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Research paper

Is photosynthetic enhancement sustained through three years of elevated CO$_2$ exposure in 175-year-old *Quercus robur*?

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Current carbon cycle models attribute rising atmospheric CO$_2$ as the major driver of the increased terrestrial carbon sink, but with substantial uncertainties. The photosynthetic response of trees to elevated atmospheric CO$_2$ is a necessary step, but not the only one, for sustaining the terrestrial carbon uptake, but can vary diurnally, seasonally and with duration of CO$_2$ exposure. Hence, we sought to quantify the photosynthetic response of the canopy-dominant species, *Quercus robur*, in a mature deciduous forest to elevated CO$_2$ (eCO$_2$) ($+$150 μmol mol^{-1} CO$_2$) over the first 3 years of a long-term free air CO$_2$ enrichment facility at the Birmingham Institute of Forest Research in central England (BIFoR FACE). Over 3000 measurements of leaf gas exchange and related biochemical parameters were conducted in the upper canopy to assess the diurnal and seasonal responses of photosynthesis during the 2nd and 3rd year of eCO$_2$ exposure. Measurements of photosynthetic capacity via biochemical parameters, derived from CO$_2$ response curves, (V_{cmax} and J_{max}) together with leaf nitrogen concentrations from the pre-treatment year to the 3rd year of eCO$_2$ exposure, were examined. We hypothesized an initial enhancement in light-saturated net photosynthetic rates (A$_{sat}$) with CO$_2$ enrichment of ≈37% based on theory but also expected photosynthetic capacity would fall over the duration of the study. Over the 3-year period, A$_{sat}$ of upper-canopy leaves was 33 $\pm$ 8% higher (mean and standard error) in trees grown in eCO$_2$ compared with ambient CO$_2$ (aCO$_2$), and photosynthetic enhancement decreased with decreasing light. There were no significant effects of CO$_2$ treatment on V_{cmax} or J_{max}, nor leaf nitrogen. Our results suggest that mature *Q. robur* may exhibit a sustained, positive response to eCO$_2$ without photosynthetic downregulation, suggesting that, with adequate nutrients, there will be sustained enhancement in C assimilated by these mature trees. Further research will be required to understand the location and role of the additionally assimilated carbon.

Keywords: CO^2 fertilization of photosynthesis, Deciduous forest, downregulation, enriched CO$_2$, Free-air CO$_2$ enrichment (FACE), photosynthetic capacity.

Introduction

Forest ecosystems cover ∼30% of the Earth's land surface, representing ∼50% of terrestrially stored carbon and account for close to 60% of total terrestrial CO$_2$ fluxes in the global carbon cycle (Luyssaert et al. 2008, Pan et al. 2011). The continual rise in atmospheric CO$_2$, overwhelmingly due to anthropogenic activity (Friedlingstein et al. 2019), increases the need to understand the terrestrial carbon feedbacks of forests in the global carbon cycle. As the foundational driver of the carbon cycle of forests (e.g., Bonan 2008), the photosynthetic response to changing atmospheric CO$_2$ is a necessary process for forests to act as long-standing carbon stores with relatively long-lived carbon (C) pools such as wood (Körner 2017) and soil (Ostle et al. 2009). The amount of forest C-uptake in the future, and subsequent C sequestration, will be crucial determinants of future atmospheric CO$_2$ concentrations. So, quantifying the

photosynthetic response under elevated CO_2 (eCO_2), especially for mature trees, is critical to understanding the carbon uptake of forests under changing atmospheric composition.

It has been widely observed that eCO_2 can have a stimulatory effect on plant photosynthesis, known as photosynthetic enhancement, at least in the short-term (weeks–months) with adequate nutrient and water availability permitting (Brodribb et al. 2020). Long-term (years to decades) photosynthetic responses to eCO_2 are less well understood and lower-than-expected responses have been observed (Ainsworth and Long 2005, Ellsworth et al. 2017). Note that, even in studies that report sustained and/or strong stimulation of photosynthesis under eCO_2, the additionally assimilated C does not necessarily translate into increased growth stimulation (Bader et al. 2013, Sigurdsson et al. 2013).

The photosynthetic process and photosynthetic response to eCO_2 is sensitive to changes in environmental variables such as temperature, light, water and availability of nutrients. For example, net photosynthesis (A_{net}) is expected to increase with exposure to eCO_2, with greatest photosynthetic enhancement expected at maximum photon flux density (Q) if Rubisco carboxylation is limiting (Sage et al. 2008). Decreases in A_{net} have been commonly associated with limitations in water and nutrient availability (Nowak et al. 2004, Ainsworth and Rogers 2007). For example, water availability has been found to increase the magnitude of eCO_2-induced photosynthetic enhancement in drier years (Nowak et al. 2004, Ellsworth et al. 2012). Thus, interannual differences in eCO_2–induced photosynthetic enhancement are to be expected as environmental conditions vary. Understanding the photosynthetic response to eCO_2 under different, real-world, environmental conditions provides information essential, but not in itself sufficient, for modeling forest productivity (Jiang et al. 2020), and predicting carbon-climate feedbacks (e.g., Cox et al. 2013, Jones et al. 2016).

Despite a significant body of research on the photosynthetic response to eCO_2 in tree seedlings and saplings (as reviewed in Medlyn et al. 1999, Ainsworth and Long 2005), fewer studies address the long-term (>1 year) photosynthetic responses in mature plantation trees (Liberloo et al. 2007, Crous et al. 2008, Uddling et al. 2009, Warren et al. 2015) and very few in mature forest-grown trees (Bader et al. 2010, Klein et al. 2016, Ellsworth et al. 2017). Currently, the dynamic vegetation components of Earth System models, which diagnose vegetation responses to environmental change, have commonly been constructed using data from eCO_2 experiments on young and/or plantation grown trees (Piao et al. 2013). Yet, it is difficult to compare, generalize, and scale results from young trees in their exponential growth phase to the response of closed-canopy mature forests (Norby et al. 2016). For example, previous work from a long-term natural experiment found CO_2 stimulation declined with tree age in *Quercus ilex* (Hättenschwiler et al. 1997). Therefore, it is plausible that model projections

are currently overestimating the photosynthetic responses of mature forests and, thence, the 'CO_2 fertilization' effect (Zhu et al. 2016). Consequently, uncertainty remains as to the magnitude of, and environmental constraints on, photosynthetic enhancement under eCO_2 in large, long-standing carbon stores such as mature forests (Norby et al. 2016, Jiang et al. 2020).

Free-air CO_2 enrichment (FACE) facilities are valuable to understand system-level responses to eCO_2 (Ainsworth and Long 2005, Terrer et al. 2019) particularly in forests (Medlyn et al. 2015, Norby et al. 2016). The development of second generation forest FACE experiments focuses on tall, mature trees grown in their own forest soil (Hart et al. 2020). To date, forest FACE experiments have observed photosynthetic enhancements ranging from 30 to 60%, depending on tree species and environmental factors (as reviewed in Nowak et al. 2004, Ainsworth and Rogers 2007). Of the few studies on closed-canopy-dominant tree species, smaller photosynthetic enhancement to eCO_2 have been observed (19–49%) than in studies conducted on younger trees (Sholtis et al. 2004, Liberloo et al. 2007, Crous et al. 2008), but the reasons behind this smaller response remain unclear.

There is evidence of a reduction in photosynthetic activity after long-term eCO_2 exposure, known as photosynthetic down-regulation (Ainsworth et al. 2004, Crous and Ellsworth 2004), but downregulation is not always observed (Curtis and Wang 1998, Herrick and Thomas 2001). Commonly photosynthetic downregulation under eCO_2 exposure is the result of decreases, either directly or indirectly, in Rubisco carboxylation (V_{cmax}; Feng et al. 2015, Wujeska-Klause et al. 2019b). However, the stimulatory effect of photosynthesis under eCO_2 may be reduced but is usually not completely removed (Leakey et al. 2009, Wujeska-Klause et al. 2019b). Photosynthetic downregulation has largely been observed in young plants (Leakey et al. 2009), with some downregulation observed in two aggrading plantation forests (Crous et al. 2008, Warren et al. 2015), commonly as a result of insufficient soil nitrogen supply (Luo et al. 2004). However, photosynthetic downregulation has largely not been observed in mature forests (Bader et al. 2010) and therefore open questions remain concerning the frequency and magnitude of photosynthetic downregulation under eCO_2 exposure in mature forests.

