

## Original Article

## Carbon source–sink limitations differ between two species with contrasting growth strategies

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## ABSTRACT

Understanding how carbon source and sink strengths limit plant growth is a critical knowledge gap that hinders efforts to maximize crop yield. We investigated how differences in growth rate arise from source–sink limitations, using a model system comparing a fast-growing domesticated annual barley (*Hordeum vulgare* cv. NFC Tipple) with a slow-growing wild perennial relative (*Hordeum bulbosum*). Source strength was manipulated by growing plants at sub-ambient and elevated CO<sub>2</sub> concentrations ([CO<sub>2</sub>]). Limitations on vegetative growth imposed by source and sink were diagnosed by measuring relative growth rate, developmental plasticity, photosynthesis and major carbon and nitrogen metabolite pools. Growth was sink limited in the annual but source limited in the perennial. RGR and carbon acquisition were higher in the annual, but photosynthesis responded weakly to elevated [CO<sub>2</sub>] indicating that source strength was near maximal at current [CO<sub>2</sub>]. In contrast, photosynthetic rate and sink development responded strongly to elevated [CO<sub>2</sub>] in the perennial, indicating significant source limitation. Sink limitation was avoided in the perennial by high sink plasticity: a marked increase in tillering and root:shoot ratio at elevated [CO<sub>2</sub>], and lower non-structural carbohydrate accumulation. Alleviating sink limitation during vegetative development could be important for maximizing growth of elite cereals under future elevated [CO<sub>2</sub>].

**Key-words:** barley; allocation; CO<sub>2</sub>; crop yield; nitrogen; photosynthesis.

## INTRODUCTION

Global population growth, economic development and climate change are exerting increasing pressure on our global food supply, raising demand that must be met, in part, by improving crop yields (Ainsworth *et al.* 2008b; Godfray *et al.* 2010; Foley *et al.* 2011; Reynolds *et al.* 2012; von Caemmerer *et al.* 2012; FAO *et al.* 2014; Ort *et al.* 2015). Increasing yield depends critically on a firm understanding of plant growth, which is in turn underpinned by the interactions between carbon and nitrogen sources and sinks (White *et al.* 2016). Sources provide net uptake of resources from the external environment, whilst sinks

cause a net internal drawdown of these resources. For carbon, mature leaves are sources, and roots are sinks, and the balance between them is achieved by well-characterized molecular crosstalk mechanisms (Smith & Stitt 2007; Lawlor & Paul 2014; White *et al.* 2016). Decades of research into the effects of elevated CO<sub>2</sub> have demonstrated that increasing source activity through a stimulation of photosynthesis often does not translate into corresponding yield increases (Long *et al.* 2006; Ainsworth *et al.* 2008a; Leakey *et al.* 2009), although this depends on the species (Yamori *et al.* 2016). Similarly, increasing sink capacity does not always translate into greater yield under field conditions (Weichert *et al.* 2010). A holistic approach to growth and yield considering both source and sink capacities is therefore essential for developing higher-yielding crop varieties (White *et al.* 2016). In this context, source strength is the product of source activity and size, with an equivalent definition for sinks (Geiger & Shieh 1993; White *et al.* 2016).

One strategy for understanding the fundamental limitations on growth is to investigate the natural diversity of growth rates in wild plants. In wild species, one of the major causes of growth rate variation is life history (Grime & Hunt 1975; Garnier 1992). Annual and perennial growth strategies enable plants to allocate resources in a way that is appropriate for their environment: annuals grow quickly and invest everything in reproduction in the first year before they die, whilst perennials grow more slowly and conserve resources for the following season (Garnier 1992; Iwasa 2000; Bennett *et al.* 2012). Annuals are typically seen as having flexible growth strategies for exploiting fluctuating environments, whereas perennials have more conservative growth strategies – that is, lower allocation to reproduction and slower growth (Atkinson *et al.* 2012, 2014). Although perennials with large storage organs may never be sink limited, annuals generally transition from sink to source limitation during development when they switch from vegetative to reproductive growth (Arp 1991), and perennials lacking large storage organs are likely to undergo this transition as well. Because perennials grow more slowly than annuals and transition to the reproductive growth stage later, they are therefore likely to be sink limited for a longer period of time.

Despite this well-developed ecological theory, we do not currently know the extent to which slower growth in perennials than annuals arises from greater source to sink limitation. Experimental manipulations of the source:sink ratio provide insights into the relative contributions of source and sink

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processes to growth rate and may be achieved through a variety of techniques including sink removal (Arp 1991), genetic modification (Ainsworth *et al.* 2004, Weichert *et al.* 2010; Zuther *et al.* 2011), source removal (von Caemmerer & Farquhar 1984, Bryant *et al.* 1998; Rogers *et al.* 1998; Eyles *et al.* 2013), inhibiting resource export from the source (Ainsworth & Bush 2011) and increasing source activity using elevated CO<sub>2</sub> (Kinsman *et al.* 1997; Masle 2000), reviewed by White *et al.* (2016). Here, we alter the atmospheric CO<sub>2</sub> concentration ([CO<sub>2</sub>]) to non-invasively manipulate the source:sink ratio in barley – elevated [CO<sub>2</sub>] to increase the source strength and sub-ambient [CO<sub>2</sub>] to decrease it – with current [CO<sub>2</sub>] as a reference against which to compare the source manipulations. This approach enables analysis of source and sink limitation under current [CO<sub>2</sub>], and strong CO<sub>2</sub> treatments are applied in order to produce marked perturbations of the system. In C<sub>3</sub> plants, [CO<sub>2</sub>] affects carbon source strength directly through one well-understood process, that is, carbon assimilation by Rubisco, and therefore avoids wounding responses and other confounding effects, which may arise from alternative approaches for source:sink manipulation. We took a holistic approach to investigating source–sink interactions, measuring the responses of development, growth, allocation, photosynthesis and key carbon and nitrogen metabolite pools on the same plants. Together, these simultaneous measurements of growth, carbon uptake and carbon utilization allowed us to diagnose source and sink limitation in our model system. For example, a high concentration of free amino acids indicates carbon source limitation (Paul & Driscoll 1997; Stitt & Krapp 1999; Isopp *et al.* 2000; Rogers *et al.* 2006), whilst a build-up of non-structural carbohydrates in leaves indicates carbon sink limitation (Rogers & Ainsworth 2006; Ainsworth & Bush 2011).

In order to elucidate physiological mechanisms underpinning differences in growth rate, this study compared domesticated annual barley (*Hordeum vulgare* cv. NFC Tipple) and a wild perennial relative (*Hordeum bulbosum*). Annual barley is sink limited during grain filling (Schnyder 1993; Bingham *et al.* 2007; Serrago *et al.* 2013), yet to our knowledge, no study of source and sink limitation during the vegetative growth stage has been made in this species. The annual barley used here is an elite agricultural spring barley from the HGCA recommended list (HGCA 2014) and has a fast-growing life-history strategy. The perennial is a wild species from Turkey, which is able to grow in diverse habitats but generally occupies nutrient-rich environments (von Bothmer 1996). CO<sub>2</sub> treatments were applied at germination and maintained until harvest, which occurred during the vegetative growth phase of the life cycle. We predicted that annual barley, which grows faster than perennial barley, would be sink limited during vegetative growth, but the perennial would be more strongly sink limited (Jaikumar *et al.* 2014). Based on this hypothesis, we would expect the fast-growing annual to show a greater increase in growth and photosynthesis in response to elevated [CO<sub>2</sub>] than the perennial (Poorter 1993; Roumet & Roy 1996). This is because the elevated [CO<sub>2</sub>] alleviates source limitation and will therefore have a greater effect in the plants which are less sink limited (Bryant *et al.* 1998; Rogers *et al.* 1998; Ainsworth *et al.* 2003). In contrast, we expected that the

more strongly sink limited, slower-growing perennial would show a greater increase in the storage of carbon-rich metabolites under elevated [CO<sub>2</sub>].

## MATERIALS AND METHODS

### Plant material and growth conditions

Seeds of *Hordeum vulgare* cv. NFC Tipple and *Hordeum bulbosum* (Accessions GRA1031 and GRA947) were obtained from Syngenta and IPK Gatersleben, respectively. Seeds were germinated on wet filter paper and transplanted to 4 L pots filled with 1:10 sand:vermiculite and topped with an additional layer of sand to aid root development of the seedlings. Plants were grown in controlled environment growth chambers (BDR 16, Conviron, Isleham, UK) at the University of Sheffield, two of which had been modified to scrub CO<sub>2</sub> using soda lime. Plants were grown in three chambers with fixed CO<sub>2</sub> levels of 180, 400 and 1500 μmol mol<sup>-1</sup> for 61 days. In order to impose strong carbon source and sink manipulations, 180 and 1500 μmol mol<sup>-1</sup> were chosen. All chambers had a 12 h photoperiod with day/night temperatures of 20/18 °C, 65% humidity and daytime light levels of 600 μmol photons m<sup>-2</sup> s<sup>-1</sup> at plant height, resulting in a daily light integral of 25.92 mol m<sup>-2</sup> day<sup>-1</sup>. Plants were kept adequately watered with 20% Long Ashton's nutrient solution. During seedling establishment, plants were watered daily – with 150 mL reverse osmosis water for 8 days and with 150 mL Long Ashton's solution thereafter. After 17 days, plants were watered three times per week with 150 mL Long Ashton's solution until 29 days old, 225 mL until 45 days old and 450 mL thereafter.

