Research Papers

Title

Morphological, biochemical and physiological traits of upper and lower canopy leaves of European beech tend to converge with increasing altitude

Running Headline

Upper and lower leaves converge with altitude

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Summary

The present work has explored for the first time acclimation of upper versus lower canopy leaves along an altitudinal gradient. We tested the hypothesis that restrictive climatic conditions associated with high altitudes reduce within-canopy variations of leaf traits.

The investigated beech (*Fagus sylvatica* L.) forest is located on the southern slope of the Hrubý Jeseník Mountains (Czech Republic). All measurements were taken on leaves from upper and bottom parts of the canopy of mature trees (>85 years old) growing at low (420 m a.s.l.), middle (720 m a.s.l.), and high (1100 m a.s.l.) altitudes.

Compared to trees at higher altitudes, those growing at low altitudes had lower stomatal conductance, slightly lower CO_2 assimilation rate (A_{max}) and leaf mass per area (LMA), and higher photochemical reflectance index, water use efficiency, and Rubisco content. Given similar stand densities at all altitudes, the different growth conditions result in a more open canopy and higher penetration of light into lower canopy with increasing altitude. Even though strong vertical gradients in light intensity occurred across the canopy at all altitudes, lower canopy leaves tended at high altitudes to acquire the same morphological, biochemical and physiological traits as did upper leaves. While elevation had no significant effect on nitrogen and carbon contents per unit leaf area, LMA, or total content of chlorophylls and epidermal flavonoids in upper leaves, these increased significantly in lower leaves at higher altitudes. The increases in N content of lower leaves were coupled with similar changes in A_{max}. Moreover, high N content coincided with high Rubisco concentrations in lower but not in upper canopy leaves.

Our results show that the limiting role of light in lower parts of the canopy is reduced at high altitudes. A great capacity of trees to adjust the entire canopy is thus demonstrated.

Key-words:

altitudinal gradient, CO_2 assimilation, flavonoids, light environment, leaf stoichiometry, LMA, Rubisco

Introduction

Climatic variation along altitudinal gradients provides an excellent and natural experimental set-up for investigating the possible impacts of climate change on terrestrial organisms and ecosystems (Körner 2007, DeFrenne et al. 2013). There are four primary atmospheric changes associated with altitude: decrease in partial pressure of gases, reduced temperature, reduced clear-sky turbidity, and higher fraction of ultraviolet radiation and precipitation. In contrast, wind velocity, soil conditions, and season length may not generally be related to altitude and may depend upon, among other things, slope orientation, topology and/or region (reviewed in Becker et al. 2007, Körner 2007).

In addition to studies on species distribution and composition of plant communities (Halbritter et al. 2013, Read et al. 2014), genomic divergence (Chapman et al. 2013), and interactions between host plant and herbivores or fungal pathogens (Hodkinson 2005), attention has also been given to the acclimation of morphological, biochemical and physiological traits of plants along an altitudinal gradient (e.g., Sakata et al. 2006, Kumar et al. 2008, Guerin et al. 2012). While these studies have focused mainly on herbaceous species and agricultural crops, possible differences in acclimation of leaves across the vertical profile of the forest canopy to growth conditions had not been studied.

Studies on deciduous forest and herbaceous species have shown an increase of leaf mass per area (LMA) and leaf nitrogen content per unit area with increasing altitude (Williams et al. 1995, Song et al. 2012). Other studies have reported increases in stomatal density, stomatal conductance, and light-saturated rate of CO_2 assimilation with increasing altitude (Hultine and Marshall 2000, Vats et al. 2009). Moreover, the maximum rates of Rubisco carboxylase activity and of photosynthetic electron transport have been shown to be higher for leaves from plants grown at high altitudes than for those grown at low altitudes (Fan et al. 2011), even as the activities of other enzymes associated with carbon assimilation have not shown significant differences with changing altitude (Kumar et al. 2008).

An exponential attenuation of solar radiation passing through a canopy leads to distinct light intensity across a vertical canopy profile. Leaves acclimate to their light environments by (i) modulation of leaf morphology, anatomy, and chloroplast ultrastructure (Boardman 1977, Lichtenthaler et al. 1981, Kubiske and Pregitzer 1997, Yano and Terashima 2001), and (ii) changes in their chemical composition, including in particular reallocation of nitrogen between photosynthetic components associated with light capture, thylakoid membrane composition, and CO_2 assimilation (Sims and Pearcy 1994, Eichelmann et al. 2005, Hikosaka 2005, Lichtenthaler et al. 2007). The thicker upper canopy leaves are characterized by lower water content, higher total chlorophyll and total carotenoid content per leaf area unit, as well as higher values for the Chl a/b ratio compared to the much thinner lower canopy leaves (Lichtenthaler et al. 2007). While upper leaves have higher rates of light-saturated CO_2 assimilation, which are associated with higher Rubisco content and stomatal conductance, lower leaves more effectively utilize low light intensities (Sims and Pearcy 1994, Urban et al. 2007). Lower canopy leaves play an important role in whole-canopy carbon fixation, particularly during cloudy days with prevailing diffuse radiation but also during hot sunny days when the stomatal conductance, CO_2 uptake and light-use efficiency of the uppermost sunlit leaves may be reduced (Urban et al. 2012a, Niinemets 2014a). It is not clear, however, how distinct growth conditions associated with different altitudes affect the vertical distribution and within-canopy variation of leaf traits.

