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DOI:

[10.1007/s00468-022-02328-7](https://doi.org/10.1007/s00468-022-02328-7)

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*Document Version*

Publisher's PDF, also known as Version of record

*Citation for published version (Harvard):*

Gardner, A, Ellsworth, DS, Pritchard, J & Mackenzie, AR 2022, 'Are chlorophyll concentrations and nitrogen across the vertical canopy profile affected by elevated CO<sub>2</sub> in mature *Quercus* trees?', *Trees*.

<https://doi.org/10.1007/s00468-022-02328-7>

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# Are chlorophyll concentrations and nitrogen across the vertical canopy profile affected by elevated CO<sub>2</sub> in mature *Quercus* trees?

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Received: 20 April 2022 / Accepted: 25 June 2022  
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## Abstract

**Key message** In mature *Q. robur*, chlorophyll varied with season and canopy height, whilst eCO<sub>2</sub>-driven changes were consistent with  $M_{\text{area}}$ , highlighting key factors for consideration when scaling photosynthetic processes and canopy N-use. Nitrogen-rich chlorophyll and carotenoid pigments are important in photosynthetic functioning. Photosynthetic pigments have been found to decrease with elevated CO<sub>2</sub> (eCO<sub>2</sub>), but few such studies have been done in aged forest trees. This study aimed to assess the effects of eCO<sub>2</sub> (150  $\mu\text{mol mol}^{-1}$  above ambient) and canopy position on chlorophyll content in mature *Quercus robur* (*Q. robur*). Over 5000 *in situ* chlorophyll absorbance measurements, alongside laboratory chlorophyll extractions, were collected on canopy-dominant *Q. robur* in the 3rd and 4th season of CO<sub>2</sub> fumigation of a free-air CO<sub>2</sub> enrichment (FACE) study in central England. Mass-based chlorophyll concentration ( $\text{Chl}_{\text{mass}}$ ,  $\text{mg g}^{-1}$ ) was significantly higher in the lower canopy compared to upper canopy foliage ( $P < 0.05$ ). In contrast, significantly higher chlorophyll content ( $\text{Chl}_{\text{area}}$ ,  $\text{mg m}^{-2}$ ) was observed in the upper canopy. eCO<sub>2</sub> did not affect  $\text{Chl}_{\text{mass}}$  but  $\text{Chl}_{\text{area}}$  significantly increased, attributable to increased leaf mass per unit area ( $M_{\text{area}}$ ,  $\text{g m}^{-2}$ ). We found no effect of eCO<sub>2</sub> on mass-based or area-based nitrogen ( $N_{\text{mass}}$ ,  $\text{mg g}^{-1}$  or  $N_{\text{area}}$ ,  $\text{g m}^{-2}$ ); however,  $N_{\text{area}}$  significantly increased with canopy height, again attributable to  $M_{\text{area}}$ . The parallel relationships between  $M_{\text{area}}$ ,  $N_{\text{area}}$  and  $\text{Chl}_{\text{area}}$  suggest the allocation of N to light harvesting is maintained with eCO<sub>2</sub> exposure as well as in the upper canopy, and that increased photosynthetic mass may help regulate the eCO<sub>2</sub> variation. An understanding of changes in the light-harvesting machinery with eCO<sub>2</sub> will be useful to assess canopy processes and, at larger scales, changes in biogeochemical cycles in future climate scenarios.

**Keywords** Leaf absorbance · Free air CO<sub>2</sub> enrichment (FACE) · Photosynthetic pigment · SPAD meter · Temperate deciduous forest

## Introduction

Large amounts of nitrogen (N) are required to maintain photosynthetic activities of plant canopies, and N availability is considered to be the cornerstone of temperate forest productivity (Norby et al. 2010; Terrer et al. 2019). Given that about 75% of total foliar N is invested in chloroplasts and up to one-third of this investment partitioned specifically to light-harvesting systems (Evans and Clarke 2019), N is an important regulator of canopy function. The key N-rich components for photosynthesis are pigments such as chlorophyll (Chl) as well as photosynthetic enzymes (Raven 2013). Chlorophyll can be divided into multiple components with chlorophyll *a* ( $\text{Chl}_a$ ) and chlorophyll *b* ( $\text{Chl}_b$ ) important for the primary reaction (i.e., light harvesting) step of photosynthesis. As  $\text{Chl}_a$  and  $\text{Chl}_b$  absorb sunlight at different wavelengths, total leaf chlorophyll ( $\text{Chl}_a + \text{Chl}_b$ ) is expected

Communicated by Victor Resco de Dios.

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to directly influence the photosynthetic capacity of plants (Croft et al. 2017; Evans and Seemann 1989; Palta 1990). Chlorophyll, therefore, intimately links N and carbon (C) availability at the cell, leaf, and plant scales in tree canopies (Li et al. 2018). These relationships are utilised in remote sensing of canopies (Asner and Martin 2008; Ollinger et al. 2013). Additionally, much of our space-based analysis of carbon cycling (e.g., Zhu et al. 2013), and remote sensing of plant health (e.g., Sims and Gamon 2002), relies on colour indices derived from the optical properties of chlorophyll.

Increasing atmospheric carbon dioxide ( $\text{CO}_2$ ) is expected to affect processes within terrestrial ecosystems, such as in forests, by increasing photosynthesis (Bader et al. 2010; Ellsworth et al. 2017; Gardner et al. 2021). However, trees grown in elevated  $\text{CO}_2$  ( $\text{eCO}_2$ ) have also been hypothesised to reallocate nitrogen (N) as a critical growth resource to maximise productivity with limited resource availability (Drake et al. 1997). To date, trees in  $\text{eCO}_2$  have been found to have lower leaf nitrogen content (per unit area;  $N_{\text{area}}$ , in  $\text{g m}^{-2}$  leaf) compared to trees grown in ambient  $\text{CO}_2$  ( $\text{aCO}_2$ ) (Ainsworth and Long 2005; Ellsworth et al. 2004). Any changes in  $N_{\text{area}}$  with future increases in ambient  $\text{CO}_2$  concentrations may have an effect on the photosynthetic apparatus and lead to changes in the tree responses to increasing  $\text{CO}_2$  (Crous et al. 2008; Major et al. 2018). The effects of  $\text{eCO}_2$  on N allocation among photosynthetic components, such as in light-harvesting processes, remain unclear (Ainsworth and Long 2005; Caldararu et al. 2020) but is important given the N limitations in temperate forests (Zaehle 2013). An understanding of changes in the light-harvesting machinery, such as photosynthetic pigments, under  $\text{eCO}_2$  is vital to assess implications on leaf and canopy processes in our future climate. Furthermore, many land surface models seek to depict the allocation of N within photosynthesis processes (Caldararu et al. 2020; Zaehle et al. 2014) and require validation in novel conditions like  $\text{eCO}_2$ . Thus, understanding how N is allocated among canopy leaves in  $\text{eCO}_2$  can help us forecast future changes in ecosystem functioning and, at larger scales, changes in linked biogeochemical cycles for C, N, and water (Medlyn et al. 2015; A. P. Walker et al. 2015).

Working at leaf scale, the abundance of chlorophyll and leaf N can both be expressed per unit mass or area of leaf, the two measures being related by leaf mass per unit area ( $M_{\text{area}}$ ; see supplementary Information; Cornelissen et al. 2003). Below, we refer to ‘content’ meaning abundance (i.e., mass) per unit area of leaf, and ‘concentration’ meaning abundance per unit mass of dry leaf.

To date, reductions in photosynthetic pigments, such as chlorophyll content ( $\text{Chl}_{\text{area}}$ ), have been found with exposure to  $\text{eCO}_2$  across a wide range of plants (Ainsworth and Long 2005; Creydt et al. 2019; Major and Mosseler, 2021 and citations therein), although not always (Rey and Jarvis 1998; Springer and Thomas 2007). Furthermore,

the magnitude of the chlorophyll reduction has ranged considerably (2–25% reduction with relatively constant  $\text{eCO}_2$ ; + 150–300  $\mu\text{mol mol}^{-1}$  above ambient) (Ainsworth and Long 2005; Warren et al. 2015). In trees,  $\text{eCO}_2$ -driven reductions in  $\text{Chl}_{\text{area}}$  have been observed in tree seedlings and saplings (Houpis et al. 1988; Oberbauer et al. 1985; Thomas 2005; Wullschlegel et al. 1992) predominantly grown in pots. Far fewer studies have been conducted on aged forest-grown trees (Warren et al. 2015; Wujeska-Klaue et al. 2019) and, therefore, questions remain as to how  $\text{eCO}_2$  may affect  $\text{Chl}_{\text{area}}$  in mature forests. Previous research has highlighted that responses of trees grown in artificial environments, such as in chambers or pots, may not reflect that of mature forest-grown trees (Norby et al. 2016). Differences in plant responses are suggested to be the result of limitations associated with these artificial environments, such as root restrictions and nutrient limitations (Berntson et al. 1993; Hättenschwiler et al. 1997). Frequently, studies that report  $\text{eCO}_2$ -driven reductions in chlorophyll content ( $\text{Chl}_{\text{area}}$ ) have found similar reductions in foliar nitrogen content ( $N_{\text{area}}$ ), suggesting that the response is dependent upon nutrient availability to the plant (Stitt and Krapp 1999) and indicates that N allocation to chlorophyll follows the pattern of total N in  $\text{eCO}_2$  (Cavender-Bares et al. 2000; Warren et al. 2015; Wujeska-Klaue et al. 2019). In contrast, other studies have found reductions in chlorophyll concentrations ( $\text{Chl}_{\text{mass}}$ ), alongside reduced  $N_{\text{mass}}$ , but not  $\text{Chl}_{\text{area}}$ , implying that  $\text{eCO}_2$ -related declines in  $\text{Chl}_{\text{mass}}$  may be the result of morphological changes in  $M_{\text{area}}$ , that is, in a dilution effect, rather than limitations to, or the reallocation of, foliar N (Epron et al. 1996; Sholtis et al. 2004). With  $\text{eCO}_2$ , plants may also alter the N allocation *within* the leaf to maximise photosynthesis; thus, one possibility may lead to  $\text{eCO}_2$ -induced reductions in N investment in the light-harvesting photosynthetic apparatus (Ainsworth and Long 2005; Caldararu et al. 2020). If this is the case,  $\text{eCO}_2$  may lead to changes in canopy functioning in the future through these changes in N investment to components of leaf function. However, both the magnitude of changes in  $\text{Chl}_{\text{mass}}$  and  $\text{Chl}_{\text{area}}$  in old forest-grown trees, and whether N allocation to chlorophyll changes with  $\text{eCO}_2$ , remain unclear due to a lack of studies conducted on this topic (Warren et al. 2015; Wujeska-Klaue et al. 2019).

