf nitrogen and leaf carbon per unit leaf area were calculated by dividing by SLA. The relative carbon isotope ratio ($\delta^{13}\text{C}$) was calculated as described in Farquhar et al. (1989). Since atmospheric $\delta^{13}\text{C}$ at the station Hohenpeißenberg at 35 km distance to the sampling locations varied during the sampling period from -8.43 ‰ (April 2012) to -7.85 ‰ (July 2012; <http://www.esrl.noaa.gov/gmd/dv/data/>), discrimination ($\Delta^{13}\text{C}$) was calculated according to Farquhar et al. (1989) from measured $\delta^{13}\text{C}$ in leaves and $\delta^{13}\text{C}$ in the atmosphere measured 1 month before the sampling of the leaf ($\Delta^{13}\text{C} = (\delta^{13}\text{C}_{\text{air}} - \delta^{13}\text{C}_{\text{sample}})/(1000 + \delta^{13}\text{C}_{\text{sample}}) \times 1000$) assuming that this was the main growth period of the leaf. $\Delta^{13}\text{C}$ is a time-integrated estimate of relative internal CO_2 concentration and

intrinsic water use efficiency (Farquhar et al. 1989; Pérez-Harguindeguy et al. 2013).

Gas exchange measurements

For a subset of four species (*Aposeris foetida*, *Knautia dipsacifolia*, *Mercurialis perennis* and *Trifolium pratense*), which frequently occurred at the study sites (for elevational range of the species, see Online Resource 1), g_s and A_{sat} were measured using Li-6400XT (LI-COR Bioscience, Lincoln, Nebraska). Irradiance was kept constant at $2000 \mu\text{mol m}^{-2} \text{s}^{-1}$, relative humidity at approximately 60 % and CO_2 concentration at 400 ppm. In August 2013, *K. dipsacifolia* and *T. pratense* were measured at 25 °C leaf temperature, in May 2014 *M. perennis* and *A. foetida* were measured at 17 °C leaf temperature due to outside weather conditions. Temperature was kept constant within a species across the elevational gradient. Measurements were conducted in the mornings (8–12 a.m.) and in the afternoons (2–6 p.m.) to avoid midday depression of photosynthesis. We optimized photosynthesis measurements for A_{sat} and g_s under the given conditions, but not for maximal conductance in a wider sense (for which we would have needed low CO_2 values) during measurements. In addition to that we did not correct for atmospheric pressure which decreases with elevation but chose a constant CO_2 concentration for all individuals to simplify the comparisons. Plants were allowed to adapt to the conditions within the cuvette for at least 10 min and measurements were taken in steady state.

Statistical analyses

In order to test whether SPI changes along the elevational gradients, we performed linear models using SPI as dependent variable and elevation, gradient (Kramer vs. Kreuzeck) and leaf side (adaxial vs. abaxial) as explanatory variables. To test for species-specific effects, we also included species alone and in interaction with elevation, leaf side and gradient and the interaction of elevation and gradient as well as of leaf side and gradient and of leaf side and elevation as covariates in these models. Full models were simplified using stepwise backward selection using ANOVA until the minimal adequate model was found as described in Crawley (2012). Model assumptions (normal distribution and constant variance in

residuals) were met in all cases. We also calculated the coefficient of variation for stomatal length and density for each species to quantify their variability.

To see whether changes in SPI are associated to changes in leaf traits we first set up four linear models using the species mean value of SPI as independent variable and the species mean values of SLA, N_{area} , C_{area} or $\Delta^{13}\text{C}$ as dependent variables. To test whether changes in SPI on the intraspecific scale follow the results of the interspecific scale, we performed four separate linear models using SLA, N_{area} , C_{area} or $\Delta^{13}\text{C}$ as dependent variable and SPI, species and the interaction thereof as independent variables. In these latter analyses, per species we included mean trait values and elevation.

To test whether SPI is a good measure for A_{sat} , we performed a linear model using A_{sat} as dependent variable and SPI, species and gradient (Kramer vs. Kreuzeck) and the two-way interactions thereof as explanatory variables. We also tested for the relation between A_{sat} and g_s using the same statistical method to see how this relation differs from the one derived from stomatal measurements. Full models were simplified following the approach described above.

