

1 Research Papers

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3 **Title**

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

6

7 **Running Headline**

8 Upper and lower leaves converge with altitude

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21

## 22 **Summary**

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

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

31 Compared to trees at higher altitudes, those growing at low altitudes had lower stomatal  
32 conductance, slightly lower CO<sub>2</sub> assimilation rate ( $A_{\max}$ ) and leaf mass per area (LMA),  
33 and higher photochemical reflectance index, water use efficiency, and Rubisco content.

34 Given similar stand densities at all altitudes, the different growth conditions result in a  
35 more open canopy and higher penetration of light into lower canopy with increasing  
36 altitude. Even though strong vertical gradients in light intensity occurred across the  
37 canopy at all altitudes, lower canopy leaves tended at high altitudes to acquire the same  
38 morphological, biochemical and physiological traits as did upper leaves. While  
39 elevation had no significant effect on nitrogen and carbon contents per unit leaf area,  
40 LMA, or total content of chlorophylls and epidermal flavonoids in upper leaves, these  
41 increased significantly in lower leaves at higher altitudes. The increases in N content of  
42 lower leaves were coupled with similar changes in  $A_{\max}$ . Moreover, high N content  
43 coincided with high Rubisco concentrations in lower but not in upper canopy leaves.

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

46

47

48 **Key-words:**

49 altitudinal gradient, CO<sub>2</sub> assimilation, flavonoids, light environment, leaf stoichiometry,

50 LMA, Rubisco

51

52 **Introduction**

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

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

71 Studies on deciduous forest and herbaceous species have shown an increase of  
72 leaf mass per area (LMA) and leaf nitrogen content per unit area with increasing  
73 altitude (Williams et al. 1995, Song et al. 2012). Other studies have reported increases  
74 in stomatal density, stomatal conductance, and light-saturated rate of CO<sub>2</sub> assimilation  
75 with increasing altitude (Hultine and Marshall 2000, Vats et al. 2009). Moreover, the  
76 maximum rates of Rubisco carboxylase activity and of photosynthetic electron transport

77 have been shown to be higher for leaves from plants grown at high altitudes than for  
78 those grown at low altitudes (Fan et al. 2011), even as the activities of other enzymes  
79 associated with carbon assimilation have not shown significant differences with  
80 changing altitude (Kumar et al. 2008).

81 An exponential attenuation of solar radiation passing through a canopy leads to  
82 distinct light intensity across a vertical canopy profile. Leaves acclimate to their light  
83 environments by (i) modulation of leaf morphology, anatomy, and chloroplast  
84 ultrastructure (Boardman 1977, Lichtenthaler et al. 1981, Kubiske and Pregitzer 1997,  
85 Yano and Terashima 2001), and (ii) changes in their chemical composition, including in  
86 particular reallocation of nitrogen between photosynthetic components associated with  
87 light capture, thylakoid membrane composition, and CO<sub>2</sub> assimilation (Sims and Pearcy  
88 1994, Eichelmann et al. 2005, Hikosaka 2005, Lichtenthaler et al. 2007). The thicker  
89 upper canopy leaves are characterized by lower water content, higher total chlorophyll  
90 and total carotenoid content per leaf area unit, as well as higher values for the Chl a/b  
91 ratio compared to the much thinner lower canopy leaves (Lichtenthaler et al. 2007).  
92 While upper leaves have higher rates of light-saturated CO<sub>2</sub> assimilation, which are  
93 associated with higher Rubisco content and stomatal conductance, lower leaves more  
94 effectively utilize low light intensities (Sims and Pearcy 1994, Urban et al. 2007).  
95 Lower canopy leaves play an important role in whole-canopy carbon fixation,  
96 particularly during cloudy days with prevailing diffuse radiation but also during hot  
97 sunny days when the stomatal conductance, CO<sub>2</sub> uptake and light-use efficiency of the  
98 uppermost sunlit leaves may be reduced (Urban et al. 2012a, Niinemets 2014a). It is not  
99 clear, however, how distinct growth conditions associated with different altitudes affect  
100 the vertical distribution and within-canopy variation of leaf traits.