In addition to the effects of  $\text{eCO}_2$ , changes to  $\text{Chl}_{\text{mass}}$  and  $\text{Chl}_{\text{area}}$  are known to occur spatially through the vertical canopy profile in forested systems as a result to changes in light (Hikosaka et al. 2016; Larcher, 2003; Richardson et al. 2002). For example,  $\text{Chl}_{\text{mass}}$  was observed to increase with decreasing height in the canopy in a mature beech forest (up to 20 m) (Scartazza et al. 2016). Equivalently,  $\text{Chl}_{\text{mass}}$  has been found to correlate negatively with increasing light levels (Lichtenthaler et al. 2007; Niinemets, 2007), whereby leaves developing under low light were found to allocate

more nitrogen to light-harvesting components, such as chlorophyll, to increase light capture efficiency (Valladares and Niinemets 2008). In contrast,  $\text{Chl}_{\text{area}}$  is found to correlate positively with increasing light levels due to changes in  $M_{\text{area}}$  outweighing changes in  $\text{Chl}_{\text{mass}}$  (Lichtenthaler et al. 2007). Both  $M_{\text{area}}$  and  $N_{\text{area}}$  have been observed to decrease from canopy top to bottom in both temperate (Ellsworth and Reich 1993; Koike et al. 2001) and tropical forests (Cavaleri et al. 2010; Kenzo et al. 2012). In broad-leaved canopies,  $N_{\text{mass}}$  often displays no, or a very small, gradient across the canopy (Ellsworth and Reich 1993; Montpied et al. 2009; Niinemets 1997). As a result, the vertical gradient in  $N_{\text{area}}$  is influenced by the gradient of  $M_{\text{area}}$  across the canopy. Therefore, it is common to observe a relationship between  $M_{\text{area}}$  and  $\text{Chl}_{\text{area}}$  both, presumably, driven by the vertical profiles of light effects on leaf development and thickness in forest canopies (Catoni et al. 2015; Ellsworth and Reich 1993; Jin et al. 2014; Wyka et al. 2012). However, the interactive effect of canopy position and  $\text{eCO}_2$  on  $\text{Chl}_{\text{area}}$  in aged forest trees has received no attention. Quantifying any interaction of responses to  $\text{eCO}_2$  and canopy position will be useful in understanding whole plant optimisation strategies (e.g., Walker et al. 2018) and whole canopy responses to future environmental change (Catoni et al. 2015).

The present study investigates  $\text{Chl}_{\text{mass}}$  and  $\text{Chl}_{\text{area}}$ ,  $N_{\text{mass}}$ ,  $N_{\text{area}}$ , and  $M_{\text{area}}$ , across the vertical profile of the canopy-dominant species, *Quercus robur*, at the Birmingham Institute of Forest Research Free Air  $\text{CO}_2$  Enrichment (BIFoR FACE) facility in the 3rd and 4th season of  $\text{eCO}_2$  fumigation. Our objectives were to quantify the effect of  $\text{eCO}_2$  and canopy position on  $\text{Chl}_{\text{mass}}$  and  $\text{Chl}_{\text{area}}$ , in addition to foliar N and  $M_{\text{area}}$ , for this *Q. robur* canopy. Our expectations were that (i) that  $\text{Chl}_{\text{mass}}$  would increase from the top to bottom of the live canopy of canopy dominants, whilst  $\text{Chl}_{\text{area}}$  would decrease along this gradient, and (ii) that  $\text{Chl}_{\text{area}}$  would be unchanged by  $\text{eCO}_2$ . Quantifying the effects of  $\text{eCO}_2$  and canopy position on  $\text{Chl}_{\text{mass}}$  and  $\text{Chl}_{\text{area}}$  in an old-aged forest tree species will provide useful information to understand the sensitivity of photosynthetic pigments to changes in the physical environment and future climate. Furthermore, an understanding of N allocation in canopy leaves in  $\text{eCO}_2$  can help us understand potential effects of  $\text{eCO}_2$  on canopy processes, biogeochemical changes, and may provide new tools for monitoring canopy status in  $\text{eCO}_2$ .

## Methods

### Site description

This study was conducted at the Birmingham Institute of Forest Research (BIFoR) Free Air Carbon Enrichment (FACE) facility located in Staffordshire (52.801°N, 2.301°W), United

Kingdom. The BIFoR FACE facility is positioned in a mature northern temperate broadleaf deciduous woodland consisting of an over storey canopy dominated by 175-year-old English oak (*Q. robur*). Within the forest, six replicate circular experimental plots of woodland (~30 m in diameter) were established and encircled by 25 m tall steel structures (Hart et al. 2020). Three of these FACE plots are ambient controls ( $\text{aCO}_2$ ) ( $\text{CO}_2$  mole fraction ca.  $405 \mu\text{mol mol}^{-1}$  at the time of measurements) and the remaining three plots are treatment plots supplied with  $\text{CO}_2$  enriched air, named elevated  $\text{CO}_2$  plots ( $\text{eCO}_2$ ) (ca.  $+150 \mu\text{mol mol}^{-1}$  above ambient so receiving ca.  $555 \mu\text{mol mol}^{-1}$ ). Across the study period, the mean day-time  $\text{CO}_2$  was  $543 (\pm 24) \mu\text{mol mol}^{-1}$  in the  $\text{eCO}_2$  plots and  $406 (\pm 13) \mu\text{mol mol}^{-1}$  in the ambient control plots (see Supplementary Fig. 1.). In each plot, canopy access to *Q. robur* trees was gained through a rope canopy access system (CAS) (Total Access Ltd., UK) (Gardner et al. 2021). One oak tree per plot ( $n=3$ ) was accessible using the CAS system as set up during this study. This facilitated in situ measurements (see details below) by allowing access to all canopy positions of each tree. Canopy positions were defined as follows: upper (top 2 m, generally between 20 and 25 m), middle (12–15 m), and lower canopy (8–10 m) for a typical 25 m *Q. robur* tree at BIFoR FACE. Further details of the site description can be found at (Hart et al. 2020) and (Gardner et al. 2021).

### Chlorophyll measurements and plant material

To assess the effect of  $\text{eCO}_2$  and canopy position, chlorophyll content measurements were taken monthly, May to October, in 2019 and 2020. Chlorophyll content was measured using a portable Minolta chlorophyll meter SPAD-502 Plus (Konica-Minolta, Japan, SPAD-502) and a subsequent in vitro calibration was conducted using chemical chlorophyll extractions (See supplemental information). SPAD readings were taken in all six plots (3 ambient replicates and 3 elevated replicates) between 10:00 and 16:00 in the defined upper, middle, and lower canopy positions of *Q. robur* (see 'Site description'). The adaxial side of the leaves were placed toward the emitting window of the instrument with the midrib and major veins being avoided and SPAD values were measured close to the midpoint of the leaf. The mean of three readings per leaf was taken to obtain one reading per leaf. In 2019, 30 leaves per canopy position per tree were measured and 15 leaves per canopy position per tree were measured in 2020.

Leaf samples were collected from the upper and lower canopy in July and August of 2019 and 2020, by arborist climbers, and stored immediately at  $-25^\circ\text{C}$ . Two upper canopy leaves, from one tree per plot, were selected for nutrient analysis (see 'Leaf N analyses' below); these trees corresponded to the measurement tree for SPAD measurements. Each leaf was

photographed on white graph paper, with a ruler for reference. Leaf area analysis was conducted using imaging software Image J (IMAGE J v1.53, National Institutes of Health, Bethesda, MD, USA).

## Leaf N analyses

Leaf material dried at 70 °C for at least 72 h, weighed for dry weight, and stored until further analysis. Known weights (~2 mg) of dried leaf fragments were ground and each sample was analysed for N using an elemental analyser interfaced with an isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK). Foliar N values were expressed as mg N per leaf dry weight ( $N_{\text{mass}}$ ) and subsequently N per area ( $N_{\text{area}}$ ).

## Statistical analysis

Statistical analyses were performed in R version 4.0.3 (R Core Team, 2020). Before statistical analysis, all data were checked for normality by inspection of the Q–Q plots and Levene's test.

SPAD-derived  $\text{Chl}_{\text{mass}}$  and  $\text{Chl}_{\text{area}}$ , over the two growing seasons of 2019 and 2020, were analysed using a linear mixed-effects model ('lmer' package). Fixed categorical factors in this model were sampling year (i.e., 2019 or 2020), sampling month, canopy position (i.e., upper, middle or lower canopy), and  $\text{CO}_2$  treatment (i.e.,  $\text{aCO}_2$  or  $\text{eCO}_2$ ). Additionally, 'M<sub>area</sub>' and 'plot' were represented as random factors, the latter as individual trees were nested within each experimental plot. An additional linear mixed-effects model analysed M<sub>area</sub>, area-based leaf nitrogen ( $N_{\text{area}}$ ) and mass-based leaf nitrogen ( $N_{\text{mass}}$ ) with sampling year, sampling month, canopy position and  $\text{CO}_2$  treatment as fixed categorical factors and 'plot' represented as a random factor. For all analyses, P values of <0.05 were considered statistically significant and type III F-statistics associated with the mixed model analysis [repeated-measures analysis of variance (ANOVA)] were reported. When a main effect was found significant, it was further tested using a post hoc Tukey's test using the R package "emmeans".

The sample size in this study was  $n=3$  replicates, which is the number of replicate treatment plots at the BIFoR FACE site, and includes within-tree replicates that were averaged per plot before analysis. All three plot replicates were sampled, except for May 2019 where replicates were reduced to two, due to logistic constraints of safe tree access.

## Results

### The empirical relationship between SPAD values and photosynthetic pigment concentrations

There were strong positive and mildly non-linear correlations ( $p < 0.001$ ) between SPAD values and extracted leaf

chlorophyll and carotenoid concentrations for mature *Q. robur* (Supplementary Fig. 2; Supplementary Table 1). The empirical relationship obtained between total  $\text{Chl}_{\text{mass}}$  and SPAD values can be represented by either linear, logarithmic, or quadratic fits (S Fig. 2a; S Table 1) according to goodness-of-fit criteria. Based on the criteria of high  $R^2$  and low RMSE, the logarithmic model was judged the best fit, whilst the quadratic and linear models performed similarly, but not as well as the logarithmic model ( $R^2$  values for all three models at 0.78).