To understand the photosynthetic responses in mature temperate deciduous forests, we evaluated the photosynthetic enhancement and potential downregulation in ca. 175-year-old canopy-dominant trees of *Q. robur* L. exposed to eCO_2 for 3 years. Considering that forest FACE experiments aim to operate for 10 years or more, we refer to these CO_2 responses as 'early' (Griffin et al. 2000). This study is among the oldest trees that have ever been examined under eCO_2. To assess the photosynthetic enhancement of the trees on daily and interannual timeframes, measurements of gas exchange and leaf biochemistry were measured in the upper oak canopy over

four growing seasons, that included one pre-treatment year (2015) and 3-treatment years (2016–2019). Our aims were to quantify the photosynthetic response to eCO$_2$ (i.e., ambient +150 µmol mol^{-1}) for mature *Q. robur* and how light level influences this response, to determine whether photosynthetic downregulation under eCO$_2$ occurred and to establish whether the relationship between leaf N and photosynthetic capacity changed in eCO$_2$. We hypothesized that net photosynthetic gas exchange, A$_{net,}$ will significantly increase with eCO$_2$ and light levels (*Q*). The greatest enhancement was expected with the highest light levels, as a result of reduced limitations in the light dependent reaction of photosynthesis, and that photosynthetic enhancement would be ≈37% following theory and reasoning in Nowak et al. (2004) (see also Supplemental Appendix 1 available as Supplementary Data at *Tree Physiology Online*). We also hypothesized that leaf nitrogen (N) will be reduced under eCO$_2$ and that photosynthetic downregulation will be observed under eCO$_2$ as a result of reduced leaf N and/or a decline in either the maximum rate of photosynthetic Rubisco carboxylation (V_{cmax}, µmol m^{-2} s^{-1}); and the maximum rate of photosynthetic electron transport (J_{max}, µmol m^{-2} s^{-1}), or both (Griffin et al. 2000).

Methods and materials

Site description

This study was conducted at the Birmingham Institute of Forest Research (BIFoR) Free Air CO$_2$ Enrichment (FACE) facility located in Staffordshire (52.801°N, 2.301°W), UK. The BIFoR FACE facility is a 'second generation' Forest FACE facility, extending the scope of first generation facilities; (see Norby et al. 2016), situated within 19 ha of mature northern temperate broadleaf deciduous woodland having a canopy height of 24–26 m. The woodland consists of an overstorey canopy dominated by English oak (*Q. robur* L.) and a dense understorey comprising mostly of hazel coppice (*Corylus avellana* L.), sycamore (*Acer pseudoplatanus* L.), and hawthorn (*Crataegus monogyna* Jacq.). *Quercus robur* (commonly known as pedunculate oak, European oak or English oak) is a common broadleaf species geographically widespread across Europe where it is both economically important and ecologically significant for many biota (Eaton et al. 2016, Mölder et al. 2019). The site was planted with the existing oak standards in the late 1840s and has been largely unmanaged for the past 30–40 years. Like most established forest of the temperate zone, the BIFoR FACE forest is under-managed.

The study site is situated within the temperature-rainfall climate space occupied by temperate forest (Sommerfeld et al. 2018, Jiang et al. 2020) and is characterized by cool wet winters and warm dry summers with a frost-free growing season from April to October. The mean January and July temperatures

were 4 and 17 °C, respectively, and the average annual precipitation for the region is 720 mm (650, 669, 646 and 818 mm, in 2015, 2017, 2018 and 2019, respectively, when the study was conducted; see Figure 1). The total N deposition load at the BIFoR FACE site is ∼ 22 Kg N/ha/year (estimate provided by S. Tomlinson at the Centre for Ecology and Hydrology, Edinburgh, UK; MacKenzie et al. 2021), representing ∼15% of the total nitrogen nutrition of temperate deciduous trees (Rennenberg and Dannenmann 2015).

BIFoR FACE consists of nine approximately circular experimental plots of woodland 30 m in diameter (Hart et al. 2020). Only the six plots with infrastructure were considered in the present study. Each 'infrastructure plot' is encircled by steel towers constructed individually to reach 2-m above the local canopy-top height. The facility uses a paired-plot design (Hart et al. 2020): three replicate plots at either ambient CO$_2$ (aCO$_2$; ca. 405 µmol mol^{-1}) and three plots supplied with CO$_2$ enriched air, termed elevated CO$_2$ plots (eCO$_2$). The latter plots were operated such that they achieved a target of +150 µmol mol^{-1} above the minimum measured in the ambient plots (i.e., concentrations in the elevated plots ca. 555 µmol mol^{-1}) as 5-min rolling averages (Hart et al. 2020; see Supplementary Figure 1 available as Supplementary Data at *Tree Physiology Online*). Elevated CO$_2$ is added from dawn (solar zenith angle, sza = −6.5°) to dusk (sza = −6.5°) throughout the growing season. Daytime exposure to eCO$_2$ was almost continuous throughout the growing season (Hart et al. 2020), with exceptions if the 15-min average wind speed was >8 m s^{-1}, or when canopy-top, 1-min average, air temperature was < 4 °C. In the latter case, gas release was resumed when the air temperature was ≥5 °C. The CO$_2$ fumigation thresholds for wind speed and temperature were selected because of the high cost of maintaining eCO$_2$ and the insignificant uptake of carbon under these conditions, respectively. The operation of the FACE system and statistical performance in terms of meeting the target CO$_2$ concentration in time and space have been described in Hart et al. (2020).

In each plot, canopy access was gained through a custombuilt canopy access system (CAS) (Total Access Ltd, UK) that was installed from the central towers with canopy measurements made from a rigged rope access system (see Supplementary Figure 2 available as Supplementary Data at *Tree Physiology Online*). This facilitated in situ gas exchange measurements by allowing access to the upper oak canopy. The hoisting system comprises of an electric winch (Harken Power Seat Compact) that lifts a harnessed (Petzl AVAO BOD 5 point harness) user vertically through the air at a predetermined fixed point to a maximum canopy height of 25 m. The system required operation from the ground by trained staff and the user is seated in a Boatswain's chair. One oak tree per plot was accessible using the CAS system as set up during this study, and all gas
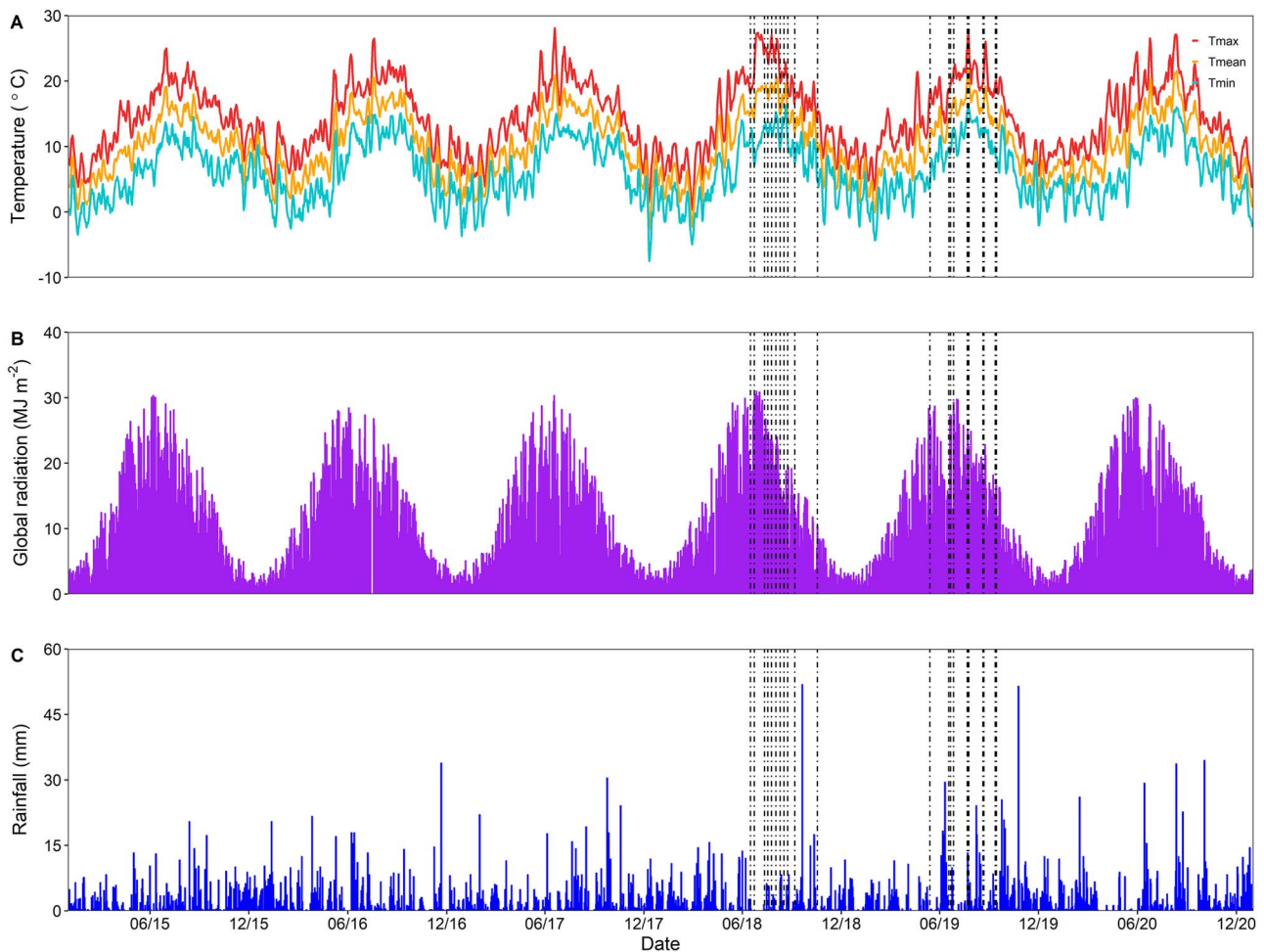
Figure 1. Time series showing the daily meteorological data at the BIFoR FACE facility covering the period of 1 January 2015–1 January 2021. Subplots are: (A) maximum, (red), mean (orange) and minimum (blue) daily air temperatures (°C), (B) global downwelling solar radiation (MJ m^{-2}) and (C) total daily precipitation (mm). Vertical dashed lines indicate diurnal sampling days. Clusters of sampling days occurred because different plots were sampled on different days in the same seasonal timeframe. Meteorological data are from RAF Shawbury, located 20 miles west of the BIFoR FACE facility, retrieved from the UK Met Office (https://www.metoffice.gov.uk/research/climate/maps-and-data/historic-station-data).

