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Are chlorophyll concentrations and nitrogen across the vertical canopy profile affected by elevated CO₂ in mature *Quercus* trees?

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Abstract

Key message In mature *Q. robur*, chlorophyll varied with season and canopy height, whilst eCO₂-driven changes were consistent with M_{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 CO₂ (eCO₂), but few such studies have been done in aged forest trees. This study aimed to assess the effects of eCO₂ (150 μmol 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 CO₂ fumigation of a free-air CO₂ enrichment (FACE) study in central England. Mass-based chlorophyll concentration (Chl_{mass}, mg g⁻¹) was significantly higher in the lower canopy compared to upper canopy foliage ($P < 0.05$). In contrast, significantly higher chlorophyll content (Chl_{area}, mg m⁻²) was observed in the upper canopy. eCO₂ did not affect Chl_{mass} but Chl_{area} significantly increased, attributable to increased leaf mass per unit area (M_{area} , g m⁻²). We found no effect of eCO₂ on mass-based or area-based nitrogen (N_{mass} , mg g⁻¹ or N_{area} , g m⁻²); however, N_{area} significantly increased with canopy height, again attributable to M_{area} . The parallel relationships between M_{area} , N_{area} and Chl_{area} suggest the allocation of N to light harvesting is maintained with eCO₂ exposure as well as in the upper canopy, and that increased photosynthetic mass may help regulate the eCO₂ variation. An understanding of changes in the light-harvesting machinery with eCO₂ will be useful to assess canopy processes and, at larger scales, changes in biogeochemical cycles in future climate scenarios.

Keywords Leaf absorbance · Free air CO₂ 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* (Chl_a) and chlorophyll *b* (Chl_b) important for the primary reaction (i.e., light harvesting) step of photosynthesis. As Chl_a and Chl_b absorb sunlight at different wavelengths, total leaf chlorophyll (Chl_a + Chl_b) is expected

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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 (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 CO_2 (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 eCO_2 have been found to have lower leaf nitrogen content (per unit area; N_{area} , in g m^{-2} leaf) compared to trees grown in ambient CO_2 (aCO_2) (Ainsworth and Long 2005; Ellsworth et al. 2004). Any changes in N_{area} with future increases in ambient CO_2 concentrations may have an effect on the photosynthetic apparatus and lead to changes in the tree responses to increasing CO_2 (Crous et al. 2008; Major et al. 2018). The effects of 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 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 eCO_2 . Thus, understanding how N is allocated among canopy leaves in 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_{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 (Chl_{area}), have been found with exposure to 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 eCO_2 ; + 150–300 $\mu\text{mol mol}^{-1}$ above ambient) (Ainsworth and Long 2005; Warren et al. 2015). In trees, eCO_2 -driven reductions in Chl_{area} have been observed in tree seedlings and saplings (Houpis et al. 1988; Oberbauer et al. 1985; Thomas 2005; Wullschleger 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 eCO_2 may affect Chl_{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 eCO_2 -driven reductions in chlorophyll content (Chl_{area}) have found similar reductions in foliar nitrogen content (N_{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 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 (Chl_{mass}), alongside reduced N_{mass} , but not Chl_{area} , implying that eCO_2 -related declines in Chl_{mass} may be the result of morphological changes in M_{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 eCO_2 plants may also alter the N allocation *within* the leaf to maximise photosynthesis; thus, one possibility may lead to 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, 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 Chl_{mass} and Chl_{area} in old forest-grown trees, and whether N allocation to chlorophyll changes with 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 eCO_2 , changes to Chl_{mass} and Chl_{area} are known to occur spatially through the vertical canopy profile in forested systems as a result of changes in light (Hikosaka et al. 2016; Larcher, 2003; Richardson et al. 2002). For example, Chl_{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, Chl_{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, Chl_{area} is found to correlate positively with increasing light levels due to changes in M_{area} outweighing changes in Chl_{mass} (Lichtenthaler et al. 2007). Both M_{area} and N_{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_{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_{area} is influenced by the gradient of M_{area} across the canopy. Therefore, it is common to observe a relationship between M_{area} and Chl_{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 $e\text{CO}_2$ on Chl_{area} in aged forest trees has received no attention. Quantifying any interaction of responses to $e\text{CO}_2$ 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 Chl_{mass} and Chl_{area} , N_{mass} , N_{area} , and M_{area} , across the vertical profile of the canopy-dominant species, *Quercus robur*, at the Birmingham Institute of Forest Research Free Air CO_2 Enrichment (BIFoR FACE) facility in the 3rd and 4th season of $e\text{CO}_2$ fumigation. Our objectives were to quantify the effect of $e\text{CO}_2$ and canopy position on Chl_{mass} and Chl_{area} , in addition to foliar N and M_{area} , for this *Q. robur* canopy. Our expectations were that (i) that Chl_{mass} would increase from the top to bottom of the live canopy of canopy dominants, whilst Chl_{area} would decrease along this gradient, and (ii) that Chl_{area} would be unchanged by $e\text{CO}_2$. Quantifying the effects of $e\text{CO}_2$ and canopy position on Chl_{mass} and Chl_{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 $e\text{CO}_2$ can help us understand potential effects of $e\text{CO}_2$ on canopy processes, biogeochemical changes, and may provide new tools for monitoring canopy status in $e\text{CO}_2$.