### Seasonal and inter-annual variations in chlorophyll

SPAD-derived measurements show that, in the two measurement years, each spring had a flush of foliage that comprised low  $\text{Chl}_{\text{mass}}$  and  $\text{Chl}_{\text{area}}$  but rapidly rose over June and July, attaining a broad  $\text{Chl}_{\text{mass}}$  maximum that persisted through summer and into early autumn (Fig. 1). A gradual decline in  $\text{Chl}_{\text{mass}}$  and  $\text{Chl}_{\text{area}}$  began in September as the leaves start to senesce. In both sampling years, first autumnal leaf tint was recorded by visual observation in late September (18<sup>th</sup> and 29<sup>th</sup> of September, respectively) and first autumnal leaf fall occurred just under a month later (13<sup>th</sup> and 26<sup>th</sup> Oct, respectively). The monthly differences for  $\text{Chl}_{\text{mass}}$  and  $\text{Chl}_{\text{area}}$  were significant (Table 1,  $P < 0.001$ ). Additionally, a significant year x month interaction was observed, where significantly lower total  $\text{Chl}_{\text{mass}}$  and  $\text{Chl}_{\text{area}}$  were reported in May and June of 2019, compared to their 2020 counterparts (Table 1,  $P < 0.05$ ) (Supplementary Fig. 3).

### Effects of canopy position and elevated $\text{CO}_2$

Common to both sampling years,  $\text{Chl}_{\text{mass}}$  was significantly higher in lower canopy leaves than in upper canopy leaves (Table 1,  $P < 0.05$ ).  $\text{Chl}_{\text{mass}}$  was 14% and 12% higher in the lower canopy leaves when compared to upper canopy in 2019 and 2020, respectively. In contrast, when expressed on an area basis ( $\text{Chl}_{\text{area}}$ ), there was significantly higher  $\text{Chl}_{\text{area}}$  in the higher canopy leaves when compared to the lower canopy leaves (Table 1,  $P < 0.05$ ). This study also found a significant month x canopy position effect for  $\text{Chl}_{\text{mass}}$ , whereby significantly lower  $\text{Chl}_{\text{mass}}$  was reported in August and May in upper canopy foliage in 2019 and 2020, respectively.

There was no effect of  $\text{eCO}_2$  on  $\text{Chl}_{\text{mass}}$  (Fig. 2, Table 1). There was a significant increase in  $\text{Chl}_{\text{area}}$ , but this effect was removed once M<sub>area</sub> had been accounted for (Fig. 2, Table 1). Additionally, there was no significant interaction between  $\text{CO}_2$  treatment x canopy position in either  $\text{Chl}_{\text{mass}}$  or  $\text{Chl}_{\text{area}}$ .

M<sub>area</sub> significantly decreased through the canopy from top to bottom (Fig. 3,  $P < 0.05$ ; Table 2) and did not significantly



**Table 1** Linear mixed-effects model showing the statistical significance of year, month, canopy position, and CO<sub>2</sub> treatment and their relevant interactions on chlorophyll concentration (Chl<sub>mass</sub>) in *Q. robur* trees

Source of variation	Chl <sub>mass</sub>			Chl <sub>area</sub>		
	DF	P value		DF	P value	
Year	1	0.083	—	1	0.13	—
Month	4	<b>&lt; 0.001</b>	***	4	<b>&lt; 0.001</b>	***
Canopy position	2	<b>0.038</b>	*	2	<b>0.017</b>	*
CO <sub>2</sub> treatment	1	0.76	—	1	0.41	—
M <sub>area</sub>	1	0.82	—	1	<b>0.013</b>	*
Year * Month	1	<b>0.0015</b>	**	1	<b>0.02</b>	*
Year * Canopy position	1	0.93	—	1	0.93	—
Year * CO <sub>2</sub> Treatment	1	0.25	—	1	0.4	—
Month * Canopy position	4	<b>0.022</b>	*	4	0.41	—
Month * CO <sub>2</sub> Treatment	4	0.52	—	4	0.45	—
Canopy position * CO <sub>2</sub> Treatment	2	0.17	—	2	0.13	—
Year * Month * Canopy position	1	0.076	—	1	0.18	—
Year * Month * CO <sub>2</sub> Treatment	1	0.37	—	1	0.47	—
Year * Canopy position * Treatment	1	0.11	—	1	0.12	—
Month * Canopy position * Treatment	4	0.16	—	4	0.27	—
Year * Month * Canopy position * Treatment	4	0.079	—	4	0.8	—

All listed factors were fixed with 'M<sub>area</sub>' and 'plot' listed as random factors. See Figs. 2 and 3 for directions and magnitudes of effect. Type III sums of squares computed using restricted maximum-likelihood estimates for *F* tests. The numerator degrees of freedom (DF) for each *F* test are shown. A post hoc Tukey's test was used to determine the significance relationships. Significance is noted in the rightmost column as (\*\*\*) = *P* < 0.001; (\*\*) = *P* < 0.01; (\*) = *P* < 0.05). *P* values < 0.05 are given in bold fonts

change with CO<sub>2</sub> treatment (Fig. 3, Table 2). M<sub>area</sub> decreased by a mean of 44% (mean ± SD: 99 ± 20 g m<sup>-2</sup>) and 53% (mean ± SD: 85 ± 14 g m<sup>-2</sup>) for eCO<sub>2</sub> and aCO<sub>2</sub>, respectively, in the lower canopy foliage when compared to upper canopy foliage. Additionally, M<sub>area</sub> increased with eCO<sub>2</sub> treatment by 16% and 23% in the upper and lower foliage, respectively. There was no effect of CO<sub>2</sub> treatment or canopy position on N<sub>mass</sub> (Fig. 3, Table 2). Additionally, there was no effect of CO<sub>2</sub> on N<sub>area</sub>; however, N<sub>area</sub> decreased by 29% and 33%, from the canopy top to bottom, in eCO<sub>2</sub> and aCO<sub>2</sub> treatments respectively (Fig. 3, *P* < 0.05; Table 2).

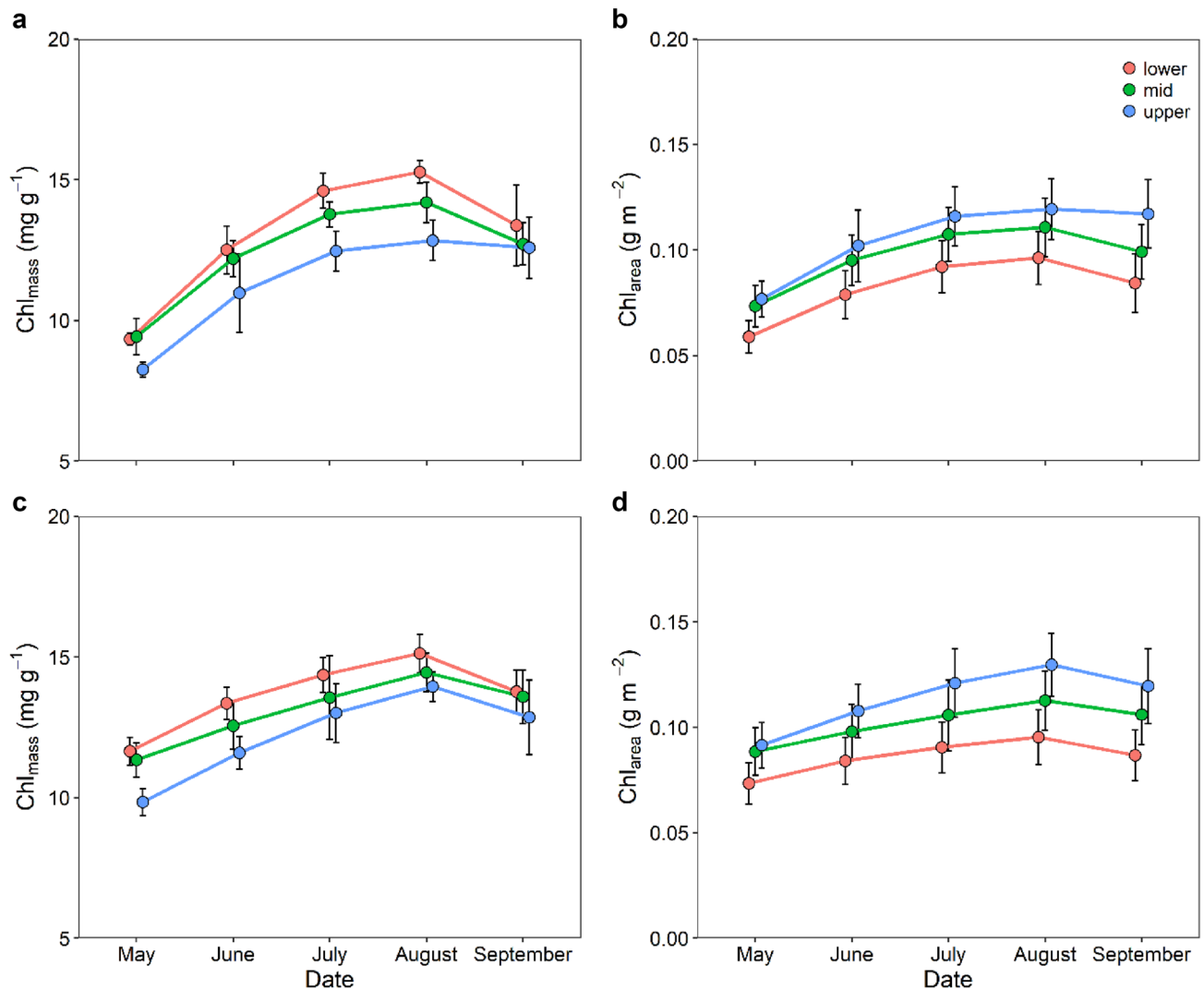
## Discussion

Very few studies have analysed the effect of eCO<sub>2</sub> on Chl<sub>mass</sub> and Chl<sub>area</sub> in aged forest-grown trees (Wujeska-Klaue et al. 2019) and fewer have also assessed the role of canopy position in the eCO<sub>2</sub> response (Crous et al. 2020; Sharwood et al. 2017). We found that both Chl<sub>mass</sub> and Chl<sub>area</sub> significantly varied in response to canopy position, but that neither Chl<sub>mass</sub> nor Chl<sub>area</sub> were affected by eCO<sub>2</sub> exposure, once M<sub>area</sub> had been accounted for. Additionally, we found no significant changes in N<sub>mass</sub> with canopy position or CO<sub>2</sub> treatment, although N<sub>area</sub> increased with eCO<sub>2</sub> and significantly increased with canopy height. We found M<sub>area</sub> increased with canopy height and with eCO<sub>2</sub>, suggesting that changes in M<sub>area</sub> rather than changes in foliar N could be attributed to

the observed changes to Chl<sub>area</sub> and N<sub>area</sub> with canopy position and eCO<sub>2</sub>. The results suggest that the allocation of N to light harvesting is maintained with eCO<sub>2</sub> exposure and in the upper canopy, but that more N is allocated to light harvesting in the lower canopy. These results are consistent with observations in other plantation and forest-grown tree species (Crous et al. 2008; Warren et al. 2015).

