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Research Article

Experimental Study of Environmental Effects: Leaf Traits of Juvenile *Fagus sylvatica*, *Acer pseudoplatanus*, and *Carpinus betulus* Are Comparable to Leaves of Mature Trees in Upper Canopies

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Morphological and functional leaf traits like leaf toughness and nutrient content are essentially influenced by the environment, especially through light and climatic conditions. Varying light conditions have been identified as a significant predictor for the variation of many leaf traits. However, the leaf acclimation to light is suggested to be of secondary importance. The aim of the experimental study was to analyse environmental effects (microclimate and soil moisture), which are present in upper canopies of forest stands, on leaf traits of juvenile *Fagus sylvatica* L. (European beech; Fagaceae), *Acer pseudoplatanus* L. (sycamore maple; Sapindaceae), and *Carpinus betulus* L. (hornbeam; Betulaceae). The experimental design managed to imitate two distinct microclimates causing different temperature and air humidity conditions. Furthermore, the irrigation treatment with different levels of applied water caused distinct soil moisture conditions in the trial pots. As a result of the treatments, leaves of *C. betulus* showed a tendency of decreased specific leaf area (SLA) caused by the treatment with warmer and drier microclimate. The environmental effect on SLA was even stronger with lower soil moisture conditions. Chlorophyll content showed lower values in treatments with higher soil moisture conditions in both greenhouses for *F. sylvatica* and *A. pseudoplatanus*. The trends are in accordance with combined effects of temperature, air humidity, and soil moisture on SLA, and increased leaf chlorophyll content caused by slight drought stress. Plants in the greenhouses were exposed to full sunlight indicating a microclimatic environment comparable to upper canopies in forest stands. The comparable SLA and chlorophyll content between leaves of mature *F. sylvatica* trees in upper canopies and juvenile trees of the greenhouses suggest similar environmental conditions instead of ontogenetic effects that are responsible for the formation of leaf trait characteristics.

1. Introduction

Morphological and functional leaf traits are essentially influenced by the environment, especially through light and climatic conditions. Changes in climate (temperature and air humidity) and light can affect leaf toughness and leaf nutrient content like nitrogen (N) and carbon (C) concentrations. While leaves of tree seedlings and saplings grow in a similar environment of the understorey, large trees need to produce leaves with a distinct development of traits that are acclimated to different environmental conditions in the canopy.

The formation of softer leaves with a thinner and larger leaf lamina, represented by a high specific leaf area (SLA), is a common response to humid environments [1–3]. In addition, high temperatures can also lead to an increase in SLA, but it strongly depends on sufficient soil moisture content [4]. Low soil moisture content rather leads to water stress situations for plants. In return, water stress can cause sclerophylly [5], resulting in thickened, hardened foliage that resists loss of moisture. Sclerophylly is based in the accumulation of phenolic compounds and lignification of leaf tissues [6, 7].

Leaf N concentrations are especially influenced by light conditions [8, 9]. Sun-exposed leaves usually show increased leaf N concentrations compared to shade leaves [10, 11]. Nonetheless, patterns of leaf N content also depend on the shade tolerance of tree species. In lower light environments, increasing the leaf N concentration is a strategy of N partitioning for more efficient light harvesting [12]. Furthermore, levels of humidity can also affect leaf nutrient concentrations. Low soil moisture conditions of dry environments, causing water stress to host plants, increase the N content in plant tissues [13].

Varying light conditions have been identified as a significant predictor for the variation of many leaf traits within forest canopies (e.g., [14, 15]). Leaf trait trends identified by Thomas [16] are likely to be influenced by the leaf acclimation both to environmental conditions (light) and to plant ontogeny (tree size). However, the leaf acclimation to light is suggested to be of secondary importance. Studies with a controlled light effect (e.g., comparison between leaves of open-grown saplings and upper canopy trees) indicate that ontogenetic changes in leaf toughness and herbivory cannot be fully accounted by environmental acclimation responses to sun and shade [17, 18]. Strong effects of tree size on leaf toughness are found independently of crown exposure [16]. Decreases in SLA and related leaf features (leaf tissue density and lignifications) at the end of tree ontogeny are also noted to be independent of sun and shade acclimation [19–22]. Furthermore, the magnitude of ontogenetic changes in traits is larger than what is documented for studies of light acclimation responses [18, 23–26].

