Plant Traits and Production Rates from CO2 Starvation to Saturation

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Chapter 4

Increases in CO_2 from Past Low to Future High Levels Result in 'Slower' Strategies on the Leaf Economic Spectrum

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Abstract

Depending on resource availability plants exhibit a specific suite of traits. At the interspecific level these traits follow the leaf economic spectrum (LES), traits related to slow turnover when resources are poor and fast turnover when resources are plentiful. Limited data shows that within species, CO_2 availability, low in the recent geologic past, high in the near future, has led to plants shifting their trait levels on the LES towards faster traits. We asked whether adjustments of physiological traits could underpin faster growth from low to high CO_2 and how these responses varied among plant types. We analysed the trait response of seedlings of up to 28 C_3 plant species grown at low (160 ppm), near-ambient (450 ppm), and high (750 ppm) CO_2. We measured growth, specific leaf area, leaf gas exchange, chemical composition and stomatal traits. On average photosynthesis was reduced by 59% at low CO_2 and increased by 14% at high CO_2 compared to ambient CO_2. Respiration decreased by 21% at low CO_2 and increased by 39% at high CO_2. Nitrogen content per mass increased by 50% at low CO_2 and decreased by 9% at high CO_2. Plants drastically increased SLA at low CO_2 so that despite lower carbon gain per area, carbon gain per unit mass was not reduced as much. Contrary to the responses to other resources, plant traits along the LES are adjusted towards the "fast" end of the spectrum (high SLA, high nitrogen content and higher photosynthesis per unit mass) at low CO_2 and towards the "slow" end with increasing CO_2. From a resource economics perspective plants thus adjust the cost for growth towards the availability of carbon and the rate of assimilation: lower respiration at low CO_2 and a lower carbon cost to grow a unit leaf area. At higher CO_2 the carbon costs increase due to increased respiration and higher leaf mass per area. This suggests that CO_2 increases from the past to the future are allowing plant species globally to combine faster growth with more robust, resource conservative leaves.

Introduction

When plant growth is limited by the availability of one resource, economic theory dictates that in successful individuals the capacity to acquire other, the acquisition of more plentiful resources should be limited; this is because it does not pay to have excess capacity in one area when resources in another cannot keep up (Bloom & Mooney 1985, Chapin *et al.* 1987). Thus, the Leaf Economic Spectrum (LES) predicts that habitats of poor resource availability host plant species that exhibit a concerted predictable suite of traits that can be linked to slow matter cycling (slow leaf turnover, low nutrient content, thick tough leaves of low specific leaf area (SLA), slow growth), while habitats where resources are plentiful host species linked to fast cycling (high leaf turnover, high nutrient content, thin "throw away" leaves of high SLA, fast growth) (Lambers & Poorter 1992, Wright *et al.* 2004, Reich 2014, Niinemets 2015).

The availability of carbon, as an important growth-limiting resource, has greatly increased from a Pleistocene low (~180ppm CO_2) (Hönisch *et al.* 2009) to current levels (~400ppm) and will increase even more moving to high levels (possibly even >800ppm) towards the end of this century (IPCC 2014). From a carbon centric point of view growth rate of plants is dependent on plant morphology related to carbon uptake and plant physiology related to carbon processing (Evans 1972, Lambers & Poorter 1992). Interestingly, studies have

shown that, within species, plant traits are strongly adjusted by carbon availability (Gerhart & Ward 2010, Temme *et al.* 2013) and that how the direction of these adjustments relates to variation in growth rate is partly opposite to what would be expected based on interspecific variation in trait-growth relations (Temme *et al.* 2015).

In a multispecies study on plant responses to a range from low to high CO_2 we confirmed that among species, at ambient CO_2 conditions, high SLA is linked with fast growth (Temme *et al.* 2015), as in previous studies (Lambers & Poorter 1992, Cornelissen, Castro Diez & Hunt 1996, Reich 2014). However, contrary to what one would expect within species, low (past) carbon availability resulted in a "faster" morphology (high SLA) compared to that at ambient CO_2 but was at the same time associated with slower growth. Correspondingly, higher (future) carbon availability resulted in a "slower" morphology (lower SLA) but also faster growth than at ambient CO_2. Together, these responses go so far as to result in a decoupling of growth rate response from morphology response from low to high CO_2 (Temme *et al.* 2015). However, how CO_2 from past low to future high affects physiological traits (gas exchange traits, stomatal traits, respiration, and chemical composition) and how leaf morphological and those physiological traits along the LES combine to affect growth performance of plants at a range of low to high CO_2 has remained an open question so far (Medlyn & McMurtrie 2005, Smith *et al.* 2012, Sack *et al.* 2013).