## Seasonal and inter-annual variations in chlorophyll

Chlorophyll (Chl<sub>area</sub> and Chl<sub>mass</sub>) increases in the first part of the growing season for both years studied, and then attains a broad maximum through July and August, before decreasing in September (S. Figure 3) (Morecroft et al. 2003). Summer maximum Chl amounts are a factor of 1.4–1.8 larger than the springtime (May) measurements. This study reported significant seasonal variation in Chl<sub>mass</sub> and Chl<sub>area</sub>, in addition to significant month × year interactions (S. Figure 4, Table 1). The lower Chl<sub>mass</sub> and Chl<sub>area</sub> observed in May and June of 2019 than in the following year (Fig. 2a vs. Figure 2c) may be attributable to inter-annual differences in leaf age in the trees at the site as a result of herbivory. In 2019, an outbreak of winter moth (*Operophtera brumata*) occurred in April and resulted in widespread defoliation across the site (Supplementary Fig. 4). The leaf regrowth likely contributed to a delay in greening of leaves in this season. There was



**Fig. 1** Mean monthly chlorophyll, expressed per unit mass ( $\text{Chl}_{\text{mass}}$ ) ( $\text{mg g}^{-1}$ ) (a, c) and per unit area ( $\text{Chl}_{\text{area}}$ ) ( $\text{g m}^{-2}$ ) (b, d), across the growing seasons of 2019 (a, b) and 2020 (c, d). Points are staggered for clarity. Colours indicate the three different canopy layers; 'upper'

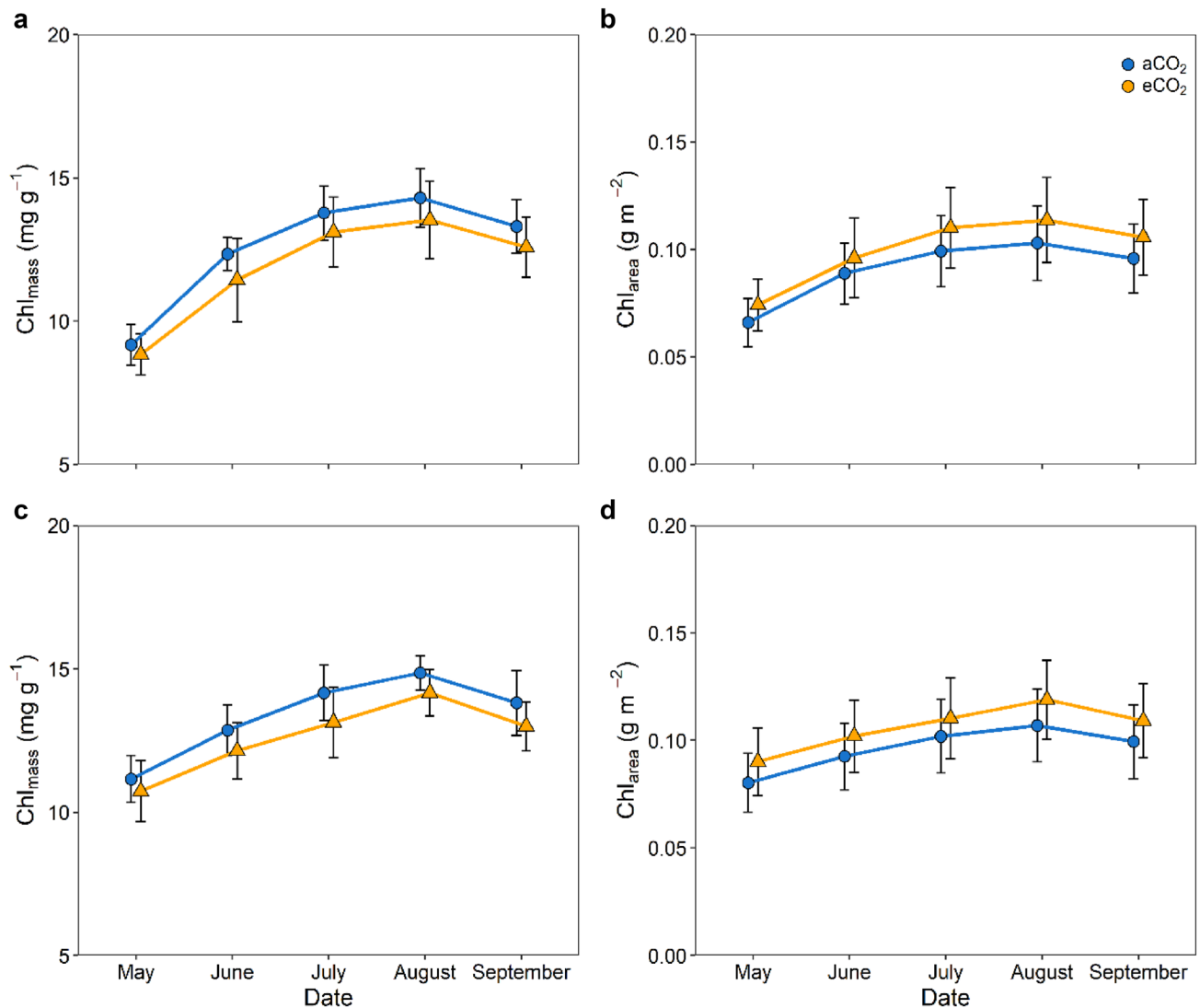
(blue), 'middle' (green), and 'lower' (red) for *Q. robur* under ambient  $\text{CO}_2$  ( $\text{aCO}_2$ ) at the BIFoR FACE facility. Each point represents the six experimental plots at BIFoR FACE

no effect of  $\text{eCO}_2$  on defoliation across the site, although this has not been explicitly quantified, but there a spatial pattern was observed where two plots (one  $\text{aCO}_2$  and one  $\text{eCO}_2$ ) were not affected by herbivory to the same extent as the others (pers. obs.). Measurements over more seasons would be required to disentangle the effects of herbivory, phenology,  $\text{eCO}_2$ , and month that are currently confounded in this study.

### The effect of $\text{eCO}_2$

Elevated  $\text{CO}_2$  treatment did not significantly affect  $\text{Chl}_{\text{mass}}$ , which has been observed in other field  $\text{eCO}_2$  experiments

on trees (Liberloo et al. 2007; Wujeska-Klaue et al. 2019). We found no significant effect of  $\text{eCO}_2$  for  $\text{Chl}_{\text{area}}$ , once  $M_{\text{area}}$  had been accounted for; that is, the mass of chlorophyll through the column of a square centimetre of leaf was unchanged. As discussed in the supplementary information, the mass fraction and areal density of a compound in a leaf are related by the leaf mass per unit area ( $M_{\text{area}}$ ). Hence, the derived  $\text{Chl}_{\text{area}}$  content can be due to a change in the measured  $\text{Chl}_{\text{mass}}$  concentration and/or a change in  $M_{\text{area}}$ . In support of this, we found that  $M_{\text{area}}$  increased with  $\text{eCO}_2$ , which is a common response of trees to  $\text{CO}_2$  enrichment (as reviewed in Ainsworth and Long 2005). For a constant areal density, increasing  $M_{\text{area}}$  leads to a decreasing dry mass fraction; that is, to a dilution effect (Epron et al. 1996; Feng



**Fig. 2** Mean monthly chlorophyll, expressed per unit mass ( $\text{Chl}_{\text{mass}}$ ) ( $\text{mg g}^{-1}$ ) and per unit area ( $\text{Chl}_{\text{area}}$ ) ( $\text{mg cm}^{-2}$ ), across the growing seasons of 2019 (**a, b**) and 2020 (**c, d**). Points are staggered for clarity. Colours indicate the CO<sub>2</sub> treatment in *Q. robur* leaves at the

BIFoR FACE facility with aCO<sub>2</sub> (blue) and eCO<sub>2</sub> (orange). Each data point consists of all three canopy positions and represents the number of replicate treatment plots at the BIFoR FACE facility ( $n=3$ )

et al. 2015). Decreases in  $\text{Chl}_{\text{mass}}$ , alongside decreases in  $N_{\text{mass}}$ , have been observed under eCO<sub>2</sub> previously and have primarily been attributed to a dilution effect as a result of increases in  $M_{\text{area}}$  (Ainsworth and Long 2005; Sholtis et al. 2004; Wullschlegel et al. 1992). However, in the present study, we did not see any reductions in  $\text{Chl}_{\text{mass}}$  or  $N_{\text{mass}}$  with eCO<sub>2</sub> suggesting the lack of a dilution effect occurring with exposure to eCO<sub>2</sub>.

Increases in  $M_{\text{area}}$  can be the result of increases in mass components that are either structural mass (such as leaf thickness and density) or photosynthetic mass (such as chlorophyll density), parameters not measured in the present study (Osnas et al. 2018). As  $M_{\text{area}}$  as a covariate was

found to account for the significant eCO<sub>2</sub> effect on  $\text{Chl}_{\text{area}}$ , this indicates that the eCO<sub>2</sub>-driven increases in  $M_{\text{area}}$  may primarily increase structural and photosynthetic mass proportionally.  $M_{\text{area}}$  is considered a key trait in plant growth (Lambers and Poorter, 1992) and an indicator of plant strategies (Poorter et al. 2009); therefore, it will be important to assess implications on  $M_{\text{area}}$  with changes in atmospheric CO<sub>2</sub>.

