CARBON SOURCE-SINK LIMITATIONS DIFFER BETWEEN TWO SPECIES WITH CONTRASTING GROWTH STRATEGIES

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CARBON SOURCE-SINK LIMITATIONS DIFFER BETWEEN TWO SPECIES WITH CONTRASTING GROWTH STRATEGIES

Running title:

Source-sink limitations vary with growth strategy

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ABSTRACT

Understanding how carbon source and sink strengths limit plant growth is a critical knowledge gap that hinders efforts to maximise 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\(_2\) concentrations ([CO\(_2\)]). 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\(_2\)] indicating that source strength was near maximal at current [CO\(_2\)]. In contrast, photosynthetic rate and sink development responded strongly to elevated [CO\(_2\)] 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\(_2\)], and lower non-structural carbohydrate accumulation. Alleviating sink limitation during vegetative development could be important for maximising growth of elite cereals under future elevated [CO\(_2\)].
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; von Caemmerer et al., 2012; Reynolds 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-characterised molecular crosstalk mechanisms (Smith and Stitt, 2007; Lawlor and Paul, 2014; White et al., 2016).

Decades of research into the effects of elevated CO$_2$ 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 and 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 and 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 – i.e. 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 or sink limitation. Experimental manipulations of the source:sink ratio provide insights into the relative contributions of source and sink 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 and Farquhar, 1984; Bryant et al., 1998; Rogers et
al., 1998; Eyles et al., 2013); inhibiting resource export from the source (Ainsworth and Bush, 2011); and increasing source activity using elevated CO$_2$ (Kinsman et al., 1997; Masle, 2000), reviewed by White et al. (2016). Here, we alter the atmospheric CO$_2$ concentration ([CO$_2$]) to non-invasively manipulate the source:sink ratio in barley – elevated [CO$_2$] to increase the source strength and sub-ambient [CO$_2$] to decrease it – with current [CO$_2$] as a reference against which to compare the source manipulations. This approach enables analysis of source and sink limitation under current [CO$_2$], and strong CO$_2$ treatments are applied in order to produce marked perturbations of the system. In C$_3$ plants, [CO$_2$] affects carbon source strength directly through one well-understood process i.e. 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 and Driscoll, 1997; Stitt and 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 and Ainsworth, 2006; Ainsworth and 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$_2$ 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, and 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$_2$] than the perennial (Poorter, 1993; Roumet and Roy, 1996). This is because the elevated [CO$_2$] 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$_2$].
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-litre 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\textsubscript{2} using soda lime. Plants were grown in three chambers with fixed CO\textsubscript{2} levels of 180 µmol mol\textsuperscript{-1}, 400 µmol mol\textsuperscript{-1} and 1500 µmol mol\textsuperscript{-1} for 61 days. 180 and 1500 µmol mol\textsuperscript{-1} were chosen in order to impose strong carbon source and sink manipulations. All chambers had a 12-hour photoperiod with day/night temperatures of 20/18°C, 65% humidity, and daytime light levels of 600 µmol photons m\textsuperscript{-2} s\textsuperscript{-1} at plant height resulting in a daily light integral of 25.92 mols m\textsuperscript{-2} day\textsuperscript{-1}. Plants were kept adequately watered with 20% Long Ashton’s nutrient solution. During seedling establishment, plants were watered daily – with 150ml Reverse Osmosis water for 8 days and with 150ml Long Ashton’s solution thereafter. After 17 days, plants were watered three times per week with 150ml Long Ashton’s solution until 29 days old, 225ml until 45 days old, and 450ml 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\textsubscript{2} level were harvested (three at dawn
and three at dusk), giving a total of 54 individuals across three weeks. In the first and 
third of these harvest weeks, six perennials from each CO2 level were harvested 
(three at dawn and three at dusk), giving a total of 36 individuals.