Our main objective was to study the plasticity and possibly different acclimation of upper and lower canopy leaves along the altitudinal gradient. To the best of our knowledge, no comprehensive study had yet been undertaken on how the morphological, biochemical and physiological traits of upper and lower canopy leaves are affected by altitudinal gradient. Therefore, we aimed to investigate the within-canopy variations in leaf structure (LMA), biochemistry (elemental stoichiometry; flavonoid, chlorophyll and Rubisco content) and functioning (CO_2 assimilation rate, stomatal conductance, photochemical reflectance index) of European beech (*Fagus sylvatica* L.) grown in a forest with prevailing beech abundance at three different altitudes. The altitudinal experiment was designed to test a hypothesis predicting that canopies respond to changing climate by altered structure that may subsequently lead to reduced within-canopy variations of morphological, biochemical and physiological leaf traits at high altitudes. Since the asymmetrical acclimation of upper and lower canopy leaves has the potential to cause a substantial change in the photosynthesis of forest canopies, this is a key issue concerning altitudinal adaptations in plant ecophysiology.

Materials and methods

Site description

The forest stand selected for this study is located on the southern slope of Mravenečník Mountain (Hrubý Jeseník Mountains, 50°2' N, 17°9' E, Czech Republic). Leaf sampling and physiological measurements were done on European beech (*Fagus sylvatica* L.) trees naturally occurring at low (L; 420 m a.s.l.), middle (M; 720 m a.s.l.), and high (H; 1100 m a.s.l.) altitudes. As calculated from 30 years of data for L, M, and H altitudes, respectively, the individual sites are characterized by gradients in mean annual air temperature (7.59, 5.94, and 3.82 °C) and mean annual sum of precipitation (753, 891, and 1083 mm). The mean monthly temperatures (2 m above the soil surface) and monthly sums of precipitation during the investigated season (2013) are shown in Fig. 1. Both meteorological parameters were measured automatically in open areas close to the investigated plots (up to 200 m distant).

Characteristics of the forest stands investigated are summarized in Table 1. A stand with mature trees (>85 years old) was selected at each altitude. The stand densities were 638, 772, and 763 trees ha^{-1} at L, M, and H altitudes, respectively. L trees had larger diameter at breast height and total tree height, basal area index, and leaf area index as compared to M and H trees. Despite similar stand density, such a structure of forest stands resulted in higher penetration of solar radiation at higher elevations as compared to low ones (Table 1). Although long-term measurements of photosynthetic photon flux (PPF) within the experimental stands could not be performed, PPF was recorded using an LAI-2200 (Li-Cor, USA) with a quantum sensor (LI-190) above the canopy and at the level of the investigated lower canopy leaves/branches. Data were collected at maximum solar elevations (10:00–14:00 LMT). Upper canopy foliage was exposed to a maximum PPF of 1500–2000 μmol m^{-2} s^{-1} irrespective of the elevation, whereas lower canopy foliage received up to 150 (H), 105 (M), and 85 (L) μmol m^{-2} s^{-1} PPF on sunny days.

Two soil samples (0–20 cm depth) were taken at each of 13 trees (at a distance ca 1 m to the west and to the east from the tree’s base) at each altitude. Soil characteristics were estimated on 2 mm fraction. Atomic-absorption spectroscopy was used to assess Ca^{2+}, Mg^{2+} and K^+ content. Content of P was determined spectrophotometrically as a molybdate–phosphate complex and total N by distillation after mineralization (Kjeldahl technique). Soil organic carbon was determined by weight loss on ignition at 530 °C. Soil elemental concentrations and stoichiometry of the plots investigated are summarized in Table 2.

Physiological measurements and sampling procedures

We evaluated biochemical and physiological parameters in leaves from the uppermost and lowest canopy layers of the beech trees. Measurements were carried out on 13 representative trees from each altitude. Two leaves per tree and canopy layer with SSW orientation were investigated. Branches with desired leaves were cut from the trees. The cut end of each branch was immediately recut under water to remove xylem embolisms and kept in water during the measurements. All branches were taken from healthy trees showing no signs of damage.