With current global change featuring rising CO_2 and increasing temperatures, most research in this field has focused on plants' response to high CO_2 (Gerhart & Ward 2010, Temme *et al.* 2013). Besides the effects of high CO_2 also the effects of low CO_2, as common for the past 10 Ma before the Industrial Revolution, should be considered in order to obtain a full picture of plants' responsiveness to CO_2. Plant physiology is strongly adjusted by CO_2 concentrations from transient to evolutionary time-scales (Medlyn & McMurtrie 2005) with potential legacy effects of evolution in a low CO_2 atmosphere (Sage & Coleman 2001, Zhu *et al.* 2004). Experiments have shown that plant trait responses to low CO_2 are far greater in magnitude than those to high CO_2 (Hattenschwiler & Korner 2000, Quirk *et al.* 2013, Temme *et al.* 2013, 2015). However, unlike for other resources we found that, among plant species, there does not appear to be a trade-off in traits relating to carbon acquisition from Pleistocene low to future high CO_2 concentration, i.e. no species appears particularly suited to either low or high CO_2 (Temme *et al.* 2015). This is likely caused by carbon availability in open vegetation varying only little in space and only slowly and gradually in time until the Industrial Revolution (Hönisch *et al.* 2009). Carbon dioxide can be a selective agent on plant functioning, as indicated for instance by the repeated evolutionary appearance of C_4 plants with carbon-concentrating mechanisms for photosynthesis (Edwards *et al.* 2010, Christin & Osborne 2014) as well as by the dominance of C_3 plants in the Holocene(Huang *et al.* 2006). However, we do not know whether the very rapid increase in CO_2 concentration since pre-industrial low levels and into the near future could show a similar trait CO_2 responsiveness to those in the Holocene (Sage & Coleman 2001, Franks *et al.* 2013).

Experiments manipulating atmospheric CO_2 levels have shown a consistent adjustment in physiological traits within species. Low CO_2 strongly reduces photosynthetic rates and increases stomatal conductance and transpiration (Gerhart & Ward 2010, Temme *et al.* 2013) whereas high CO_2 increases photosynthetic rate and decreases stomatal conductance and transpiration to a far lesser extent (Ainsworth & Rogers 2007, Norby & Zak 2011). At low

and high CO_2, relative to ambient CO_2, plants' leaf area ratio (leaf area per plant mass, LAR) is strongly adjusted, following the response of one of its components, SLA (Temme *et al.* 2013, 2015). Plants drastically increase LAR at low CO_2 and decrease it at high CO_2. Under unchanged unit leaf rate (plant biomass production rate per unit leaf area, ULR) this would be expected to lead to faster growth at low CO_2 and slower growth at high CO_2, as relative growth rate (RGR) is the product of LAR and ULR (Lambers & Poorter 1992). However, in reality quite the opposite happens because ULR, which is related to photosynthetic rates, is itself also affected by CO_2 availability (Poorter & Navas 2003). Thus CO_2 starvation leads to reduced growth (Temme *et al.* 2015) and excess CO_2 enhances growth (Poorter & Navas 2003, Ainsworth & Rogers 2007, Norby & Zak 2011). This would imply stronger shifts in ULR in response to CO_2 that are only partially compensated for by altered leaf morphology and/or allocation (SLA, LAR).

Gas exchange and chemical composition are the key traits underlying biomass production per unit leaf rate (Evans & Poorter 2001, Poorter, Lambers & Evans 2014). Important in plants' physiological response to CO_2 are the stomata as the entry point of CO_2 into the leaf. Over past geological cycles of shifts in CO_2, stomata have been relatively small and numerous during periods of low CO_2 (promoting high maximum stomatal conductance) and large but low in density during periods of high CO_2 (low maximum stomatal conductance) (Woodward 1987, Franks & Beerling 2009, de Boer *et al.* 2012). A higher stomatal conductance allows for greater carbon uptake at low CO_2 levels whereas at high CO_2 a developmentally lower maximum stomatal conductance allows for greater fine-tuning of stomatal conductance (Drake, Froend & Franks 2013).

Besides by gas exchange leaf chemical composition is strongly altered by CO_2, with greater nitrogen content at low CO_2 (Temme *et al.* 2013). This may reflect greater investment into RuBisCO in order to facilitate the drawdown of CO_2 in photosynthesis via a stronger diffusion gradient. On the other hand the increase of nitrogen at low CO_2 might also be due to luxury consumption of nitrogen by small carbon-starved plants (Chapin 1980). At higher CO_2 nitrogen content is reduced. This reduction could be due to increased carbohydrate accumulation and/or redistribution of nitrogen towards other growth limiting steps (Díaz *et al.* 1993). Alternatively, closure of stomata to reduce transpiration at higher CO_2 limits the water flux through the roots, thereby possibly reducing N uptake (ref). A comprehensive understanding of the linkages between gas exchange, stomatal traits, chemical composition and growth performance across the range from low to high CO_2 is still missing however, especially because empirical data on plant responses to low CO_2 are still scarce (Gerhart & Ward 2010, Franks *et al.* 2013, Temme *et al.* 2013).