Relative growth rate, root:shoot ratio and tillering

RGR was calculated based on the plant mass estimated from weekly imaging of 
above ground biomass, beginning when plants were two weeks old. Plants were 
photographed (PowerShot G9, Canon, Tokyo, Japan) six times, from the side against 
a white background, with the plant rotated 60 degrees 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 mm2 
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 (Fig. S1; r² = 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 timepoints by differentiation, where more than three timepoints 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. 51 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 hours between 30 minutes after dawn and 30 minutes 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 above. 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 – i.e. the carbon source activity. This value was multiplied by the projected shoot area to estimate the total daily photosynthesis in the whole shoot – i.e. the carbon source strength.
**Metabolites**

Plants were harvested from 47 to 61 DAG, within one hour before dawn and one hour before dusk. Samples were flash frozen in liquid nitrogen, stored at -80°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 randomised 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, low degree of polymerisation (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). 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 P³ Precision Pipetting Platform, Perkin Elmer, Waltham, MA, USA).
The pellets from the ethanol extraction were heated to 95°C in 0.1M NaOH, to solubilise protein. A commercially available protein assay kit (Pierce BCA protein assay kit, Thermoscientific, 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 neutralised with HCl.

For the starch and high degree of polymerisation (HDP) fructan assay, starch and HDP fructans from 40 µl aliquots of the suspended pellet material were digested using enzymes in 60 µl 0.05M acetate buffer as follows. Starch: 0.17U well⁻¹ amylloglucosidase (EC 3.2.1.3) and 0.1U well⁻¹ α-amylase (EC 3.2.1.1); starch and HDP fructan: 0.1U well⁻¹ exo-inulinase (EC 3.2.1.80), 0.1U well⁻¹ endo-inulinase (EC 3.2.1.7), 0.17U well⁻¹ amylloglucosidase and 0.1U well⁻¹ α-amylase. Plates were incubated overnight at 37°C. 40 µl of the supernatant from the overnight digest was transferred to each well of a 96-well microplate. 262µg ATP well⁻¹, 349µg NADP well⁻¹ and 3.6U well⁻¹ glucose-6-phosphate dehydrogenase (EC 1.1.1.49, grade II) were added in a buffer of 0.1M HEPES/KOH, 3mM MgCl₂, ph7.0 to initiate the reaction. Microplates were centrifuged for 1 minute to remove bubbles then inserted into a plate reader and the NADPH associated with the carbohydrates in the sample was measured at A₃₄₀ (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 starch assay values for starch from the starch and fructan
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. 15μl 0.1M sodium borate buffer, 90μl fluorescamine and 100μl water were combined with 2μl of ethanol extract in a black 96-well microplate (Nunc MicroWell, Thermo Fisher Scientific, Waltham, MA, USA). Following a 5 minute dark incubation, fluorescence (360nm excitation, 460nm emission, 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. First, 0.005U well\(^{-1}\) nitrate reductase (EC 1.7.1.3) and 50 nmol NADPH in 0.11M potassium phosphate buffer were added to a 10μl aliquot of the ethanol extract. Each microplate was shaken. Following a 30 minute incubation (dark, room temperature), 20μl 0.25mM phenazine methosulfate was added to each well. Plates were shaken again and incubated for a further 20 minutes. 45μl 1% w/v sulfanilamide in 5% phosphoric acid followed by 45μl 0.02% N(1-Naphthyl)ethylendiamine dihydrochloride was added to each well. Following another shake and a 5 minute incubation, \(A_{540}\) 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 (Fernie et al., 2011).