The aim of the experimental study was to analyse environmental effects, which are present in upper canopies of forest stands, on leaf traits of juvenile *Fagus sylvatica* L. (European beech; Fagaceae), *Acer pseudoplatanus* L. (sycamore maple; Sapindaceae), and *Carpinus betulus* L. (hornbeam; Betulaceae). The two following hypotheses were tested: (1) higher temperatures (and lower air humidity) and lower soil moisture conditions increase leaf toughness, and (2) lower soil moisture conditions increase the leaf N content.

2. Materials and Methods

2.1. Set-Up. Plant individuals of *Fagus sylvatica* L. (European beech; Fagaceae), *Acer pseudoplatanus* L. (sycamore maple; Sapindaceae), and *Carpinus betulus* L. (hornbeam; Betulaceae) with an age of 2 years and a height about 50–80 cm (Müller Münchehof, Seesen, Germany) were planted in trial pots in August 2013 (see Figure 1(a)). A mixture of 50% soil (Fruhstorfer Erde Typ T, HAWITA, Vechta, Germany) and 50% sand (Estrich sand, grain size = 0–2 mm, Tönsmeier, Hildesheim, Germany) was used as substrate. Two greenhouses (size: 6x28 m) were installed with different UV permeable greenhouse films (FVG EURO 4 and FVG Sun 5 Clear ST, FVG Professional Gardening, Dernbach, Germany) in March 2014, creating distinct climatic conditions (see Figure 1(b)). A plant protection product (Micula, Biofa AG, Münsingen, Germany) was applied to the trees in April 2014 against eggs and individuals of sap-sucking insects that potentially occurred on the tree individuals avoiding herbivory on the experimental foliage material. All trial pots were protected against insects with mosquito nets.

Table 1: Treatment codes representing the combination of temperature and irrigation levels that were used in the experimental study.

Treatment	Lower irrigation	Higher irrigation
Lower temperature	LTLW	LTHW
Higher temperature	HTLW	HTHW

The experimental design consisted of 10 trial pots for each tree species in greenhouses 1 and 2. Manipulations of microclimate and soil moisture were used as treatments for the trial pots. Trial pots in greenhouses 1 and 2 were labelled with the codes LT (lower temperature) and HT (higher temperature) for the microclimate treatment, respectively. Irrigation codes LW (lower water amount) and HW (higher water amount) were added to the trial pots in each greenhouse according to the soil moisture treatment. Overall, four different treatments with five replicates existed for each tree species (see Table 1).

Trial pots were irrigated two or three times a week. The different amounts of irrigation levels were orientated to maximum and minimum values of the precipitation gradient of a field study (Artern: 59 $\mathrm{l/m^2}$; Wahlsburg: 75 $\mathrm{l/m^2}$) by Stiegel *et al.* [27]. The area of a trial pot was 0.03 $\mathrm{m^2}$. Therefore, the amount of water was adapted to the size of the trial pot resulting in 1.77 l and 2.25 l per month for lower and higher water irrigation treatments, respectively. Based on tougher conditions in the trial pots compared to forest sites, trees were always irrigated when soil was dry to avoid withering of individuals. A total amount of 4.96 l and 7.08 l water per month (about three times higher than at the forest sites) was used for irrigation of each trial pot with lower and higher water irrigation, respectively.

2.2. Measurements. Greenhouse measurements took place in June 2014. Microclimate was assessed as air temperature and relative air humidity. Microclimatic data were measured every hour with data loggers (iButton, Model DS1923, Maxim Integrated, California, USA). Data loggers were installed in the centers at about 1.5 m height in both greenhouses. Soil moisture was measured as volumetric water content (% v $\mathrm{v^{-1}}$) with a soil moisture sensor (FieldScout TDR 100 Soil Moisture Meter, Aurora, Illinois, USA) using 7.5 cm long rods. Five measurements of soil moisture were taken for every trial pot during the experimental period to calculate mean values for statistical analyses.