Previously, eCO<sub>2</sub>-driven reductions in  $\text{Chl}_{\text{area}}$  have been suggested to be the result of eCO<sub>2</sub>-driven reductions in  $N_{\text{area}}$  and, therefore, lead to a reduced N investment in the photosynthetic apparatus (Ainsworth and Long 2005; Drake et al. 1997). However, there have not been many measurements



**Fig. 3** Mean **a** leaf mass per area ( $M_{\text{area}}$ ) ( $\text{g m}^{-2}$ ), **b** nitrogen per mass ( $N_{\text{mass}}$ ) ( $\text{mg g}^{-1}$ ), **c** nitrogen per area ( $N_{\text{area}}$ ) ( $\text{g m}^{-2}$ ), and **d** chlorophyll per area ( $\text{Chl}_{\text{area}}$ ) ( $\text{g m}^{-2}$ ) in the upper (20–23 m) and lower (8–11 m) canopy positions. Data points represent true replicates ( $n=3$ ). Colours and shapes indicate the  $\text{CO}_2$  treatment in *Q. robur* leaves at the BIFoR FACE facility with  $\text{aCO}_2$  (blue circles) and  $\text{eCO}_2$  (orange triangles)

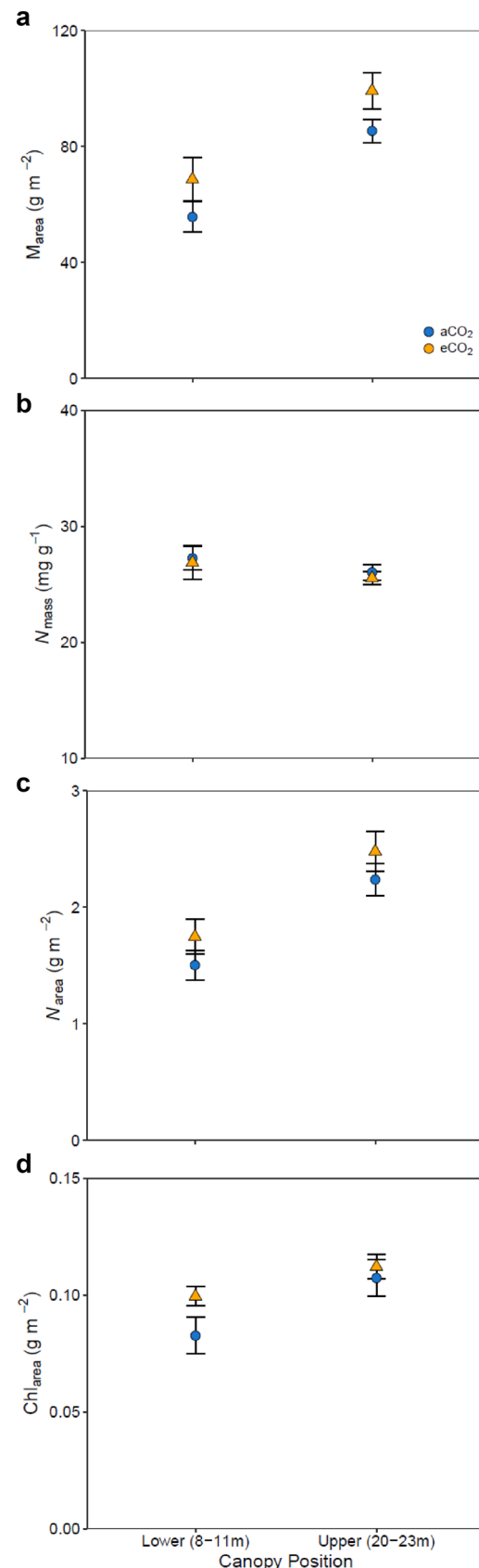
to test this hypothesis, particularly for aged forests in whose canopies N allocation to light harvesting is important. Foliar N had been maintained in the first three years of  $\text{CO}_2$  fumigation at the BIFoR FACE site (Gardner et al. 2021), so we expected to see no  $\text{eCO}_2$ -driven reductions in foliar N in the present study, as was indeed the case. In the present study, we found  $N_{\text{area}}$  either increased or remained unchanged in response to  $\text{eCO}_2$ , suggesting that there were no limitations to the nitrogen supply (Warren et al. 2015). If the allocation of N to chlorophyll, with  $\text{eCO}_2$ , follows that of total N, we would therefore expect  $\text{Chl}_{\text{area}}$  to follow the relationship of  $N_{\text{area}}$ , as observed.

It has previously been suggested that N may be reallocated with exposure to  $\text{eCO}_2$  away from light-harvesting apparatus to other processes (Caldararu et al. 2020; Drake et al. 1997). This has primarily been suggested in instances where nutrients have or may become limiting and can lead to reductions in photosynthetic capacity (Crous et al. 2008; Major et al. 2018). Our results suggest that this is not the case in sesquicentennial *Q. robur*, as  $\text{Chl}_{\text{area}}$  was not reduced with  $\text{eCO}_2$  exposure. Instead, the results indicate that N availability is in excess to meet the requirements for leaf processes, such as for photosynthetic apparatus (Warren et al. 2015) and, therefore, the tree is able to maintain leaf stoichiometry with  $\text{eCO}_2$ . This is supported by previous analysis at this site that found the maintenance of foliar N with  $\text{eCO}_2$  in addition to a sustained 33% photosynthetic enhancement in mature *Q. robur* (Gardner et al. 2021).

The data in the present study are from the first 4 years of  $\text{eCO}_2$  exposure, and future seasons of  $\text{eCO}_2$  fumigation could lead to a progressive decline in soil N over time. A progressive decline or nutrient limitation has been previously observed in other FACE experiments and can lead to reductions in  $N_{\text{area}}$  that consequently affect  $\text{Chl}_{\text{area}}$  (Rey and Jarvis 1997; Warren et al. 2015). An understanding of N allocation in canopy leaves in  $\text{eCO}_2$  can help us understand potential effects of  $\text{eCO}_2$  on canopy processes, biogeochemical changes (given that N–C cycles are coupled in the biota of an ecosystem), and may provide new tools for monitoring canopy status in  $\text{eCO}_2$ .

### The effect of canopy position

This study reported significantly higher  $\text{Chl}_{\text{mass}}$  in the lower canopy, compared to the upper canopy ( $P < 0.05$ , Fig. 1,



**Table 2** Linear mixed model showing the statistical significance of CO<sub>2</sub> treatment and canopy position, and their interaction, on leaf mass per area ( $M_{\text{area}}$ ), nitrogen per mass ( $N_{\text{mass}}$ ), and nitrogen per area ( $N_{\text{area}}$ ) in *Q. robur* trees

Source of variation	DF	$M_{\text{area}}$ <i>P</i> value	$N_{\text{mass}}$ <i>P</i> value	$N_{\text{area}}$ <i>P</i> value
CO <sub>2</sub> treatment	1	0.33	0.40	0.35
Canopy position	1	<b>0.003**</b>	0.16	<b>0.006**</b>
Canopy position * CO <sub>2</sub> treatment	1	0.93	0.29	0.57

All listed factors were fixed with ‘plot’ listed as a random factor. See Fig. 4 for direction and magnitudes of effect. Type III sums of squares computed using restricted maximum-likelihood estimates for *F* tests. The numerator degrees of freedom (DF) for each *F* test are shown. A post hoc Tukey’s test was used to determine the significance relationships. Significance is noted as (\*\*=*P* < 0.01; \*=*P* < 0.05). *P* values < 0.05 are given in bold fonts

Table 1), in agreement with previous studies of plantation and forest-grown trees in eCO<sub>2</sub> (Crous et al. 2008; Liberloo et al. 2007; Scartazza et al. 2016). Increases in photosynthetic pigments are expected with increasing depth in the canopy and have been attributed to decreasing light availability through the canopy (Hikosaka et al. 2016; Niinemets 2007). This is due to increased requirement of light capture in shaded leaves and therefore a greater allocation of resources to the light-harvesting apparatus (Lennon et al. 2021; Lichtenthaler and Buschmann 2001; Sharwood et al. 2017). In contrast, upper canopies naturally receive high light levels and thus require less N investment in their light-harvesting capability (Sharwood et al. 2017). Previous research suggests lower  $\text{Chl}_{\text{mass}}$  in upper canopy leaves may occur, because the leaves have thicker cell walls, lower leaf water content, and higher dry weight (Lichtenthaler and Buschmann 2001). In support of this, the present study found upper canopy leaves to have significantly higher leaf mass per unit area ( $M_{\text{area}}$ ), compared to leaves from the lower canopy (Fig. 3) (Ellsworth and Reich 1993; Norby et al. 2021). Therefore, the decline in  $\text{Chl}_{\text{mass}}$  with increasing canopy height is likely attributable to a dilution effect as a result of the increases in  $M_{\text{area}}$  (Epron et al. 1996; Houpius et al. 1988; Sholtis et al. 2004; Wulschleger et al. 1992).

Similarly, we found  $\text{Chl}_{\text{area}}$  significantly increased with increasing canopy height, paralleling the response of both  $M_{\text{area}}$  and  $N_{\text{area}}$ . Increases in  $M_{\text{area}}$  and  $N_{\text{area}}$  with increasing canopy height has been found previously (Fig. 3) (Crous and Ellsworth 2004; Jin et al. 2014; Norby et al. 2021). Our results suggest that  $M_{\text{area}}$  leads to the changes in  $\text{Chl}_{\text{area}}$  with canopy position and has been found previously (Herrick and Thomas 1999; Liberloo et al. 2007). The upper canopy leaves are primarily associated with higher rates of photosynthesis and therefore greater amount of nitrogen per leaf unit area and, assuming there is no photosynthetic

downregulation or N reallocation occurring, would present similar increases  $\text{Chl}_{\text{area}}$ . This suggests that there are enough nutrients to support the photosynthetic process (i.e., no nitrogen limitation present) and is supported by previous findings (Warren et al. 2015). In support of this, previous research has shown that the structural adjustments in  $M_{\text{area}}$ , as a result of light gradients, are responsible for much of the variation regarding photosynthetic capacity (Niinemets, 2007). These results support the importance of accurate representation of  $M_{\text{area}}$  through the vertical canopy that is parameterised in vegetation models.