**Statistical Methods**

Analysis was performed using R (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 [CO₂] than the annual species

Relative growth rate (RGR; the efficiency of whole plant dry mass increase obtained from calibration of shoot area, and measured in g g⁻¹ day⁻¹) 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 CO₂ levels (Fig. 1). Stepwise model selection was used to choose fixed effects, and the effects of species and [CO₂] 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 [CO₂] and species. In both species, the increase in RGR was greater between 180 and 400 µmol mol⁻¹ CO₂ than between 400 and 1500 µmol mol⁻¹ CO₂. Peak RGR in the annual increased by 17.1% between 180 and 400 µmol mol⁻¹ CO₂, but only 5.0% between 400 and 1500 µmol mol⁻¹ CO₂. Peak RGR in the perennial increased by 20.5% between 180 and 400 µmol mol⁻¹ CO₂, but only 6.0% between 400 and 1500 µmol mol⁻¹ CO₂. However the difference between annual and perennial remained relatively consistent: peak RGR in the annual was 21.1%, 17.7% and 16.7% higher than in the perennial, at 180, 400 and 1500 µmol mol⁻¹ CO₂ 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 µmol mol\(^{-1}\) CO\(_2\), whereas the number of
tillers in the annual increased by just 15% across the same CO\(_2\) range (Table 1). This
highly significant species x [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 [CO\(_2\)] in perennial than
annual barley. In the annual, the root:shoot ratio increased by only 2.8% between
180 and 400 µmol mol\(^{-1}\) CO\(_2\), whilst in the perennial it increased 11.6% between 180
and 400 µmol mol\(^{-1}\), and 4.2% between 400 and 1500 µmol mol\(^{-1}\) (Table 1). This
highly significant species x [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 [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 [CO\(_2\)] (Fig.
2B). In the annual plants, the daily photosynthetic rate increased by 87% between
180 and 400 µmol mol\(^{-1}\) CO\(_2\), but only by 13% between 400 and 1500 µmol mol\(^{-1}\). In
contrast, in the perennial it increased 58% between 180 and 400 µmol mol\(^{-1}\), but 75%
between 400 and 1500 µmol mol\(^{-1}\). This led to a significant species x [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, i.e. carbon source
strength (Fig. 2C) is greater than the difference in the rate per unit area, i.e. carbon
source activity (Fig. 2B). In the annual, the whole shoot daily photosynthetic rate
increased by 177% between 180 and 400 µmol mol$^{-1}$ CO$_2$, but only 25% between 400 and 1500 µmol mol$^{-1}$. In contrast, in the perennial it increased 528% between 180 and 400 µmol mol$^{-1}$, and 123% between 400 and 1500 µmol mol$^{-1}$. There was a highly significant species x [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 g C g$^{-1}$ 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 g C plant$^{-1}$ day$^{-1}$ (Fig. 3B), these clusters are no longer present, and a positive correlation between photosynthesis and growth is seen. The ratio increases with [CO$_2$] (Fig. 3C) and with plant age at harvest (Fig. 3D). There was a highly significant interaction of the harvest week x [CO$_2$] ($F_{(4,61)} = 16$, p<0.001), such that the photosynthesis:growth ratio is greater at higher [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 utilised 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 [CO$_2$] was increased from 180 to 1500 µmol mol$^{-1}$ (Fig. 4). However, in the leaves, perennial barley showed a stronger TNC response than the annual when [CO$_2$] was increased from 180 to 1500 µmol mol$^{-1}$ (Fig. 4). Across all TNC data, there was a significant organ type x time of day x species interaction ($F_{(2,120)} = 9.2, p<0.001$); a significant organ type x time of day x [CO$_2$] interaction ($F_{(4,120)} = 18, p<0.001$); a significant organ x species x [CO$_2$] interaction ($F_{(4,120)} = 22, p<0.001$); and a significant organ x harvest week x [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 µmol mol$^{-1}$ CO$_2$, 57% greater in the annual at 400 µmol mol$^{-1}$ CO$_2$, but approximately equal at 1500 µmol mol$^{-1}$ CO$_2$ (Fig. 4A). In the leaf sheath, TNC was 29% greater in the annual than the perennial at 180 µmol mol$^{-1}$ CO$_2$, 57% greater in the annual at 400 µmol mol$^{-1}$ CO$_2$, and 25% greater in the annual at 1500 µmol mol$^{-1}$ CO$_2$ (Fig. 4B). In the root, TNC was 35% greater in the annual than the perennial at 180 µmol mol$^{-1}$ CO$_2$, 56% greater in the annual at 400 µmol mol$^{-1}$ CO$_2$, and 97% greater in the annual at 1500 µmol mol$^{-1}$ CO$_2$ (Fig. 4C). In both species, TNC concentration is highest at 1500 µmol mol$^{-1}$ CO$_2$ 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 for respiration, export or growth at night. These differentials are much greater in the leaf than in leaf sheath or root (Fig. 5), since 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 µmol mol\(^{-1}\) CO\(_2\), yet there is little difference in TNC differentials in leaf sheath and root, across the CO\(_2\) 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 µmol mol\(^{-1}\) CO\(_2\) (Fig. 5).