exchange measurements were made on unshaded leaves within the top 2 m of each tree canopy on dominant trees.

For this study, the sample size used throughout the study ($n = 3$) represents the number of replicate experimental plots at BIFoR FACE and includes within-tree replicates that were averaged per plot before analysis. All the three replicates were sampled for the majority of campaigns, except for September 2018 and June 2019 where replicates were reduced to two due to logistic constraints, weather and safe tree access.

Gas exchange measurements

All gas exchange measurements were conducted in situ on upper canopy oak leaves using either a Li-6400XT or Li-6800 portable photosynthesis system (LiCOR, Lincoln, NE, USA) to quantify photosynthetic performance at BIFoR FACE. Measurement campaigns focused on two different types of measurements: (i) instantaneous diurnal measurements, at prevailing

environmental conditions (2018 and 2019), and (ii) net assimilation rate-intercellular CO_2 concentration ($A–C_i$) measurements (includes pre-treatment, 2015; 1st year, 2017; and 3rd year, 2019, of CO_2 fumigation). Measurements were conducted in all six experimental plots with infrastructure, on one chosen candidate tree per plot. The target tree remained the same for all treatment years (2017, 2018 and 2019) but a different tree was measured during the pre-treatment period in 2015. This change was because the plot infrastructure, which determined the CAS system, was not constructed until 2016.

When reporting treatment effects from the present study, we report the *mean enhancement* or *treatment effect*:

$$100 \cdot \frac{\Delta A_i}{A_{i,a}} = 100 \left(\frac{A_{i,e} - A_{i,a}}{A_{i,a}} \right) \qquad (1)$$

where $A_{i,x}$ is a measure of gas exchange ($i = $ 'net' or 'sat', see below) at ambient (a) or elevated (e) CO_2 mixing ratios. When

comparing our results with other studies using different eCO_2 treatments, we report the sensitivity to eCO_2, following Keenan et al. (2016):

$$\text{Sensitivity} = \frac{c_a}{\text{GPP}} \frac{\partial \text{GPP}}{\partial c_a} \approx \frac{\Delta A_i}{A_{i,a}} \cdot \frac{c_a}{\Delta c_a} \qquad (2)$$

where c_a is the aCO_2 mixing ratio and Δc_a is the treatment size (e.g., +150 µmol mol^{-1} as in our case). For the conditions of the present study (see 'Diurnal measurements' section, below), $c_a/\Delta c_a = 392/150 = 2.61$, and we use net photosynthesis instead of GPP. Hence, our theoretical predicted photosynthetic enhancement (Nowak et al. 2004; see Supplemental Appendix 1 available as Supplementary Data at *Tree Physiology Online*) for the +150 µmol mol^{-1} increase in CO_2 (i.e., $\approx 37\%$; Hart et al. 2020), is equivalent to expecting a sensitivity to eCO_2 of unity.

Diurnal measurements

Near the canopy-top, in situ diurnal measurements of gas exchange were conducted on upper canopy oak leaves on 11 and 12 separate summer days of 2018 and 2019, respectively. Measurements of gas exchange (e.g., net CO_2 photosynthetic assimilation rates, A_{net}) were made using a Li-6800 equipped with the default clear Propafilm (Innovia Films Inc., Atlanta, GA) window chamber head, which allowed for natural sunlight to illuminate the leaf. Measurements were conducted in one pair of plots (i.e., one eCO_2 plot and its paired aCO_2 plot) on each sampling day. Therefore, each full campaign ($n = 3$) took 3 days to complete, with the exception of September 2018 and June 2019 where only two replicate plots could be measured. A total of four diurnal campaigns were conducted in both 2018 and 2019, providing a total of 3426 data points. Five to six healthy leaves were randomly selected in the same oak tree per plot, every 30–40 min across the time course of the day for gas exchange measurements, swapping between aCO_2 and eCO_2 plots.

Measurements were made at the respective growth CO_2 of aCO_2 ($\sim$405 µmol mol^{-1}) or +150 µmol mol^{-1} aCO_2 ($\sim$555 µmol mol^{-1}) for eCO_2 plots, along with other environmental variables such as relative humidity (RH); air temperature (T_{air}) and quanta of photosynthetically active radiation (PAR). Measurements were confined to the youngest fully expanded leaves of the leader branch within reaching distance of the CAS system. Measurements were confined to the first flush of leaves across the season for consistency in leaf age. Expanding leaves, judged from color and texture, were avoided for measurements, as they had not matured in terms of chlorophyll and formation of the photosynthetic apparatus. Once a leaf was inside the chamber, the Li-6800 head was gently positioned and held constant at an angle towards the sun. This was to ensure sun exposure on the leaf, to minimize shading of the chamber head on the measured leaf and to reduce variation across the leaf measurements. Measurements were recorded after an initial stabilization period (typically $\sim$40 seconds to 1 minute), to meet programmed stability parameters. This allowed for instantaneous steady-state photosynthesis to be captured, yet avoided chamber-related increases in leaf temperature (Parsons et al. 1998). Care was taken to ensure conditions matched those outside the chamber before each measurement was taken. The daily mean RH inside the leaf chamber was between 50 and 77% for all measurements. The mean C_a values in the LiCOR chamber head were 390 ± 0.9 and 538 ± 2.7 µmol mol^{-1}, in 2018, and 393 ± 1.0 and 545 ± 4.8 µmol mol^{-1}, in 2019, for aCO_2 and eCO_2, respectively. The mean CO_2 treatments were, therefore, +148 ± 2.8 µmol mol^{-1} in 2018 and +152 ± 4.9 in 2019, and were not statistically different. The gas exchange systems were calibrated before each growing season.

A–C_i curves

A–C_i curves were conducted in three growing seasons: pre-treatment year (2015), in the 1st year of CO_2 fumigation (2017) and third year of CO_2 fumigation (2019). Measurements were either conducted on attached branches in situ (2015 and 2019) or on detached branches harvested by climbers (2017) using a portable open gas exchange system that incorporated a controlled environment leaf chamber (Li-6400XT and LI-6800, LICOR, Inc., Lincoln, NE, USA). Detached branches were transferred to researchers on the ground immediately after excision, where they were placed in a bucket of water to minimize desiccation. Branches were re-cut under water and allowed to stabilize, before starting measurements. Measurement on detached branches was conducted no longer than 45 min after collection. Previous studies investigating measurements of gas exchange on severed or attached branches found no significant differences between the two methods (Bader et al. 2016, Verryckt et al. 2020). A–C_i curves were measured at a Q of 1800 µmol m^{-2} s^{-1} (in 2015 and 2019) or 1200 µmol m^{-2} s^{-1} (in 2017) and at a leaf temperature of 25 °C. Before each curve, a stabilization period of between 5 and 10 min was used depending on the prevailing environmental conditions and each curve took an average of 40 min. Light-saturated net photosynthesis (A_{sat}) was estimated from A–C_i curves at growth [CO_2]. The CO_2 concentrations were changed in 12–14 steps starting at the respective growth [CO_2]; every 100 µmol mol^{-1} down to 50 µmol mol^{-1} (near the photosynthetic CO_2 compensation point), then increasing to 1800 µmol mol^{-1} in roughly 200 µmol mol^{-1} increment steps. Five to six replicate A–C_i curves on different leaves per CO_2 treatment were measured per day. Measurements were taken between 09:00–11:00 and 14:00–17:00 to avoid potential midday stomatal closure (Valentini et al. 1995). Measurements were made using the treatment pair arrangement of one aCO_2 and one eCO_2 plot per day ($n = 3$).