Approximately 0.06 g of leaf fresh weight was sampled for analysis of Rubisco (ribulose-1,5-bisphosphate carboxylase/oxygenase) enzyme content. After determining the projection leaf area using a portable leaf area meter (Li-3000A, Li-Cor, USA), the samples were immediately frozen in liquid nitrogen. Rubisco content was determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using a Mini-PROTEAN 3 system (Bio-Rad, USA), as described by Urban et al. (2012b), using purified Rubisco protein (Sigma-Aldrich) as a standard. The quantification of individual bands was performed on an HP Scanjet 5590P running the program Advanced Image Data Analyzer, version 3.23.001 (Raytest, Germany).

The elemental analyses of C and N were made using an automatic analyser (Flash 2000, Thermo Scientific, USA). Leaf samples for elemental analyses (ca 100 mg) were stored in liquid nitrogen after determination of projected leaf area. Before analysis, each sample was dried to a constant mass in a drying oven (80 °C) for ca 2 days. The leaf mass per area (LMA) ratio was defined as the ratio between leaf dry mass and projected leaf area. Leaf moisture (Lm) in leaf samples was calculated as the ratio of leaf fresh minus dry weight divided by leaf dry weight.

Light-saturated rates of CO_2 assimilation (A_{max}) and stomatal conductance (G_{Smax}) were determined under ambient CO_2 concentration (385 ± 5 μmol mol^{-1}) and constant microclimatic conditions (leaf temperature: 25 ± 1 °C, relative air humidity: 55 ± 3%) using a Li-6400XT gas exchange system (Licor, Lincoln, NE, USA). Constant saturating irradiance (1200 μmol m^{-2} s^{-1}) was provided by LED light source of a LI-6400-02B (Li-Cor, Lincoln, NE). In our previous studies (Lichtenthaler et al. 2007, Urban et al. 2007) we had shown that such PPF is sufficient to saturate CO_2 assimilation rate in upper canopy leaves of many tree species, while it has no photoinhibitory effects on lower canopy leaves. The *in vivo* contents of epidermal flavonols and chlorophylls (Chl a+b) were determined by Dualex 4 Flav (Force-A, Orsay, France). Leaf reflectance spectra were measured in the wavelength range 350–2500 nm using a FieldSpec 4 HiRes spectroradiometer (ASD Inc., Boulder, CO, USA) coupled with leaf clip reflectance probe (ASD Inc.). Three reflectance spectra per leaf were taken. The photochemical reflectance index (PRI) was subsequently derived. This index expresses an association with photosynthetic light use-efficiency and it is defined as the ratio of reflectance (R) at 531 and 570 nm wavelengths: PRI = (R531 − R570) / (R531 + R570) (Gamon et al. 1992, Peñuelas et al. 1995).

All measurements and samplings were done during the extended noon period (10:00–14:00 LMT) and at two stages of growing season, the first characterized by active growth (9–10 July) and the second by early senescence (16–17 September).

Statistical analyses

Before the analysis of variance, the normality of data for individual parameters was tested using the Kolmogorov–Smirnov test for normality. A two-way fixed-effect ANOVA model was used for the general analysis of altitude and leaf position effects (see Supplementary Table 1).

To compare the data within graphs, a two-way ANOVA followed by a multiple range test was performed to investigate the effects of altitude and leaf position within canopy on biochemical, physiological, and morphological parameters. Tukey's post-hoc ($P < 0.05$) test was used. All statistical tests were done using Statistica 12 (StatSoft, Tulsa, USA).

Results

Leaf structure and C:N stoichiometry

The vertical position of a leaf within the canopy had a major effect on LMA and Lm (Supplementary Table 1). Significantly lower LMA values were found in lower as compared to upper canopy leaves. While the LMA values of upper leaves did not differ with altitude, LMA values of lower leaves increased with rising altitude. This pattern was similar for both measurement dates (Fig. 2a). Lm values were significantly higher in lower than in upper canopy leaves at all altitudes and in both seasons. Lm of lower leaves tended to decrease with altitude whereas Lm of upper leaves increased with altitude (Fig. 2b).

Both nitrogen (N_{area}) and carbon (C_{area}) content per unit leaf area were closely related to LMA ($R^2 = 0.926$ and 0.996 for N_{area} and C_{area}, respectively; $P < 0.01$; data not shown). The content of N_{area} and C_{area} as well as the C:N ratio were higher in upper than lower canopy leaves in both sampling periods, but these markedly increased with altitude in lower canopy leaves (Fig. 3a–d). Accordingly, differences in C:N ratio (Fig. 3e,f) between uppermost and bottom leaves tended to be smaller or to disappear at higher altitudes (M and H) while large within-canopy differences were observed at low altitude (L).