Specific leaf area ($\mathrm{cm^2\ g^{-1}}$) was assessed as an indicator for leaf toughness. It relates the area of a fresh leaf to its dry mass, and low SLA values are linked to structural defences [28]. Fully developed leaves were collected for all tree individuals in June 2014 (four leaves of *F. sylvatica* and *C. betulus* and three leaves of *A. pseudoplatanus*). All fresh leaves were scanned with a flat-bed scanner (CanoScan LiDE 110, Canon, Krefeld, Germany), analysing their areas with the computer image analysis system WinFOLIA (Régent Instruments Inc., Ville de Québec, QC, Canada). Then, each foliage sample was dried (70°C, 48 h) and weighed for calculation of SLA that

Table 2: Climatic conditions represented by temperature and relative air humidity during day (5 am–9 pm; n = 1020) and midday (11 am–2 pm; n = 60) in greenhouses 1 and 2 in June 2014. Values represent the median and interquartile ranges (IQR = first quartile, third quartile). Uppercase letters indicate significant differences of temperature and relative air humidity between the greenhouses using Mann-Whitney *U* test ($p < 0.05$).

Climate	**Temperature** (°C)		**Relative air humidity** (%)	
	Greenhouse 1	Greenhouse 2	Greenhouse 1	Greenhouse 2
Day	21.4 (16.7,27.9)^{A}	22.7 (17.2,30.0)^{B}	58 (40,80)^{A}	55 (37,79)^{A}
Midday	26.5 (22.3,31.5)^{A}	29.3 (24.1,35.9)^{B}	41 (33,52)^{A}	37 (28,49)^{B}

(a) (b)

Figure 1: Experimental study with the (a) trail pots harbouring *Fagus sylvatica*, *Acer pseudoplatanus*, and *Carpinus betulus* tree individuals (b) in greenhouses 1 (left) and 2 (right) at the installation site at the Samelsonplatz, Hildesheim.

was used as the mean per individual for further analyses of leaf toughness.

The chlorophyll content of leaves correlates with leaf N content [29], because up to 75% of N content is located in chloroplasts [30]. Chlorophyll content, as an indicator for leaf N content, was measured with a CCM-200 plus Chlorophyll Content Meter (Opti-Sciences Inc., Hudson, NH, USA). From each tree individual, four chlorophyll values measured as chlorophyll content index (CCI) were taken in June 2014. Average values were calculated for each treatment per tree species (n = 20). Since *A. pseudoplatanus* did not survive the treatment with higher temperature and lower water irrigation, neither SLA nor chlorophyll content could be assessed.

2.3. Statistics. For significant comparisons of measured parameters (microclimate, soil moisture conditions, and leaf traits) in the greenhouses, statistical analyses were performed with R Version 3.4.1 [31]. Statistical distributions of the parameters were assessed with the Shapiro-Wilk test. Depending on the statistical distributions, further tests for microclimate and soil moisture were performed with Mann-Whitney *U* test and *t*-test, respectively. Comparisons of SLA and chlorophyll content between the treatments were performed with ANOVA or Kruskal-Wallis and suitable post-hoc test using the R package *pgrimess* [32].