Photosynthesis measurements and metabolite harvests were carried out three times in consecutive weeks, between 46 and 61 days after germination (DAG). In each of these harvest weeks, six annuals from each CO<sub>2</sub> level were harvested (three at dawn and three at dusk), giving a total of 54 individuals across 3 weeks. In the first and third of these harvest weeks, six perennials from each CO<sub>2</sub> level were harvested (three at dawn and three at dusk), giving a total of 36 individuals.

### Relative growth rate, root:shoot ratio and tillering

Relative growth rate (RGR) was calculated based on the plant mass estimated from weekly imaging of above ground biomass, beginning when plants were 2 weeks old. Plants were photographed (PowerShot G9, Canon, Tokyo, Japan) six times from the side against a white background, with the plant rotated 60° between successive photographs. A scale bar of known length was included for calibration. Leaf area in pixels was obtained for each photograph using Image J (U. S. National Institutes of Health, Bethesda, Maryland, USA, <http://imagej.nih.gov/ij/>), and converted to mm<sup>2</sup> using the area of the scale bar. A batch of 29 additional plants, not used in the main study, was also photographed weekly. At nine time points between 33 and 60 DAG, individuals from this batch were separated into leaf, leaf sheath and root and oven-dried to provide a calibration curve for leaf area and dry mass for each species. These curves were then used to predict dry mass for each plant

for each set of six photographs (Supporting Information Fig. S1;  $r^2 = 0.97$  for species-based calibration). Individual growth curves showing predicted dry mass over time were obtained for each plant using a nonlinear mixed-effects model obtained by stepwise selection and used to estimate RGR at multiple time points by differentiation, where more than three time points had been measured.

The calibration was also used to predict root:shoot ratio for the plants in the main study. For each of the oven-dried individuals, the relative contribution of shoot and root to whole plant mass was recorded, and the mean fraction of root and shoot was calculated. This was then applied to the plant mass predicted for the plants in the main study using calibration of image data, to give an estimate of root:shoot ratio for each individual.

Tillers were counted the day before metabolite harvests were carried out.

### Photosynthesis

Diurnal measurements of photosynthesis were made the day before plants were harvested for metabolite assays. Fifty-one annual and 26 perennial individuals were measured (the remaining plants were too small for gas exchange measurements to be performed). Instantaneous net photosynthetic rate was measured every 3.5 h between 30 min after dawn and 30 min before dusk, using the LI-6400XT Portable Photosynthesis System (Li-Cor Biosciences, Lincoln, NE, USA). Net photosynthetic rate was measured *in situ* within growth chambers, under the ambient environmental conditions of each chamber described earlier. These measurements were used to obtain a curve of photosynthesis during the photoperiod for each plant, and the area underneath was integrated to give a daily rate of net carbon fixation per unit area – that is, the carbon source activity. This value was multiplied by the projected shoot area to estimate the total daily photosynthesis in the whole shoot – that is, the carbon source strength.

### Metabolites

Plants were harvested from 47 to 61 DAG, within 1 h before dawn and 1 h before dusk. Samples were flash frozen in liquid nitrogen, stored at  $-80^\circ\text{C}$  and freeze-dried prior to analysis. For small plants, the entire plant was harvested; for larger plants, representative samples of leaf, leaf sheath and root from both young and old tissue were harvested. In the first week, plants were harvested at 47, 48 and 49 DAG. In the second week, plants were harvested at 52, 53 and 54 DAG. In the third week, plants were harvested at 59, 60 and 61 DAG. Three replicates from each species from one chamber were harvested on each date, with the exception of the second week when only annuals were harvested. The order of chambers was randomized in each of the three harvest weeks.

Metabolite analysis was carried out at Brookhaven National Laboratory. Metabolites were extracted from the freeze-dried ground tissue using sequential ethanol extractions.

Ethanol-soluble carbohydrates (glucose, fructose, sucrose and low degree of polymerization (LDP) fructan) were

analysed using a continuous enzymatic substrate assay as described previously (Ainsworth *et al.* 2007) adapted for measuring sucrose in the presence of LDP fructans (Harrison *et al.* 1997), and 1U each of exo- and endo-inulinases was added to each well to quantify LDP fructans after glucose, fructose and sucrose had been measured. All biochemical analysis was conducted in standard 96-well microplates (Microtest Plate 96-Well Flat Bottom, Sarstedt, Nümbrecht, Germany), using a robotic liquid handling system (Evolution P<sup>3</sup> Precision Pipetting Platform, Perkin Elmer, Waltham, MA, USA).

The pellets from the ethanol extraction were heated to  $95^\circ\text{C}$  in 0.1 M NaOH, to solubilize protein. A commercially available protein assay kit (Pierce BCA protein assay kit, ThermoFisher, Rockford, IL, USA) based on the Lowry method was used to measure protein content (Lowry *et al.* 1951) using BSA as a standard. Following the protein assay, samples were neutralized with HCl.

For the starch and high degree of polymerization (HDP) fructan assay, starch and HDP fructans from  $40\ \mu\text{L}$  aliquots of the suspended pellet material were digested using enzymes in  $60\ \mu\text{L}$  0.05 M acetate buffer as follows. Starch: 0.17U well<sup>-1</sup> amyloglucosidase (EC 3.2.1.3) and 0.1U well<sup>-1</sup>  $\alpha$ -amylase (EC 3.2.1.1); starch and HDP fructan: 0.1U well<sup>-1</sup> exoinulinase (EC 3.2.1.80), 0.1U well<sup>-1</sup> endoinulinase (EC 3.2.1.7), 0.17U well<sup>-1</sup> amyloglucosidase and 0.1U well<sup>-1</sup>  $\alpha$ -amylase. Plates were incubated overnight at  $37^\circ\text{C}$ . A total of  $40\ \mu\text{L}$  of the supernatant from the overnight digest was transferred to each well of a 96-well microplate. A total of  $262\ \mu\text{g}$  ATP well<sup>-1</sup>,  $349\ \mu\text{g}$  NADP well<sup>-1</sup> and  $3.6\ \text{U}$  well<sup>-1</sup> glucose-6-phosphate dehydrogenase (EC 1.1.1.49, grade II) were added in a buffer of 0.1 M HEPES/KOH, 3 mM  $\text{MgCl}_2$ , pH7.0 to initiate the reaction. Microplates were centrifuged for 1 min to remove bubbles and then inserted into a plate reader, and the NADPH associated with the carbohydrates in the sample was measured at  $A_{340}$  (ELx808, BioTek, Winooski, VT, USA).

Starch and HDP fructans were assayed by sequentially adding 1U enzyme in HEPES buffer to each well as follows, using the rationale for the soluble carbohydrates assay (Ainsworth *et al.* 2007). Starch: hexokinase (EC 2.7.1.1); starch and HDP fructan: hexokinase, phosphoglucose-isomerase (EC 5.3.1.9). HDP fructan values were obtained by subtracting the values for starch from the values for combined starch and fructan. This approach was necessary because preliminary recovery experiments had shown that digesting fructan using exoinulinase and endoinulinase also degraded a small amount of starch, leading to an artificially high value for fructan content which was corrected using the approach described here. Starch and HDP fructan content was measured as nmol hexose equivalents using a standard glucose curve loaded on each plate.

Total free amino acids were quantified using fluorescamine:  $15\ \mu\text{L}$  0.1 M sodium borate buffer,  $90\ \mu\text{L}$  fluorescamine and  $100\ \mu\text{L}$  water were combined with  $2\ \mu\text{L}$  of ethanol extract in a black 96-well microplate (Nunc MicroWell, Thermo Fisher Scientific, Waltham, MA, USA). Following a 5 min dark incubation, fluorescence (360 nm excitation, 460 nm emission and 40 nm bandwidth) was measured (Synergy HT, BioTex, Winooski, VT, USA) and converted into nmol amino groups using a standard glutamate curve loaded on each plate.

The Griess reaction was used to quantify free nitrate. Firstly, 0.005U well<sup>-1</sup> nitrate reductase (EC 1.7.1.3) and 50nmol NADPH in 0.11M potassium phosphate buffer were added to a 10 $\mu$ L aliquot of the ethanol extract. Each microplate was shaken. Following a 30min incubation (dark, room temperature), 20 $\mu$ L 0.25mM phenazine methosulfate was added to each well. Plates were shaken again and incubated for a further 20min, and 45 $\mu$ L 1% w/v sulfanilamide in 5% phosphoric acid followed by 45 $\mu$ L 0.02% N(1-Naphthyl)ethylenediamine dihydrochloride was added to each well. Following another shake and a 5min incubation, A<sub>540</sub> was measured (ELx808, BioTex, Winooski, VT, USA) and converted into nmol nitrate using a standard nitrate curve loaded on each plate.