How the concerted trait shifts in morphology and physiology of individual species in response to CO_2 levels did and will affect community composition requires broader generalizations. Scaling up individual species' responses to ecosystems is generally done by classifying species by functional type, including growth form strategy (Chapin *et al.* 1996). Whether and how different plant types, notably grasses, forbs and woody species, respond consistently to CO_2 will provide a basis for (back-)predicting potential shifts in community composition from the low CO_2 past to the high CO_2 near future (Prentice & Harrison 2009). Importantly in this context, dynamic global vegetation models that are coupled to global circulation models to predict biogeochemical cycling and climate at different CO_2 regimes,

use similar functional types and traits (Sitch *et al.* 2008, Verheijen *et al.* 2015a, b). In relation to carbon availability different plant functional types show different responses in traits to low CO_2 (Temme *et al.* 2013, 2015) versus high CO_2 (Poorter & Navas 2003, Ainsworth & Rogers 2007, Norby & Zak 2011) but, again, information on physiological responses to low CO_2 is still minimal and based on a very small number of species (Temme *et al.* 2013).

With humanity increasingly altering the carbon cycle at an unprecedented rate through fossil fuel emissions and land use change, understanding plants' response to future conditions becomes increasingly important. Given plants' >10Ma year evolution in a low CO_2 atmosphere, understanding how plants function at low CO_2 could shed light on how plants will respond to future high CO_2 environments. We therefore asked ourselves the following questions:

1. How are leaf physiological traits related to plant carbon uptake affected by CO_2 from past low to future high levels and are there consistent differences in the response among plant types?

2. How do the changes in leaf traits in response to low versus high CO_2 serve to underpin the changes in plant physiology in order to maintain sufficient levels of carbon uptake?

3. As there is an apparent decoupling of plant morphology from growth rate across the range from low to high CO_2, is there a similar decoupling for plant physiological traits or does a greater investment in photosynthetic machinery still lead to higher growth rates regardless of CO_2?

We sought to answer these questions through an experimental screening of seedlings of up to 28 temperate C_3 plant species, ranging broadly in phylogeny and functional type, for their responses to low and high CO_2. This paper presents the physiological response of 28 plant species and its effect on plant growth, complementary to a previous study on the effect of CO_2 on plant morphology and its effect on growth (Temme *et al.* 2015).

Methods

Plants of up to 28 different species (**Appendix Table 3.1**), which consisted of 6 woody species (2 trees, 4 shrubs), 16 forb species, and 6 grasses were grown at 160 ppm (low: range 150-180 ppm), 450 ppm (near-ambient) and 750 ppm (high) CO_2 at 70% relative humidity and a 19-22°C day-night with a 10 hr photoperiod at ~350umol PAR. Seeds of these species were germinated and grown as described in Temme *et al.* 2015. Seeds were germinated on wet paper and transplanted to experimental conditions shortly after germination. Total daily photon flux was comparable to that of an average March day in the Netherlands, which is when several of the species would have naturally germinated and started to grow (Temme *et al.* 2015).

Growth measurements were carried out as described in Temme *et al.* 2015. In brief, a representative subsample of individuals was harvested for biomass measurement after the expansion of the first leaf. Three weeks later the remaining individuals were harvested and plant biomass and RGR determined according to Hoffman & Poorter (Hoffmann &

Poorter 2002). Replication varied between 4 and 10 (mean 7). SLA was determined from a representative leaf of each plant at final harvest. To determine nitrogen and carbon content all leaves were separated from stems after harvest and oven-dried and ground in a ball mill (Retch MM200, Hahn, Germany). Three to four µg of leaf powder was then analysed for C&N content using a dry combustion element analyser (Carlo Erba NA1500, Rodana, Italy).

Photosynthesis was measured within the last 2 days prior to final harvest and from at least one hour after "daylight". One fully developed leaf per individual (4 individuals per species/treatment) was placed in a LiCor 6400 infrared gas analyser and allowed to acclimate to the cuvette, set similar to growth conditions, for two minutes in the light after which three measurements were taken and averaged to take into account any error introduced by the analyser. Subsequently the red-blue light source (LI-6400-02B) was turned off and the leaf was allowed to acclimate for three minutes in the dark after which three measurements were taken again to measure respiration. The area inside the leaf cuvette was marked and removed at final harvest. When leaves did not fill the total cuvette area (6 cm2) the portion of the leaf that could be placed inside the cuvette was scanned using a Cannon LiDe 110 scanner. Leaf area was then measured using ImageJ v1.47. Net area-based photosynthesis (Anet) and respiration rates (R) and stomatal conductance (gs) were then calculated by using the correct area in the gas analyser equations.