All statistical analyses were computed in R 3.1.0 (R Development Core Team 2014).

Results

Changes in SPI along the elevational gradients

SPI varied with elevation, although its response was highly species-specific ($R^2 = 0.93$, $F_{86, 663} = 103.6$, $p < 0.001$; Fig. 1). Most of the species increased their SPI with elevation (e.g. *Centaurea jacea* and *Potentilla erecta*), although some species decreased their SPI (as for example *Buphthalmum salicifolium* and *Parnassia palustris*). Thus, the reaction of SPI along the elevational gradient was not uniform but depended on species, the gradient (Kramer or Kreuzeck), the interactions of elevation and species, species and gradient, elevation and leaf side as well as species and leaf side. Whereas size of stomata remained rather constant (mean coefficient of variation within species: 0.18), larger variations in densities (mean coefficient of variation within species: 0.63) existed. The mean coefficient of variation for total SPI was rather low (0.20) due to low variation of stomata size and a

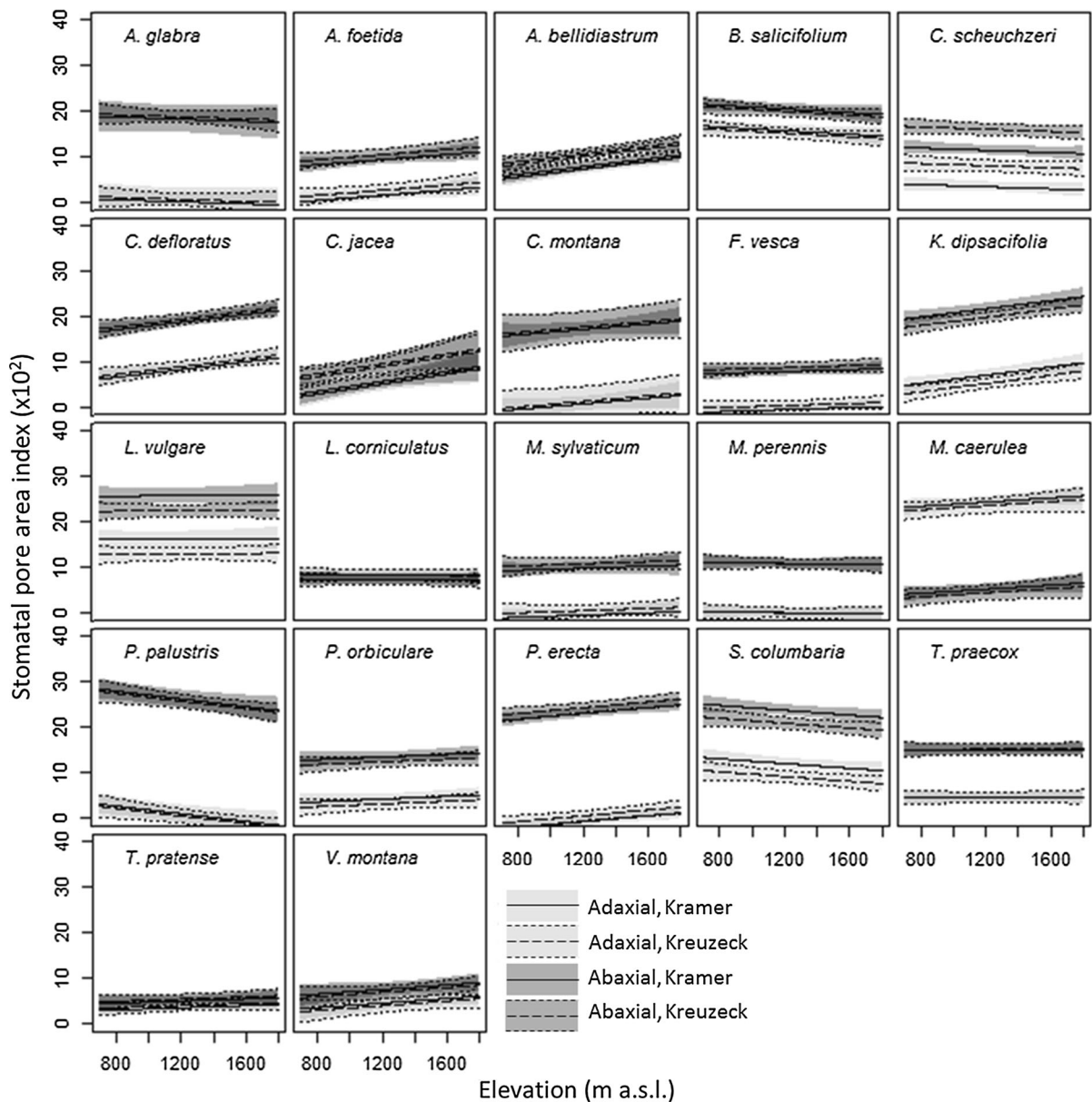


Fig. 1 The stomatal pore area index (SPI) as depending on elevation, gradient, species and leaf side ($R^2 = 0.93$, $F_{86,663} = 103.6$, $p < 0.001$). The 95 % confidence interval of

the regression is shown (adaxial in light grey and abaxial in dark grey). For abbreviations of species names see Online Resource 1

negative relation between stomatal size and density (see Online Resource 3 and 4).

Relation of SPI to other plant functional traits on inter- and intraspecific levels

There was no significant relation of the species mean values of SPI and $\Delta^{13}\text{C}$ ($R^2 = 0.01$,

$p = 0.64$, $F_{1, 20} = 0.23$). However, when analyzing the relation of SPI and $\Delta^{13}\text{C}$ including species-specific changes, we found mainly negative relations of SPI to $\Delta^{13}\text{C}$. The strength of the relationship between SPI and $\Delta^{13}\text{C}$ differed between species, but only *P. palustris* and *Scabiosa columbaria* showed a positive relation ($R^2 = 0.53$, $p < 0.001$, $F_{43, 347} = 9.15$; Fig. 2a). Results of