Metabolite data were expressed per g carbohydrate-corrected dry weight, obtained by subtracting the mass of total non-structural carbohydrate (the sum of glucose, fructose, sucrose, LDP and HDP fructan and starch) from the dry mass of each sample. Technical and analytical replicates were run for all assays (Ferne *et al.* 2011).

### Statistical methods

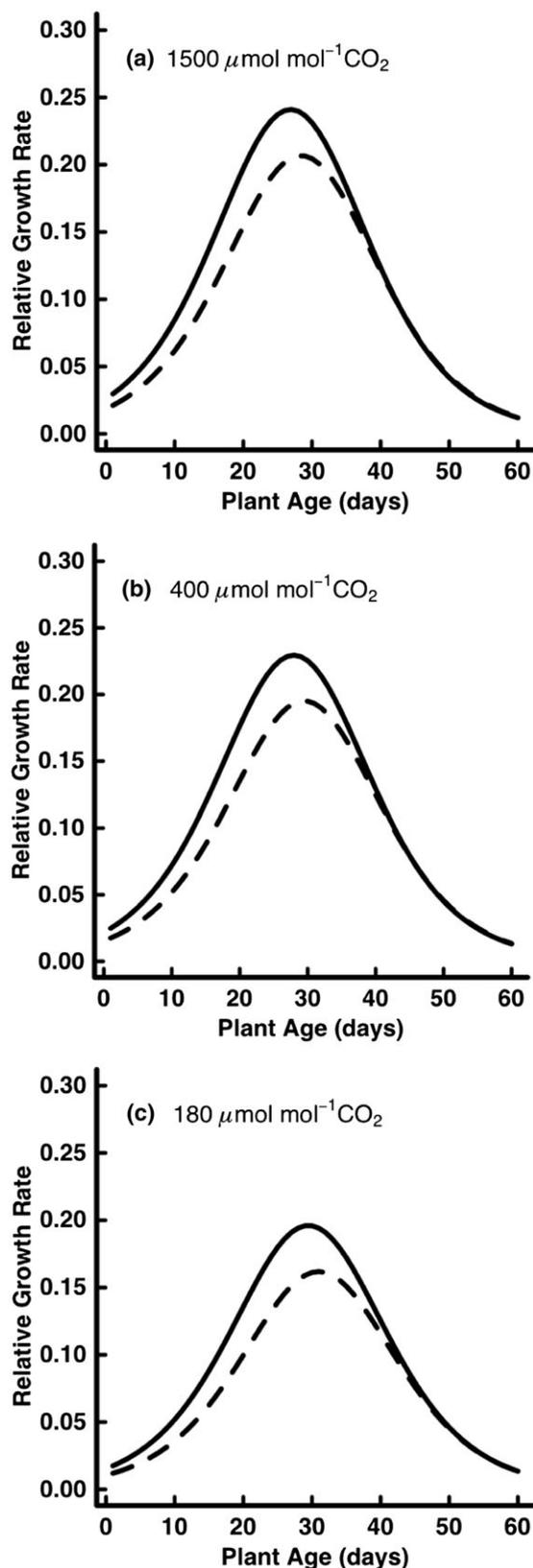
Analysis was performed using R (RCore Team, 2015). Analysis of variance models incorporating error terms reflecting the split–split plot design of the experiment were carried out for each variable measured. Logarithmic transformations were performed on all data prior to analysis to improve the fit of the models.

For photosynthesis, TNC, amino acids and amino acid:sucrose (Figs 2,4,6 & 7), small error bars are present but are obscured by symbols.

## RESULTS

### Perennial barley shows greater developmental plasticity in response to elevated [CO<sub>2</sub>] than the annual species

Relative growth rate (the efficiency of whole plant dry mass increase obtained from calibration of shoot area, and measured in g g<sup>-1</sup> day<sup>-1</sup>) was obtained from individual growth curves by differentiation and represents the sink activity of plant growth. RGR was higher in the annual than perennial plants, and greater at higher CO<sub>2</sub> levels (Fig. 1). Stepwise model selection was used to choose fixed effects, and the effects of species and [CO<sub>2</sub>] on the maximum plant size and the time to reach half size were each highly significant ( $P < 0.001$ ) – although these are additive effects with no significant interaction between [CO<sub>2</sub>] and species. In both species, the increase in RGR was greater between 180 and 400  $\mu$ mol mol<sup>-1</sup> CO<sub>2</sub> than between 400 and 1500  $\mu$ mol mol<sup>-1</sup> CO<sub>2</sub>. Peak RGR in the annual increased by 17.1% between 180 and 400  $\mu$ mol mol<sup>-1</sup> CO<sub>2</sub> but only 5.0% between 400 and 1500  $\mu$ mol mol<sup>-1</sup> CO<sub>2</sub>. Peak RGR in the perennial increased by 20.5% between 180 and 400  $\mu$ mol mol<sup>-1</sup> CO<sub>2</sub>, but only 6.0% between 400 and 1500  $\mu$ mol mol<sup>-1</sup> CO<sub>2</sub>. However, the difference between annual and perennial remained relatively consistent: peak RGR



**Figure 1.** Relative growth rate is higher in annual (solid line) than perennial (dashed line) barley, and greater at higher [CO<sub>2</sub>]. Relative growth rate is daily gain in dry mass relative to whole plant dry mass, g g<sup>-1</sup> day<sup>-1</sup>. (a), elevated [CO<sub>2</sub>]: 1500  $\mu$ mol mol<sup>-1</sup>; (b), current [CO<sub>2</sub>]: 400  $\mu$ mol mol<sup>-1</sup>; (c), sub-ambient [CO<sub>2</sub>]: 180  $\mu$ mol mol<sup>-1</sup>.

in the annual was 21.1%, 17.7% and 16.7% higher than in the perennial, at 180, 400 and 1500  $\mu\text{mol mol}^{-1}$   $\text{CO}_2$ , respectively.

The modular nature of plant body plans means that, in order for RGR to increase, plants must either increase the biomass of existing organs or initiate new structures through branching (tillering, in the case of grasses). Tillering in the perennial increased by 163% between 180 and 1500  $\mu\text{mol mol}^{-1}$   $\text{CO}_2$ , whereas the number of tillers in the annual increased by just 15% across the same  $\text{CO}_2$  range (Table 1). This highly significant species  $\times$   $[\text{CO}_2]$  interaction ( $F_{(1, 80)} = 56$ ,  $P < 0.001$ ) indicates greater developmental plasticity in the perennial.

Root:shoot ratio also showed a larger response to increasing  $[\text{CO}_2]$  in perennial than annual barley. In the annual, the root:shoot ratio increased by only 2.8% between 180 and 400  $\mu\text{mol mol}^{-1}$   $\text{CO}_2$ , whilst in the perennial, it increased 11.6% between 180 and 400  $\mu\text{mol mol}^{-1}$ , and 4.2% between 400 and 1500  $\mu\text{mol mol}^{-1}$  (Table 1). This highly significant species  $\times$   $[\text{CO}_2]$  interaction ( $F_{(1, 77)} = 24$ ,  $P < 0.001$ ) provides further evidence of greater developmental plasticity in the perennial.

### Perennial barley also shows a greater photosynthetic response to elevated $[\text{CO}_2]$ than the annual species

Annual barley generally has a higher photosynthetic rate than the perennial, but the photosynthetic rate in the perennial shows a much stronger response to  $[\text{CO}_2]$  (Fig. 2b). In the annual plants, the daily photosynthetic rate increased by 87% between 180 and 400  $\mu\text{mol mol}^{-1}$   $\text{CO}_2$ , but only by 13% between 400 and 1500  $\mu\text{mol mol}^{-1}$ . In contrast, in the perennial, it increased 58% between 180 and 400  $\mu\text{mol mol}^{-1}$ , but 75% between 400 and 1500  $\mu\text{mol mol}^{-1}$ . This led to a significant species  $\times$   $[\text{CO}_2]$  interaction:  $F_{(1, 67)} = 4.9$ ,  $P < 0.05$ ; Fig. 2b. Because the annual is a larger plant than the perennial, the difference in whole shoot photosynthetic rate, that is, carbon source strength (Fig. 2c) is greater than the difference in the rate per unit area, that is, carbon source activity (Fig. 2b). In the annual, the whole shoot daily photosynthetic rate increased by 177% between 180 and 400  $\mu\text{mol mol}^{-1}$   $\text{CO}_2$ , but only 25% between 400 and 1500  $\mu\text{mol mol}^{-1}$ . In contrast, in the perennial, it increased 528% between 180 and 400  $\mu\text{mol mol}^{-1}$ , and 123%

between 400 and 1500  $\mu\text{mol mol}^{-1}$ . There was a highly significant species  $\times$   $[\text{CO}_2]$  interaction:  $F_{(1, 66)} = 18$ ,  $P < 0.001$ ; Fig. 2c.