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

Free amino acids are an indicator of source limitation (Paul and Driscoll, 1997; Stitt and 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, since 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 µmol mol\(^{-1}\) CO\(_2\), which implies a carbon source limitation, and decreases as [CO\(_2\)] increases (Fig. 6). Before dawn, amino acid concentration is 41% greater in the perennial than the annual at 180 µmol mol\(^{-1}\) CO\(_2\), 127% greater in the perennial at 400 µmol mol\(^{-1}\) CO\(_2\), and 12% greater in the perennial at 1500 µmol mol\(^{-1}\) CO\(_2\) (Fig. 6A). Before dusk, amino acid concentration is 67% greater in the perennial than the annual at 180 µmol mol\(^{-1}\) CO\(_2\), 47% greater in the perennial at 400 µmol mol\(^{-1}\) CO\(_2\), and 64% greater in the perennial at 1500 µmol mol\(^{-1}\) CO\(_2\) (Fig. 6B).

There was a highly significant organ x species x [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 CO\(_2\) levels is seen for free nitrate (data shown in summary form in Fig. 8) and there
was also a significant organ x species x $[\text{CO}_2]$ 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 since sucrose accumulates during the day, and decreases with $[\text{CO}_2]$; for leaves, there is a significant species x $[\text{CO}_2]$ x 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 synthesises the metabolite data, expressed as ratios relative to 400 $\mu$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 $\mu$mol mol$^{-1}$ and higher at 1500 $\mu$mol mol$^{-1}$, compared to 400 $\mu$mol mol$^{-1}$ $\text{CO}_2$ (Fig. 8), with short- and long-chain fructans representing the major stores for carbon 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 $\mu$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 utilising available amino acids (Fig. 6, 7). At 180 $\mu$mol mol$^{-1}$ $\text{CO}_2$, growth becomes more source limited, with lower carbohydrate and higher nitrate and amino acid concentrations compared to 400 $\mu$mol mol$^{-1}$ (Figs. 4, 6, 8A,B), whilst at 1500 $\mu$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.
(Figs. 8A,B), since 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 µmol mol$^{-1}$ CO$_2$ 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 µmol mol$^{-1}$ CO$_2$, the perennial remains source limited, so levels of free nitrate and amino acids generally do not increase relative to 400 µmol mol$^{-1}$ (Fig. 8C,D). Just as the perennial shows a greater response of tillering and root allocation (Table 1) and photosynthesis (Fig. 2B) than the annual between 400 and 1500 µmol mol$^{-1}$ CO$_2$, as this alleviates source limitation, it also shows a more dramatic decrease in free amino acids and amino acid:sucrose (Figs. 6, 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 µmol 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 [CO$_2$], and even the perennial becomes more sink limited at elevated [CO$_2$].
DISCUSSION

Developmental plasticity in the perennial enables extra CO\textsubscript{2} to be utilised in growth, suggesting source limitation