The present study found no significant interaction of CO<sub>2</sub> treatment and canopy position for  $\text{Chl}_{\text{mass}}$  or  $\text{Chl}_{\text{area}}$ . The lack of interaction of CO<sub>2</sub> treatment and canopy position indicates that future changes in atmospheric CO<sub>2</sub> may not influence plant traits differently across the canopy compared with the relationships in the present day. Additionally, there was no effect of eCO<sub>2</sub> on the pattern of  $N_{\text{area}}$  with canopy height which suggests that eCO<sub>2</sub> has no effect on N content integrated through the depth of the leaf. Similar results have been reported previously (Norby et al. 2021; Takeuchi et al. 2001), and so, we provide further confidence for the representation of  $N_{\text{area}}$  as a constant with exposure to eCO<sub>2</sub> in models.

## Conclusion

In mature *Q. robur* canopies considering season, canopy height, and CO<sub>2</sub> treatment,  $\text{Chl}_{\text{mass}}$  varied significantly with most of these factors. This suggests that canopy chlorophyll is highly dynamic in oak canopies, which needs to be considered in remote-sensing measures using canopy light absorbance or greenness as proxies for light-use efficiency. There were eCO<sub>2</sub>-driven increases in  $\text{Chl}_{\text{area}}$ , that were consistent with eCO<sub>2</sub>-driven increases in  $M_{\text{area}}$ , suggesting that increased cell stacking and chlorophyll density in oak leaves may help regulate the eCO<sub>2</sub> response and within-canopy variation. The results suggest that season and canopy position are particularly important to consider for scaling leaf-to-ecosystem light harvesting and canopy N-use and photosynthetic processes.

**Supplementary Information** The online version contains supplementary material available at <https://doi.org/10.1007/s00468-022-02328-7>.

**Acknowledgements** We gratefully acknowledge Professor Richard J. Norby for the useful comments and discussion on an earlier version of the manuscript. We thank Professor Christine Foyer and Dr Barbara Karpinski for laboratory space and advice regarding chlorophyll extractions. AG gratefully thanks Dr Angeliki Kourmouli for assistance during chlorophyll extractions. We thank the BIFoR technical team for canopy access, provision of correlative data, and site operations. AG gratefully acknowledges funding by the John Horseman trust.

The BIFoR FACE facility is supported by the JABBS foundation, the University of Birmingham, the John Horseman Trust, the Ecological Continuity Trust, and private donations. ARMK acknowledges support from the U.K. Natural Environment Research Council through grant (NE/S015833/1) which also facilitated DSE's participation.

**Author contributions** ARMK, DE, and AG designed the study; AG collected the data. AG organised the datasets under the supervision of DSE, with input from ARMK; AG and DSE designed and performed the statistical analyses, with input from ARMK. AG and DSE wrote the first draft of the paper. All authors contributed to the manuscript revision, and read and approved the submitted version.

**Funding** AG is funded by the John Horseman trust. The BIFoR FACE facility is supported by the JABBS foundation, the University of Birmingham, the John Horseman Trust, the Ecological Continuity Trust, and private donations. ARMK acknowledges support from the U.K. Natural Environment Research Council through grant (NE/S015833/1) which also facilitated DSE's participation.

## Declarations

**Conflict of interest** None declared.

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## References

- Ainsworth EA, Long SP (2005) What have we learned from 15 years of free-air CO<sub>2</sub> enrichment (FACE)? A meta-analytic review of the responses of photosynthesis, canopy properties and plant production to rising CO<sub>2</sub>. *New Phytol* 165(2):351–372. <https://doi.org/10.1111/j.1469-8137.2004.01224.x>
- Arnon DI (1949) Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris*. *Plant Physiol* 24(1):1–15. <https://doi.org/10.1104/pp.24.1.1>
- Asner GP, Martin RE (2008) Spectral and chemical analysis of tropical forests: scaling from leaf to canopy levels. *Remote Sens Environ* 112(10):3958–3970. <https://doi.org/10.1016/j.rse.2008.07.003>
- Bader MKF, Siegwolf R, Körner C (2010) Sustained enhancement of photosynthesis in mature deciduous forest trees after 8 years of free air CO<sub>2</sub> enrichment. *Planta* 232(5):1115–1125. <https://doi.org/10.1007/s00425-010-1240-8>
- Berntson GM, McConnaughay KDM, Bazzaz FA (1993) Elevated CO<sub>2</sub> alters deployment of roots in “small” growth containers. *Oecologia* 94:558–564
- Caldararu S, Thum T, Yu L, Zaehle S (2020) Whole-plant optimality predicts changes in leaf nitrogen under variable CO<sub>2</sub> and nutrient availability. *New Phytol* 225(6):2331–2346. <https://doi.org/10.1111/nph.16327>
- Catani R, Gratani L, Sartori F, Varone L, Granata MU (2015) Carbon gain optimization in five broadleaf deciduous trees in response to light variation within the crown: correlations among morphological, anatomical and physiological leaf traits. *Acta Bot Croat* 74(1):71–94. <https://doi.org/10.1515/botcro-2015-0010>
- Cavaleri MA, Oberbauer SF, Clark DB, Clark DA, Ryan MG (2010) Height is more important than light in determining leaf morphology in a tropical forest. *Ecology* 91(6):1730–1739. <https://doi.org/10.1890/09-1326.1>
- Cavender-Bares J, Potts M, Zacharias E, Bazzaz FA (2000) Consequences of CO<sub>2</sub> and light interactions for leaf phenology, growth, and senescence in *Quercus rubra*. *Glob Change Biol* 6(8):877–887. <https://doi.org/10.1046/j.1365-2486.2000.00361.x>
- Cornelissen JHC, Lavorel S, Garnier E, Díaz S, Buchmann N, Gurvich DE, Reich PB, Ter Steege H, Morgan HD, Van Der Heijden MGA, Pausas JG, Poorter H (2003) A handbook of protocols for standardised and easy measurement of plant functional traits worldwide. *Aust J Bot* 51(4):335–380. <https://doi.org/10.1071/BT02124>
- Coste S, Baraloto C, Leroy C, Marcon E, Renaud A, Richardson AD, Roggy JC, Schimann H, Uddling J, Hérault B (2010) Assessing foliar chlorophyll contents with the SPAD-502 chlorophyll meter: a calibration test with thirteen tree species of tropical rainforest in French Guiana. *Ann for Sci* 67(6):607–607. <https://doi.org/10.1051/forest/2010020>
- Creydt M, Vuralhan-Eckert J, Fromm J, Fischer M (2019) Effects of elevated CO<sub>2</sub> concentration on leaves and berries of black elder (*Sambucus nigra*) using UHPLC-ESI-QTOF-MS/MS and gas exchange measurements. *J Plant Physiol* 234–235:71–79. <https://doi.org/10.1016/j.jplph.2019.01.005>
- Croft H, Chen JM, Luo X, Bartlett P, Chen B, Staebler RM (2017) Leaf chlorophyll content as a proxy for leaf photosynthetic capacity. *Glob Change Biol* 23(9):3513–3524. <https://doi.org/10.1111/gcb.13599>
- Crous KY, Ellsworth DS (2004) Canopy position affects photosynthetic adjustments to long-term elevated CO<sub>2</sub> concentration (FACE) in aging needles in a mature *Pinus taeda* forest. *Tree Physiol* 24(9):961–970. <https://doi.org/10.1093/treephys/24.9.961>
- Crous KY, Walters MB, Ellsworth DS (2008) Elevated CO<sub>2</sub> concentration affects leaf photosynthesis-nitrogen relationships in *Pinus taeda* over nine years in FACE. *Tree Physiol* 28(4):607–614. <https://doi.org/10.1093/treephys/28.4.607>
- Crous KY, Campy CE, Lopez R, Cano F, Ellsworth D (2020) Canopy position affects photosynthesis and anatomy in mature *Eucalyptus* trees in elevated CO<sub>2</sub>. *Tree Physiol*. <https://doi.org/10.1093/treephys/tpaa117>
- Donnelly A, Yu R, Rehberg C, Meyer G, Young EB (2020) Leaf chlorophyll estimates of temperate deciduous shrubs during autumn senescence using a SPAD-502 meter and calibration with extracted chlorophyll. *Ann for Sci*. <https://doi.org/10.1007/s13595-020-00940-6>
- Drake BG, González-Meler MA, Long SP (1997) More efficient plants: a consequence of rising atmospheric CO<sub>2</sub>? *Annu Rev Plant Biol* 48:609–639. <https://doi.org/10.1146/annurev.arplant.48.1.609>
- Ellsworth DS, Reich PB (1993) Canopy structure and vertical patterns of photosynthesis and related leaf traits in a deciduous forest. *Oecologia* 96(2):169–178. <https://doi.org/10.1007/BF00317729>
- Ellsworth DS, Reich PB, Naumburg ES, Koch GW, Kubiske ME, Smith SD (2004) Photosynthesis, carboxylation and leaf nitrogen responses of 16 species to elevated pCO<sub>2</sub> across four free-air CO<sub>2</sub> enrichment experiments in forest, grassland and desert. *Glob Change Biol* 10(12):2121–2138. <https://doi.org/10.1111/j.1365-2486.2004.00867.x>
- Ellsworth DS, Anderson IC, Crous KY, Cooke J, Drake JE, Gherlenda AN, Gimeno TE, Macdonald CA, Medlyn BE, Powell JR, Tjoelker MG, Reich PB (2017) Elevated CO<sub>2</sub> does not increase