Leaf carbon and nitrogen

Oak leaves were collected from the top of the canopy in each month, May–November in 2015 and 2019, by arborist climbers, and stored immediately at $-25\,°C$. Two upper canopy leaves, from one tree per plot, were selected for elemental analyses, these trees corresponded to the measurement tree for leaf gas exchange. 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) and the fresh weight was recorded. Each leaf was oven dried at $70\,°C$ for at least 72 h, re-weighed for dry weight and the leaf mass per unit area was calculated. Dried leaf fragments were ground and each sample (~2 mg) was enclosed in a tin capsule. Samples were analyzed for $\delta^{13}C$, total C and total N using an elemental analyzer interfaced with an isotope ratio mass spectrometer (Sercon Ltd, Cheshire, UK).

Statistical analysis

All 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, and residuals from model fitting were checked for evidence of heteroscedasticity. Hourly averages of diurnal measurements were analyzed using a linear mixed effects model ('lmer' package). Fixed categorical factors in this model were CO_2 treatment (i.e., aCO_2 or eCO_2), sampling month and sampling year (i.e., 2018 or 2019), in addition to their interactions. In addition, 'time of day' and 'plot' were represented as random factors, the latter as individual trees were nested within each experimental plot. Type III F-statistics associated with the mixed model analysis (repeated-measures analysis of variance, ANOVA) were reported. Statistically significant CO_2 treatment differences among groups were further tested with Tukey's post hoc test using the R package 'emmeans' ($P < 0.05$ reported as significant). To investigate the dependence of photosynthetic enhancement with variation of light, the diurnal gas exchange data, with leaf temperature, $T_{leaf} > 18\,°C$, and vapor pressure deficit (D), $D < 2.2$ kPa, were sub-divided into four light (Q) categories, each sampled about equally. The Q classes were chosen based on the characteristic response of A_{net} to light as follows: $Q < 250$; $250 \leq Q < 500$; $500 \leq Q < 1000$ and $Q \geq 1000\,\mu mol\,m^{-2}\,s^{-1}$. CO_2 treatment, year and Q category were then used as parameters in the ANOVA.

The photosynthetic CO_2 response (A–C_i) curves were fit with the model of Farquhar et al. (1980) to estimate the apparent maximum rate of photosynthetic Rubisco carboxylation (V_{cmax}, $\mu mol\,m^{-2}\,s^{-1}$) and the apparent maximum rate of photosynthetic electron transport (J_{max}, $\mu mol\,m^{-2}\,s^{-1}$) using 'Plantecophys' package in R (Duursma 2015). The model-fitting was undertaken to provide insight into photosynthetic capacity and its response to long-term exposure to elevated

$[CO_2]$ (Rogers and Ellsworth 2002). We tested for outliers by examining the J_{max}/V_{cmax} ratio, RMSE values and standard errors (SE) for fits of J_{max} and V_{cmax}, all of which indicate violations to the theory for fitting these curves (Sharkey et al. 2007). Visual inspection of each A–C_i curve with outliers allowed us to identify any incomplete curves and/or mechanical failures and those curves were subsequently removed. This accounted for $< 10\%$ of the data, leaving a total of 86 A–C_i curves across the 3-sampling years in the analysis.

Results

Measurement conditions

Overall, diurnal measurements were conducted on dry, sunny days (Figure 1), and environmental conditions (Q and T_{leaf}) were consistent between aCO_2 and eCO_2 across the two growing seasons of diurnal measurements (Figures 2A, B and 3A, B). Q levels were largely comparable between CO_2 treatments although cloud and temperature conditions were more variable among sampling days and campaigns in 2018 than in 2019.

Leaf temperature was more stable than Q with lower variability across the diurnal sampling, high similarity between sampling days, and high consistency between CO_2 treatments. There were differences of up to $15\,°C$ in midday measurements of T_{leaf}, between months, suggesting a seasonal influence as would be expected from the site's mid-latitude location, with differences more prominent in 2019 than 2018. The highest T_{leaf} values were observed in July with a common seasonal decline after this campaign.

Analysis of the diurnal dataset showed the range of mean daily A_{net} was similar between years, however the highest mean daily A_{net} ($12.2\,\mu mol\,m^{-2}\,s^{-1}$) was reported in 2018. Contrasting seasonal patterns were observed between the sampling years of 2018 and 2019, with decreases in mean daily A_{net} across the growing season observed in 2018 compared with increases in A_{net} in 2019. In both sampling years, we observed a significant enhancement of A_{net} when exposed to eCO_2 ($P < 0.05$, Table 1 and Figures 2 and 3). Here, we did not observe any significant effect of either season or sampling year on A_{net} (Table 1). Therefore, from measurements of A_{net} collected from the diurnal dataset, a mean eCO_2-driven photosynthetic enhancement (i.e., $100.\Delta A_i/A_{i,a}$) of $23 \pm 4\%$ was observed across the 2-year period of this study.

Photosynthesis and variation in photon flux density (Q)

This study analyzed the role of measurement Q affecting A_{net} and its response to eCO_2 in separate growing seasons to investigate photosynthetic enhancement values at different light conditions. In each light category (see section Methods, above), the light conditions between the CO_2 treatments were statistically comparable (Figure 4, see Supplementary Table S1
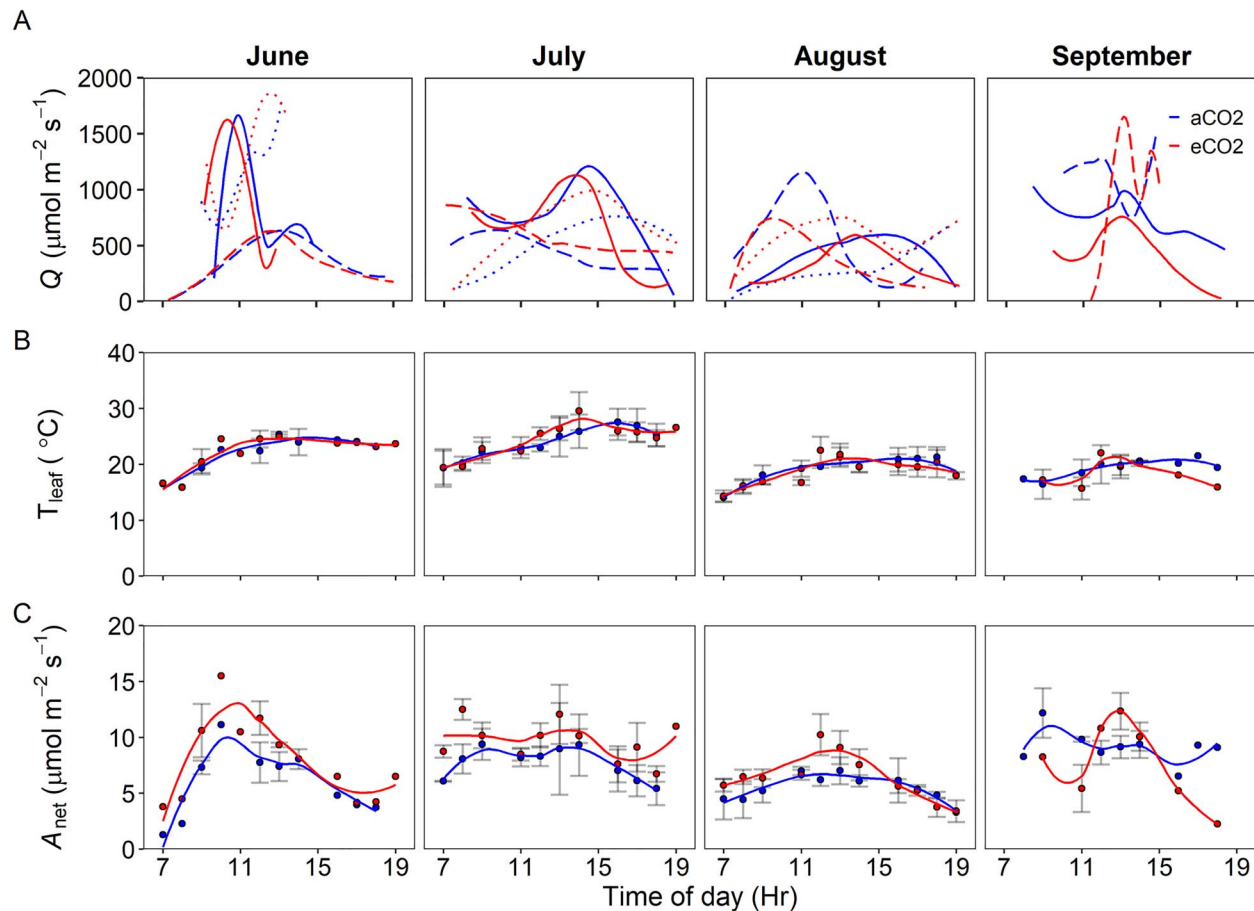
Figure 2. In situ diurnal measurements of (A) Q (µmol m^{-2} s^{-1}), (B) hourly mean T_{leaf} (°C) and (C) hourly mean A$_{net}$ (µmol m^{-2} s^{-1}); each fitted with an LOESS regression, at BIFoR FACE in 2018 from the upper $Q. robur$ canopy. Error bars indicate $n = 3$, with the exception of September where only two replicate plots were measured and not all time points were replicated. The line types in (A) represent replicate plot pairings of; plots 1 and 3 (dotted), plots 2 and 4 (solid) and plots 5 and 6 (long-dash) and the two colors represent the CO$_2$ treatments of aCO$_2$ (blue) and eCO$_2$ (red).