3. Results

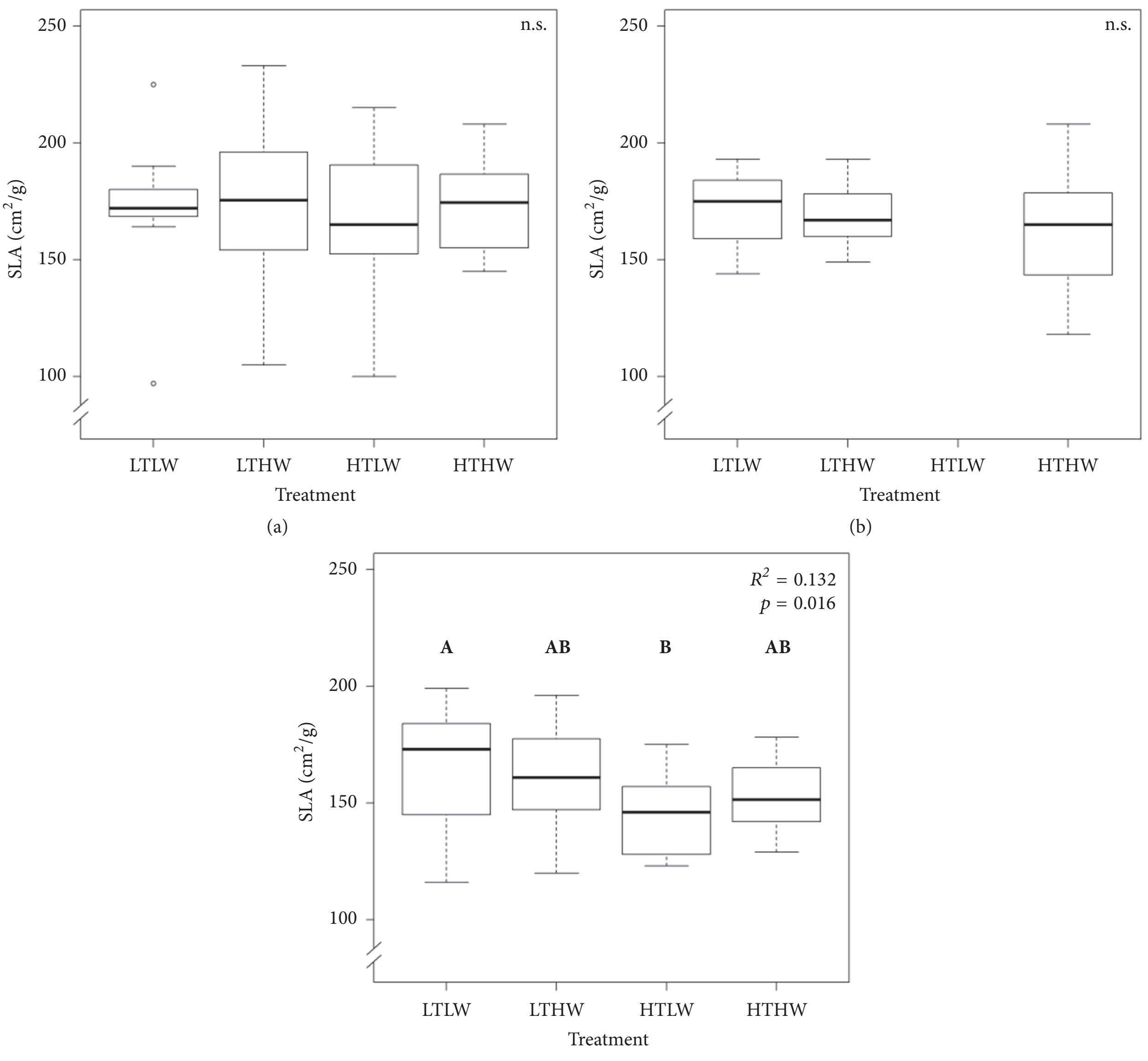
Climatic conditions represented by temperature and relative air humidity differed between the two greenhouses (see Table 2). On average, temperature was increased about 1.3°C and 2.8°C, and relative air humidity decreased about 3% and 4% in greenhouse 2 compared to greenhouse 1 during day and midday, respectively. Humidity was only significantly distinct between the two greenhouses considering midday values.

Table 3: Comparisons of soil moisture conditions (volumetric water content) in the trial pots between lower and higher irrigation treatments for all three tree species (n = 10, per species). Presented are mean values with standard deviation. Uppercase letters indicate significant differences using *t*-test (*F. sylvatica*: $p = 0.003$, df = 13; *A. pseudoplatanus*: $p = 0.028$, df = 18; *C. betulus*: $p = 0.079$, df = 18).

Trial pot species	**Soil moisture** (% v v^{-1})	
	Lower irrigation	Higher irrigation
Fagus sylvatica	18.9 (± 2.2)^{A}	24.6 (± 4.3)^{B}
Acer pseudoplatanus	21.6 (± 3.9)^{A}	25.3 (± 3.0)^{B}
Carpinus betulus	19.6 (± 2.4)^{A}	22.4 (± 4.3)^{A}

Soil moisture conditions were increased through the treatment of higher irrigation for all tree species in both greenhouses. Average values of volumetric water content were increased about 6%, 4%, and 3% in the higher irrigated trial pots of *F. sylvatica*, *A. pseudoplatanus*, and *C. betulus*, respectively. Statistical analyses revealed significant differences for trial pots containing *F. sylvatica* and *A. pseudoplatanus* trees (see Table 3).