101 Our main objective was to study the plasticity and possibly different acclimation  
102 of upper and lower canopy leaves along the altitudinal gradient. To the best of our  
103 knowledge, no comprehensive study had yet been undertaken on how the morphological,  
104 biochemical and physiological traits of upper and lower canopy leaves are affected by  
105 altitudinal gradient. Therefore, we aimed to investigate the within-canopy variations in  
106 leaf structure (LMA), biochemistry (elemental stoichiometry; flavonoid, chlorophyll  
107 and Rubisco content) and functioning (CO<sub>2</sub> assimilation rate, stomatal conductance,  
108 photochemical reflectance index) of European beech (*Fagus sylvatica* L.) grown in a  
109 forest with prevailing beech abundance at three different altitudes. The altitudinal  
110 experiment was designed to test a hypothesis predicting that canopies respond to  
111 changing climate by altered structure that may subsequently lead to reduced within-  
112 canopy variations of morphological, biochemical and physiological leaf traits at high  
113 altitudes. Since the asymmetrical acclimation of upper and lower canopy leaves has the  
114 potential to cause a substantial change in the photosynthesis of forest canopies, this is a  
115 key issue concerning altitudinal adaptations in plant ecophysiology.

116

117

## 118 **Materials and methods**

### 119 *Site description*

120 The forest stand selected for this study is located on the southern slope of Mravenečník  
121 Mountain (Hrubý Jeseník Mountains, 50°2' N, 17°9' E, Czech Republic). Leaf sampling  
122 and physiological measurements were done on European beech (*Fagus sylvatica* L.)  
123 trees naturally occurring at low (L; 420 m a.s.l.), middle (M; 720 m a.s.l.), and high (H;  
124 1100 m a.s.l.) altitudes. As calculated from 30 years of data for L, M, and H altitudes,  
125 respectively, the individual sites are characterized by gradients in mean annual air

126 temperature (7.59, 5.94, and 3.82 °C) and mean annual sum of precipitation (753, 891,  
127 and 1083 mm). The mean monthly temperatures (2 m above the soil surface) and  
128 monthly sums of precipitation during the investigated season (2013) are shown in Fig.  
129 1. Both meteorological parameters were measured automatically in open areas close to  
130 the investigated plots (up to 200 m distant).

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

145         Two soil samples (0–20 cm depth) were taken at each of 13 trees (at a distance  
146 ca 1 m to the west and to the east from the tree's base) at each altitude. Soil  
147 characteristics were estimated on 2 mm fraction. Atomic-absorption spectroscopy was  
148 used to assess Ca<sup>2+</sup>, Mg<sup>2+</sup> and K<sup>+</sup> content. Content of P was determined  
149 spectrophotometrically as a molybdate–phosphate complex and total N by distillation  
150 after mineralization (Kjeldahl technique). Soil organic carbon was determined by

151 weight loss on ignition at 530 °C. Soil elemental concentrations and stoichiometry of  
152 the plots investigated are summarized in Table 2.

153

#### 154 *Physiological measurements and sampling procedures*

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

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

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



176 projected leaf area. Leaf moisture (Lm) in leaf samples was calculated as the ratio of  
177 leaf fresh minus dry weight divided by leaf dry weight.