available as Supplementary Data at *Tree Physiology Online*). Mean, median and interquartile range of A$_{net}$ increased with increasing Q class for both sampling years and CO$_2$ treatments (Figure 4A and Table 2). We observed no significant effect of year for A$_{net}$ in this study, but we did observe a larger variation in A$_{net}$ in 2019, when compared with 2018 (Table 2 and Figure 4A). Values of mean A$_{net}$ ranged from 4.6 ± 0.3 µmol m^{-2} s^{-1}, at the lowest Q level with a mean of 150 µmol m^{-2} s^{-1}, to 11.5 ± 0.7 µmol m^{-2} s^{-1} at highest Q (mean Q of 1360 µmol m^{-2} s^{-1}). In addition, in both sampling years A$_{net}$ was significantly higher under eCO$_2$ conditions when compared with aCO$_2$ ($P < 0.05$, Table 2 and Figure 4A).

Consistent with our hypothesis, we observed mean eCO$_2$-driven photosynthetic enhancement to increase with increasing Q, with the largest enhancement observed at highest Q in both sampling years, 30 ± 9% and 35 ± 13%, for 2018 and 2019, respectively (Figure 4B). In 2018, eCO$_2$-driven photosynthetic enhancement ranged from 7 ± 10%, in the lowest Q class, to 30 ± 9%, in the highest Q class (Figure 4B). A similar positive relationship between eCO$_2$-driven photosynthetic enhancement

and Q was present in 2019 with enhancement ranging from 11 ± 6%, in the lowest Q class, to 35 ± 13%, in the highest Q class (Figure 4B). There was no significant effect of year (Table 2) and therefore the mean eCO$_2$-driven photosynthetic enhancement at light saturation (i.e., in the highest Q class) was on average 33 ± 8% across the 2-sampling years. Our results report that the mean eCO$_2$-driven photosynthetic enhancement of light-saturated A$_{net}$ (A$_{sat}$) in both sampling years was consistent, within error (using 95% confidence intervals), of the theoretical predicted enhancement based on proportion of CO$_2$ increase ($\approx$37 ± 6%), indicating a sensitivity to eCO$_2$ (Eq. (2), above) of close to unity for A$_{sat}$.

Photosynthetic capacity and foliar nitrogen

The seasonal and interannual biochemical changes in $Q. robur$ were assessed via differences in leaf apparent maximum CO$_2$ carboxylation capacity (V_{cmax}) and apparent maximum electron transport capacity for RuBP regeneration (J_{max}; Figure 5) to assess the photosynthetic capacity in the initial years of the long-term experiment. Initially, we tested for differences between
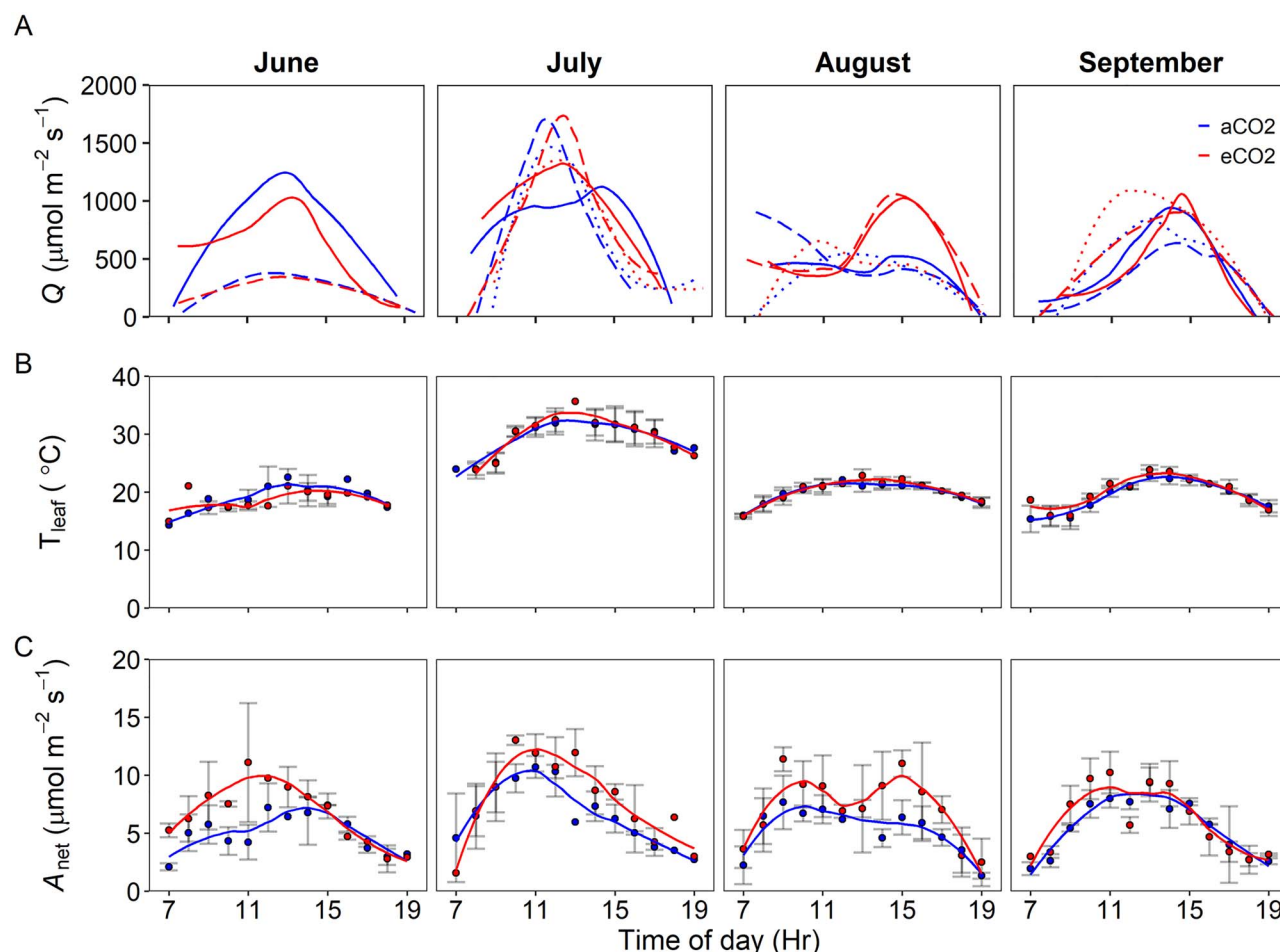
Figure 3. In situ diurnal measurements of (A) Q (μmol m^{-2} s^{-1}), (B) hourly mean T_{leaf} (°C) and (C) hourly mean A_{net} (μmol m^{-2} s^{-1}); each fitted with an LOESS regression, at BIFoR FACE in 2019 from the upper *Q. robur* canopy. Error bars indicate $n = 3$, the exception of June where only two replicate plots were measured and not all time points were replicated. The line types in (A) represent replicate plot pairings of; plots 1 and 3 (dotted), plots 2 and 4 (solid) and plots 5 and 6 (long-dash) and the two colors represent the CO_2 treatments of aCO_2 (blue) and eCO_2 (red).

the year of sampling and found no statistical difference of either V_{cmax} or J_{max} between the 3-sampling years (2015, 2017 and 2019; Figure 5, see Supplementary Table S2 available as Supplementary Data at *Tree Physiology Online*). This study found no significant effects of CO_2 enrichment on V_{cmax} or J_{max} across the 2 years of CO_2 enrichment, i.e., the 1st and 3rd years, and no significant effect of season between the 3 measurement years (Figure 5 and Table 3). However, this study did observe a significant effect of month for the variable V_{cmax} in 2019, whereby an increase in V_{cmax} was observed with progression of the growing season (Figure 5A and Table 3). Thus, this study observed no statistical evidence to suggest photosynthetic downregulation of either V_{cmax} or J_{max} under eCO_2 across the 3 years of eCO_2 exposure in *Q. robur*.