The ratio of photosynthesis to growth is higher in the annual than the perennial (Fig. 3), seen in the plots of individuals (Fig. 3a,b) and means (Fig. 3c,d) with a highly significant effect of species:  $F_{(1, 61)} = 25$ ,  $P < 0.001$ . When expressed in  $\text{g C g}^{-1} \text{ day}^{-1}$  (Fig. 3a), growth shows three clusters corresponding to the decreasing values of RGR as time progresses over the three harvests. When expressed in  $\text{g C plant}^{-1} \text{ day}^{-1}$  (Fig. 3c), these clusters are no longer present, and a positive correlation between photosynthesis and growth is seen. The ratio increases with  $[\text{CO}_2]$  (Fig. 3c) and with plant age at harvest (Fig. 3d). There was a highly significant interaction of the harvest week  $\times$   $[\text{CO}_2]$  ( $F_{(4, 61)} = 16$ ,  $P < 0.001$ ), such that the photosynthesis: growth ratio is greater at higher  $[\text{CO}_2]$ , but this trend becomes less pronounced at later harvests.

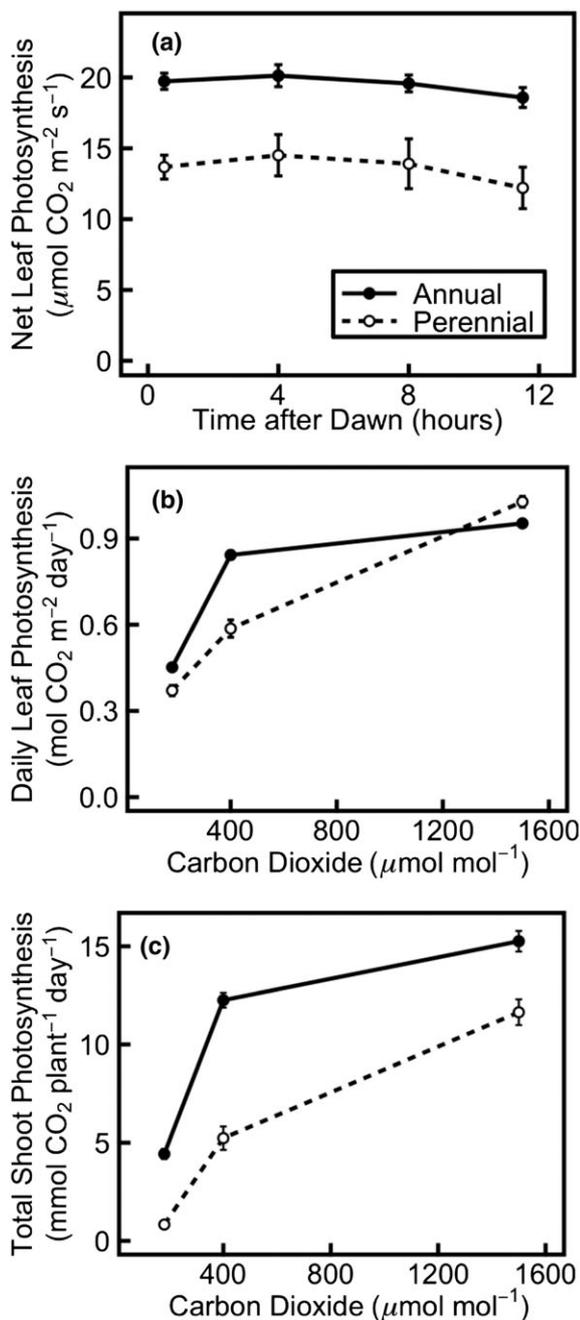
### Annual barley accumulates more non-structural carbohydrates than the perennial species

Pre-dawn measurements indicate the basal level of carbohydrates in plant organs, when metabolites accumulated during the previous photoperiod have been utilized for respiration, exported or consumed by growth at night. Before dawn, annual barley had a higher concentration of total non-structural carbohydrates (TNC, the sum of glucose, fructose, sucrose, fructan and starch) than the perennial and showed a greater accumulation of TNC in leaf sheaths and roots when  $[\text{CO}_2]$  was increased from 180 to 1500  $\mu\text{mol mol}^{-1}$  (Fig. 4). However, in the leaves, perennial barley showed a stronger TNC response than the annual when  $[\text{CO}_2]$  was increased from 180 to 1500  $\mu\text{mol mol}^{-1}$  (Fig. 4). Across all TNC data, there was a significant organ type  $\times$  time of day  $\times$  species interaction ( $F_{(2, 120)} = 9.2$ ,  $P < 0.001$ ), a significant organ type  $\times$  time of day  $\times$   $[\text{CO}_2]$  interaction ( $F_{(4, 120)} = 18$ ,  $P < 0.001$ ), a significant organ  $\times$  species  $\times$   $[\text{CO}_2]$  interaction ( $F_{(4, 120)} = 22$ ,  $P < 0.001$ ) and a significant organ  $\times$  harvest week  $\times$   $[\text{CO}_2]$  interaction ( $F_{(8, 120)} = 4.5$ ,  $P < 0.001$ ). In the leaf, TNC was 114% greater in the annual than the perennial at 180  $\mu\text{mol mol}^{-1}$   $\text{CO}_2$ , 57% greater in the annual at 400  $\mu\text{mol mol}^{-1}$   $\text{CO}_2$ , but approximately equal at 1500  $\mu\text{mol mol}^{-1}$   $\text{CO}_2$  (Fig. 4a). In the leaf sheath, TNC was 29% greater in the annual than the perennial at

**Table 1.** Responses of tillering and root:shoot ratio to increasing  $[\text{CO}_2]$ .

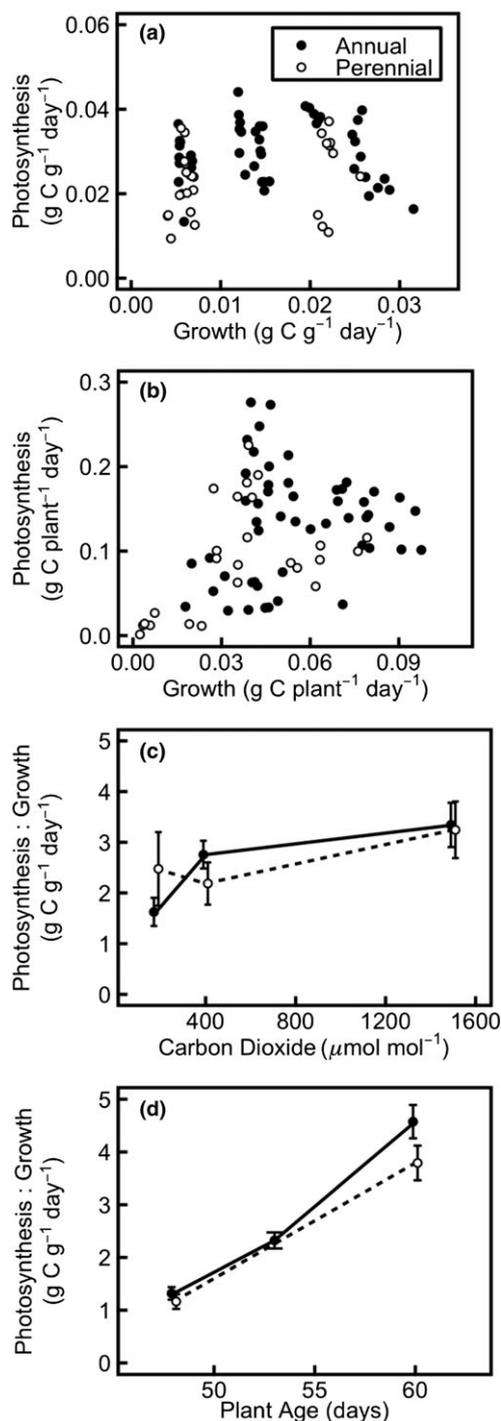
	Annual, 180 $\mu\text{mol mol}^{-1}$ $\text{CO}_2$	Annual, 400 $\mu\text{mol mol}^{-1}$ $\text{CO}_2$	Annual, 1500 $\mu\text{mol mol}^{-1}$ $\text{CO}_2$	Perennial, 180 $\mu\text{mol mol}^{-1}$ $\text{CO}_2$	Perennial, 400 $\mu\text{mol mol}^{-1}$ $\text{CO}_2$	Perennial, 1500 $\mu\text{mol mol}^{-1}$ $\text{CO}_2$
Tillers (mean)	<b>13.3</b>	<b>13.4</b>	<b>15.3</b>	<b>12.4</b>	<b>17.4</b>	<b>32.6</b>
Tillers (S.E.)	0.753	0.506	0.676	1.275	1.341	2.464
Root:shoot ratio (mean)	<b>0.529</b>	<b>0.544</b>	<b>0.547</b>	<b>0.466</b>	<b>0.520</b>	<b>0.542</b>
Root:shoot ratio (S.E.)	0.00321	0.00190	0.00187	0.0174	0.00768	0.00309

Annual barley shows very limited tillering and root:shoot ratio responses to increasing  $\text{CO}_2$  concentration, whilst the perennial shows a dramatic increase in tillering and a significant increase in root:shoot ratio. Data shown are obtained from 54 annual and 36 perennial individuals across the three treatments. Tillers were counted directly, whilst root:shoot ratio was estimated non-destructively using imaging. Means (bold) and their associated standard errors (S.E.) are reported to three-significant figures (annual  $n = 18$ , perennial  $n = 12$ ).