Effect of altitude on CO_2 assimilation and stomatal conductance

The altitudinal patterns of A_{max} and G_{Smax} changed during the season (Fig. 4a–d). While in July the highest A_{max} and G_{Smax} values were achieved at the middle altitude (M), in September both parameters gradually increased with rising altitudes. These differences were not statistically significant, however. Upper canopy leaves showed higher A_{max} compared to lower canopy leaves in both sampling periods and at all altitudes. The $A_{\text{max}}/G_{\text{Smax}}$ ratio (Fig. 4e,f) – also referred to as intrinsic water use efficiency – was not influenced by leaf position within the canopy, but it did show gradual decrease with rising altitude.

The close relationships ($R^2 = 0.51$; $P < 0.01$; calculated for the whole dataset) between A_{max} and G_{Smax} (Fig. 5) revealed that changes in CO_2 assimilation relate to changes in stomatal conductance. Distinct relationships were found for upper and lower canopy leaves, however, thus reflecting also the effect of leaf structure and biochemical composition on CO_2 assimilation rate. Furthermore, a clear decrease in the asymptote of this relationship, represented by the parameter a in the hyperbolic function applied (Fig. 5), was found at the end of summer.

Lower PRI values, associated with photosynthetic light-use efficiency, were observed in upper as compared to lower canopy leaves all through the vegetation season (Fig. 6). Particularly in September (Fig. 6b), PRI tended to decrease in both upper and lower leaves with increasing altitudes.

N partitioning and CO_2 assimilation

Rubisco content per unit leaf area ($\text{Rubisco}_{\text{area}}$) was significantly higher in upper than lower canopy leaves at all altitudes. $\text{Rubisco}_{\text{area}}$ tended to decrease with altitude in upper leaves while slightly increasing in lower canopy leaves. These patterns were more pronounced in July (Fig. 7; Supplementary Table 1). The relationship between N_{area} and $\text{Rubisco}_{\text{area}}$, analysed for the whole dataset, shows exponential growth (Fig. 8). However, different relationships can be recognized for individual altitudes. Particularly at high N_{area} contents ($N_{\text{area}} \geq 2.0$ g m^{-2}), which are typical for upper canopy leaves, a lower amount of nitrogen is allocated to Rubisco at high as compared to low altitudes. This is reflected in the higher value of the b exponent in the exponential model applied (Fig. 8).

The relationship between A_{max} and $\text{Rubisco}_{\text{area}}$ shows relatively high variation (caused by G_{Smax}), and relationships for individual altitudes can be differentiated (Fig. 9). At the lowest altitude, the A_{max} values reached the asymptotic level at relatively lower concentrations of Rubisco as compared to the M and H altitudes (reflected by the lower parameter a in the exponential model applied).

Total chlorophyll content estimated in upper and lower canopy leaves tended to converge at high altitude in both periods (Fig. 10a,b). Generally, leaves from the canopy bottom show lower content of epidermal flavonols than do the upper leaves (Fig. 10c,d). Moreover, flavonol content determined by fluorescence technique tended to increase with altitude in lower canopy leaves while no such differences were found in upper leaves. A close link between carbon metabolism and the accumulation of flavonols has been revealed by the relationship between carbon content in leaves and epidermal flavonols (R^2 = 0.88–0.89; data not shown). This relationship showed the asymptotic nature of flavonol accumulation at C_{area} above 30 g m^{-2}. The relationship was slightly shifted by time, with higher content of flavonols occurring in the later season. The nitrogen balance index (NBI), calculated as the ratio of chlorophylls to flavonoids, decreased with altitude in lower canopy leaves while remaining relatively constant in upper leaves (Fig. 10e,f).

Discussion

At similar tree density per hectare, the different growth conditions along the altitudinal gradient resulted in a more open canopy at high altitudes. This was reflected in lower LAI values and subsequently increased penetration of light into the canopy (Table 1). Similarly, Lowman (1986) had reported that warm temperate forests have higher LAI as compared to cold temperate forests and that this results in lower transmission of light through the canopy (5.2% versus 7.5%). Canopy structure thus has a key effect on the penetration of solar beams into lower canopy depths. At highest sun elevations, the lower canopy leaves of H, M, and L trees investigated received up to 150, 105, and 85 μmol m^{-2} s^{-1} PPF, respectively, whereas the uppermost leaves were exposed to a maximum PPF of 2000 μmol m^{-2} s^{-1} in clear sky conditions at all altitudes. The crowns of all trees were thus considerably differentiated into a sunlit and a shaded part at all altitudes investigated.