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 ($a\text{CO}_2$) (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 CO_2 enriched air, named elevated CO_2 plots ($e\text{CO}_2$) (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 CO_2 was $543 (\pm 24) \mu\text{mol mol}^{-1}$ in the $e\text{CO}_2$ 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 $e\text{CO}_2$ 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°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_{mass}) and subsequently N per area (N_{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 Chl_{mass} and Chl_{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 CO_2 treatment (i.e., a CO_2 or e CO_2). Additionally, M_{area} 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_{area} , area-based leaf nitrogen (N_{area}) and mass-based leaf nitrogen (N_{mass}) with sampling year, sampling month, canopy position and 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 Chl_{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 Chl_{mass} and Chl_{area} but rapidly rose over June and July, attaining a broad Chl_{mass} maximum that persisted through summer and into early autumn (Fig. 1). A gradual decline in Chl_{mass} and Chl_{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 (18th and 29th of September, respectively) and first autumnal leaf fall occurred just under a month later (13nd and 26th Oct, respectively). The monthly differences for Chl_{mass} and Chl_{area} were significant (Table 1, $P < 0.001$). Additionally, a significant year x month interaction was observed, where significantly lower total Chl_{mass} and Chl_{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 CO_2

Common to both sampling years, Chl_{mass} was significantly higher in lower canopy leaves than in upper canopy leaves (Table 1, $P < 0.05$). Chl_{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 (Chl_{area}), there was significantly higher Chl_{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 Chl_{mass} , whereby significantly lower Chl_{mass} was reported in August and May in upper canopy foliage in 2019 and 2020, respectively.

There was no effect of e CO_2 on Chl_{mass} (Fig. 2, Table 1). There was a significant increase in Chl_{area} , but this effect was removed once M_{area} had been accounted for (Fig. 2, Table 1). Additionally, there was no significant interaction between CO_2 treatment x canopy position in either Chl_{mass} or Chl_{area} .

M_{area} 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 CO₂ treatment and their relevant interactions on chlorophyll concentration (Chl_{mass}) in *Q. robur* trees

Source of variation	Chl _{mass}			Chl _{area}		
	DF	<i>P</i> value		DF	<i>P</i> value	
Year	1	0.083	–	1	0.13	–
Month	4	< 0.001	***	4	< 0.001	***
Canopy position	2	0.038	*	2	0.017	*
CO ₂ treatment	1	0.76	–	1	0.41	–
M _{area}	1	0.82	–	1	0.013	*
Year * Month	1	0.0015	**	1	0.02	*
Year * Canopy position	1	0.93	–	1	0.93	–
Year * CO ₂ Treatment	1	0.25	–	1	0.4	–
Month * Canopy position	4	0.022	*	4	0.41	–
Month * CO ₂ Treatment	4	0.52	–	4	0.45	–
Canopy position * CO ₂ Treatment	2	0.17	–	2	0.13	–
Year * Month * Canopy position	1	0.076	–	1	0.18	–
Year * Month * CO ₂ 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_{area}’ 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 CO₂ treatment (Fig. 3, Table 2). M_{area} decreased by a mean of 44% (mean ± SD: 99 ± 20 g m⁻²) and 53% (mean ± SD: 85 ± 14 g m⁻²) for eCO₂ and aCO₂, respectively, in the lower canopy foliage when compared to upper canopy foliage. Additionally, M_{area} increased with eCO₂ treatment by 16% and 23% in the upper and lower foliage, respectively. There was no effect of CO₂ treatment or canopy position on N_{mass} (Fig. 3, Table 2). Additionally, there was no effect of CO₂ on N_{area}; however, N_{area} decreased by 29% and 33%, from the canopy top to bottom, in eCO₂ and aCO₂ treatments respectively (Fig. 3, *P* < 0.05; Table 2).