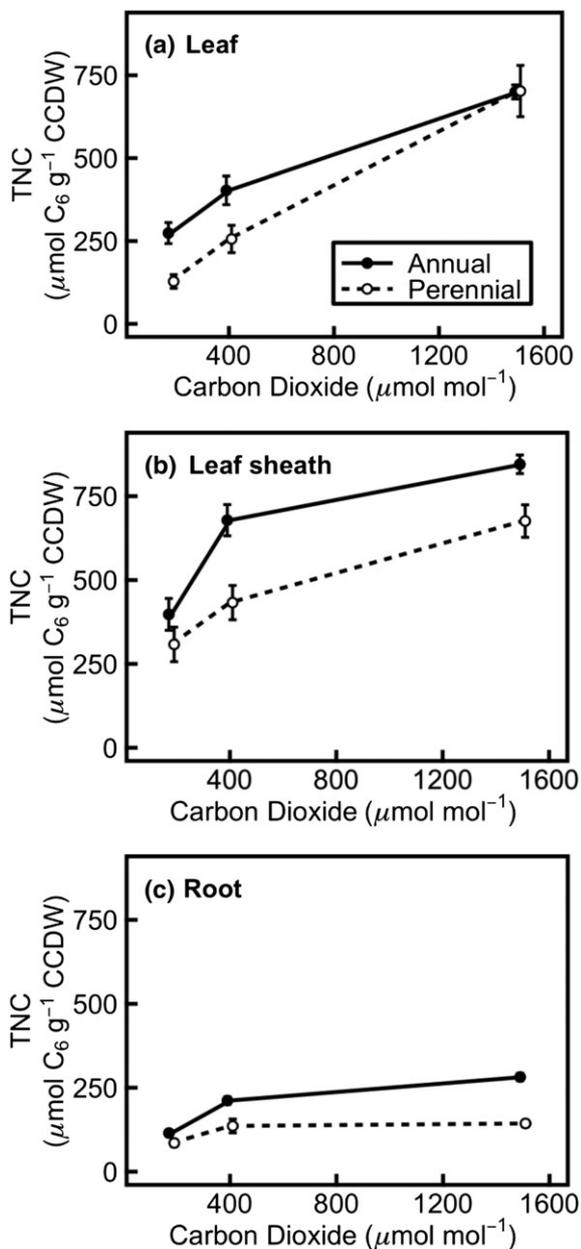


**Figure 2.** Perennial barley (dashed line) has a more pronounced photosynthetic response to elevated  $[\text{CO}_2]$  than annual barley (solid line). (a) Diurnal time course of net leaf photosynthesis in annuals and perennials grown at  $400 \mu\text{mol mol}^{-1} \text{ CO}_2$ . (b) Daily rate of net leaf photosynthesis per unit area obtained from integrating curves (e.g. (a)); (c) total daily photosynthesis in the whole shoot, obtained by multiplying the daily rate (b) by projected shoot area. Data show mean  $\pm$  SE ([a]: annual  $n = 18$ , perennial  $n = 9$ ; [b]: at 180, 400 and  $1500 \mu\text{mol mol}^{-1} \text{ CO}_2$ , annual  $n = 15, 18$  and  $18$ , perennial  $n = 5, 9$  and  $12$ ; [c]: at 180, 400 and  $1500 \mu\text{mol mol}^{-1} \text{ CO}_2$ , annual  $n = 15, 18$  and  $18$ , perennial  $n = 4, 9$  and  $12$ ).

$180 \mu\text{mol mol}^{-1} \text{ CO}_2$ , 57% greater in the annual at  $400 \mu\text{mol mol}^{-1} \text{ CO}_2$  and 25% greater in the annual at  $1500 \mu\text{mol mol}^{-1} \text{ CO}_2$  (Fig. 4b). In the root, TNC was 35% greater in the annual than the perennial at  $180 \mu\text{mol mol}^{-1}$



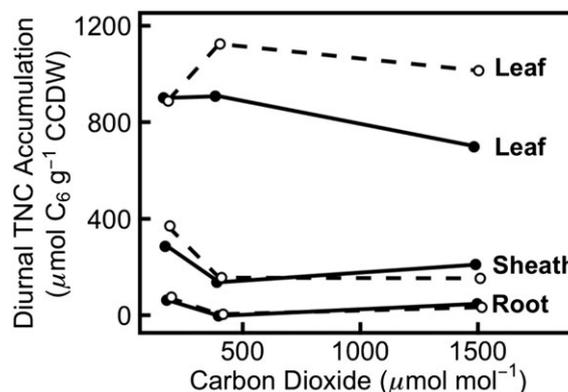
**Figure 3.** The ratio of photosynthesis to growth is higher in annual than perennial barley and greater at higher  $[\text{CO}_2]$  and in older plants. (a) Source activity versus sink activity, plotted as photosynthesis and growth for individual plants at all times and  $\text{CO}_2$  levels, expressed in  $\text{g C g}^{-1} \text{ day}^{-1}$ , showing three clusters along the x-axis corresponding to the three-harvest times with relative growth rate decreasing as time progresses; (b) source strength versus sink strength, plotted as photosynthesis and growth for individual plants at all times and  $\text{CO}_2$  levels expressed in  $\text{g C plant}^{-1} \text{ day}^{-1}$ ; (c), photosynthesis:growth ratio in the three  $[\text{CO}_2]$  treatments; (d) changes in the photosynthesis:growth ratio with respect to the mean plant age at harvest. Data show mean  $\pm$  SE ([c] and [d]: at 180, 400 and  $1500 \mu\text{mol mol}^{-1} \text{ CO}_2$ , annual  $n = 15, 18$  and  $18$ , perennial  $n = 4, 9$  and  $12$ ).



**Figure 4.** Pre-dawn concentrations of total non-structural carbohydrates (TNC) are higher in annual (solid line) than perennial (dashed line) barley. (a) leaf; (b) leaf sheath; (c) root. The overall CO<sub>2</sub> response is greater for perennials in the leaf but greater for annuals in the leaf sheath and root. Data are expressed in  $\mu\text{mol}$  glucose equivalents per g carbohydrate-corrected dry weight (CCDW). Data show mean  $\pm$  SE (annual  $n=9$ , perennial  $n=6$ ).

CO<sub>2</sub>, 56% greater in the annual at 400  $\mu\text{mol mol}^{-1}$  CO<sub>2</sub> and 97% greater in the annual at 1500  $\mu\text{mol mol}^{-1}$  CO<sub>2</sub> (Fig. 4c). In both species, TNC concentration is highest at 1500  $\mu\text{mol mol}^{-1}$  CO<sub>2</sub> suggesting that sinks are replete under these conditions.