Our results show great capacity of *F. sylvatica* trees to adjust the morphological, biochemical and physiological traits of the entire canopy. We found evidence supporting the hypothesis that the climatic conditions along the altitudinal gradient modulate the structure of forest canopies and thereby alter the local light environment. In particular, the limiting role of low light intensities is pronounced under the favourable climate conditions of low altitudes. A less limiting role of light was meanwhile observed under climate-limiting conditions of high altitudes, where the canopies achieve lower LAI values (Table 1). As discussed below, such asymmetrical acclimation resulted in a convergence of morphological, biochemical and physiological traits of upper and lower canopy leaves with increasing altitude.

Leaf mass area (LMA) and leaf N stoichiometry

It has been reported that LMA and N_{area} increase with altitude in some functional groups like forbs and angiosperm trees but do not vary in conifers (Williams et al. 1995, Read et al. 2014). Our results for *F. sylvatica* show increasing LMA, N_{area}, and C_{area} with rising altitude in lower canopy leaves but not in upper leaves (Figs 2 and 3). To the best of our knowledge, such an asymmetrical response has not previously been reported.

LMA is significantly modulated by, among other factors, light intensity, temperature and nutrient availability (Poorter et al. 2009), i.e., by variables that decrease with canopy depth and altitude. Higher transmittance of light into lower canopy depths at high altitudes (Table 1) is likely the most important reason for increased LMA of lower canopy leaves. In addition, however, developmental constraints on high-elevation plants may decouple leaf N content from soil N content due to restricted root activity at low temperatures (Pregitzer et al. 2000) or the dilution of N and other nutrients in leaf tissues may be inhibited due to restricted growth (Körner 1989, 2007). These hypotheses are supported, respectively, by the relatively low differences of N_{area} values in leaves (Fig. 3) which are in contrast to the large differences in total N content in soil across the altitudinal gradient (Table 2) or by higher N_{area} values in lower canopy *F. sylvatica* leaves at high elevations (Fig. 3). Finally, the decrease in temperature with higher altitudes may additionally contribute to an increase in LMA of lower canopy leaves. For example, Atkin et al. (2006) had noted that lowland *Plantago* species grown at low temperatures increased LMA. This was associated with increased photosynthetic capacity, thus demonstrating cold acclimation of lowland species.

Accumulation of chlorophylls and flavonols

We found a decrease in total chlorophyll content in upper canopy leaves at the highest altitude studied, which is in accordance with the literature (Roblek et al. 2008, Prakash et al. 2011). The total chlorophyll content in lower canopy leaves presented the opposite trend, however, as it increased with altitude. Accordingly, upper and lower canopy leaves at the highest altitude had approximately the same amount of chlorophylls (Fig. 10). Evans and Poorter (2006) had found that changes in LMA and nitrogen partitioning between proteins and photosynthetic pigments within leaves are closely coupled in the process of light acclimation. Plants grown in low-light conditions partitioned a larger fraction of leaf nitrogen into light-harvesting proteins and proteins associated with effective photochemical reactions on thylakoid membrane (Boardman 1977, Seemann et al. 1987, Sims and Pearcy 1994). In contrast to lower canopy leaves, upper leaves invest nitrogen primarily into photosynthetic enzymes and hence have greater demand for carbon dioxide per unit area (Körner and Diemer 1987).

In addition, we observed a significant increase in flavonol content in lower canopy leaves along the altitudinal gradient, whereas no differences between altitudes were observed in upper canopy leaves (Fig. 10). We found strong relationships between C_{area} (Fig. 3c,d) and accumulation of epidermal flavonols, which is in accordance with the previous finding that the biosynthesis of flavonoids, particularly phenylpropanoid-derived compounds, is closely related to carbon–nutrient balance (Koricheva et al. 1998, Peñuelas and Estiarte 1998). The synthesis of carbon-based secondary metabolites is further determined by specific demands (e.g., osmolytes under drought stress, antioxidants under ozone stress) induced by an unfavourable growth environment. The synthesis of flavonoids, tannins, and hydroxycinnamate esters, among other metabolites, may thus represent an alternative pathway for the dissipation of excessive radiation energy and consequently may contribute to enhanced antioxidant capacity of the cell (Grace and Logan 2000), particularly under the stress conditions of high elevations.

Changes in Rubisco content

Rubisco content per unit leaf area in upper and lower canopy leaves tended to converge at higher altitudes (Fig. 7), particularly due to reduced Rubisco content in upper canopy leaves. This is consistent with a gradually decreased allocation of N to Rubisco in upper canopy leaves with increasing altitude (Fig. 8). It is consistent, too, with previous findings that cold acclimation of plants, including induction of antifreeze proteins and changes in the membrane composition (Janda et al. 2007), represent an important sink of nitrogen.