species-specific correlations of SPI and $\Delta^{13}\text{C}$ are given in the Online Resource 5.

The species mean of N_{area} and the species mean values of SPI showed no significant correlation ($R^2 = 0.00$, $p = 0.94$, $F_{1, 20} = 0.01$). The relationship between N_{area} and SPI became significant when including species-specific changes and differed in strength and direction between species ($R^2 = 0.67$, $p < 0.001$, $F_{43, 347} = 17.69$; Fig. 2b). Most of the species showed positive relationships between N_{area} and SPI on this intraspecific scale, However seven out of the 22 species, namely *C. jacea*, *Leucanthemum vulgare*, *Melampyrum sylvaticum*, *Molinia caerulea*, *Phyteuma orbiculare*, *S. columbaria* and *Thymus praecox* showed negative relationships. Results of species-specific correlations of SPI and N_{area} are given in the Online Resource 5.

There was a positive relation between the species mean values for SPI and C_{area} with a slope of 0.19 ($R^2 = 0.15$, $p < 0.1$, $F_{1, 20} = 3.51$). Also on an

intraspecific level, most species increased C_{area} with increasing SPI values, only *C. jacea*, *S. columbaria* and *T. praecox* showed a negative relationship between SPI and C_{area} ($R^2 = 0.79$, $p < 0.001$, $F_{43, 347} = 30.98$; Fig. 2c). Results of species-specific correlations of SPI and C_{area} are given in the Online Resource 5.

SLA was negatively related to SPI on both, the inter- ($R^2 = 0.19$, $p < 0.001$, $F_{1, 20} = 4.58$) and intraspecific scale. Intraspecifically, there was only a species-effect on the intercept but not on the slope, i.e. all species decreased SLA with increasing SPI ($R^2 = 0.73$, $p < 0.001$, $F_{22, 368} = 44.56$; Fig. 2d). However, the strength of the relationship between SPI and SLA differed on the inter- and intraspecific scale (slope of -0.33 vs. -0.27 respectively). Results of species-specific correlations of SPI and SLA are given in the Online Resource 5.