Consistent with previous research, this study observed a strong positive linear relationship between J_{max} and V_{cmax}, which remained unchanged across CO_2 treatments and growing season ($r^2 = 0.75$ ambient; $r^2 = 0.71$ elevated; see Supplementary Figure 3 available as Supplementary Data at *Tree*

Table 1. Linear mixed-effects model analysis for photosynthesis with CO_2 treatment (CO_2) using the diurnal dataset, sampling month (Month) and sampling year (Year) as fixed factors and random effects of 'plot' and 'time'. 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 test was used to determine the significance relationships. Significance of CO_2 treatment is noted in the rightmost column as (* = $P < 0.05$).

Parameter	df	P-value
CO_2	1	**0.044***
Month	3	0.14
Year	1	0.31
CO_2 * Month	3	0.18
CO_2 * Year	1	0.18
Month * Year	3	0.43
CO_2 * Month * Year	3	0.079

Physiology Online). In addition, no eCO_2-induced decreases in either area-based foliar nitrogen (N_a) or mass-based foliar nitrogen (N_m) were observed (Figure 5C and D and Table 3) across
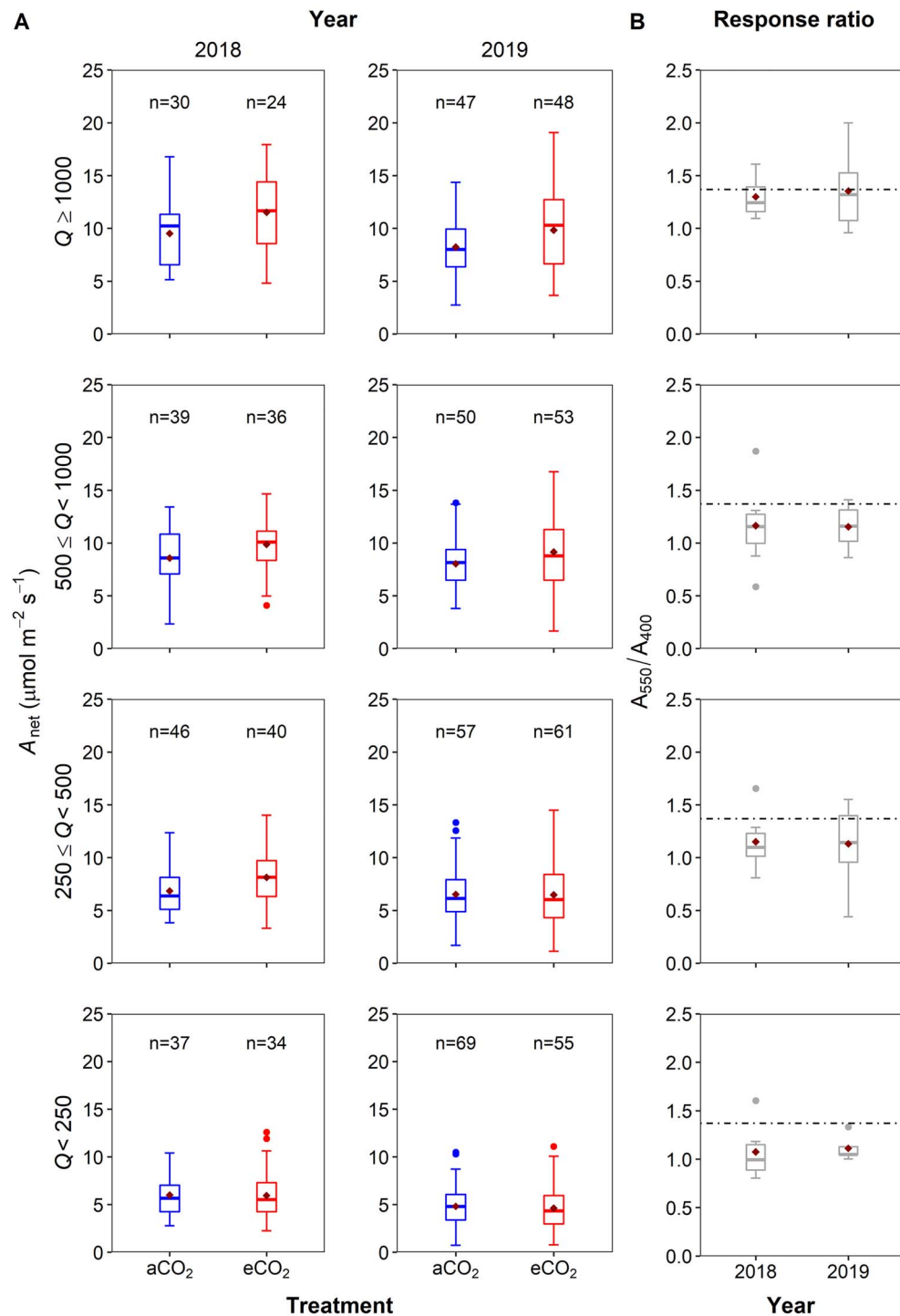
Figure 4. (A) The distribution of net photosynthesis (A$_{net}$; μmol m^{-2} s^{-1}) in each of the four photon flux density (Q) categories (Q < 250; 250 ≥ Q < 500; 500 ≥ Q < 1000 and Q ≥ 1000 μmol m^{-2} s^{-1}) for years 2018 (left) and 2019 (right). Whiskers denote the 5%ile and 95%ile; outliers are plotted as individual points (filled circles). The box denotes the interquartile range and the bar denotes the median with the number of data points above each boxplot. The mean is also plotted as a diamond symbol. Data use diurnal gas exchange measurements in the upper canopy oak trees at the BIFoR FACE facility with T$_{leaf}$ > 18 °C and D < 2.2 kPa, in eCO$_2$ (red) or aCO$_2$ (blue) treatments. Red diamonds indicate the mean A$_{net}$ values. (B) Boxplots of the enhancement response ratio (A$_{550}$/A$_{400}$) (gray) for each year, and predicted enhancement ratio (dashed line) (1.37) following Nowak et al. (2004).

the study period. No change in foliar nitrogen is corroborative of the results in Figure 5 and also suggests the absence of

photosynthetic downregulation under eCO$_2$ in mature *Q. robur* in the first 3 years of the long-term experiment.

Table 2. Linear mixed-effects model parameters for prediction of A_{net} with variation in photo flux density (Q). Type III sums of squares computed using restricted maximum likelihood estimates for F-tests. The numerator df for each F-test are shown. A post-hoc Tukey's test was used to determine the significance relationships. Significance is noted in bold in the rightmost column as (*** = $P < 0.001$; ** = $P < 0.01$ and * = $P < 0.05$).

Parameter	df	P-value
CO_2	1	**0.016***
Year	1	0.062
Q	3	<**0.001***
CO_2 * Year	1	0.97
CO_2 * Q	3	**0.011***
Year * Q	3	**0.0078**
CO_2 * Year * Q	3	0.13

The instantaneous response ratio (2015) and the longer-term response ratio (2017 and 2019) were calculated using the light-saturated A_{net} (i.e., A_{sat}) values at growth CO_2 from the A–C_i datasets (Figure 6B). There was no significant difference between the measurement years in either A_{sat} or the response ratio suggesting comparability between the instantaneous response ratio and the longer-term response ratio (see Supplementary Table S3 available as Supplementary Data at *Tree Physiology Online*). A significant treatment effect was observed for A_{sat} (Figure 6A and Table 3) in all 3-sampling years, with a mean eCO_2-driven photosynthetic enhancement of $24 \pm 2\%$, $31 \pm 7\%$ and $32 \pm 11\%$ in 2015, 2017 and 2019, respectively, under eCO_2 when compared with aCO_2. A significant effect of month on A_{sat} was observed in 2019, with A_{sat} increasing with the progression of the growing season (Table 3 and Figure 6A). The photosynthetic enhancement observed from our A–C_i curve datasets are consistent with the values obtained in the diurnal dataset ($33 \pm 8\%$, Figure 5) but is lower than the theoretical predicted enhancement calculated via CO_2 increase (37%) (see Supplemental Appendix 1 available as Supplementary Data at *Tree Physiology Online*). In summary, the consistency in the two separate measurements (i.e., diurnal and A–C_i curves) support the finding of sustained eCO_2-driven photosynthetic enhancement in mature *Q. robur* across the first 3 years of the BIFoR FACE experiment.