Although the paradigm of N-based photosynthetic machinery assumes that N-containing enzymes are fully active, several studies have shown that Rubisco may not be fully active in naturally growing leaves (Eichelmann et al. 2005, Urban et al. 2012b). The relatively low $A_{\max}$ at high Rubisco contents observed in trees at low altitudes indicates the Rubisco to be in enzymatically inactive forms. This may imply that inactive Rubisco serves as nitrogen storage, especially in upper canopy leaves of trees growing at low altitudes (Fig. 9). Similarly, Sakata et al. (2006) had reported an impairment of Rubisco content and its activity in upper canopy leaves of *Aconogonum weyrichii* along an altitudinal gradient as well as during the vegetation season.

The activity of photosynthetic enzymes – in contrast to light absorption – is reduced at low temperatures and thus leads to an increased risk of photo-oxidative damage (Tsonev and Hikosaka 2003). Therefore, plants adapt to the low growth temperatures associated with high altitudes by increasing the Chl a/b and Rubisco/Chls ratios (Strand et al. 1999), reallocating nitrogen to fructose-1,6-phosphatase (Hikosaka 2005) or to antifreeze proteins (Yeh et al. 2000), raising the de-epoxidation state of xanthophyll pigments (Molina-Montenegro et al. 2012), and/or accumulating UV-screening pigments in the epidermis (Koricheva et al. 1998, Filella and Peñuelas 1999, Roblek et al. 2008). These mechanisms thus document a strong modulatory effect of growth temperature on plant/leaf acclimation to a local radiation regime.

CO_2 assimilation rate and photochemical efficiency

The aforementioned changes in morphological and biochemical traits of leaves consequently result in a convergence of physiological functions of upper and lower canopy leaves at higher altitudes, and in particular of assimilation capacity ($A_{\max}$; Fig. 4a,b) and light-use efficiency as measured by the proxy PRI (Fig. 6). The rate of CO_2 uptake, however, was significantly controlled by stomatal conductance (Fig. 5). Stomatal density, and presumably stomatal conductance, generally increases with altitude (Körner and Cochrane 1985, Vats et al. 2009), but this was confirmed for both upper and lower canopy leaves only at the end of the vegetation season (Fig. 4c,d). In July, the hottest and driest period, the highest stomatal conductance was found for altitude M, likely the site with the greatest local water availability.

As PRI has been associated with photosynthetic light use-efficiency (Gamon et al. 1992, Peñuelas et al. 1995), our results suggest a trend, at least in September, towards lower light-use efficiency at high altitudes (Fig. 6). This phenomenon has been observed previously for *Quercus ilex* in areas of the Iberian Peninsula (Filella and Peñuelas 1999).

In our previous studies (Lichtenthaler et al. 2007, Urban et al. 2007), we have shown that leaves of *F. sylvatica* respond to insufficient light conditions primarily by reduced LMA, which leads to significantly higher $A_{\max}$ per leaf weight unit in lower canopy leaves of *F. sylvatica* as compared to upper canopy leaves. Similarly, little variation in the mass-based traits LMA, N_{area}, and $A_{\max}$ was found in a study of *Quercus ilex* by Niinemets (2014b). Such acclimation to low light intensities is regarded as a typical response of shade-intolerant species (Kubiske and Pregitzer 1997). Nevertheless, restricted growth conditions associated with high altitudes have a potential to stimulate biochemical acclimation (represented for example by changes in N_{area}, chlorophyll and Rubisco contents) in lower canopy leaves of *F. sylvatica*.

Conclusion

Generally, with increasing altitude lower canopy leaves tended to acquire the same traits as upper canopy leaves. Nevertheless, there were strong vertical gradients in light intensity across a canopy at all altitudes investigated. Under similar stand density, restrictive growth conditions result in a more open canopy and higher penetration of light into lower canopy with increasing altitude. An asymmetrical acclimation of upper and lower canopy leaves thus resulted in a convergence of their morphological (LMA), biochemical (N_{area}, C_{area}, Chls, Flavs, $\mathrm{Rubisco}_{\mathrm{area}}$), and physiological ($A_{\max}$, G_{Smax}, PRI) traits with increasing altitude. The beech forest responded mainly by changing the traits of lower canopy leaves along the elevation gradient and thus showed a great capacity for the tree to adjust its entire canopy to cope with changing conditions. Such plasticity in the acclimation of leaves has the potential to cause a substantial change in the photosynthesis of forest canopies and in their contribution to the overall carbon balance of vegetation.