Table 1 gives a summarizing overview on all trait–trait correlations with respective species effects.

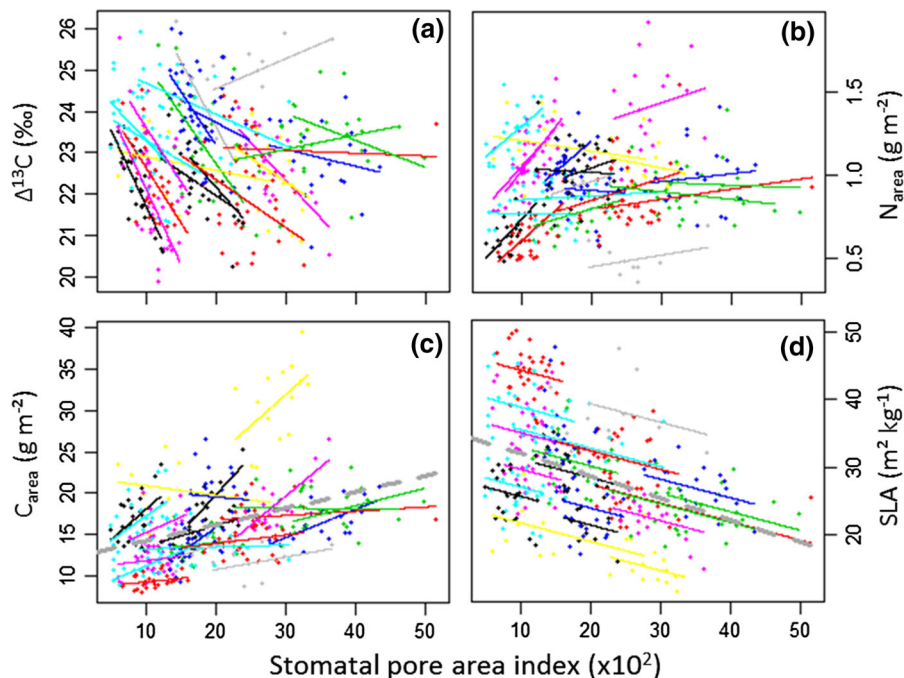


Fig. 2 Changes in values of stomatal pore area index (SPI) in relation to selected functional traits as dependent on species (colours are representing species, each line is the regression line of one species) and on species mean values of the traits (*dashed grey line*, drawn if relations were significant). SPI in relation to **a** $\Delta^{13}\text{C}$ as depending on species ($R^2 = 0.53$, $p < 0.001$, $F_{43, 347} = 9.15$), n.s. for species mean values, **b** leaf nitrogen

(N_{area}) as dependent on species ($R^2 = 0.67$, $p < 0.001$, $F_{43, 347} = 17.69$), n.s. for species mean values, **c** leaf carbon (C_{area}) as dependent on species ($R^2 = 0.79$, $p < 0.001$, $F_{43, 347} = 30.98$) and on species mean values ($R^2 = 0.15$, $p < 0.1$, $F_{1, 20} = 3.51$) and **d** specific leaf area (SLA) as dependent on species ($R^2 = 0.73$, $p < 0.001$, $F_{22, 368} = 44.56$) and species mean values ($R^2 = 0.19$, $p < 0.001$, $F_{1, 20} = 4.58$)

The relationship between SPI, A_{sat} and g_s

SPI, species, gradient (Kramer and Kreuzek) and the interaction of species and gradient had significant effects on A_{sat} ($R^2 = 0.57$, $F_{8, 380} = 64.15$, $p < 0.001$; Fig. 3). Overall, we found a positive relation of SPI to A_{sat} . The relation of SPI with g_s was similar to that of SPI with A_{sat} although the latter relationship was slightly stronger. g_s , species and the interactions of g_s and species as well as species and gradient had a significant influence on A_{sat} ($R^2 = 0.69$, $F_{11, 379} = 77.36$, $p < 0.001$, see Online Resource 6). The relation of g_s and SPI was weak and even differed in direction between species ($R^2 = 0.58$, $F_{12, 376} = 43.35$, $p < 0.001$) which might have been, however, due to sampling protocols. For the relation of A_{sat} to elevation see Online Resource 6.