178 Light-saturated rates of CO<sub>2</sub> assimilation ( $A_{\max}$ ) and stomatal conductance  
179 ( $G_{S\max}$ ) were determined under ambient CO<sub>2</sub> concentration ( $385 \pm 5 \mu\text{mol mol}^{-1}$ ) and  
180 constant microclimatic conditions (leaf temperature:  $25 \pm 1 \text{ }^\circ\text{C}$ , relative air humidity:  
181  $55 \pm 3\%$ ) using a Li-6400XT gas exchange system (Licor, Lincoln, NE, USA). Constant  
182 saturating irradiance ( $1200 \mu\text{mol m}^{-2} \text{ s}^{-1}$ ) was provided by LED light source of a LI-  
183 6400-02B (Li-Cor, Lincoln, NE). In our previous studies (Lichtenthaler et al. 2007,  
184 Urban et al. 2007) we had shown that such PPF is sufficient to saturate CO<sub>2</sub> assimilation  
185 rate in upper canopy leaves of many tree species, while it has no photoinhibitory effects  
186 on lower canopy leaves. The *in vivo* contents of epidermal flavonols and chlorophylls  
187 (Chl a+b) were determined by Dualex 4 Flav (Force-A, Orsay, France). Leaf reflectance  
188 spectra were measured in the wavelength range 350–2500 nm using a FieldSpec 4  
189 HiRes spectroradiometer (ASD Inc., Boulder, CO, USA) coupled with leaf clip  
190 reflectance probe (ASD Inc.). Three reflectance spectra per leaf were taken. The  
191 photochemical reflectance index (PRI) was subsequently derived. This index expresses  
192 an association with photosynthetic light use-efficiency and it is defined as the ratio of  
193 reflectance (R) at 531 and 570 nm wavelengths:  $\text{PRI} = (\text{R}_{531} - \text{R}_{570}) / (\text{R}_{531} + \text{R}_{570})$   
194 (Gamon et al. 1992, Peñuelas et al. 1995).

195 All measurements and samplings were done during the extended noon period  
196 (10:00–14:00 LMT) and at two stages of growing season, the first characterized by  
197 active growth (9–10 July) and the second by early senescence (16–17 September).

198

199 *Statistical analyses*

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

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

209

210

## 211 **Results**

### 212 *Leaf structure and C:N stoichiometry*

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

221 Both nitrogen ( $N_{\text{area}}$ ) and carbon ( $C_{\text{area}}$ ) content per unit leaf area were closely  
222 related to LMA ( $R^2 = 0.926$  and  $0.996$  for  $N_{\text{area}}$  and  $C_{\text{area}}$ , respectively;  $P < 0.01$ ; data  
223 not shown). The content of  $N_{\text{area}}$  and  $C_{\text{area}}$  as well as the C:N ratio were higher in upper  
224 than lower canopy leaves in both sampling periods, but these markedly increased with

225 altitude in lower canopy leaves (Fig. 3a–d). Accordingly, differences in C:N ratio (Fig.  
226 3e,f) between uppermost and bottom leaves tended to be smaller or to disappear at  
227 higher altitudes (M and H) while large within-canopy differences were observed at low  
228 altitude (L).

229

### 230 *Effect of altitude on CO<sub>2</sub> assimilation and stomatal conductance*

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

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

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

250

251 *N partitioning and CO<sub>2</sub> assimilation*

252 Rubisco content per unit leaf area (Rubisco<sub>area</sub>) was significantly higher in upper than  
253 lower canopy leaves at all altitudes. Rubisco<sub>area</sub> tended to decrease with altitude in upper  
254 leaves while slightly increasing in lower canopy leaves. These patterns were more  
255 pronounced in July (Fig. 7; Supplementary Table 1). The relationship between N<sub>area</sub> and  
256 Rubisco<sub>area</sub>, analysed for the whole dataset, shows exponential growth (Fig. 8). However,  
257 different relationships can be recognized for individual altitudes. Particularly at high  
258 N<sub>area</sub> contents (N<sub>area</sub> ≥ 2.0 g m<sup>-2</sup>), which are typical for upper canopy leaves, a lower  
259 amount of nitrogen is allocated to Rubisco at high as compared to low altitudes. This is  
260 reflected in the higher value of the *b* exponent in the exponential model applied (Fig. 8).

261 The relationship between A<sub>max</sub> and Rubisco<sub>area</sub> shows relatively high variation  
262 (caused by G<sub>Smax</sub>), and relationships for individual altitudes can be differentiated (Fig.  
263 9). At the lowest altitude, the A<sub>max</sub> values reached the asymptotic level at relatively  
264 lower concentrations of Rubisco as compared to the M and H altitudes (reflected by the  
265 lower parameter *a* in the exponential model applied).