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Table 1. Tree age and mean values *(standard deviations)* of total tree height (Height), stem diameter at breast height (DBH), basal area index (BAI), and leaf area index (LAI) of European beech (*Fagus sylvatica*) trees growing at low (L; 400 m a.s.l.), middle (M; 750 m a.s.l.), and high (H; 1100 m a.s.l.) altitudes. Transmittance (Tr) of photosynthetic photon flux (PPF) was calculated as the ratio of PPFs above the canopy to those at the level of lower canopy leaves/branches investigated at maximum solar elevations (10:00–14:00 LMT) and clear sky conditions. Different letters denote significantly different values at $P < 0.05$ ($n = 13$). BAI and LAI were estimated using an LAI-2200 optical plant canopy analyser (Li-Cor, USA) and represent the area of branches and main stems and the total area of leaves per m^2 of land, respectively. Different superscript letters denote significantly different values at $P < 0.05$ ($n = 13$).

Altitude	**Age**	**Height**	**DBH**	**BAI**	**LAI**	**Tr**
	years	m	m	$m^2\ m^{-2}$	$m^2\ m^{-2}$	%
L	95+	27^a *(3.0)*	0.51^a *(0.09)*	2.3^a *(0.2)*	12.5 *(1.03)*	4.3
M	85+	19^b *(3.8)*	0.33^b *(0.09)*	1.4^b *(0.2)*	11.4 *(1.69)*	6.3
H	100+	21^b *(1.8)*	0.37^b *(0.04)*	1.1^b *(0.1)*	7.2 *(0.04)*	8.4

Table 2. Mean values *(minimum–maximum values)* of organic carbon (Corg), total nitrogen (N), phosphorus (P), calcium (Ca), magnesium (Mg), and potassium (K) content in the soils of three experimental plots located at low (L), middle (M), and high (H) altitudes. Different superscript letters denote significantly different values at $P < 0.05$ ($n = 13$).

Altitude	Corg	N	P	Ca	Mg	K	C:N	C:P	N:P
	g kg^{-1}	g kg^{-1}	mg kg^{-1}	mg kg^{-1}	mg kg^{-1}	mg kg^{-1}	g g^{-1}	g mg^{-1}	g mg^{-1}
L	35^{a} *(27-53)*	1.5^{a} *(0.9-2.3)*	3.96^{b} *(0.55-8.50)*	715^{b} *(220-1079)*	80^{b} *(50-106)*	73^{a} *(48-98)*	24.0	8.9	0.4
M	75^{b} *(59-99)*	3.8^{b} *(2.7-5.7)*	0.85^{a} *(0.00-5.05)*	799^{b} *(381-1383)*	75^{b} *(47-126)*	120^{b} *(71-188)*	20.0	87.6	4.5
H	109^{c} *(65-152)*	4.7^{c} *(2.2-6.1)*	1.84^{a} *(0.00-6.05)*	336^{a} *(258-497)*	49^{a} *(30-73)*	142^{b} *(85-208)*	23.1	59.2	2.6

Figure Legend

Fig. 1. Annual courses of monthly mean air temperature (2 m above the ground) and monthly sums of precipitation measured on an open area close to the investigated plots in 2013. The plots are located along the altitudinal gradient: low (L; 400 m a.s.l.), middle (M; 750 m a.s.l.), and high (H; 1100 m a.s.l.).

Fig. 2. Leaf mass per area ratio (LMA; **a**, **b**) and leaf moisture (Lm; **c**, **d**) in upper canopy (open columns) and lower canopy (opaque columns) leaves of European beech (*Fagus sylvatica*) growing at low (L; 400 m a.s.l.), middle (M; 750 m a.s.l.), and high (H; 1100 m a.s.l.) altitudes. Columns represent means, and error bars show standard deviations ($n = 13$ trees). Identical superscript letters indicate homogeneous groups with statistically non-significant differences ($P > 0.05$).

Fig. 3. Elemental analyses of total nitrogen (N_{area}; **a**, **b**) and carbon (C_{area}; **c**, **d**) contents per unit leaf area in upper canopy (open columns) and lower canopy (opaque columns) leaves of European beech (*Fagus sylvatica*) growing at low (L; 400 m a.s.l.), middle (M; 750 m a.s.l.), and high (H; 1100 m a.s.l.) altitudes. Columns represent means, and error bars show standard deviations ($n = 13$ trees). Identical superscript letters indicate homogeneous groups with statistically non-significant differences ($P > 0.05$).

Fig. 4. Light-saturated (1200 µmol m^{-2} s^{-1}) rate of CO_2 assimilation (A_{max}; **a**, **b**), stomatal conductance (G_{Smax}; **c**, **d**), and intrinsic water use efficiency (A_{max}/G_{Smax}; **e**, **f**) in upper canopy (open columns) and lower canopy (opaque columns) leaves of European beech (*Fagus sylvatica*) growing at low (L; 400 m a.s.l.), middle (M; 750 m a.s.l.), and high (H; 1100 m a.s.l.) altitudes. Columns represent means, and error bars

show standard deviations ($n = 13$ trees). Identical superscript letters indicate homogeneous groups with statistically non-significant differences ($P > 0.05$).