On a subset of 8 species stomatal sizes and densities were measured on a nail polish imprint taken from a single typical full-grown leaf per individual (4 individuals per species per CO_2 treatment) prior to harvest. Clear nail polish was applied directly on the leaf and allowed to dry for 45 minutes. After drying nail polish films were removed by sticking on clear tape and peeling from the leaf. Nail polish films of abaxial and adaxial leaf sides were mounted on a glass slide and an image was taken using CellSens Entry v1.7.1 via an Olympus SC_30 camera attached to an Olympus CX41 microscope at 10x optical magnification. Per leaf side up to five (fewer when quality of nail polish film was low, generally not so) 640µm by 320µm (204800 µm²) viewing frames were saved at a resolution of 0.1024µm² per pixel. Numbers of epidermal cells and stomata were subsequently counted using Adobe Photoshop CS5.5 and ImageJ v1.47 using the CellCounter plugin. Occasionally poor image quality prevented counting of the full frame and a smaller area of good quality was counted. Per leaf side 15 stomata were measured for stomatal pore size, guard cell length and guard cell width. This was measured live using CellSens Entry v1.7.1 at a resolution of 80nm per pixel length (0.0064µm² pixel^{-1}) at 40x optical magnification. Stomatal size traits (pore size, guardcell width/length) as well as densities of all frames were averaged per individual plant. Using stomatal density, maximum pore aperture and guard cell width as a proxy for pore depth we calculated maximum stomatal conductance (Parlange & Waggoner 1970, Dow, Bergmann & Berry 2014).

Statistics were carried out using R version 3.1.2 running in RStudio version 0.98. As in Temme *et al.* (2015), CO_2 effects on traits and species differences in traits were tested by comparing the shift in trait value to the trait value at ambient CO_2. To improve normality and minimise skew, trait values were natural log (ln) transformed prior to analysis. The difference in ln-transformed trait level from ln-transformed ambient level was then the relative shift in trait level via $e^{ln\ transformed\ difference}$-1. This approach had the added benefit that a halving

or a doubling in trait value from ambient had the same ln-transformed difference. Per species we averaged the trait response of individual replicates per treatment. These shifts in species trait values at low or high CO_2 (compared to ambient CO_2) were then tested by one-sample t-tests. Differences between plant types were determined by two-sample t-tests on species trait shift with Bonferroni correction for the three comparisons made (forb-grass, forb-woody, grass-woody). We used standard major axis regression in R using the package SMATR v3.4 to test the relationship between shifts in SLA and shifts in photosynthesis and respiration per unit mass. In addition we also tested if the interspecific relationship between relative growth rate and nitrogen content changed with CO_2 concentration.

Results

The plants generally responded strongly to the CO_2 treatments with all species having lower photosynthetic rates per area at low CO_2 and higher at high CO_2 (when compared to ambient CO_2), combined with strong adjustments in nitrogen content per unit mass and photosynthetic nitrogen use efficiency. Leaf size occasionally precluded measurements of gas exchange when leaves did not fit the LiCor 6400 cuvette. Nevertheless we could measure gas exchange for 17 species and nitrogen content for the full species set (see **Appendix Table 3.1** for details)

Physiological traits and CO$_2$

CO_2 concentration strongly influenced photosynthesis and respiration rates. Plant photosynthesis per area at growth conditions (A_{net}, $\mu mol \cdot m^{-2} \cdot s^{-1}$) was strongly affected by CO_2 (**Fig. 4.1a**) with species at low CO_2 on average having a 59%±4 (17 species, p<0.001) lower A_{net} compared to that at ambient CO_2. Plant types did not respond significantly differently (possibly due to small sample size). Per type the reduction for forb species was 64%±6 (9 species, p<0.001) followed by grasses (58%±7; 5 species, p<0.01) and woody species 45%±10; 3 species, p<0.1). At high CO_2 A_{net} was stimulated far less than it was reduced at low CO_2 with species on average having increased A_{net} by 14%±3 (16 species, p<0.001). Again different plant types did not respond differently. Small sample sizes and large spread of CO_2 effect among species likely contributed to non-significance for the stimulation trend for grasses and woody species. Respiration per unit leaf area (r_{net}, $\mu mol \cdot m^{-2} \cdot s^{-1}$) followed a similar pattern to A_{net} (**Fig. 4.1b**), although the reduction at low CO_2 was less strong (21%±7; 15 species, p<0.05). However, per plant type, possibly due to large spread and small sample size, no significant results were found. At high CO_2 r_{net} was increased by 39%±13 (15 species, p<0.01). Again, likely due to small sample sizes and large spread we found only a significant effect for forb r_{net} by 45%±20 (9 species, p<0.05).