Discussion

Very few studies have analysed the effect of eCO₂ on Chl_{mass} and Chl_{area} in aged forest-grown trees (Wujeska-Klaue et al. 2019) and fewer have also assessed the role of canopy position in the eCO₂ response (Crous et al. 2020; Sharwood et al. 2017). We found that both Chl_{mass} and Chl_{area} significantly varied in response to canopy position, but that neither Chl_{mass} nor Chl_{area} were affected by eCO₂ exposure, once M_{area} had been accounted for. Additionally, we found no significant changes in N_{mass} with canopy position or CO₂ treatment, although N_{area} increased with eCO₂ and significantly increased with canopy height. We found M_{area} increased with canopy height and with eCO₂, suggesting that changes in M_{area} rather than changes in foliar N could be attributed to

the observed changes to Chl_{area} and N_{area} with canopy position and eCO₂. The results suggest that the allocation of N to light harvesting is maintained with eCO₂ 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_{area} and Chl_{mass}) 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_{mass} and Chl_{area}, in addition to significant month × year interactions (S. Figure 4, Table 1). The lower Chl_{mass} and Chl_{area} 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

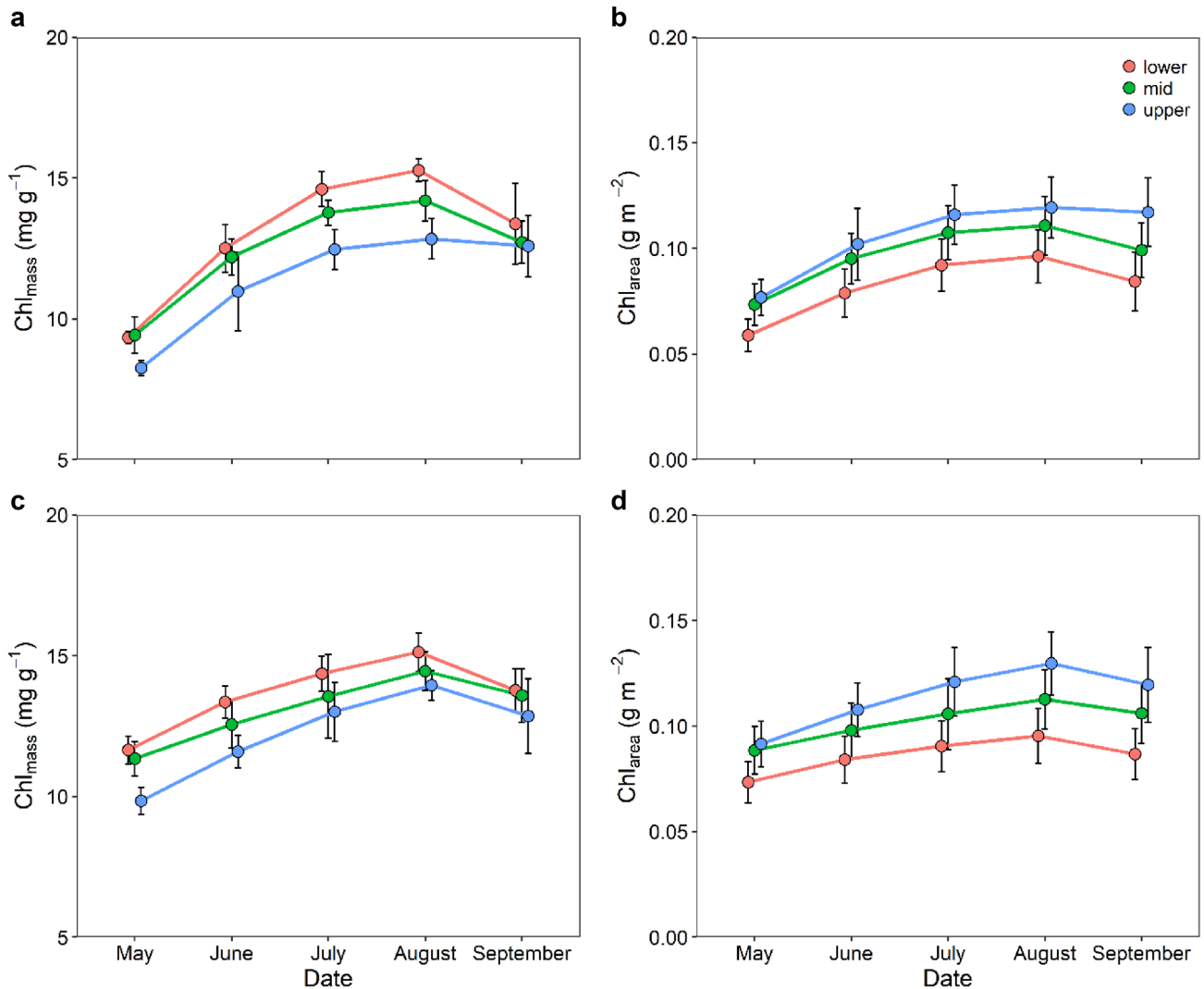