Subtracting the mean pre-dawn values from the mean pre-dusk values provides a differential of TNC (Fig. 5), which represents the amount of carbon accumulated during the photoperiod, and is equivalent to the amount of carbon available

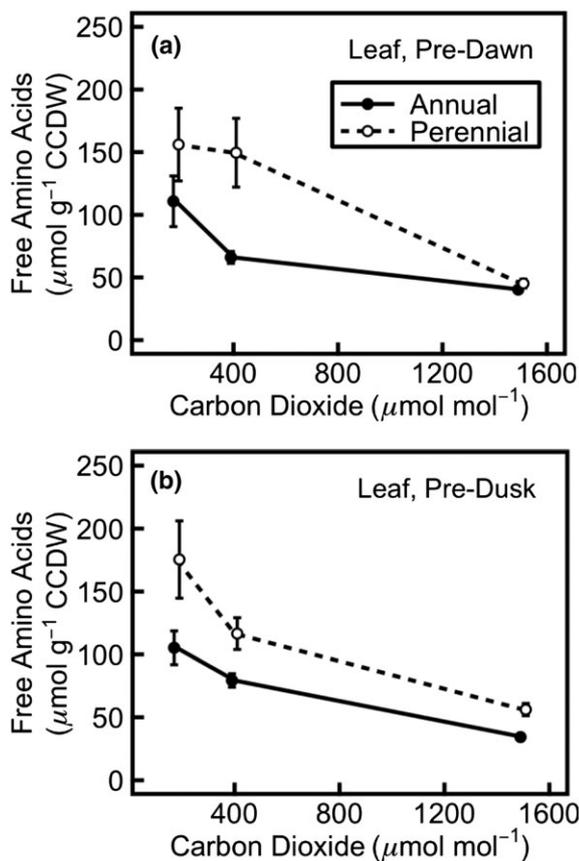


**Figure 5.** The diurnal accumulation of total non-structural carbohydrates (TNC), equivalent to the carbon pool available for nocturnal use or export in the leaf, leaf sheath and root, in annual barley (solid line) and perennial barley (dashed line). Data show the mean pre-dawn concentrations subtracted from mean pre-dusk concentrations, expressed in  $\mu\text{mol}$  glucose equivalents per g carbohydrate-corrected dry weight (CCDW). Different plants were harvested at dawn and dusk, so standard errors cannot be calculated for these data (raw data presented in the Supporting Information Table S1).

for respiration, export or growth at night. These differentials are much greater in the leaf than in leaf sheath or root (Fig. 5), because diurnal fluctuations in leaves are more tightly coupled to the diurnal activity of photosynthesis than the distal sinks of leaf sheath and root. The perennial shows a greater TNC differential than the annual in leaves at 400 and 1500  $\mu\text{mol mol}^{-1}$  CO<sub>2</sub>, yet there is little difference in TNC differentials in leaf sheath and root, across the CO<sub>2</sub> concentrations (Fig. 5). Therefore, whilst the basal pre-dawn level of TNC is higher in annuals (Fig. 4), the diurnal accumulation of TNC is greater in perennials for leaves at 400 and 1500  $\mu\text{mol mol}^{-1}$  CO<sub>2</sub> (Fig. 5).

### Perennial barley accumulates more free amino acids than the annual species

Free amino acids are an indicator of carbon source limitation (Paul & Driscoll 1997; Stitt & Krapp 1999; Isopp *et al.* 2000). A high-free amino acid concentration or high-amino acid:sucrose ratio reflects a surplus of available nitrogen for biosynthesis, because source-limited plants lack sufficient carbon to use along with this nitrogen for growth and development. The perennial has a higher concentration of free amino acids than the annual (Fig. 6). In both annual and perennial, free amino acid concentration is highest at 180  $\mu\text{mol mol}^{-1}$  CO<sub>2</sub>, which implies a carbon source limitation, and decreases as [CO<sub>2</sub>] increases (Fig. 6). Before dawn, amino acid concentration is 41% greater in the perennial than the annual at 180  $\mu\text{mol mol}^{-1}$  CO<sub>2</sub>, 127% greater in the perennial at 400  $\mu\text{mol mol}^{-1}$  CO<sub>2</sub> and 12% greater in the perennial at 1500  $\mu\text{mol mol}^{-1}$  CO<sub>2</sub> (Fig. 6a). Before dusk, amino acid concentration is 67% greater in the perennial than the annual at 180  $\mu\text{mol mol}^{-1}$  CO<sub>2</sub>, 47% greater in the perennial at 400  $\mu\text{mol mol}^{-1}$  CO<sub>2</sub> and 64% greater in the perennial at 1500  $\mu\text{mol mol}^{-1}$  CO<sub>2</sub> (Fig. 6b).

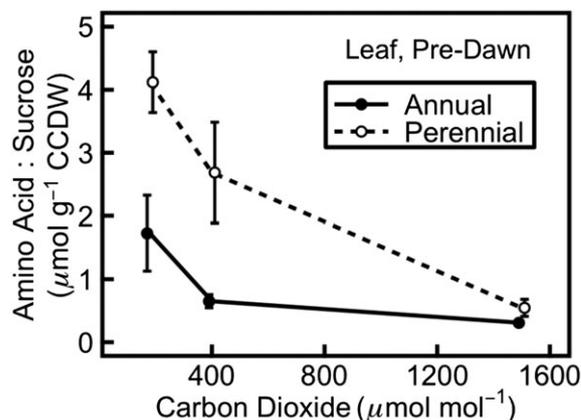


**Figure 6.** Free amino acid concentration is higher in perennial barley (dashed line) than annual barley (solid line). (a) pre-dawn; (b) pre-dusk. Data are expressed in  $\mu\text{mol}$  amino groups per g carbohydrate-corrected dry weight (CCDW). Data show mean  $\pm$  SE (annual  $n = 9$ , perennial  $n = 6$ ).

There was a highly significant organ  $\times$  species  $\times$   $[\text{CO}_2]$  interaction for free amino acid concentration:  $F_{(4, 117)} = 9.3$ ,  $P < 0.001$ . A similar trend for the two species and three  $\text{CO}_2$  levels is seen for free nitrate (data shown in summary form in Fig. 8), and there was also a significant organ  $\times$  species  $\times$   $[\text{CO}_2]$  interaction for these data:  $F_{(4, 116)} = 6.9$ ,  $P < 0.001$ . The perennial also has a higher free amino acid:sucrose ratio than the annual (Fig. 7), indicative of carbon source limitation. This ratio is higher pre-dawn because sucrose accumulates during the day, and the amino acid:sucrose ratio decreases with  $[\text{CO}_2]$ ; for leaves, there is a significant species  $\times$   $[\text{CO}_2]$   $\times$  time of day interaction:  $F_{(1, 70)} = 13$ ,  $P < 0.001$ .

### Metabolite data reveal source limitation in the perennial and sink limitation in the annual

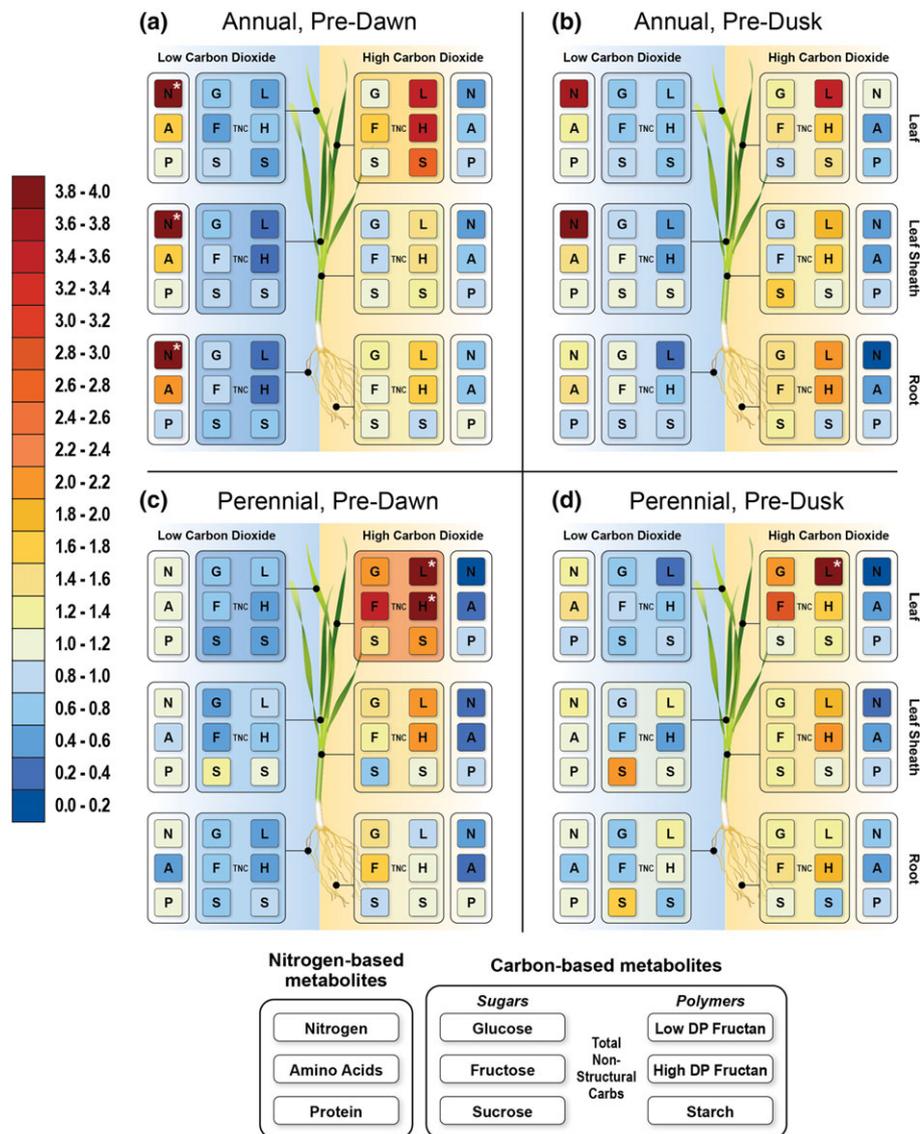
Figure 8 synthesizes the metabolite data, expressed as ratios relative to  $400 \mu\text{mol mol}^{-1}$   $\text{CO}_2$ , in each compartment (leaf, sheath and root), for each species and time of day. In general, the amount of each non-structural carbohydrate was lower at  $180 \mu\text{mol mol}^{-1}$  and higher at  $1500 \mu\text{mol mol}^{-1}$ , compared with  $400 \mu\text{mol mol}^{-1}$   $\text{CO}_2$  (Fig. 8), with short-chain and long-chain fructans representing the major stores for carbon