Surprisingly stomatal conductance (g_s, $mmol \cdot m^{-2} \cdot s^{-1}$) was not affected by low compared to ambient CO_2 (**Fig. 4.1c**). Across species there was a varied response with some species having higher g_s and others with lower g (**Appendix 3.1**)$_s$ resulting in average g_s remaining the same across species. At high CO_2 g_s was significantly lower across all species, (20%±8; 16 species, p<0.05). The combined effect of CO_2 on A_{net} and g_s resulted in a shift in intrinsic water use efficiency, A_{net} over g_s (iWUE, $\mu mol\ CO_2 \cdot mmol\ H_2O^{-1} \cdot s^{-1}$), which at low CO_2 closely followed the effect on A_{net} at low CO_2 but exceeded the effect on A_{net} at high CO_2
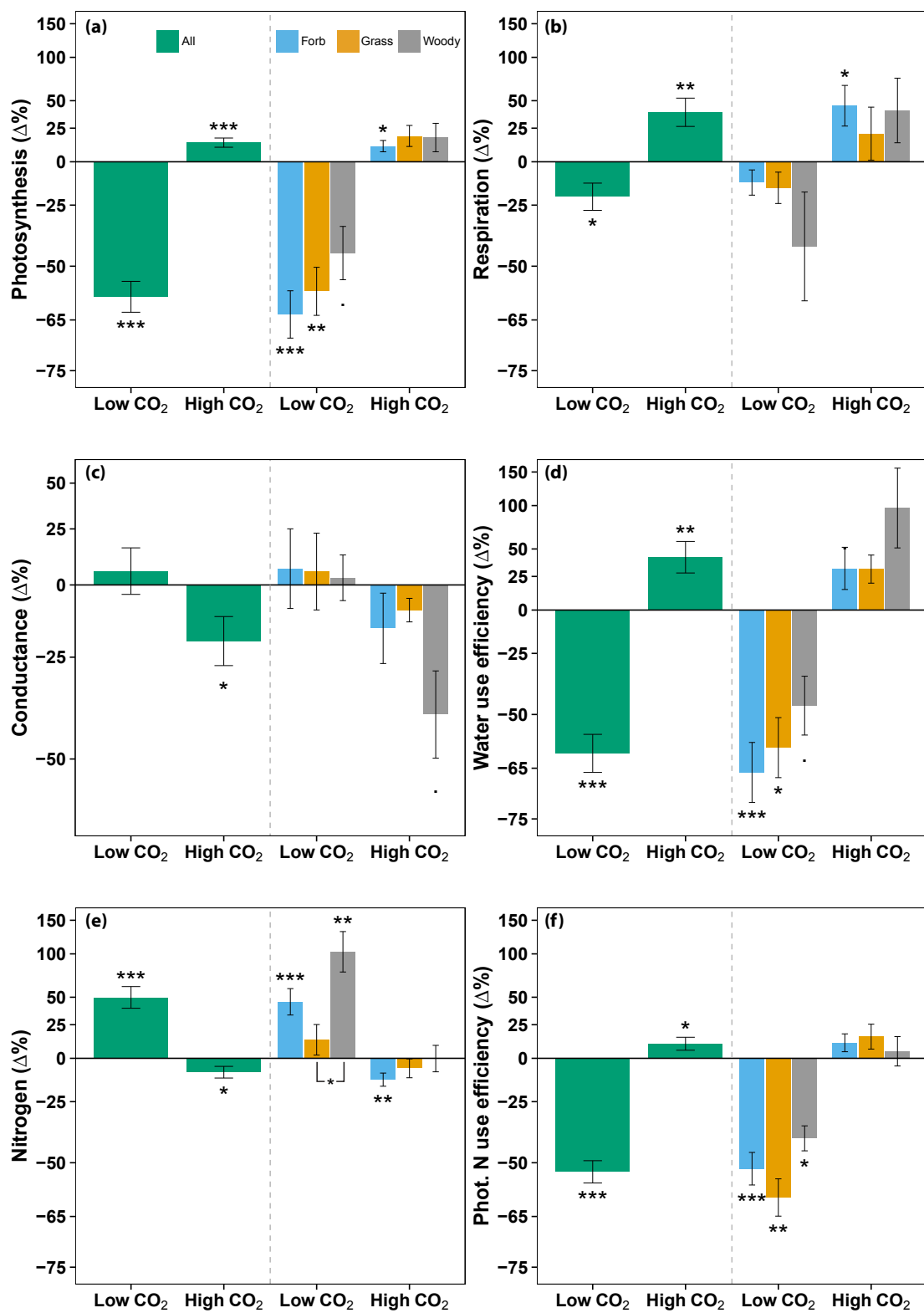
(left) Figure 4.1. Relative shifts in trait values at low or high CO_2 compared to ambient CO_2 for forb, grass and woody species. Bars indicate percentage shift in trait value at low CO_2 (160 ppm) and high (750 ppm) CO_2 compared to trait value at ambient (450 ppm) CO_2. Axes are natural log transformed so that the size of the bars at a 50% decrease or a 100% increase is the same (reflecting a factor 2 adjustment). Green bars: all species, blue bars: forb species, orange bars: grass species, grey bars: woody species. Error bars give standard error of the mean. *s near error bars indicate t-test significance of difference from zero. *s opposite bars indicate significance of 2-sample t-test, with Bonferroni correction, between linked types. *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$. **(a)** Photosynthetic rate at growth conditions (Anet, $\mu mol \cdot m^{-2} \cdot s^{-1}$). **(b)** Respiration at growth conditions (rnet, , $\mu mol \cdot m^{-2} \cdot s^{-1}$). **(c)** Stomatal conductance (gs, $mmol \cdot m^{-2} \cdot s^{-1}$), note the different axis scaling. **(d)** Intrinsic water use efficiency, Anet/gs (iWUE, $mmol\ H_2O \cdot \mu mol\ CO_2^{-1}$). **(e)** Nitrogen content, (N_g, g N·g leaf^{-1}). **(f)** Photosynthetic nitrogen use efficiency (PNUE, $\mu mol\ CO_2 \cdot \mu mol\ N^{-1} \cdot s^{-1}$).