Fig. 1 Mean monthly chlorophyll, expressed per unit mass (Chl_{mass}) (mg g^{-1}) (a, c) and per unit area (Chl_{area}) (g m^{-2}) (b, d), across the growing seasons of 2019 (a, b) and 2020 (c, d). Points are staggered for clarity. Colours indicate the three different canopy layers; 'upper'

(blue), 'middle' (green), and 'lower' (red) for *Q. robur* under ambient CO_2 (aCO_2) at the BIFoR FACE facility. Each point represents the six experimental plots at BIFoR FACE

no effect of 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 aCO_2 and one 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, eCO_2 , and month that are currently confounded in this study.

The effect of eCO_2

Elevated CO_2 treatment did not significantly affect Chl_{mass} , which has been observed in other field eCO_2 experiments

on trees (Liberloo et al. 2007; Wujeska-Klaue et al. 2019). We found no significant effect of eCO_2 for Chl_{area} , once M_{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_{area}). Hence, the derived Chl_{area} content can be due to a change in the measured Chl_{mass} concentration and/or a change in M_{area} . In support of this, we found that M_{area} increased with eCO_2 , which is a common response of trees to CO_2 enrichment (as reviewed in Ainsworth and Long 2005). For a constant areal density, increasing M_{area} leads to a decreasing dry mass fraction; that is, to a dilution effect (Epron et al. 1996; Feng