Increasing [CO\textsubscript{2}] increases the availability of photosynthetic substrate and suppresses photorespiration (Farquhar \textit{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 [CO\textsubscript{2}] has the opposite effects. The stronger photosynthetic, tillering and root partitioning responses of perennial than annual barley to increasing [CO\textsubscript{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 µmol mol\textsuperscript{-1} CO\textsubscript{2}). The ratio of photosynthesis to growth is higher in annual barley (Fig. 3), a further indication of sink limitation, and increases at higher [CO\textsubscript{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 [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- or carbon-acquiring tissues (Stitt and Krapp, 1999; Freschet et al., 2015). Under elevated [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, since 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 CO$_2$ levels (Fig. 1). In combination, these results suggest that the combined response of sink strength (growth and respiration) to [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 annual, whereby the use of additional resources is maximised 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 µmol mol$^{-1}$ 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 maximised 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 and 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 utilising 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 upregulate its sink capacity (Geiger and 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 are 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 isotopic CO$_2$ in a series of staged harvests could enable diurnal carbon utilisation to be tracked (e.g. Ferrieri et al., 2013).

Both species are carbon sink limited at elevated [CO$_2$]; leaf sucrose is a key driver of phloem loading for photosynthate export (Ainsworth and Bush, 2011), yet the increase in TNC at elevated [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 [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 and 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 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 maximise the use of available CO$_2$ for growth. We therefore expected the annual to be less sink limited than the perennial during vegetative growth, especially since it is adapted for fertilised 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 capitalise on rising [CO$_2$] 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 (Álvaro *et al.*, 2008; Acreche and Slafer, 2009; Peterhansel and 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$_2$] 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 maximise growth when CO$_2$ 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$_2$] for photosynthetic rate per unit leaf area and leaf TNC concentration, even the maximal values at 1500 µmol mol$^{-1}$ CO$_2$ never exceed those of the annual, implying intrinsic physiological or developmental limits that are common to both species. The annual is unable to utilise more photosynthate than it acquires at 400 µmol mol$^{-1}$ CO$_2$ by increasing partitioning to tillers and roots; the perennial has greater developmental flexibility and is able to utilise the additional photosynthate acquired at the highest CO$_2$ 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 [CO₂], 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 [CO₂], 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 optimised for growth at current [CO₂] and that future elevated [CO₂] 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 [CO₂]. The holistic approach taken here enables a broad view of 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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FIGURE LEGENDS & TABLE

Figure 1. Relative growth rate is higher in annual (solid line) than perennial (dashed line) barley, and greater at higher [CO$_2$]. Relative growth rate (RGR) is daily gain in dry mass relative to whole plant dry mass, g g$^{-1}$ day$^{-1}$. A, elevated [CO$_2$]: 1500 µmol mol$^{-1}$; B, current [CO$_2$]: 400 µmol mol$^{-1}$; C, sub-ambient [CO$_2$]: 180 µmol mol$^{-1}$.

Figure 2. Perennial barley (dashed line) has a more pronounced photosynthetic response to elevated [CO$_2$] than annual barley (solid line). A, diurnal timecourse of net leaf photosynthesis in annuals and perennials grown at 400 µmol mol$^{-1}$ 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 ± SE (A: annual n=18, perennial n=9; B: at 180, 400, 1500 µmol mol$^{-1}$ CO$_2$, annual n=15, 18, 18, perennial n=5, 9, 12; C: at 180, 400, 1500 µmol mol$^{-1}$ CO$_2$, annual n=15, 18, 18, perennial n=4, 9, 12).

Figure 3. The ratio of photosynthesis to growth is higher in annual than perennial barley and greater at higher [CO$_2$] and in older plants. A, source activity vs sink activity, plotted as photosynthesis and growth for individual plants at all times and CO$_2$ levels, expressed in g C g$^{-1}$ day$^{-1}$, showing three clusters along the x-axis corresponding to the three harvest times with RGR decreasing as time progresses;
B, source strength vs sink strength, plotted as photosynthesis and growth for individual plants at all times and CO₂ levels expressed in g C plant⁻¹ day⁻¹; C, photosynthesis: growth ratio in the three [CO₂] treatments; D, changes in the photosynthesis: growth ratio with respect to the mean plant age at harvest. Data show mean ± SE (C and D: at 180, 400, 1500 µmol mol⁻¹ CO₂, annual n=15, 18, 18, perennial n=4, 9, 12).