(**Fig. 4.1d**). iWUE was reduced at low CO_2 by 61%±5 (17 species, $p<0.001$) across species and increased by 42%±15 (16 species, $p<0.01$) at high CO_2. This suggests increased water demand proportional to decreased photosynthesis at low CO_2 but a greater than proportional decrease in water demand at high CO_2 due to the combined effect of CO_2 increasing A_{net} and decreasing g_s.

Plant leaf nitrogen content per unit mass (N_g, g N·g leaf^{-1}) increased sharply at low (as compared to ambient) CO_2 (**Fig. 4.1e**) with species having on average a 50%±11 (25 species, $p<0.001$) higher N_g. This response was different among plant types with grasses showing no significant increase and woody plants showing a 103%±27 (6 species, $p<0.01$) increase, although with large spread among species. At high CO_2 species on average had a 9%±4 reduction in N_g (20 species, $p<0.05$) though only forbs showed a significantly different N_g from that at ambient CO_2 with a reduction of 13% (12 species, $p<0.01$). Photosynthetic nitrogen use efficiency (PNUE, $\mu mol\ CO_2 \cdot \mu mol\ N^{-1} \cdot s^{-1}$) followed the shifts in A_{net} and N_g with CO_2 (**Fig 4.1f**) and was reduced by 53%±3 (17 species, $p<0.001$) at low CO_2 and increased by 10%±5 (10 species, $p<0.05$) at high CO_2.