- eucalypt forest productivity on a low-phosphorus soil. *Nat Clim Chang* 7(4):279–282. <https://doi.org/10.1038/nclimate3235>
- Epron D, Liozon R, Mousseau M (1996) Effects of elevated CO<sub>2</sub> concentration on leaf characteristics and photosynthetic capacity of beech (*Fagus sylvatica*) during the growing season. *Tree Physiol* 16(4):425–432. <https://doi.org/10.1093/treephys/16.4.425>
- Evans, J. R., and Seemann, J. R. (1989). The allocation of protein nitrogen in the photosynthetic apparatus: costs, consequences, and control. *Photosynthesis, January 1989*, 18–205. [https://www.researchgate.net/profile/John\\_Evans6/publication/285010849\\_The\\_allocation\\_of\\_protein\\_nitrogen\\_in\\_the\\_photosynthetic\\_apparatus\\_Costs\\_consequences\\_and\\_control/links/58046a0008ae1c5148d0a05b/The-allocation-of-protein-nitrogen-in-the-photosyntheti](https://www.researchgate.net/profile/John_Evans6/publication/285010849_The_allocation_of_protein_nitrogen_in_the_photosynthetic_apparatus_Costs_consequences_and_control/links/58046a0008ae1c5148d0a05b/The-allocation-of-protein-nitrogen-in-the-photosyntheti)
- Evans JR, Clarke VC (2019) The nitrogen cost of photosynthesis. *J Exp Bot* 70(1):7–15. <https://doi.org/10.1093/jxb/ery366>
- Feng Z, Rütting T, Pleijel H, Wallin G, Reich PB, Kammann CI, Newton PCD, Kobayashi K, Luo Y, Uddling J (2015) Constraints to nitrogen acquisition of terrestrial plants under elevated CO<sub>2</sub>. *Glob Change Biol* 21(8):3152–3168. <https://doi.org/10.1111/gcb.12938>
- Gardner A, Ellsworth DS, Crous KY, Pritchard J, MacKenzie AR (2021) Is photosynthetic enhancement sustained through three years of elevated CO<sub>2</sub> exposure in 175-year-old *Quercus robur*? *Tree Physiol*, C. <https://doi.org/10.1093/treephys/tpab090>
- Hart KM, Curioni G, Blaen P, Harper NJ, Miles P, Lewin KF, Nagy J, Bannister EJ, Cai XM, Thomas RM, Krause S, Tausz M, MacKenzie AR (2020) Characteristics of free air carbon dioxide enrichment of a northern temperate mature forest. *Glob Change Biol* 26(2):1023–1037. <https://doi.org/10.1111/gcb.14786>
- Hättenschwiler S, Miglietta F, Raschi A, Körner C (1997) Thirty years of in situ tree growth under elevated CO<sub>2</sub>: a model for future forest responses? *Glob Change Biol* 3(5):463–471. <https://doi.org/10.1046/j.1365-2486.1997.00105.x>
- Hawkins TS, Gardiner ES, Comer GS (2009) Modeling the relationship between extractable chlorophyll and SPAD-502 readings for endangered plant species research. *J Nat Conserv* 17(2):123–127. <https://doi.org/10.1016/j.jnc.2008.12.007>
- Herrick JD, Thomas RB (1999) Effects of CO<sub>2</sub> enrichment on the photosynthetic light response of sun and shade leaves of canopy sweetgum trees (*Liquidambar styraciflua*) in a forest ecosystem. *Tree Physiol* 19(12):779–786. <https://doi.org/10.1093/treephys/19.12.779>
- Hikosaka K, Niinemets Ü, Anten NP (2016) Canopy photosynthesis: from basics to applications, 1st edn. Springer, Netherlands
- Hoel BO (1998) Use of a hand-held chlorophyll meter in winter wheat: evaluation of different measuring positions on the leaves. *Acta Agric Scand Sect B Soil Plant Sci* 48(4):222–228. <https://doi.org/10.1080/09064719809362502>
- Houpis JLJ, Surano KA, Cowles S, Shinn JH (1988) Chlorophyll and carotenoid concentrations in two varieties of *Pinus ponderosa* seedlings subjected to long-term elevated carbon dioxide. *Tree Physiol* 4(2):187–193. <https://doi.org/10.1093/treephys/4.2.187>
- Jin D, Cao X, Ma K (2014) Leaf functional traits vary with the adult height of plant species in forest communities. *J Plant Ecol* 7(1):68–76. <https://doi.org/10.1093/jpe/rtt023>
- Kenzo T, Yoneda R, Sano M, Araki M, Shimizu A, Tanaka-Oda A, Chann S (2012) Variations in leaf photosynthetic and morphological traits with tree height in various tree species in a Cambodian tropical dry evergreen forest. *Japan Agric Res Quart* 46(2):167–180. <https://doi.org/10.6090/jarq.46.167>
- Koike T, Kitao M, Maruyama Y, Mori S, Lei TT (2001) Leaf morphology and photosynthetic adjustments among deciduous broad-leaved trees within the vertical canopy profile. *Tree Physiol* 21(12–13):951–958. <https://doi.org/10.1093/treephys/21.12-13.951>
- Larcher W (2003) *Physiological plant ecology : ecophysiology and stress physiology of functional groups* (4th edition). Springer
- Lennon AM, Lewis VR, Farrell AD, Umaharan P (2021) Photochemical responses to light in sun and shade leaves of *Theobroma cacao* L. (West African Amelonado). *Sci Hortic* 276:109747. <https://doi.org/10.1016/j.scienta.2020.109747>
- Li Y, He N, Hou J, Xu L, Liu C, Zhang J, Wang Q, Zhang X, Wu X (2018) Factors influencing leaf chlorophyll content in natural forests at the biome scale. *Front Ecol Evol*. <https://doi.org/10.3389/fevo.2018.00064>
- Liberloo M, Tulva I, Raïm O, Kull O, Ceulemans R (2007) Photosynthetic stimulation under long-term CO<sub>2</sub> enrichment and fertilization is sustained across a closed *Populus* canopy profile (EUROFACE). *New Phytol* 173(3):537–549. <https://doi.org/10.1111/j.1469-8137.2006.01926.x>
- Lichtenthaler HK, Buschmann C (2001) Chlorophylls and carotenoids: measurement and characterization by UV-VIS spectroscopy. *Curr Prot Food Anal Chem*. <https://doi.org/10.1002/0471142913.faf0403s01>
- Lichtenthaler HK, Ač A, Marek MV, Kalina J, Urban O (2007) Differences in pigment composition, photosynthetic rates and chlorophyll fluorescence images of sun and shade leaves of four tree species. *Plant Physiol Biochem* 45(8):577–588. <https://doi.org/10.1016/j.plaphy.2007.04.006>
- Loh FCW, Grabosky JC, Bassuk NL (2002) Using the SPAD 502 meter to assess chlorophyll and nitrogen content of Benjamin fig and cottonwood leaves. *Hort Technology* 12(4):682–686
- Major JE, Mosseler A (2021) Chlorophyll pigment and needle macronutrient responses and interactions to soil moisture and atmospheric CO<sub>2</sub> treatments of eight pine and spruce species. *Trees – Struct Funct* 35(6):2069–2085. <https://doi.org/10.1007/s00468-021-02173-0>
- Major JE, Mosseler A, Malcolm JW (2018) Genetic variation among pines and spruces in assimilation efficiencies and photosynthetic regulation under elevated CO<sub>2</sub>. *Trees - Struct Funct* 32(1):215–229. <https://doi.org/10.1007/s00468-017-1625-4>
- Marengo RA, Antezana-Vera SA, Nascimento HCS (2009) Relationship between specific leaf area, leaf thickness, leaf water content and SPAD-502 readings in six Amazonian tree species. *Photosynthetica* 47(2):184–190. <https://doi.org/10.1007/s11099-009-0031-6>
- Markwell J, Osterman JC, Mitchell JL (1995) Calibration of the Minolta SPAD-502 leaf chlorophyll meter. *Photosynth Res* 46(3):467–472. <https://doi.org/10.1007/BF00032301>
- Medlyn BE, Zaehle S, De Kauwe MG, Walker AP, Dietze MC, Hanson PJ, Hickler T, Jain AK, Luo Y, Parton W, Prentice IC, Thornton PE, Wang S, Wang YP, Weng E, Iversen CM, McCarthy HR, Warren JM, Oren R, Norby RJ (2015) Using ecosystem experiments to improve vegetation models. *Nat Clim Chang* 5(6):528–534. <https://doi.org/10.1038/nclimate2621>
- Montpied P, Granier A, Dreyer E (2009) Seasonal time-course of gradients of photosynthetic capacity and mesophyll conductance to CO<sub>2</sub> across a beech (*Fagus sylvatica* L.) canopy. *J Exp Bot* 60(8):2407–2418. <https://doi.org/10.1093/jxb/erp093>
- Morecroft MD, Stokes VJ, Morison JIL (2003) Seasonal changes in the photosynthetic capacity of canopy oak (*Quercus robur*) leaves: the impact of slow development on annual carbon uptake. *Int J Biometeorol* 47(4):221–226. <https://doi.org/10.1007/s00484-003-0173-3>
- Niinemets Ü (1997) Distribution patterns of foliar carbon and nitrogen as affected by tree dimensions and relative light conditions in the canopy of *Picea abies*. *Trees* 11(3):144–154. <https://doi.org/10.1007/pl00009663>
- Niinemets Ü (2007) Photosynthesis and resource distribution through plant canopies. *Plant, Cell Environ* 30(9):1052–1071. <https://doi.org/10.1111/j.1365-3040.2007.01683.x>