**Figure 7.** Ratio of free amino acids to free sucrose is higher in perennial barley (dashed line) than annual barley (solid line) at  $180$  and  $400 \mu\text{mol mol}^{-1}$   $\text{CO}_2$  in leaves pre-dawn. This is an indicator of carbon source limitation. Metabolites are expressed in  $\mu\text{mol}$  amino groups and  $\mu\text{mol}$  sucrose per g carbohydrate-corrected dry weight (CCDW), respectively. The ratio is lower at higher  $[\text{CO}_2]$ . Data show mean  $\pm$  SE (annual  $n = 9$ , perennial  $n = 6$ ).

at elevated  $\text{CO}_2$  (Fig. 8). In contrast, free nitrate, free amino acid and protein levels tended to show the opposite trend (especially for the annual, Fig. 8a,b). At  $400 \mu\text{mol mol}^{-1}$ , growth in the annual shows strong evidence of sink limitation, shown by a high rate of photosynthesis (Fig. 2), high TNC accumulation (Fig. 4) and low amino acid concentration and amino acid:sucrose ratio – indicating that sufficient carbon skeletons are available for utilizing available amino acids (Fig. 6 & 7). At  $180 \mu\text{mol mol}^{-1}$   $\text{CO}_2$ , growth becomes more source limited, with lower carbohydrate and higher nitrate and amino acid concentrations compared with  $400 \mu\text{mol mol}^{-1}$  (Figs 4,6 & 8a,b), whilst at  $1500 \mu\text{mol mol}^{-1}$   $\text{CO}_2$ , growth becomes more sink limited, with higher carbohydrate and lower nitrate and lower amino acid concentrations (Figs 4,6 & 8a,b). This trend is seen at both times of day but is most pronounced before dawn (Fig. 8a,b), because carbon skeletons and reductants from photosynthesis are required to incorporate free nitrate into amino acids and to assimilate amino acids into proteins. As a result, the levels of these metabolites decrease during the day as carbohydrates build up. Although this trend is seen in all organ types, it is most pronounced in leaves, where photosynthesis is strongly coupled to changes in carbon and nitrogen metabolism.

In contrast to the annual, at  $400 \mu\text{mol mol}^{-1}$ , the perennial shows strong evidence of source limitation, having a lower rate of photosynthesis than the annual (Fig. 2), low-TNC accumulation (Fig. 4) and high amino acid concentrations and amino acid:sucrose ratio (Figs 6 & 7). At  $180 \mu\text{mol mol}^{-1}$   $\text{CO}_2$ , the perennial remains source limited, so levels of free nitrate and amino acids generally do not increase relative to  $400 \mu\text{mol mol}^{-1}$  (Fig. 8c,d). Just as the perennial shows a greater response of tillering and root allocation (Table 1) and photosynthesis (Fig. 2) than the annual between  $400$  and  $1500 \mu\text{mol mol}^{-1}$   $\text{CO}_2$ , as this alleviates source limitation, it also shows a more dramatic decrease in free amino acids and amino acid:sucrose (Figs 6



**Figure 8.** Carbon-based and nitrogen-based metabolites in annual and perennial barley at pre-dawn and pre-dusk harvests. Data are ratios of metabolite concentrations in leaf, leaf sheath and root of annual and perennial barley at 180 relative to 400  $\mu\text{mol mol}^{-1}$   $\text{CO}_2$ , and 1500 relative to 400  $\mu\text{mol mol}^{-1}$   $\text{CO}_2$ . Blue denotes a decrease and orange/red an increase compared with 400  $\mu\text{mol mol}^{-1}$ , according to the colour scale on the left; a ratio of 1 signifies no change. Low and high DP refer to the degree of polymerization in short-chain and long-chain fructans, respectively. (a) annual pre-dawn; (b) annual pre-dusk; (c) perennial pre-dawn; (d) perennial pre-dusk. Exceptionally high ratios, indicated by asterisks, are as follows: annual pre-dawn (a) at 180  $\mu\text{mol mol}^{-1}$   $\text{CO}_2$ , nitrate in leaf is 6.5 $\times$  concentration at 400  $\mu\text{mol mol}^{-1}$ , nitrate in leaf sheath is 7.7 $\times$  concentration at 400  $\mu\text{mol mol}^{-1}$  and nitrate in root is 5.5 $\times$  concentration at 400  $\mu\text{mol mol}^{-1}$ ; perennial pre-dawn (c), in leaf at 1500  $\mu\text{mol mol}^{-1}$   $\text{CO}_2$ , low-DP fructan is 23.4 $\times$  concentration at 400  $\mu\text{mol mol}^{-1}$  and high DP fructan is 6.3 $\times$  concentration at 400  $\mu\text{mol mol}^{-1}$ ; perennial pre-dusk (d), in leaf at 1500  $\mu\text{mol mol}^{-1}$   $\text{CO}_2$ , low-DP fructan is 4.8 $\times$  concentration at 400  $\mu\text{mol mol}^{-1}$ .

& 7) as it is better able than the annual to pair additional sugars from photosynthesis with existing free amino acids to bring about a growth response. However, at 1500  $\mu\text{mol mol}^{-1}$ , growth in the perennial transitions to become sink limited and the plants have a high carbohydrate content, and low nitrate and low amino acid concentrations (Figs 4,6 & 8a,b). Thus, the treatments imposed are sufficiently strong that even the annual becomes more source limited at low  $[\text{CO}_2]$ , and even the perennial becomes more sink limited at elevated  $[\text{CO}_2]$ .

## DISCUSSION

### Developmental plasticity in the perennial enables extra $\text{CO}_2$ to be utilized in growth, suggesting source limitation

Increasing  $[\text{CO}_2]$  increases the availability of photosynthetic substrate and suppresses photorespiration (Farquhar *et al.* 1980). This increases the potential rate of carbon uptake into the plant, increasing source strength, alleviating source limitation and increasing the source:sink ratio. Conversely,

decreasing  $[\text{CO}_2]$  has the opposite effects. The stronger photosynthetic, tillering and root partitioning responses of perennial than annual barley to increasing  $[\text{CO}_2]$  (Table 1; Fig. 2) suggest that the source is more limiting for growth than the sink in this species during the vegetative stage. This response is not seen to such a great extent in the annual, suggesting that its growth is primarily sink limited and constrained by developmental potential; as a consequence, the annual is operating at near-maximum source activity under current ambient conditions ( $400 \mu\text{mol mol}^{-1} \text{CO}_2$ ). The ratio of photosynthesis to growth is higher in annual barley (Fig. 3), a further indication of sink limitation, and increases at higher  $[\text{CO}_2]$  and as plants become older and leave the exponential phase of growth. Furthermore, the developmental plasticity seen in the perennial, via its ability to increase tillering and root partitioning in response to greater carbon source strength, suggests that it is better able than the domesticated annual crop to adapt to fluctuating environmental conditions. In general, selective breeding of crops has resulted in plants with fewer tillers because, although additional non-flowering tillers provide a selective advantage through competition in wild plants, they reduce the yield of crop stands by diverting resources away from flowering tillers. To an extent, domesticated barley has retained its tillering capacity (Doust 2007; Sang 2009). However, under experimental conditions, the perennial barley was far readier to increase tillering in response to increased  $[\text{CO}_2]$  than the annual crop.

Altering the root:shoot ratio enables plants to increase access to the most limiting resources by adjusting allocation to nitrogen-acquiring or carbon-acquiring tissues (Stitt & Krapp 1999; Freschet *et al.* 2015). Under elevated  $[\text{CO}_2]$ , nitrogen becomes more limiting for growth, making an increase in root:shoot ratio advantageous. The perennial was better able to make this plastic adjustment to growth (Table 1). However, its greater relative increase in allocation to roots (Table 1) would have also tended to offset its growth response, because roots are heterotrophic and root respiration represents a significant carbon sink. This greater allocation to a respiratory carbon sink may explain why the perennial still showed a similar increase in RGR to the annual at higher  $\text{CO}_2$  levels (Fig. 1). In combination, these results suggest that the combined response of sink strength (growth and respiration) to  $[\text{CO}_2]$  was stronger in the perennial than annual. Increasing root allocation enabled the perennial to take up more nitrogen, further increasing its ability to match carbon skeletons with amino acids for growth.