Stomatal traits were surprisingly unresponsive to CO_2. In a subset of 8 C_3 forbs from the total species set we found that species showed a clear scaling relation from a limited number of large stomata to a large number of small stomata (**Fig. 4.2a**). However, the effect of CO_2 on this scaling relation was very limited. Guard cell size was largely unresponsive to CO_2 with only minor variation with growth CO_2 and no consistent direction across species (**Fig. 4.2b**). Stomatal density of both leaf sides taken together (only *Geranium* and *Clinopodium* were hypostomatous) was also largely unresponsive to CO_2 (**Fig. 4.2c**). However, there was a slight trend of lower stomatal densities at low CO_2. When viewed separately the abaxial leaf side stomatal density was significantly lower (19%±7; 8 species, $p<0.05$) at low CO_2 across all 8 species. Changes in epidermal cell density (mm^{-1}), pore size, guard cell width (μm) and maximum stomatal conductance calculated from stomatal density and stomatal pore traits varied inconsistently with CO_2 across species (**Appendix 3.3**)**.** At low CO_2 however there was a correlation between maximum stomatal conductance and actual measured stomatal conductance (**Appendix 3.4**) suggesting pores were more constrained.
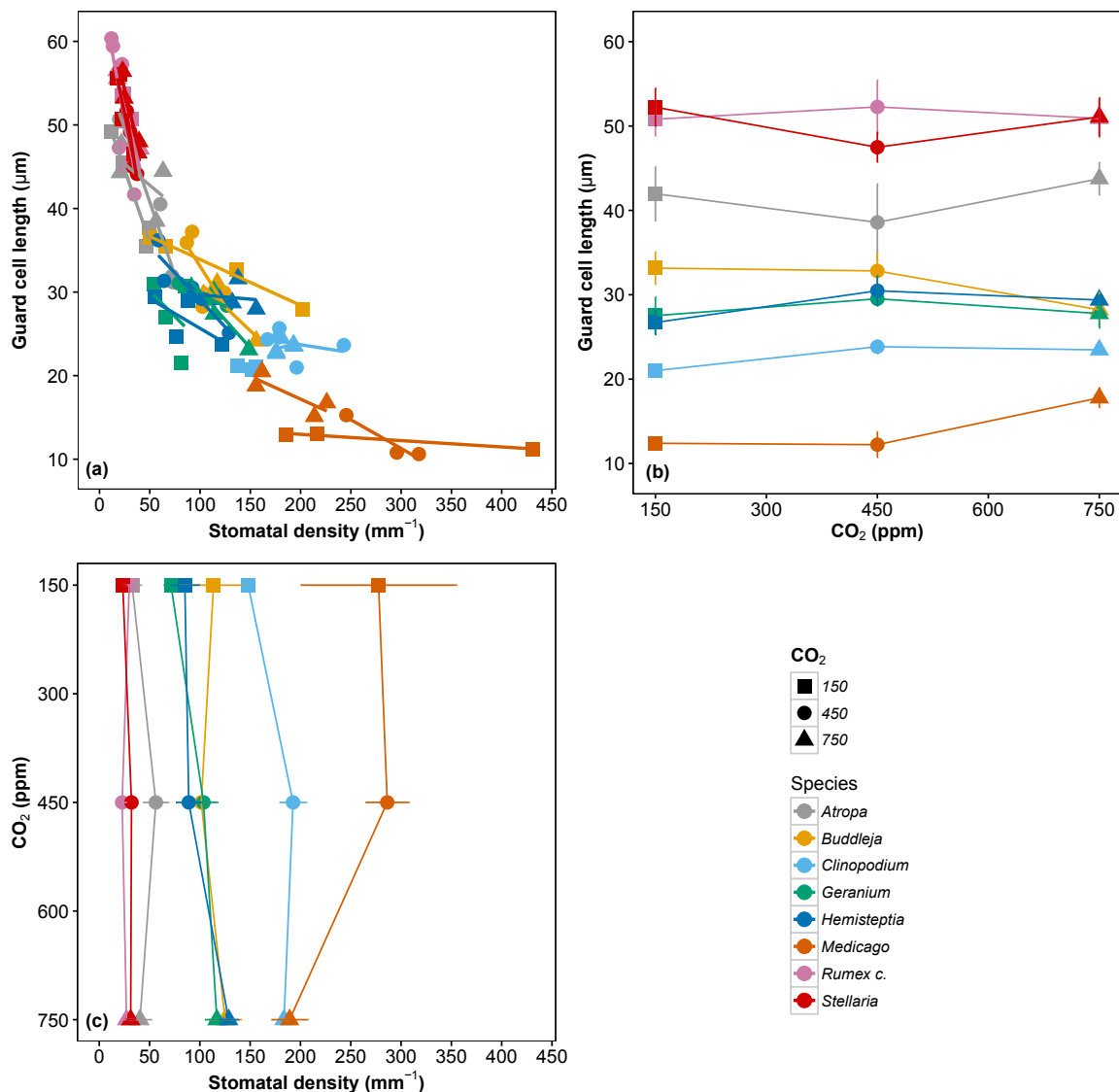
Figure 4.2. Effect of CO_2 on the relationship between stomatal guard cell size and stomatal density. **(a)** Average stomatal density (mm^{-1}) of adaxial and abaxial leaf sides and length of abaxial stomatal guard cells (μm) in relation to growth at low (160ppm) ambient (450ppm) and high (750ppm) CO_2 of 8 C_3 forbs. Each point represents the average density of 10 counting frames and 30 stomata of a single leaf (5/15 abaxial and adaxial) of an individual. Colours represent different species with symbols representing CO_2 treatment. Squares: Low CO_2, circles: ambient CO_2, triangles: High CO_2. **(b)** Species average (n=3-4) guard cell length at low, ambient and high CO_2. **(c)** Species average (n=3-4) stomatal density at low, ambient and high CO_2.