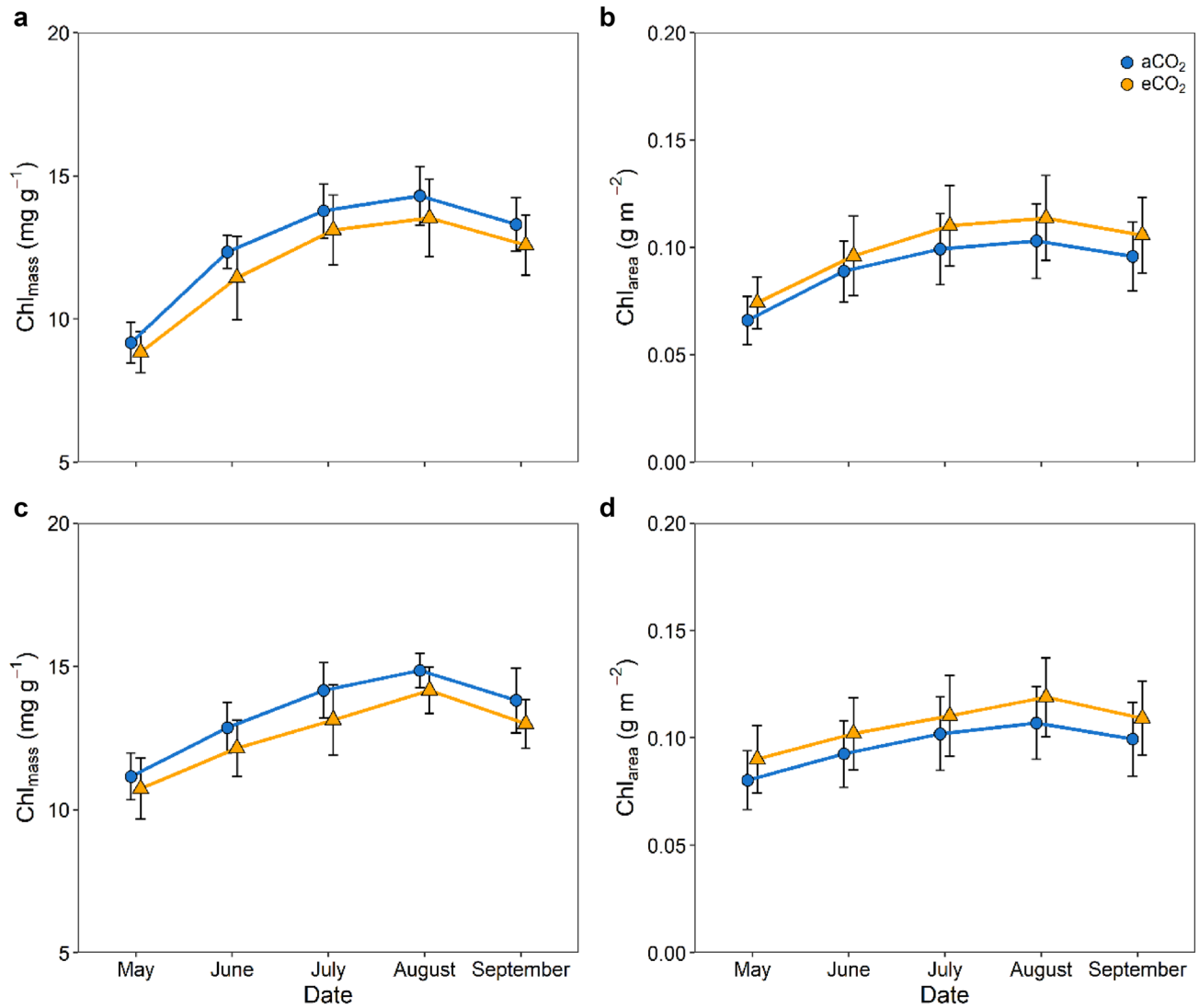


Fig. 2 Mean monthly chlorophyll, expressed per unit mass (Chl_{mass}) (mg g^{-1}) and per unit area (Chl_{area}) (mg cm^{-2}), across the growing seasons of 2019 (**a, b**) and 2020 (**c, d**). Points are staggered for clarity. Colours indicate the CO_2 treatment in *Q. robur* leaves at the

BIFoR FACE facility with aCO_2 (blue) and eCO_2 (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 Chl_{mass} , alongside decreases in N_{mass} , have been observed under eCO_2 previously and have primarily been attributed to a dilution effect as a result of increases in M_{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 Chl_{mass} or N_{mass} with eCO_2 suggesting the lack of a dilution effect occurring with exposure to eCO_2 .

Increases in M_{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_{area} as a covariate was

found to account for the significant eCO_2 effect on Chl_{area} , this indicates that the eCO_2 -driven increases in M_{area} may primarily increase structural and photosynthetic mass proportionally. M_{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_{area} with changes in atmospheric CO_2 .

Previously, eCO_2 -driven reductions in Chl_{area} have been suggested to be the result of eCO_2 -driven reductions in N_{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_{area}) (g m^{-2}), **b** nitrogen per mass (N_{mass}) (mg g^{-1}), **c** nitrogen per area (N_{area}) (g m^{-2}), and **d** chlorophyll per area (Chl_{area}) (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 CO_2 treatment in *Q. robur* leaves at the BIFoR FACE facility with aCO_2 (blue circles) and 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 CO_2 fumigation at the BIFoR FACE site (Gardner et al. 2021), so we expected to see no eCO_2 -driven reductions in foliar N in the present study, as was indeed the case. In the present study, we found N_{area} either increased or remained unchanged in response to eCO_2 , suggesting that there were no limitations to the nitrogen supply (Warren et al. 2015). If the allocation of N to chlorophyll, with eCO_2 , follows that of total N, we would therefore expect Chl_{area} to follow the relationship of N_{area} , as observed.

It has previously been suggested that N may be reallocated with exposure to 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 Chl_{area} was not reduced with 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 eCO_2 . This is supported by previous analysis at this site that found the maintenance of foliar N with 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 eCO_2 exposure, and future seasons of 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_{area} that consequently affect Chl_{area} (Rey and Jarvis 1997; Warren et al. 2015). An understanding of N allocation in canopy leaves in eCO_2 can help us understand potential effects of 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 eCO_2 .