Fig. 5. Relationship between light-saturated rate of CO_2 assimilation (A_{max}) and stomatal conductance (G_{Smax}) in upper canopy (open circles) and lower canopy (opaque circles) leaves of European beech (*Fagus sylvatica*) growing at low (L; 400 m a.s.l.), middle (M; 750 m a.s.l.), and high (H; 1100 m a.s.l.) altitudes. The hyperbolic functions ($y = a / (1 + \exp(-(x - x0) / b))$) were fitted separately for upper ($a = 16.35$, $b = 0.073$, $x0 = 0.083$, $R^2 = 0.89$, $P < 0.01$ in July and $a = 10.36$, $b = 0.035$, $x0 = 0.075$, $R^2 = 0.74$, $P < 0.01$ in September) and lower canopy leaves ($a = 11.08$, $b = 0.037$, $x0 = 0.068$, $R^2 = 0.66$, $P < 0.01$ in July and $a = 9.37$, $b = 0.062$, $x0 = 0.095$, $R^2 = 0.82$, $P < 0.01$ in September).

Fig. 6. Photochemical reflectance index (PRI) estimated on the basis of full reflectance spectra (350–2500 nm) in upper canopy (open columns) and lower canopy (opaque columns) leaves of European beech (*Fagus sylvatica*) growing at low (L; 400 m a.s.l.), middle (M; 750 m a.s.l.), and high (H; 1100 m a.s.l.) altitudes. Columns = means, error bars = standard deviations, $n = 13$ (trees). Identical superscript letters indicate homogeneous groups with statistically non-significant differences ($P > 0.05$).

Fig. 7. Total content of Rubisco enzyme (ribulose-1,5-bisphosphate carboxylase/oxygenase) per unit leaf area in upper canopy (open columns) and lower canopy (opaque columns) leaves of European beech (*Fagus sylvatica*) growing at low (L; 400 m a.s.l.), middle (M; 750 m a.s.l.), and high (H; 1100 m a.s.l.) altitudes. Columns = means, error bars = standard deviations, $n = 13$ (trees). Identical superscript

letters indicate homogeneous groups with statistically non-significant differences ($P >$ 0.05).

Fig. 8. Relationship between total Rubisco content per unit leaf area and total nitrogen content per unit leaf area (N_{area}) in upper canopy (u.c.; circles) and lower canopy (l.c.; triangles) leaves of European beech (*Fagus sylvatica*) growing at low (L; 400 m a.s.l.), middle (M; 750 m a.s.l.), and high (H; 1100 m a.s.l.) altitudes. The exponential function $y = a * \exp(b * x)$ was fitted to the data irrespective of leaf position within a canopy and time of season for low ($a = 1.18$, $b = 0.996$, $R^2 = 0.88$; $P < 0.01$), middle ($a = 1.92$, $b = 0.676$, $R^2 = 0.67$, $P < 0.01$), and high ($a = 1.40$, $b = 0.743$, $R^2 = 0.60$, $P < 0.05$) altitudes.

Fig. 9. Relationship between light-saturated rate of CO_2 assimilation (A_{max}) and total Rubisco content per unit leaf area in upper canopy (u.c.; circles) and lower canopy (l.c.; triangles) leaves of European beech (*Fagus sylvatica*) growing at low (L; 400 m a.s.l.), middle (M; 750 m a.s.l.), and high (H; 1100 m a.s.l.) altitudes. The exponential rise to maximum function ($y = a * (1 - \exp(-b * x))$) was fitted to the data irrespective of leaf position within a canopy and time of season for low ($a = 7.75$, $b = 0.362$, $R^2 = 0.40$, $P < 0.01$), middle ($a = 19.04$, $b = 0.096$, $R^2 = 0.59$, $P < 0.01$), and high ($a = 10.18$, $b = 0.326$, $R^2 = 0.26$, $P < 0.01$) altitudes.

Fig. 10. Total chlorophyll (a+b) content (Chls; **a**, **b**), epidermal content of flavonols (Flavs; **c**, **d**), and nitrogen balance index (NBI; **e**, **f**) in upper canopy (open columns) and lower canopy (opaque columns) leaves of European beech (*Fagus sylvatica*) growing at low (L; 400 m a.s.l.), middle (M; 750 m a.s.l.), and high (H; 1100 m a.s.l.) altitudes. Columns = means, error bars = standard deviations, *n* = 13 (trees). Identical

superscript letters indicate homogeneous groups with statistically non-significant differences ($P > 0.05$). Chlorophyll content was estimated on the basis of differential transmission for two near-infrared wavelengths. Epidermal content of flavonols was estimated based on the ratio of chlorophyll fluorescence induced by UV and red light. NBI was determined as the Chls/Flavs ratio.