- Norby RJ, Warren JM, Iversen CM, Medlyn BE, McMurtrie RE (2010) CO<sub>2</sub> enhancement of forest productivity constrained by limited nitrogen availability. *Proc Natl Acad Sci USA* 107(45):19368–19373. <https://doi.org/10.1073/pnas.1006463107>
- Norby RJ, De Kauwe MG, Domingues TF, Duursma RA, Ellsworth DS, Goll DS, Lapola DM, Luus KA, Mackenzie AR, Medlyn BE, Pavlick R, Rammig A, Smith B, Thomas R, Thonicke K, Walker AP, Yang X, Zaehle S (2016) Model-data synthesis for the next generation of forest free-air CO<sub>2</sub> enrichment (FACE) experiments. *New Phytol* 209(1):17–28. <https://doi.org/10.1111/nph.13593>
- Norby RJ, Warren JM, Iversen CM, Childs J, Jawdy SS, Walker AP (2021) Forest stand and canopy development unaltered by 12 years of CO<sub>2</sub> enrichment. *Tree Physiol*. <https://doi.org/10.1093/treephys/tpab107>
- Oberbauer SF, Strain BR, Fetcher N (1985) Effect of CO<sub>2</sub>-enrichment on seedling physiology and growth of two tropical tree species. *Physiol Plant* 65(4):352–356. <https://doi.org/10.1111/j.1399-3054.1985.tb08658.x>
- Ollinger SV, Reich PB, Frolking S, Lepine LC, Hollinger DY, Richardson AD (2013) Nitrogen cycling, forest canopy reflectance, and emergent properties of ecosystems. *Proc Natl Acad Sci USA* 110(27):2437. <https://doi.org/10.1073/pnas.1304176110>
- Osnas JLD, Katabuchi M, Kitajima K, Wright SJ, Reich PB (2018) Divergent drivers of leaf trait variation within species, among species, and among functional groups. *Proc Natl Acad Sci USA*. <https://doi.org/10.1073/pnas.1803989115>
- Palta JP (1990) Leaf chlorophyll content. *Remote Sens Rev* 5(1):207–213. <https://doi.org/10.1080/02757259009532129>
- Percival GC, Keary IP, Noviss K (2008) The potential of a chlorophyll content SPAD meter to quantify nutrient stress in foliar tissue of sycamore (*Acer pseudoplatanus*), English oak (*Quercus robur*), and European beech (*Fagus sylvatica*). *Arboric Urban Forestry* 34(2):89–100
- Pinkard EA, Patel V, Mohammed C (2006) Chlorophyll and nitrogen determination for plantation-grown *Eucalyptus nitens* and *E. globulus* using a non-destructive meter. *Forest Ecol Manag* 223(1–3):211–217. <https://doi.org/10.1016/j.foreco.2005.11.003>
- Poorter H, Niinemets Ü, Poorter L, Wright IJ, Villar R (2009) Causes and consequences of variation in leaf mass per area (LMA): a meta-analysis. *New Phytol* 182(3):565–588. <https://doi.org/10.1111/j.1469-8137.2009.02830.x>
- Porra RJ, Thompson WA, Kriedemann PE (1989) Determination of accurate extinction coefficients and simultaneous equations for assaying chlorophylls a and b extracted with four different solvents: verification of the concentration of chlorophyll standards by atomic absorption spectroscopy. *Biochem Biophys Acta* 975:384–394. <https://doi.org/10.1213/ANE.0000000000001681>
- Raven JA (2013) Rubisco: still the most abundant protein of earth? *New Phytol* 198(1):1–3. <https://doi.org/10.1111/nph.12197>
- Rey A, Jarvis PG (1997) Growth response of young birch trees (*Betula pendula* Roth.) after four and a half years of CO<sub>2</sub> exposure. *Ann Bot* 80(6):809–816. <https://doi.org/10.1006/anbo.1997.0526>
- Rey A, Jarvis PG (1998) Long-term photosynthetic acclimation to increased atmospheric CO<sub>2</sub> concentration in young birch (*Betula pendula*) trees. *Tree Physiol* 18(7):441–450. <https://doi.org/10.1093/treephys/18.7.441>
- Richardson AD, Duigan SP, Berlyn GP, Richardson AD (2002) An evaluation of noninvasive methods to estimate foliar chlorophyll content. *New Phytol* 153:185–194
- Richardson AD, Keenan TF, Migliavacca M, Ryu Y, Sonnentag O, Toomey M (2013) Climate change, phenology, and phenological control of vegetation feedbacks to the climate system. *Agric for Meteorol* 169:156–173. <https://doi.org/10.1016/j.agrformet.2012.09.012>
- Scartazza A, Di Baccio D, Bertolotto P, Gavrichkova O, Matteucci G (2016) Investigating the European beech (*Fagus sylvatica* L) leaf characteristics along the vertical canopy profile: leaf structure, photosynthetic capacity light energy dissipation and photoprotection mechanisms. *Tree Physiol* 36(9):1060–1076. <https://doi.org/10.1093/treephys/tpw038>
- Sharwood RE, Crous KY, Whitney SM, Ellsworth DS, Ghannoum O (2017) Linking photosynthesis and leaf N allocation under future elevated CO<sub>2</sub> and climate warming in *Eucalyptus globulus*. *J Exp Bot* 68(5):1157–1167. <https://doi.org/10.1093/jxb/erw484>
- Sholtis JD, Gunderson CA, Norby RJ, Tissue DT (2004) Persistent stimulation of photosynthesis by elevated CO<sub>2</sub> in a sweetgum (*Liquidambar styraciflua*) forest stand. *New Phytol* 162(2):343–354. <https://doi.org/10.1111/j.1469-8137.2004.01028.x>
- Sims DA, Gamon JA (2002) Relationships between leaf pigment content and spectral reflectance across a wide range of species, leaf structures and developmental stages. *Remote Sens Environ* 81(2–3):337–354. [https://doi.org/10.1016/S0034-4257\(02\)00010-X](https://doi.org/10.1016/S0034-4257(02)00010-X)
- Springer CJ, Thomas RB (2007) Photosynthetic responses of forest understory tree species to long-term exposure to elevated carbon dioxide concentration at the Duke Forest FACE experiment. *Tree Physiol* 27(1):25–32. <https://doi.org/10.1093/treephys/27.1.25>
- Stitt M, Krapp A (1999) The interaction between elevated carbon dioxide and nitrogen nutrition: the physiological and molecular background. *Plant, Cell Environ* 22(6):583–621. <https://doi.org/10.1046/j.1365-3040.1999.00386.x>
- Takeuchi Y, Kubiske ME, Isebrands JG, Pregitzer KS, Hendrey G, Karnosky DF (2001) Photosynthesis, light and nitrogen relationships in a young deciduous forest canopy under open-air CO<sub>2</sub> enrichment. *Plant, Cell Environ* 24(12):1257–1268. <https://doi.org/10.1046/j.0016-8025.2001.00787.x>
- Terrer C, Jackson RB, Prentice IC, Keenan TF, Kaiser C, Vicca S, Fisher JB, Reich PB, Stocker BD, Hungate BA, Peñuelas J, McCallum I, Soudzilovskaia NA, Cernusak LA, Talhelm AF, Van Sinderk K, Piao S, Newton PCD, Hovenden MJ, Franklin O (2019) Nitrogen and phosphorus constrain the CO<sub>2</sub> fertilization of global plant biomass. *Nat Clim Chang* 9(9):684–689. <https://doi.org/10.1038/s41558-019-0545-2>
- Thomas SC (2005) Increased leaf reflectance in tropical trees under elevated CO<sub>2</sub>. *Glob Change Biol* 11(2):197–202. <https://doi.org/10.1111/j.1529-8817.2005.00907.x>
- Toomey M, Friedl MA, Frolking S, Hufkens K, Klosterman S, Sonnentag O, Baldocchi DD, Bernacchi CJ, Biraud SC, Bohrer G, Brzostek E, Burns SP, Coursolle C, Hollinger DY, Margolis HA, McCaughey H, Monson RK, Munger JW, Pallardy S, Richardson AD (2015) Greenness indices from digital cameras predict the timing and seasonal dynamics of canopy-scale photosynthesis. *Ecol Appl* 25(1):99–115. <https://doi.org/10.1890/14-0005.1>
- Uddling J, Gelang-Alfredsson J, Piikki K, Pleijel H (2007) Evaluating the relationship between leaf chlorophyll concentration and SPAD-502 chlorophyll meter readings. *Photosynth Res* 91(1):37–46. <https://doi.org/10.1007/s1120-006-9077-5>
- Valladares F, Niinemets Ü (2008) Shade tolerance, a key plant feature of complex nature and consequences. *Annu Rev Ecol Evol Syst* 39:237–257. <https://doi.org/10.1146/annurev.ecolsys.39.110707.173506>
- Walker AP, Zaehle S, Medlyn BE, De Kauwe MG, Asao S, Hickler T, Parton W, Ricciuto DM, Wang Y-P, Wärlind D, Norby RJ (2015) Predicting long-term carbon sequestration in response to CO<sub>2</sub> enrichment: How and why do current ecosystem models differ? *Global Biogeochem Cycles* 29(3):288–306. <https://doi.org/10.1002/2014GB004995>
- Walker BJ, Drewry DT, Slattery RA, VanLoocke A, Cho YB, Ort DR (2018) Chlorophyll can be reduced in crop canopies with little penalty to photosynthesis. *Plant Physiol* 176(2):1215–1232. <https://doi.org/10.1104/pp.17.01401>
- Warren JM, Jensen AM, Medlyn BE, Norby RJ, Tissue DT (2015) Carbon dioxide stimulation of photosynthesis in *Liquidambar*



- styraciflua* is not sustained during a 12-year field experiment. AoB Plants. <https://doi.org/10.1093/aobpla/plu074>
- Wujeska-Klaus A, Crous KY, Ghannoum O, Ellsworth DS (2019) Leaf age and eCO<sub>2</sub> both influence photosynthesis by increasing light harvesting in mature *Eucalyptus tereticornis* at EucFACE. Environ Exp Bot 167(August):103857. <https://doi.org/10.1016/j.envexpbot.2019.103857>
- Wullschlegel SD, Norby RJ, Hendrix DL (1992) Carbon exchange rates, chlorophyll content, and carbohydrate status of two forest tree species exposed to carbon dioxide enrichment. Tree Physiol 10(1):33–43. <https://doi.org/10.1093/treephys/10.1.21>
- Wyka TP, Oleksyn J, Żytowski R, Karolewski P, Jagodziński AM, Reich PB (2012) Responses of leaf structure and photosynthetic properties to intra-canopy light gradients: a common garden test with four broadleaf deciduous angiosperm and seven evergreen conifer tree species. Oecologia 170(1):11–24. <https://doi.org/10.1007/s00442-012-2279-y>
- Zaehle S (2013) Terrestrial nitrogen-carbon cycle interactions at the global scale. Phil Trans R Society b: Biol Sci. <https://doi.org/10.1098/rstb.2013.0125>
- Zaehle S, Medlyn BE, De Kauwe MG, Walker AP, Dietze MC, Hickler T, Luo Y, Wang YP, El-Masri B, Thornton P, Jain A, Wang S, Warland D, Weng E, Parton W, Iversen CM, Gallet-Budynek A, McCarthy H, Finzi A, Norby RJ (2014) Evaluation of 11 terrestrial carbon-nitrogen cycle models against observations from two temperate free-air CO<sub>2</sub> enrichment studies. New Phytol 202(3):803–822. <https://doi.org/10.1111/nph.12697>
- Zhu Z, Bi J, Pan Y, Ganguly S, Anav A, Xu L, Samanta A, Piao S, Nemani RR, Myneni RB (2013) Global data sets of vegetation leaf area index (LAI)3g and fraction of photosynthetically active radiation (FPAR)3g derived from global inventory modeling and mapping studies (GIMMS) normalized difference vegetation index (NDVI3G) for the period 1981 to 2. Remote Sensing 5(2):927–948. <https://doi.org/10.3390/rs5020927>

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