Discussion

There are ample data on the short-term enhancement of photosynthesis by eCO_2 in young trees using a variety of experimental set-ups from tree chambers to FACE experiments (e.g., Ainsworth and Rogers 2007, Crous et al. 2008), but few data for mature forest-grown trees with multi-year CO_2 exposure in a FACE setting. For mature trees, available evidence suggests that there are significant increases in light-saturated A_{net} (Körner et al. 2005, Ellsworth et al. 2017) but there have
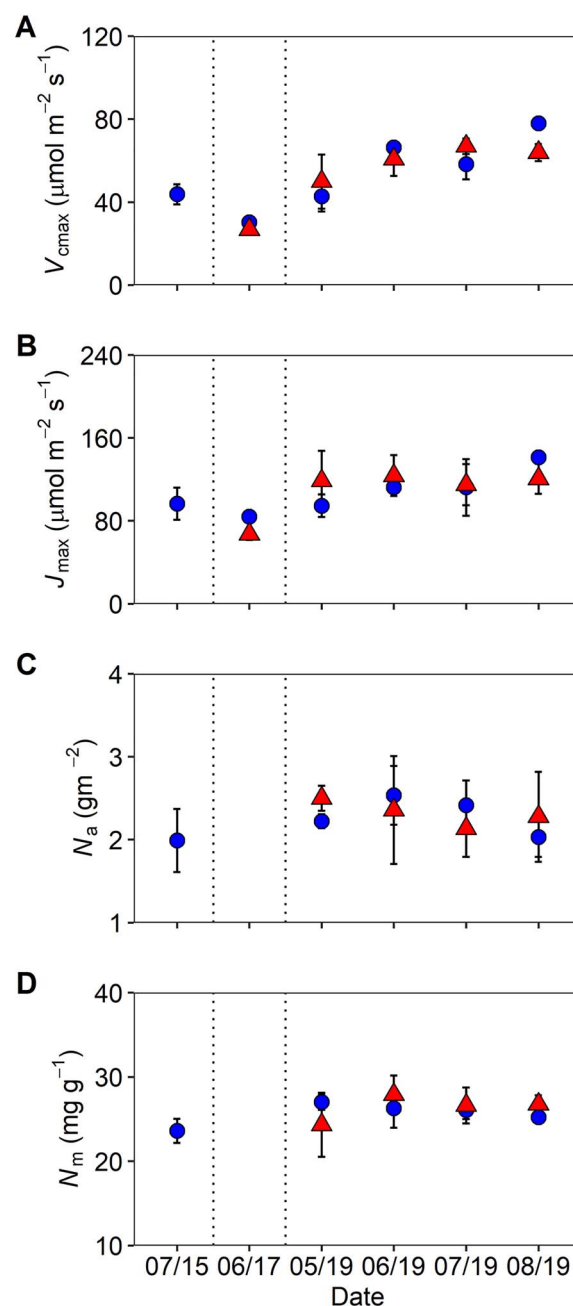
Figure 5. Maximum rates of (A) carboxylation (V_{cmax}) and (B) electron transport (J_{max}), in addition to (C) area-based (N_a) and (D) mass based (N_m) leaf nitrogen of upper canopy *Q. robur* from 2015 to 2019 at BIFoR FACE. Means ($\pm$ SE) of whole-plot averages ($n = 3$) for ambient (blue circles) and elevated (red triangles) CO_2 treatments. Dashed line indicates the separation of sampling years with campaigns labelled 'month/year', as follows: Pre-treatment ('07/15'); 1st year ('06/17') and the 3rd year ('05/19'–'08/19') of CO_2 fumigation. Data points may obscure error bars.

been mixed results regarding the magnitude of photosynthetic enhancement (range 13–49% per 100 ppm of CO_2 increase) and occurrence of photosynthetic downregulation in mature forest-grown trees (Crous et al. 2008, Bader et al. 2010,

Table 3. Linear mixed-effects model analysis for V_{cmax}, J_{max}, net photosynthesis (A_{net}), area-based leaf nitrogen (N_a) and mass-based leaf nitrogen (N_m) with CO_2 treatment (CO_2) and sampling month (month) as fixed factors and random effects of 'plot' and 'time'. Type III sums of squares computed using restricted maximum likelihood estimates for F-tests. The numerator df for each F-test are shown. Significance is noted in boldface as (* $P < 0.05$).

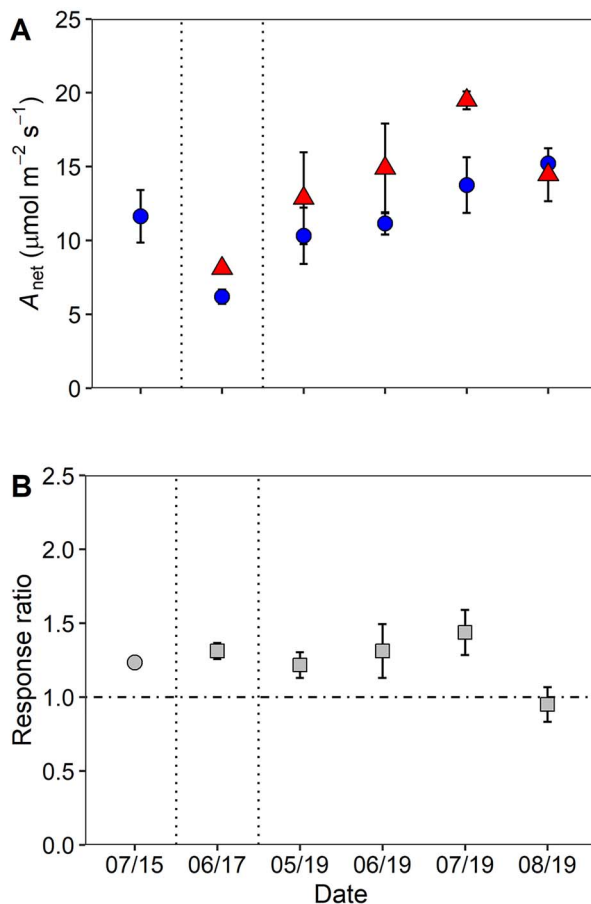| Parameter	df	V_{cmax} P-value	J_{max} P-value	A_{net} P-value	N_m P-value	N_a P-value
CO₂	1	0.70	0.37	**0.042***	0.42	0.64
Month	3	**0.02***	0.15	**0.034***	0.93	0.052
CO₂ * Month	3	0.20	0.57	0.33	0.69	0.11

Figure 6. (A) Net photosynthesis (A_{net}) at growth CO_2 and (B) instantaneous (2015) and longer-term (2017 and 2019) response ratios in the upper oak canopy using the A–C_i curve data. Means ($\pm$ SD) of the plots per treatment are shown across six sampling campaigns for aCO_2 (blue circles), eCO_2 (red triangles) and either the instantaneous (gray squares) or longer-term response ratio (grey circles). Dashed line indicate the separation of sampling years with campaigns labelled as follows; pre-treatment ('07/15'), 1st year ('06/17') and the 3rd year ('05/19'–'08/19') of CO_2 fumigation.

2016, Warren et al. 2015, Ellsworth et al. 2017). In this study, we predicted a theoretical A_{net} enhancement of 37% for the 150 μmol mol⁻¹ increase in CO_2 at BIFoR FACE following reasoning in Nowak et al. (2004; see Supplemental Appendix 1 available as Supplementary Data at *Tree Physiology Online*).