Discussion

The current study used SPI as an integrated measure of stomata density and guard cell length, both important in regulating gas exchange in plant species. SPI did not only differ in magnitude between species but it also varied along elevation in a species-specific manner. Whereas for most of the species (64 %) SPI increased with increasing elevation, for some species (32 %) we could even report a decreasing SPI. Accordingly, relationships between SPI and leaf traits were most obvious on an intraspecific level and differed between inter- and intraspecific scale which may have important consequences also for macroecological and modelling studies. On a subset of four species we could confirm that the SPI is related to changes in photosynthesis along elevational gradients, though this relationship seemed to be determined by differences between species-specific changes in A_{sat} going along with changes in SPI.

Changes in SPI along elevational gradients

The changes of SPI along the elevational gradient were mainly induced by changes in stomatal densities, whereas stomatal size remained comparably constant within species. Increases in stomatal density has previously been attributed to higher irradiance and shorter growth phases of the plants and lower CO_2 concentrations (due to a decrease of atmospheric

Table 1 Statistical parameters of the models for leaf traits depending on SPI based on species mean values ('inter') and models including means per elevation and species as a factor ('intra')

Organizational level	$\Delta^{13}\text{C}$		N_{area}		C_{area}		SLA	
	Inter	Intra	Inter	Intra	Inter	Intra	Inter	Intra
SPI	$R^2 = 0.01$ n.s. $F_{1, 20} = 0.23$	$R^2 = 0.53^{***}$ $F_{43, 347} = 9.15$	$R^2 = 0.00$ n.s. $F_{1, 20} = 0.01$	$R^2 = 0.67^{***}$ $F_{43, 347} = 17.69$	$R^2 = 0.15^+$ $F_{1, 20} = 3.51$	$R^2 = 0.79^{***}$ $F_{43, 347} = 30.98$	$R^2 = 0.19^{***}$ $F_{1, 20} = 4.58$	$R^2 = 0.73^{***}$ $F_{22, 368} = 44.56$
Species	n.s.	*	n.s.	***	***	***	***	***
SPI: species	–	***	–	***	–	***	–	n.s.

Significant results are displayed with bold letters
Significances are indicated as *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ and $^+ p < 0.1$

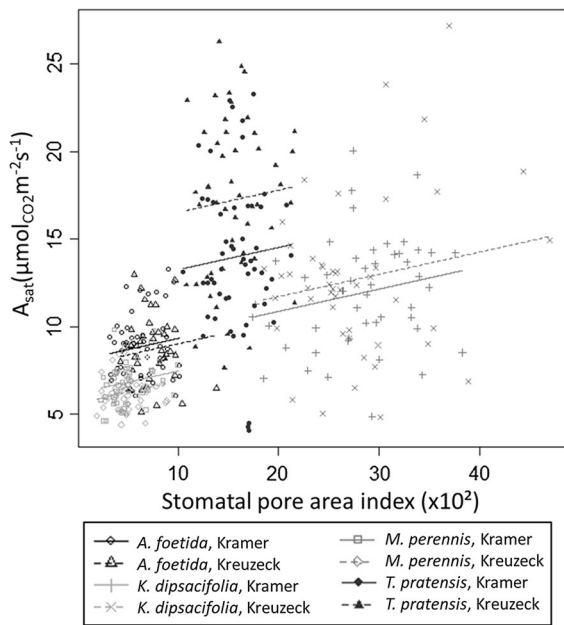


Fig. 3 Relation of the stomatal pore area index (SPI) to maximum light saturated net photosynthetic rate at ambient CO_2 concentration (A_{sat} ; $R^2 = 0.57$, $F_{8, 380} = 64.15$, $p < 0.001$). Colours represent species, *solid lines* represent Kramer gradient and *dashed lines* represent Kreuzeck

pressure) in high compared to low elevational sites (Körner et al. 1986; Körner 2003). In addition, Nogués et al. (1999) argued that higher UV-B radiation limits stomatal opening and increases densities. Since UV radiation increases with elevation, this would favour higher stomatal densities in higher elevation. Changes in stomatal densities are known to show variable trends in the reaction to elevation and are therefore thought to depend rather on local conditions such as light availability and moisture content than on elevation itself (Körner et al. 1986; Qiang et al. 2003). However, we had only chosen individuals from a similar environment for a certain species to minimize this source of variation, so we assume that this cannot be the essential reason for this highly species-specific pattern.