266 Total chlorophyll content estimated in upper and lower canopy leaves tended to  
267 converge at high altitude in both periods (Fig. 10a,b). Generally, leaves from the canopy  
268 bottom show lower content of epidermal flavonols than do the upper leaves (Fig. 10c,d).  
269 Moreover, flavonol content determined by fluorescence technique tended to increase  
270 with altitude in lower canopy leaves while no such differences were found in upper  
271 leaves. A close link between carbon metabolism and the accumulation of flavonols has  
272 been revealed by the relationship between carbon content in leaves and epidermal  
273 flavonols ( $R^2 = 0.88\text{--}0.89$ ; data not shown). This relationship showed the asymptotic  
274 nature of flavonol accumulation at C<sub>area</sub> above 30 g m<sup>-2</sup>. The relationship was slightly

275 shifted by time, with higher content of flavonols occurring in the later season. The  
276 nitrogen balance index (NBI), calculated as the ratio of chlorophylls to flavonoids,  
277 decreased with altitude in lower canopy leaves while remaining relatively constant in  
278 upper leaves (Fig. 10e,f).

279

280

## 281 **Discussion**

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

294 Our results show great capacity of *F. sylvatica* trees to adjust the morphological,  
295 biochemical and physiological traits of the entire canopy. We found evidence  
296 supporting the hypothesis that the climatic conditions along the altitudinal gradient  
297 modulate the structure of forest canopies and thereby alter the local light environment.  
298 In particular, the limiting role of low light intensities is pronounced under the  
299 favourable climate conditions of low altitudes. A less limiting role of light was

300 meanwhile observed under climate-limiting conditions of high altitudes, where the  
301 canopies achieve lower LAI values (Table 1). As discussed below, such asymmetrical  
302 acclimation resulted in a convergence of morphological, biochemical and physiological  
303 traits of upper and lower canopy leaves with increasing altitude.

304

#### 305 *Leaf mass area (LMA) and leaf N stoichiometry*

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

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

325 at low temperatures increased LMA. This was associated with increased photosynthetic  
326 capacity, thus demonstrating cold acclimation of lowland species.

327

### 328 *Accumulation of chlorophylls and flavonols*

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

342 In addition, we observed a significant increase in flavonol content in lower  
343 canopy leaves along the altitudinal gradient, whereas no differences between altitudes  
344 were observed in upper canopy leaves (Fig. 10). We found strong relationships between  
345  $C_{\text{area}}$  (Fig. 3c,d) and accumulation of epidermal flavonols, which is in accordance with  
346 the previous finding that the biosynthesis of flavonoids, particularly phenylpropanoid-  
347 derived compounds, is closely related to carbon–nutrient balance (Koricheva et al. 1998,  
348 Peñuelas and Estiarte 1998). The synthesis of carbon-based secondary metabolites is  
349 further determined by specific demands (e.g., osmolytes under drought stress,

350 antioxidants under ozone stress) induced by an unfavourable growth environment. The  
351 synthesis of flavonoids, tannins, and hydroxycinnamate esters, among other metabolites,  
352 may thus represent an alternative pathway for the dissipation of excessive radiation  
353 energy and consequently may contribute to enhanced antioxidant capacity of the cell  
354 (Grace and Logan 2000), particularly under the stress conditions of high elevations.

355

### 356 *Changes in Rubisco content*

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

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

373         The activity of photosynthetic enzymes – in contrast to light absorption – is  
374 reduced at low temperatures and thus leads to an increased risk of photo-oxidative



375 damage (Tsonev and Hikosaka 2003). Therefore, plants adapt to the low growth  
376 temperatures associated with high altitudes by increasing the Chl a/b and Rubisco/Chls  
377 ratios (Strand et al. 1999), reallocating nitrogen to fructose-1,6-phosphatase (Hikosaka  
378 2005) or to antifreeze proteins (Yeh et al. 2000), raising the de-epoxidation state of  
379 xanthophyll pigments (Molina-Montenegro et al. 2012), and/or accumulating UV-  
380 screening pigments in the epidermis (Koricheva et al. 1998, Filella and Peñuelas 1999,  
381 Roblek et al. 2008). These mechanisms thus document a strong modulatory effect of  
382 growth temperature on plant/leaf acclimation to a local radiation regime.