After 3 years of eCO_2 exposure in mature temperate oak forest, net photosynthetic rates of upper canopy foliage from *Q. robur* were on average 23 $\pm$ 4% higher, based on the diurnal dataset, in the trees exposed to eCO_2 when compared with control plots (Figures 2–4; Tables 1 and 3). The eCO_2-driven photosynthetic enhancement observed is substantially lower than the theoretical expected enhancement of 37%, likely due to diurnal and seasonal variation in prevailing environmental conditions such as lower air temperatures, lower light conditions and varying vapor pressure deficits. Only considering light-saturated A_{net} (A_{sat}) from the diurnal dataset, our mean photosynthetic enhancement is greater than the average diurnal enhancement, at 33 $\pm$ 8% rather than 23%. Furthermore, our independent estimate of A_{sat} enhancement based on the A–C_i curve data is 32 $\pm$ 11%, which is comparable within error (using 95% confidence intervals) to both the A_{sat} value from the diurnal measurements and the hypothesized enhancement of 37%. A slight stomatal closure in eCO_2 could have contributed to the slightly lower photosynthetic enhancement than the hypothesized enhancement of 37% (see Supplemental Appendix 1 available as Supplementary Data at *Tree Physiology Online*). However, our average light-saturated photosynthetic enhancement is generally lower than previously reported values in canopy-dominant trees from other forest FACE experiments (Bader et al. 2010, 42–48%; Crous et al. 2008, 40–68%; Liberloo et al. 2007, 49% and Sholtis et al. 2004, 44%), but is somewhat higher than the value of 19% from the EucFACE experiment on mature *Eucalyptus* trees (Ellsworth et al. 2017). The lower photosynthetic enhancement observed at EucFACE was likely due to lower nutrient availability compared with BIFoR (Crous et al. 2015), although there were other differences such as the tree species and prevailing temperatures that would also affect the magnitude of the photosynthetic enhancement.

The role of environmental conditions for photosynthetic enhancement

Consistent with our initial hypothesis, we observed significantly higher A_{net} and a 24% higher photosynthetic enhancement under the highest light conditions at BIFoR FACE (i.e., $Q > 1000$ μmol m⁻² s⁻¹) compared with the lowest light category. Thus, a negative linear relationship was observed for

both A_{net} and eCO_2-induced photosynthetic enhancement with decreasing light levels. Our results are consistent with previous research on mature trees that observed an effect of light on the magnitude of CO_2-driven stimulation of photosynthesis (Bader et al. 2016), suggesting variation in light should be considered when assessing the response to eCO_2. Consequently, the relationship of A_{net} and CO_2 treatment effect with light intensity is important when scaling upper canopy data both across diurnal periods of light limitation and extending to the whole canopy, of shaded and sunlit leaves, to avoid overestimating canopy-scale photosynthesis by temperate forests.

It has been previously suggested that larger photosynthetic enhancement may be expected in low light environments (Hättenschwiler 2001, Norby and Zak 2011). For example, deep shaded tree seedlings displayed greater photosynthetic gains than those in moderate shade (photosynthetic enhancement of 97% and 47%, respectively) with exposure to eCO_2 (Kitao et al. 2015). In light-limited environments, higher CO_2 concentrations can increase the apparent quantum yield and reduce the light compensation point leading to enhanced carbon uptake (Larcher, 2003, Kitao et al. 2015). Hättenschwiler (2001) found large interspecific variability and, in *Quercus,* that greater photosynthetic responses to CO_2 occurred under higher light when compared with low light. However, both Kitao et al. (2015) and Hättenschwiler (2001) studied tree seedlings in contrast to upper canopy leaves of a canopy-dominant species in the present study. Although shade leaves were not measured here, the results here from the top of the tree canopy provide an important benchmark for the magnitude of photosynthetic enhancement by eCO_2 in a mature oak forest.

In addition to light intensity, the photosynthetic response of *Q. robur* varied across the growing season, as has been observed in many other trees (Tissue et al. 1999, Rogers and Ellsworth 2002, Sholtis et al. 2004). Here, A_{sat} (derived from the $A-C_i$ dataset) in both CO_2 treatments increased about 50% from early in the season (May), to the middle of the season (July); yet, the relative response ratio to eCO_2 was stable throughout this period at 32%. In addition, when assessing the diurnal dataset, we found contrasting seasonal patterns between 2018 and 2019, with decreases in A_{net} across the growing season observed in 2018 compared with increases in A_{net} in 2019, likely due to drier and warmer conditions in 2018. Previous research has identified reductions in photosynthesis across the season is largely associated with drier conditions (Gunderson et al. 2002), which support the results observed in the present study. This suggests that the influence of soil water availability on the seasonal pattern in oak physiology is critical for determining seasonal C-uptake by mature forests and should be further investigated in mature *Q. robur* to improve longer term carbon-climate models (see Limousin et al. 2013).

Previous research has identified eCO_2-driven photosynthetic responses observed in seedlings and saplings may not reflect the photosynthetic responses of mature forest-grown trees (Hättenschwiler et al. 1997). The present study provided a unique opportunity to assess the eCO_2-driven photosynthetic responses in 175-year-old canopy-dominant trees and found lower photosynthetic stimulation than the many previous studies on tree seedlings and younger trees (e.g., Curtis and Wang 1998, Sholtis et al. 2004, Ainsworth and Long 2005, Liberloo et al. 2007, Crous et al. 2008). The age dependency of CO_2 responsiveness to photosynthesis in trees (Turnbull et al. 1998, Wujeska-Klause et al. 2019a), highlights the importance of long-term experiments, such as the present study and others in understanding potential variable responses across the lifetime of a tree, vital for accurate climate-carbon modeling of forests.

Did changes to photosynthetic capacity or leaf biochemistry occur under eCO_2?

In some studies, a time-dependent decline in the magnitude of eCO_2-induced photosynthetic enhancement, i.e., photosynthetic downregulation, has been observed (Cure and Acock 1986, Gunderson and Wullschleger 1994). Here, we hypothesized that there may be reductions in V_{cmax}, J_{max} and leaf N, particularly in the 3rd year of eCO_2 exposure (Luo et al. 2004). Our analysis of the 86 $A-C_i$ curves collected in this experiment revealed no decrease in the rate of V_{cmax} or J_{max}, indicating that there were no significant changes in the photosynthetic capacity of *Q. robur* over the first 3 years of exposure to eCO_2. A lack of photosynthetic downregulation has also been found in similar seasonally deciduous species, including the closely related species *Quercus petraea* (Bader et al. 2010), in addition to *Liquidambar styraciflua, Populus spp.* and *Betula papyrifera* (Herrick and Thomas 2001, Sholtis et al. 2004, Liberloo et al. 2007, Uddling et al. 2009). An apparent lack of downregulation has also been observed in other mature forest-grown species (Bader et al. 2010, Ellsworth et al. 2017).

As nitrogen is required for the synthesis and maintenance of photosynthetic proteins, eCO_2-driven photosynthetic downregulation has been associated with declines in foliar N (as reviewed in Medlyn et al. 1999) and soil N-limitations (e.g., Rogers and Ellsworth 2002, Crous et al. 2008, Warren et al. 2015). The current study on *Q. robur* did not find any changes in either mass- or area-based leaf nitrogen across the study period, indicating there are no reductions to photosynthetic capacity (Figure 5). This corroborates the findings from the V_{cmax} and J_{max} parameters, supporting the suggestion for sustained photosynthesis in *Q. robur* over the first 3 years of exposure to eCO_2. Hence, there were no changes to the ratio of J_{max} to V_{cmax}, indicating that the relationship between carboxylation and light-harvesting processes was not affected by CO_2 treatment, as found in previous studies (Medlyn et al. 1999, Crous et al. 2008), including the closely related species, *Q. petraea* (Bader

et al. 2010). These results may point to soil nutrient availability not yet limiting the photosynthetic processes in this forest system. The BIFoR FACE site receives moderately high atmospheric N deposition (~22 Kg N/ha/yr) thought to represent 15% of the total nitrogen nutrition of temperate deciduous trees, likely preventing ecosystem N-limitation at present (Rennenberg and Dannenmann 2015). Therefore, with adequate N deposition in the soil, sustained photosynthetic enhancement was observed in the first 3 years of eCO_2 exposure at BIFoR FACE.

Conclusions

After 3 years of eCO_2 exposure in a temperate deciduous forest at the BIFoR FACE facility, photosynthetic enhancement of mature *Q. robur* leaves at the top of the canopy was sustained across all years and was $33 \pm 8\%$ (mean $\pm$ SE) at light saturation, close to the theoretical expectation. The magnitude of photosynthetic enhancement was significantly affected by light conditions with higher enhancement at higher light. We found no evidence of photosynthetic downregulation under eCO_2 and no declines in leaf nitrogen in the upper canopy. The lack of evidence for downregulation suggest there are sufficient soil nutrients for *Q. robur* to maintain a relatively high photosynthetic enhancement under eCO_2 conditions, at least to this point in the eCO_2 experiment. Much further work remains to determine the movement and allocation of this enhanced C-uptake in the forest. Our results are consistent with a sustained, positive C-uptake response to rising atmospheric CO_2 in a mature deciduous forest tree species, provided adequate nutrients are available.

Supplementary data

Supplementary data for this article are available at *Tree Physiology* Online.

Conflict of interest

None declared.

Author contributions

ARMK, JP and AG designed the study; AG, KYC and DSE collected the data. AG organized the datasets under the supervision of DSE, with input from ARMK; AG and DSE designed and performed the statistical analyses, with input from KYC and 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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