Relations of changes in SPI with changes in other leaf traits

The higher $\Delta^{13}\text{C}$ values are, the higher is the CO_2 concentration in the substomatal cavity (high c_i/c_a) which consequently leads to a decline in water use

efficiency (Farquhar et al. 1989; Guehl et al. 1994, 1995; Osório et al. 1998). As $\Delta^{13}\text{C}$ and thus c_i/c_a decreased with increasing SPI, relative substomatal CO_2 concentration decreased the larger the total pore area per leaf area became. This unexpected pattern was especially visible on the intraspecific level, but was covered when analyzing species mean values, maybe due to the different intensities of species, with which $\Delta^{13}\text{C}$ decreased with an increase in SPI, hiding the overall trend. However, a low c_i/c_a can be attributed to low stomata opening or alternatively, to high photosynthesis rates. The negative correlation between SPI and c_i/c_a may also indicate that an increase in photosynthesis rates was responsible for the decrease in c_i/c_a with elevation rather than a decrease in stomata opening. This also relates to the fact that N_{area} increased and that SLA consistently decreased when SPI increased in most of the species. The decrease in $\Delta^{13}\text{C}$ with increasing SPI was especially pronounced for species with lower SPI values, whereas for species possessing high SPI values there seemed to be little change. Also for N_{area} , low-SPI species showed a stronger reaction than high-SPI species which supports our explanation. High SPI-species are less limited by stomatal restrictions of gas exchange and thus show no clear pattern. Interestingly, the regression between SPI and $\Delta^{13}\text{C}$ was not significant for species mean values highlighting the fact that this relation is not universal but depends on species.

The positive relation of N_{area} and SPI in most species is in line with our hypothesis, since previous studies showed that there was a positive relation of leaf nitrogen to photosynthesis rates and stomatal conductance (Evans 1989; Bond et al. 1999) as leaf nitrogen is predominantly located in the proteins of the Calvin cycle and thylakoids (Evans 1989). However, despite this very clear relation on the intraspecific level, we could not detect any relationship on the interspecific scale. This supports the findings of Li et al. (2015) who clearly showed for subtropical forest trees and shrubs that leaf nitrogen (as a leaf economics trait) and stomata density (as a hydraulic trait) are decoupled on an interspecific level and with Evans (1989) who also found differences between species in the relation of photosynthesis rates and leaf nitrogen. This highlights again the importance of investigating trait–trait correlations on intraspecific levels as well for a better understanding of eco-physiological relationships and

processes that may remain hidden in simple trait–trait correlations based on species mean values.

The detected positive relation of SPI and C_{area} on the intraspecific scale is in accordance with the negative relation of SLA and SPI as high-SLA species usually possess low C_{area} values due to thinner and less dense leaves (Pérez-Harguindeguy et al. 2013). The same relation between SPI and C_{area} was found when looking at species mean values although this latter relation was weaker.

SLA was the only trait for which comparable relationships to SPI were detected on both the intra- and interspecific scale. SLA as the ratio of leaf area to dry weight, is by definition mainly influenced by leaf thickness, i.e. leaves with low SLA values are thicker and/or denser per unit area than leaves with higher SLA (Pérez-Harguindeguy et al. 2013). Since no differences in the reaction on the interspecific compared to the intraspecific level could be detected, the relation between SPI and SLA seems to be the most robust relationship of the ones examined.

The detected discrepancies in trait–trait correlations on the intra- versus interspecific levels illustrate that our general understanding of the association of the traits does not apply on every organizational level. Even in the case of the relations between SPI and C_{area} or SLA, which could be detected on both scales, the use of species means led to different slopes on both levels. This highlights the fact that caution should be paid when transferring rules from one scale to the other but it may also lead to a better understanding of trait–trait relations with respect to plant functioning. The different plant ecological strategies which are reflected by the high variability on an intraspecific scale could facilitate species coexistence, increase species diversity and enhance ecosystem stability (Kraft et al. 2008; Adler et al. 2013; Pillar et al. 2013; Li et al. 2015).