383

#### 384 *CO<sub>2</sub> assimilation rate and photochemical efficiency*

385 The aforementioned changes in morphological and biochemical traits of leaves  
386 consequently result in a convergence of physiological functions of upper and lower  
387 canopy leaves at higher altitudes, and in particular of assimilation capacity ( $A_{\max}$ ; Fig.  
388 4a,b) and light-use efficiency as measured by the proxy PRI (Fig. 6). The rate of CO<sub>2</sub>  
389 uptake, however, was significantly controlled by stomatal conductance (Fig. 5).  
390 Stomatal density, and presumably stomatal conductance, generally increases with  
391 altitude (Körner and Cochrane 1985, Vats et al. 2009), but this was confirmed for both  
392 upper and lower canopy leaves only at the end of the vegetation season (Fig. 4c,d). In  
393 July, the hottest and driest period, the highest stomatal conductance was found for  
394 altitude M, likely the site with the greatest local water availability.

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

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

410

411

## 412 **Conclusion**

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

424 photosynthesis of forest canopies and in their contribution to the overall carbon balance  
425 of vegetation.

426

427

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436

437

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588

589

590 **Table 1.** Tree age and mean values (*standard deviations*) of total tree height (Height),  
591 stem diameter at breast height (DBH), basal area index (BAI), and leaf area index (LAI)  
592 of European beech (*Fagus sylvatica*) trees growing at low (L; 400 m a.s.l.), middle (M;  
593 750 m a.s.l.), and high (H; 1100 m a.s.l.) altitudes. Transmittance (Tr) of photosynthetic  
594 photon flux (PPF) was calculated as the ratio of PPFs above the canopy to those at the  
595 level of lower canopy leaves/branches investigated at maximum solar elevations  
596 (10:00–14:00 LMT) and clear sky conditions. Different letters denote significantly  
597 different values at  $P < 0.05$  ( $n = 13$ ). BAI and LAI were estimated using an LAI-2200  
598 optical plant canopy analyser (Li-Cor, USA) and represent the area of branches and  
599 main stems and the total area of leaves per  $m^2$  of land, respectively. Different  
600 superscript letters denote significantly different values at  $P < 0.05$  ( $n = 13$ ).

601

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

602

603

604 **Table 2.** Mean values (*minimum–maximum values*) of organic carbon (Corg), total  
605 nitrogen (N), phosphorus (P), calcium (Ca), magnesium (Mg), and potassium (K)  
606 content in the soils of three experimental plots located at low (L), middle (M), and high  
607 (H) altitudes. Different superscript letters denote significantly different values at  $P <$   
608 0.05 ( $n = 13$ ).

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

609

610

611 **Figure Legend**

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

616

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

623

624 **Fig. 3.** Elemental analyses of total nitrogen ( $N_{\text{area}}$ ; **a, b**) and carbon ( $C_{\text{area}}$ ; **c, d**) contents  
625 per unit leaf area in upper canopy (open columns) and lower canopy (opaque columns)  
626 leaves of European beech (*Fagus sylvatica*) growing at low (L; 400 m a.s.l.), middle (M;  
627 750 m a.s.l.), and high (H; 1100 m a.s.l.) altitudes. Columns represent means, and error  
628 bars show standard deviations ( $n = 13$  trees). Identical superscript letters indicate  
629 homogeneous groups with statistically non-significant differences ( $P > 0.05$ ).