Our findings suggest a more opportunistic growth strategy in the perennial than the annual, whereby the use of additional resources is maximized via partitioning into more branches above ground and roots below ground. In contrast, the annual appears to be highly constrained in its ability to develop larger sinks at  $400 \mu\text{mol mol}^{-1} \text{CO}_2$  (Table 1; Fig. 2), and unable to increase these to the same extent as the perennial. It thus seems that the strategy of the annual is for maximal growth under current  $[\text{CO}_2]$  – and as a result it is sink limited. The annual has been subjected to intense selective breeding that has maximized growth under current ambient  $\text{CO}_2$  conditions but suppressed its developmental plasticity, and growth during the vegetative phase is largely unresponsive to increased  $[\text{CO}_2]$ .

### The annual accumulates carbohydrates whilst having low amino acids, suggesting carbon sink limitation

The metabolite data reinforce the pattern of source limitation in the perennial and sink limitation in the annual seen in the growth and photosynthesis data. The annual has higher TNC concentration, and lower amino acid concentration and amino acid:sucrose ratio than the perennial, indicating an excess of carbon that cannot be invested in growth (Figs 4,6,7 & 8). Although many studies into the relationship between amino acid accumulation and carbon source limitation have focused on a single species (Paul & Driscoll 1997; Isopp *et al.* 2000), the use of the amino acid:sucrose ratio, which is a more robust measurement, confirms the trend seen for free amino acids and is one of several lines of evidence pointing towards greater carbon source limitation in the perennial. The lower basal level of TNC in the perennial (Fig. 4) suggests that this species is highly efficient at utilizing the carbon acquired each day – by developing new sinks or enlarging existing ones, seen in the strong tillering response to elevated  $[\text{CO}_2]$  (Table 1), or by increasing TNC storage in the leaf sheath (Fig. 4). Developing new sinks such as tillers increases sink size, whilst increasing storage in existing sink organs increases sink activity; both enable the plant to up-regulate its sink capacity (Geiger & Shieh 1993; White *et al.* 2016). The high rate of tillering and root allocation in the perennial translates to a higher sink capacity and high demand for photosynthate which could explain the high accumulation of carbohydrates in these organs. As a consequence, the large quantity of leaf carbohydrates accumulated during the day is likely to be exported to developing tillers or other sinks, in addition to the carbon sink of maintenance respiration at night; in future work, the use of isotopically labelled  $\text{CO}_2$  in a series of staged harvests could enable diurnal carbon utilization to be tracked (e.g. Ferrieri *et al.* 2013).

Both species are carbon sink limited at elevated  $[\text{CO}_2]$ ; leaf sucrose is a key driver of phloem loading for photosynthate export (Ainsworth & Bush 2011), yet the increase in TNC at elevated  $[\text{CO}_2]$  seen here is primarily driven by increases in storage carbohydrates (fructans and starch, Fig. 8) and not transport carbohydrates (sucrose). This provides evidence that the carbohydrate accumulation at elevated  $[\text{CO}_2]$  arises from sink limitation rather than reflecting the increased phloem loading of recent photosynthate. Indeed, the increased accumulation of carbohydrates will feed back on phloem transport throughout the plant and phloem loading in the leaf (Ainsworth & Bush 2011), and high foliar TNC concentration is thus an indicator of sink limitation in both species. The fact that TNC does not accumulate in roots of the perennial under elevated  $\text{CO}_2$  suggests that carbon transport may be more limiting in this species.

It is interesting to note that the negative correlation between starch and biomass observed in a range of accessions of *Arabidopsis* (Sulpice *et al.* 2009) is not borne out by the data of this study – rather, the fast-growing annual species has a higher rate of carbohydrate accumulation despite having greater biomass. However, the physiology and metabolism of *Arabidopsis* do not always map onto those of crop plants

(White *et al.* 2016), for example, the relationship between protein and starch found by Sulpice *et al.* (2009) was uncoupled in these data. Growth in plants with different life forms and life histories may be subject to different constraints; in slow-growing *Arabidopsis* accessions, growth is slow because it is sink limited, whereas in perennial barley, growth is slower than the annual because it is source limited and therefore uncorrelated with carbohydrate content.

### Ecological strategies and intrinsic limits to growth

The typical growth strategy of wild annual plants can be caricatured as 'live fast, die young', leading to the expectation of a growth strategy that is primarily source limited during the lifetime of the plant, and that enables the annual to maximize the use of available CO<sub>2</sub> for growth. We therefore expected the annual to be less sink limited than the perennial during vegetative growth, especially because it is adapted for fertilized soils. In contrast, we expected the perennial to have a more conservative growth habit, 'live slow, live long', which limits photosynthesis and growth but is opportunistic, being better adapted for the possibility of low nutrients in a variable environment yet able to capitalize on rising [CO<sub>2</sub>] by increasing storage when substrates are available. Although plants are typically sink limited during the vegetative stage and transition to source limitation at reproduction (Arp 1991), many crops are co-limited by sinks and sources during grain filling (Álvarez *et al.* 2008; Acreche & Slafer 2009; Peterhansel & Offermann 2012; Slewinski 2012). We anticipated that during the vegetative stage, the 'live slow' perennial would be more sink limited than the 'live fast' annual (Jaikumar *et al.* 2014). The results confounded these expectations.

The perennial adopts more of a 'live fast' strategy than anticipated; perennials generally store carbon for future use (Atkinson *et al.* 2012), yet here, the perennial showed a dramatic increase in growth under elevated [CO<sub>2</sub>] rather than an increase in storage, indicating source limitation. Coming from a fluctuating natural environment, and being able to grow in a variety of habitats including roadsides, ditches and rich grassy meadows and at varying altitudes (von Bothmer 1996), this species has the plasticity to maximize growth when CO<sub>2</sub> is abundant. However, a perennial confined to unproductive habitats might be expected to display slower growth and greater sink limitation.

Although the perennial displays a greater response to [CO<sub>2</sub>] for photosynthetic rate per unit leaf area and leaf TNC concentration, even the maximal values at 1500 μmol mol<sup>-1</sup> CO<sub>2</sub> never exceed those of the annual, implying intrinsic physiological or developmental limits are common to both species. The annual is unable to utilize more photosynthate than it acquires at 400 μmol mol<sup>-1</sup> CO<sub>2</sub> by increasing partitioning to tillers and roots; the perennial has greater developmental flexibility and is able to utilize the additional photosynthate acquired at the highest CO<sub>2</sub> concentration but never exceeds the maximum rates of growth and photosynthesis seen in the annual (Figs 1 & 2).

The developmental plasticity of the annual species appears to have been altered through selective breeding such that it cannot adapt to live faster when conditions allow, and the results of this study show that it is sink limited during vegetative growth even under elevated [CO<sub>2</sub>], in addition to being sink limited during reproduction (Schnyder 1993; Bingham *et al.* 2007; Serrago *et al.* 2013). It thus seems that the sink strength of barley will limit yield of this important crop in the current global context of rising atmospheric [CO<sub>2</sub>], and a concerted effort to increase sink strength would be a vital part of breeding programmes in order to increase yield.

### CONCLUSIONS

Contrary to expectations, these results indicate that annual barley is more sink limited, and perennial barley is more source limited during the vegetative growth stage. Our findings show that annual barley germplasm is optimized for growth at current [CO<sub>2</sub>] and that future elevated [CO<sub>2</sub>] may be unlikely to facilitate yield increases in this species; the lack of developmental plasticity in the annual means that new sinks are not readily initiated, which could result in a critical lack of flexibility for developing additional grain sinks and thus increasing yield under elevated [CO<sub>2</sub>]. The holistic approach taken here enables a broad view of the source–sink balance to be taken, encompassing measurements of resource acquisition, storage, allocation to growth and plant development, in a model system of congeneric species. In order to draw firm conclusions of agricultural relevance, it will be vital to extend such research: including nitrogen as well as carbon source–sink manipulations, following source–sink processes throughout crop development to their impact on yield; investigating these processes in a wider range of cereal varieties and wild species; and carrying out agronomically relevant experiments in the field.

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## SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

**Figure S1:** Log mass against log area, for the additional subset of annual and perennial barley plants harvested throughout the duration of the study in order to establish a calibration between mass and area for the plants in the main study.  $r^2 = 0.97$ , meaning that differences in species and plant area account for 97% of variation in plant mass. Lines show linear regression of area and mass. Log mass = 1.53 log area – 13.11 (annual); log mass = 1.06 log area – 8.37 (perennial).

**Figure S2:** Growth curves for all individuals in the main study used to obtain relative growth rate. Log predicted mass, obtained using mass-area calibration from additional plants (shown in Fig. S1), is plotted against plant age. Blue lines show the overall mean using fixed effects from model; pink lines show growth curves for individual plants. Each plant has a unique ID: A denotes annual individuals and PM and PE denote the two accessions of perennial individuals; numbers were assigned to individuals at random.