Relations between SPI and A_{sat}

The relationship between SPI and A_{sat} was weak. The relationships of the two factors combined in SPI, namely stomatal density and stomatal size, to A_{sat} were even insignificant (analysis not shown). This weakness of the relationships may be due to plants which have recently suffered from stress. This would not be reflected in a low SPI but might have decreased A_{sat} and g_s values. Also leaf ageing could be a possible

explanation for decreases in A_{sat} which would not be reflected in the SPI that remains stable over leaf age (once leaves are fully developed); yet with the choice of measuring only plants at full flowering we tried to cope with this problem. Although temperatures differed between species due to sampling campaigns in two different seasons, temperature was kept constant within species. This makes the relationships comparable on an intraspecific scale. Since atmospheric pressure declines with increasing elevation (by around 1 % for every 100 m of elevation) and depends on local weather conditions, we calculated internal CO_2 partial pressures based on the atmospheric pressure measured in parallel to photosynthesis rates and found that they also decrease with elevation which has negative effects on photosynthesis rates (Körner and Diemer 1994; Körner and Pelaez Menendez-Riedl 1989; Körner 2003). However, previous studies showed that reduced partial pressure of CO_2 is partially compensated by faster diffusion through stomata and intercellular spaces in the “thinner” air (at constant temperatures) and by the oxygenase activity of RubisCO and thus photorespiration is also reduced by the simultaneous decline of O_2 partial pressure (Körner and Diemer 1994; Körner 2003). In addition to that, a screening of alpine and lowland plants measured at equal partial pressures revealed that alpine plants display higher photosynthesis rates at the same internal CO_2 partial pressures than lowland plants and lower internal CO_2 partial pressures at the same external CO_2 partial pressures due to a higher specific capacity to fix CO_2 via greater mesophyll thickness and N_{area} (Körner and Diemer 1994; Körner and Pelaez Menendez-Riedl 1989; Körner 2003) which we could confirm as $\Delta^{13}\text{C}$, C_{area} and SLA decreased due to increased leaf thickness and N_{area} increased along the elevational gradient (see Online Resource 2).

The finding that SPI is a weak yet significant proxy of A_{sat} is especially of interest for studies focussing on large species sets being carried out in remote areas, as the method of measuring stomata is easier, cheaper and less time consuming than measuring photosynthesis rates themselves. However, more studies are needed to establish a clearer relationship between A_{sat} and SPI including a larger species set to confirm our results based on this limited data set.

Genetic variability as well as common garden or greenhouse experiments under controlled conditions

could be executed scrutinizing our results and further investigating the reasons for this highly species-specific performance. Overall, we were able to show differences in adaptations in SPI along the elevational gradients and in relation to other plant functional traits. The relation of SPI to SLA was the most consistent displaying no difference when comparing the relation between and within species. Trait databases, which report only species mean values, cannot capture that trait–trait relations also differ on the intraspecific level in addition to the interspecific level which has implications for modelling work.

Acknowledgments The authors would like to thank the members of the Institute of Botany at the University of Regensburg, especially Patrizia König, Nina Berndt, Désirée Dotter, Melanie Hahn, Günther Kolb, Stefanie Meier, Sabine Moll, Sergey Rosbakh and Jessica Rossow for practical assistance and valuable discussions. We would also like to thank Thijs Pons and Manfred Küppers as well as two anonymous reviewers for valuable advice and comments on the manuscript. Rudi Schäufele at Technische Universität München in Freising carried out the isotope measurements. This work was supported by the Elitförderung des Landes Bayern via provision of a scholarship (granted to SFB) and the University of Regensburg. We also acknowledge support from the Bavarian State Forest Enterprise and the district government of upper Bavaria. CR acknowledges financial support of the DFG (RO3842/3-1) and the DFG-Research Centre for integrative Biodiversity Research (iDiv).

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