Average SLA values were highest for *F. sylvatica* (173 cm^{2} g^{-1} ± 25.9 SD), intermediate for *A. pseudoplatanus* (167 cm^{2} g^{-1} ± 20.3 SD), and lowest for *C. betulus* (155 cm^{2} g^{-1} ± 20.8 SD). Differences of SLA between the treatments (microclimate and soil moisture) were only present for *C. betulus* but not for *F. sylvatica* and *A. pseudoplatanus* (see Figure 2). Specific leaf area of *C. betulus* was decreased in warmer temperatures of greenhouse 2 compared to greenhouse 1 but



(a)

(b)

(c)

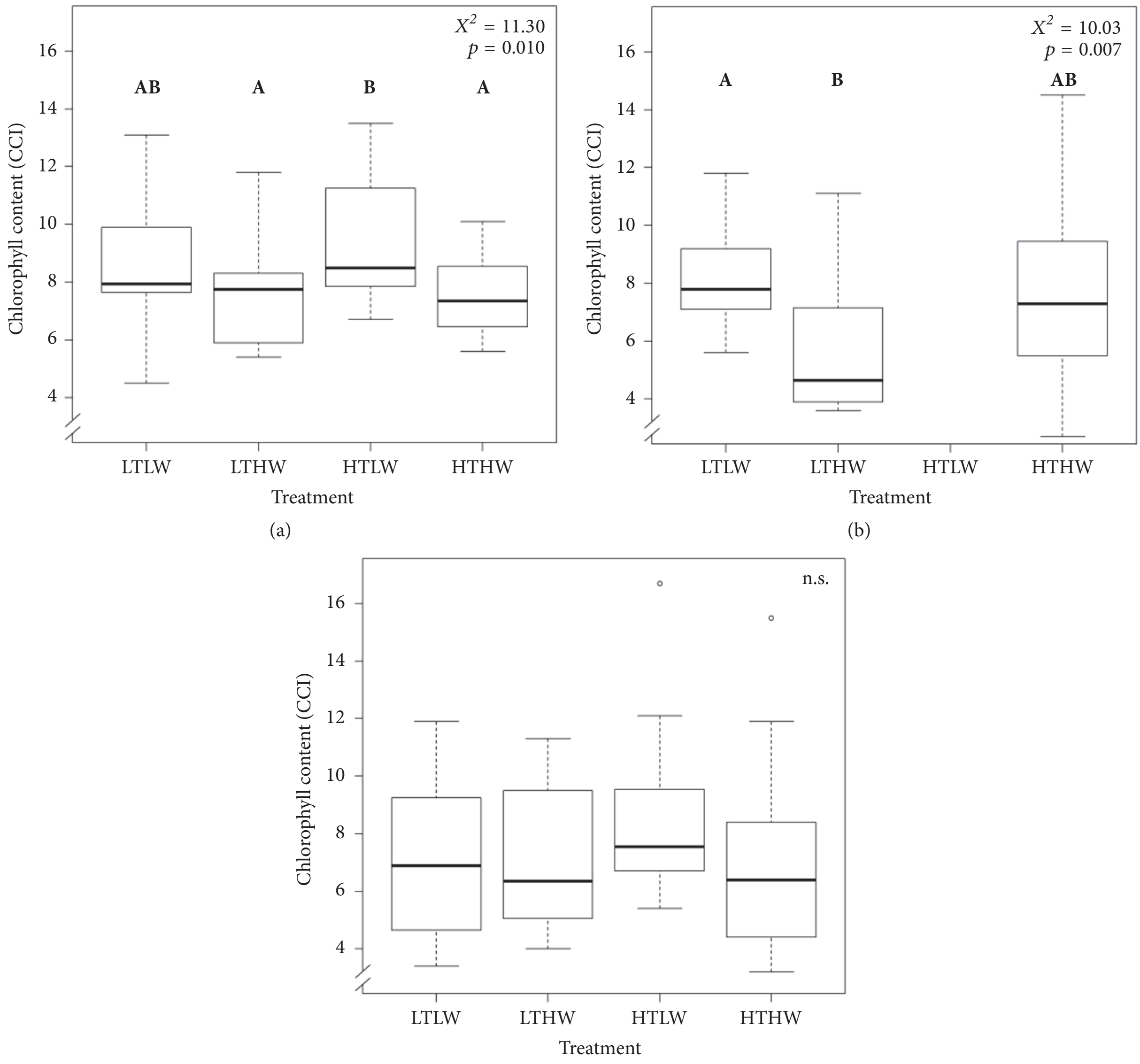
Figure 2: Specific leaf area (SLA) of (a) *Fagus sylvatica* (n = 20), (b) *Acer pseudoplatanus* (n = 15), and (c) *Carpinus betulus* (n = 18) for the different treatments in the greenhouse experiment. Boxplots are marked with uppercase letters indicating significant differences or with "n.s." for nonsignificant differences using (a) Kruskal-Wallis and post hoc test ($p < 0.05$; df = 3) and (b-c) ANOVA and Tukey's HSD ($p < 0.05$, df = 3). Treatments: LTLW, lower temperature and lower irrigation; LTHW, lower temperature and higher irrigation; HTLW, higher temperature and lower irrigation; HTHW, higher temperature and higher irrigation.