630

631 **Fig. 4.** Light-saturated ( $1200 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) rate of  $\text{CO}_2$  assimilation ( $A_{\text{max}}$ ; **a, b**),  
632 stomatal conductance ( $G_{\text{Smax}}$ ; **c, d**), and intrinsic water use efficiency ( $A_{\text{max}}/G_{\text{Smax}}$ ; **e, f**)  
633 in upper canopy (open columns) and lower canopy (opaque columns) leaves of  
634 European beech (*Fagus sylvatica*) growing at low (L; 400 m a.s.l.), middle (M; 750 m  
635 a.s.l.), and high (H; 1100 m a.s.l.) altitudes. Columns represent means, and error bars

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

638

639 **Fig. 5.** Relationship between light-saturated rate of CO<sub>2</sub> assimilation ( $A_{\max}$ ) and  
640 stomatal conductance ( $G_{S\max}$ ) in upper canopy (open circles) and lower canopy (opaque  
641 circles) leaves of European beech (*Fagus sylvatica*) growing at low (L; 400 m a.s.l.),  
642 middle (M; 750 m a.s.l.), and high (H; 1100 m a.s.l.) altitudes. The hyperbolic functions  
643 ( $y = a / (1 + \exp(-(x - x_0) / b))$ ) were fitted separately for upper ( $a = 16.35$ ,  $b = 0.073$ ,  
644  $x_0 = 0.083$ ,  $R^2 = 0.89$ ,  $P < 0.01$  in July and  $a = 10.36$ ,  $b = 0.035$ ,  $x_0 = 0.075$ ,  $R^2 = 0.74$ ,  
645  $P < 0.01$  in September) and lower canopy leaves ( $a = 11.08$ ,  $b = 0.037$ ,  $x_0 = 0.068$ ,  $R^2 =$   
646  $0.66$ ,  $P < 0.01$  in July and  $a = 9.37$ ,  $b = 0.062$ ,  $x_0 = 0.095$ ,  $R^2 = 0.82$ ,  $P < 0.01$  in  
647 September).

648

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

655

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

661 letters indicate homogeneous groups with statistically non-significant differences ( $P >$   
662 0.05).

663

664 **Fig. 8.** Relationship between total Rubisco content per unit leaf area and total nitrogen  
665 content per unit leaf area ( $N_{\text{area}}$ ) in upper canopy (u.c.; circles) and lower canopy (l.c.;  
666 triangles) leaves of European beech (*Fagus sylvatica*) growing at low (L; 400 m a.s.l.),  
667 middle (M; 750 m a.s.l.), and high (H; 1100 m a.s.l.) altitudes. The exponential function  
668  $y = a * \exp(b * x)$  was fitted to the data irrespective of leaf position within a canopy and  
669 time of season for low ( $a = 1.18$ ,  $b = 0.996$ ,  $R^2 = 0.88$ ;  $P < 0.01$ ), middle ( $a = 1.92$ ,  $b =$   
670  $0.676$ ,  $R^2 = 0.67$ ,  $P < 0.01$ ), and high ( $a = 1.40$ ,  $b = 0.743$ ,  $R^2 = 0.60$ ,  $P < 0.05$ ) altitudes.  
671

672 **Fig. 9.** Relationship between light-saturated rate of  $\text{CO}_2$  assimilation ( $A_{\text{max}}$ ) and total  
673 Rubisco content per unit leaf area in upper canopy (u.c.; circles) and lower canopy (l.c.;  
674 triangles) leaves of European beech (*Fagus sylvatica*) growing at low (L; 400 m a.s.l.),  
675 middle (M; 750 m a.s.l.), and high (H; 1100 m a.s.l.) altitudes. The exponential rise to  
676 maximum function ( $y = a * (1 - \exp(-b * x))$ ) was fitted to the data irrespective of leaf  
677 position within a canopy and time of season for low ( $a = 7.75$ ,  $b = 0.362$ ,  $R^2 = 0.40$ ,  $P <$   
678  $0.01$ ), middle ( $a = 19.04$ ,  $b = 0.096$ ,  $R^2 = 0.59$ ,  $P < 0.01$ ), and high ( $a = 10.18$ ,  $b = 0.326$ ,  
679  $R^2 = 0.26$ ,  $P < 0.01$ ) altitudes.

680

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

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