**Figure 4.** Pre-dawn concentrations of total non-structural carbohydrates (TNC) are higher in the annual (solid line) than perennial (dashed line) barley. A, leaf; B, leaf sheath; C, root. The overall CO₂ response is greater for perennials in the leaf, but greater for annuals in the leaf sheath and root. Data are expressed in µmol glucose equivalents per g carbohydrate-corrected dry weight (CCDW). Data show mean ± SE (annual n=9, perennial n=6).

**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 µmol 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 Table S1).

**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
µmol amino groups per g carbohydrate-corrected dry weight (CCDW). Data show mean ± SE (annual n=9, perennial n=6).

**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 µmol mol\(^{-1}\) CO\(_2\) in leaves pre-dawn. This is an indicator of carbon source limitation. Metabolites are expressed in µmol amino groups and µmol sucrose per g carbohydrate-corrected dry weight (CCDW), respectively. The ratio is lower at higher [CO\(_2\)]. Data show mean ± SE (annual n=9, perennial n=6).

**Figure 8.** Carbon- 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 µmol mol\(^{-1}\) CO\(_2\), and 1500 relative to 400 µmol mol\(^{-1}\) CO\(_2\). Blue denotes a decrease and orange/red an increase compared to 400 µmol 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 polymerisation in short- 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 µmol mol\(^{-1}\) CO\(_2\), nitrate in leaf is 6.5x concentration at 400 µmol mol\(^{-1}\), nitrate in leaf sheath is 7.7x concentration at 400 µmol mol\(^{-1}\) and nitrate in root is 5.5x concentration at 400 µmol mol\(^{-1}\); perennial pre-dawn (C), in leaf at 1500 µmol mol\(^{-1}\) CO\(_2\), low DP fructan is 23.4x concentration at 400 µmol mol\(^{-1}\) and high DP fructan is 6.3x concentration at 400 µmol mol\(^{-1}\); perennial pre-dusk (D), in leaf at 1500 µmol mol\(^{-1}\) CO\(_2\), low DP fructan is 4.8x concentration at 400 µmol mol\(^{-1}\).
Table 1. Responses of tillering and root:shoot ratio to increasing [CO₂]. Annual barley shows very limited tillering and root:shoot ratio responses to increasing CO₂ 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 and their associated standard errors (S.E.) are reported to three significant figures (annual n=18, perennial n=12).

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Figure 8. Carbon- 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 µmol mol⁻¹ CO₂, and 1500 relative to 400 µmol mol⁻¹ CO₂. Blue denotes a decrease and orange/red an increase compared to 400 µmol 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 polymerisation in short- 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 µmol mol⁻¹ CO₂, nitrate in leaf is 6.5x concentration at 400 µmol mol⁻¹, nitrate in leaf sheath is 7.7x concentration at 400 µmol mol⁻¹ and nitrate in root is 5.5x concentration at 400 µmol mol⁻¹; perennial pre-dawn (C), in leaf at 1500 µmol mol⁻¹ CO₂, low DP fructan is 23.4x concentration at 400 µmol mol⁻¹ and high DP fructan is 6.3x concentration at 400 µmol mol⁻¹; perennial pre-dusk (D), in leaf at 1500 µmol mol⁻¹ CO₂, low DP fructan is 4.8x concentration at 400 µmol mol⁻¹.
Patterns of metabolite accumulation in annual and perennial barley grown at sub-ambient and elevated carbon dioxide.

203x241mm (300 x 300 DPI)
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.