only differed significantly with the lower irrigation treatment compared to the other three treatment combinations.

Chlorophyll contents were on average similar for all tree species with values ranging between 7.1 CCI ± 2.7 SD (*A. pseudoplatanus*), 7.4 CCI ± 2.7 SD (*C. betulus*), and 8.2 CCI ± 2.0 SD (*F. sylvatica*). Depending on the experimental irrigation treatment, leaf chlorophyll content differed significantly for *F. sylvatica* and *A. pseudoplatanus* but not for *C. betulus* (see Figure 3). *Carpinus betulus* leaves showed lower chlorophyll content in treatments with higher soil moisture conditions in both greenhouses. Chlorophyll content of trees with the same irrigation treatment did not differ significantly between the two greenhouses.

4. Discussion

The experimental design managed to imitate two distinct microclimates causing different temperature and air humidity conditions. Furthermore, the irrigation treatment with different levels of applied water caused distinct soil moisture conditions in the trial pots. Different soil moisture conditions implicated varying water availability for the tree individuals.



(a)

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FIGURE 3: Chlorophyll content of (a) *Fagus sylvatica* (n = 20), (b) *Acer pseudoplatanus* (n= 15), and (c) *Carpinus betulus* (n = 20) for the different treatments in the greenhouse experiment. Boxplots are marked with uppercase letters indicating significant differences using Kruskal-Wallis and post hoc test ($p < 0.05$; df = 3) or with "n.s." for nonsignificant differences. Treatments: LTLW, lower temperature and lower irrigation; LTHW, lower temperature and higher irrigation; HTLW, higher temperature and lower irrigation; HTHW, higher temperature and higher irrigation.

As a result of the treatments, *C. betulus* showed varying leaf toughness (indicated by SLA), and *F. sylvatica* and *A. pseudoplatanus* differed in chlorophyll content (indicating leaf N content).

Plants in the greenhouses were exposed to full sunlight indicating a microclimatic environment comparable to upper canopies of mixed deciduous forest stands in Central Germany [27]. Average day temperatures in upper canopies of the field study are 1.5 and 2.8°C lower and relative air humidity conditions are 11 and 14% higher compared to greenhouses 1 and 2 of this experimental study, respectively. The magnitude of differences in temperature and relative air humidity between lower and upper canopies (1.1°C and 5%, respectively) of the forest stands [27] is comparable to the difference between the two greenhouses of this study (1.3°C and 3%, respectively).

The SLA of all three juvenile tree species (*F. sylvatica*, *A. pseudoplatanus*, and *C. betulus*) in this experimental greenhouse study is similar to SLA values of mature *F. sylvatica* in upper canopies, representing less than half of the SLA values that were found for the tree species in the understorey of forest stands [27]. The comparable leaf toughness between upper canopies (mature trees) and the greenhouses (juvenile trees) suggests similar environmental conditions instead of ontogenetic effects that are responsible for the formation of leaf trait characteristics. Upper canopies and the greenhouses were both exposed to full sunlight, and leaves of juvenile trees in the greenhouses with low SLA values exhibit the typical pattern of sun leaves. Generally, a decline of SLA can be induced by light conditions because light increases the leaf thickness [33]. Thicker sun leaves of *F. sylvatica* are characterized by a lower SLA compared to the much thinner blades of shade leaves [34].