The effect of canopy position

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

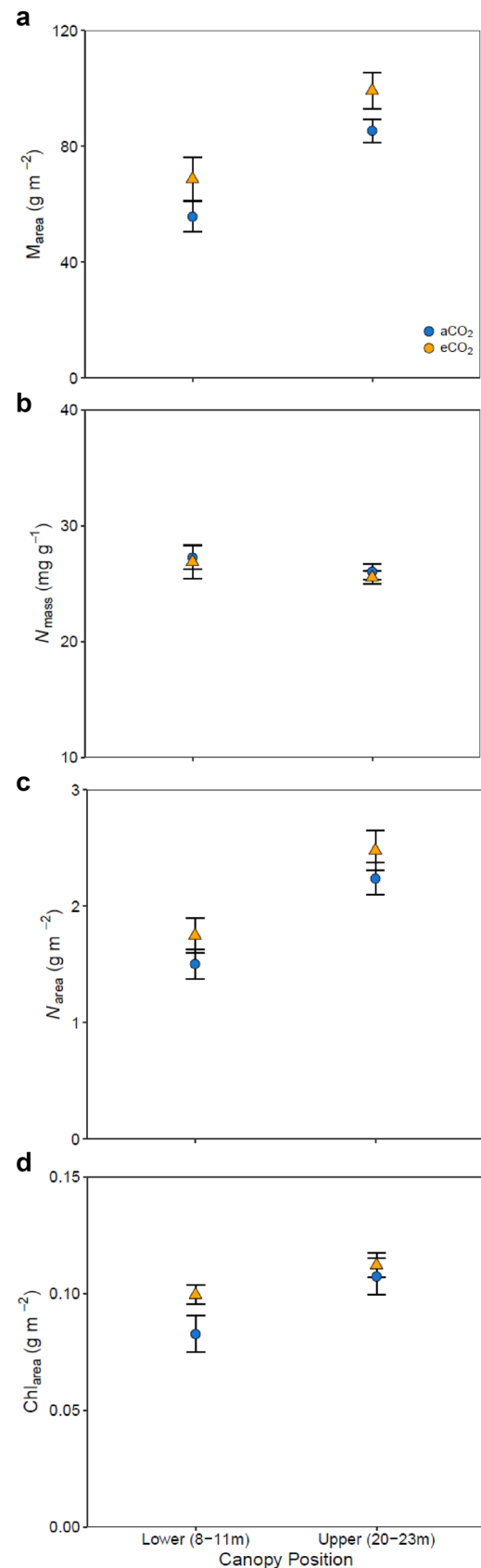


Table 2 Linear mixed model showing the statistical significance of CO₂ treatment and canopy position, and their interaction, on leaf mass per area (M_{area}), nitrogen per mass (N_{mass}), and nitrogen per area (N_{area}) in *Q. robur* trees

Source of variation	DF	M_{area} <i>P</i> value	N_{mass} <i>P</i> value	N_{area} <i>P</i> value
CO ₂ treatment	1	0.33	0.40	0.35
Canopy position	1	0.003**	0.16	0.006**
Canopy position * CO ₂ 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 eCO₂ (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 Chl_{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_{area}), compared to leaves from the lower canopy (Fig. 3) (Ellsworth and Reich 1993; Norby et al. 2021). Therefore, the decline in Chl_{mass} with increasing canopy height is likely attributable to a dilution effect as a result of the increases in M_{area} (Epron et al. 1996; Houpius et al. 1988; Sholtis et al. 2004; Wullschleger et al. 1992).

Similarly, we found Chl_{area} significantly increased with increasing canopy height, paralleling the response of both M_{area} and N_{area} . Increases in M_{area} and N_{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_{area} leads to the changes in Chl_{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 Chl_{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_{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_{area} through the vertical canopy that is parameterised in vegetation models.

The present study found no significant interaction of CO₂ treatment and canopy position for Chl_{mass} or Chl_{area} . The lack of interaction of CO₂ treatment and canopy position indicates that future changes in atmospheric CO₂ may not influence plant traits differently across the canopy compared with the relationships in the present day. Additionally, there was no effect of eCO₂ on the pattern of N_{area} with canopy height which suggests that eCO₂ 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_{area} as a constant with exposure to eCO₂ in models.

Conclusion

In mature *Q. robur* canopies considering season, canopy height, and CO₂ treatment, Chl_{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 eCO₂-driven increases in Chl_{area} that were consistent with eCO₂-driven increases in M_{area} , suggesting that increased cell stacking and chlorophyll density in oak leaves may help regulate the eCO₂ 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.

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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.

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Declarations

Conflict of interest None declared.

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