Leaf chlorophyll content was decreased about 50% in the experimental study compared to values that were measured in the field study [27]. As part of the photosynthesis, chlorophyll content is dependent on the regulating influence of light conditions. Studies present contrasting results concerning patterns of chlorophyll content based on light conditions. Sun leaves of *F. sylvatica* show higher chlorophyll content than shade leaves [35], in contrast to shade leaves of other tree species that contain more chlorophyll than sun leaves [36]. High light conditions in the greenhouses might have caused the low chlorophyll content in leaves of the three tree species. In addition, this effect of decreasing chlorophyll content can be enhanced through impacts of water supply.

Leaves of *C. betulus* showed a tendency of decreased SLA caused by the treatment with warmer and drier microclimate. The environmental effect on SLA was even stronger with lower soil moisture conditions, thus supporting the first hypothesis. This trend is in accordance with combined effects of temperature and air humidity on SLA [1–3], considering also the important factor of soil moisture [4]. According to basic plant physiology, drought experiments with potted tree seedlings or saplings show a reduction in SLA with decreasing water supply [37, 38]. In contrast, *F. sylvatica* and *A. pseudoplatanus* did not demonstrate significant changes in SLA caused by the experimental treatments. While *F. sylvatica* and *A. pseudoplatanus* are most abundant in forest communities where soil drought is rare, *C. betulus* grows also in regions with regular or episodic summer drought [39]. *Carpinus betulus* is known to have smaller water flux levels per tree in contrast to greater water flux levels encountered in *F. sylvatica* and *A. pseudoplatanus* [40].

Generally, drought sensitivity varies between different tree species. Indeed, *C. betulus* reveals a lower drought sensitivity compared to *F. sylvatica* and *A. pseudoplatanus* [40]. Potentially, *C. betulus* reacted as an adaptation strategy with lower SLA, leading to an increase in leaf toughness, for the survival in a warm and dry environment, which was manipulated by the experimental treatments. This would also be in accordance with the range for SLA, which increases in the sequence *A. pseudoplatanus* < *F. sylvatica* < *C. betulus* [41]. Regarding *A. pseudoplatanus*, the fact that tree individuals grown under the treatment with lower water supply in the warmer and drier greenhouse did not survive has to be taken into account. Compared to the other two tree species, leaves of *A. pseudoplatanus* had the largest size, which potentially resulted in a higher water demand leading to the desiccation of the tree individuals.

In the experimental study, differences of chlorophyll content did not exist between the two greenhouses but regarding the irrigation treatment, leaves showed lower chlorophyll content in treatments with higher soil moisture conditions in both greenhouses. Significant differences were identified for the chlorophyll content of *F. sylvatica* and *A. pseudoplatanus*. The physiological state of plants, which influences photosynthetic processes, is strongly affected by the water supply. Impacts of drought stress on the variation of chlorophyll content have been well studied. Generally, drought stress can limit plant growth through variations of chlorophyll content, respiration, and nutrient metabolism [42]. Chlorophyll content decreases significantly through drought stress situations [43–45]. However, a slight drought stress can increase leaf chlorophyll content [46]. Potentially, the lower irrigation treatment caused minor drought stress conditions for the tree individuals of *F. sylvatica* and *A. pseudoplatanus* reacting with increased chlorophyll content. This is also in accordance with a higher drought sensitivity of *F. sylvatica* and *A. pseudoplatanus* compared to *C. betulus* [40]. In conclusion, the patterns of chlorophyll content, indicating leaf N concentrations, would support the second hypothesis for *F. sylvatica* and *A. pseudoplatanus* that lower soil conditions lead to an increase in leaf N content.

5. Conclusions

The comparable specific leaf area and chlorophyll content between leaves of mature *F. sylvatica* trees in upper canopies of forest stands and juvenile trees of the experimental greenhouse study suggest similar environmental conditions instead of ontogenetic effects that are responsible for the formation of leaf trait characteristics.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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