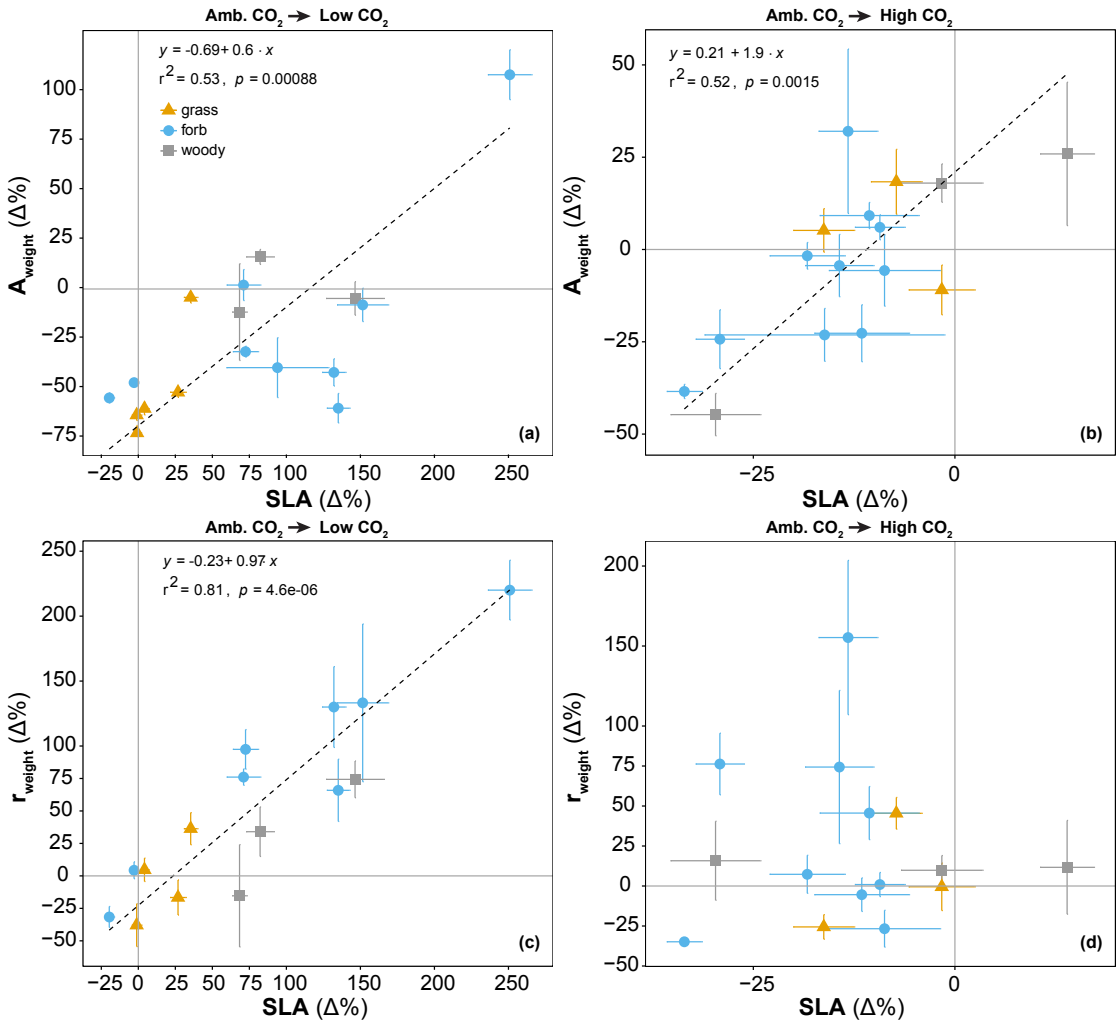
Figure 4.3. Shifts in mass-based photosynthesis and respiration versus shifts in SLA. Left panels indicate shifts between ambient (450 ppm) CO_2 and low (150-180ppm) CO_2, right panels shifts between ambient and high CO_2. Each point represents a species with bars denoting SE, orange circles: grasses, blue circles: forbs, grey squares: woody species. Dashed line is the standard major axis regression of shift in specific leaf area (SLA, m^2 leaf · g leaf^{-1}) versus photosynthetic rate and dark respiration per leaf mass ($\mu mol·g^{-1}·s^{-1}$). Dark grey lines indicate 0% change in trait. (SLA data from Temme et al. 2015)

Morphological traits complement physiology

Standard major axis regression revealed how species shifts in SLA influenced their photosynthetic carbon gain and respiratory carbon loss per unit mass (**Fig. 4.3**). At low CO_2 plant photosynthetic rate per area was reduced (**Fig. 4.1a**). However, many plant species concurrently drastically increased their SLA. With a greater area capable of carbon uptake per unit mass invested in leaf tissue photosynthetic rate per unit mass (A_{mass} $\mu mol·g^{-1}·s^{-1}$) was reduced by 38%±8 (17 species, p<0.01), i.e. less than per area photosynthesis, which

across all species was reduced by 59%±4 (**Fig. 4.1a**). Furthermore there was a clear relationship between the extent of SLA increase and the reduction in A_{mass} (**Fig. 4.3a**). Species that had a greater increase in their SLA had a smaller reduction in A_{mass} (r^2=0.54, p<0.001) even going so far as to have a higher A_{mass} than at ambient for the forb *Atropa belladonna*. While plants increased their SLA at low CO_2, thus incorporating less mass per area, the thinning of the leaf did not appear to come at the expense of energy demanding leaf tissue as there was an almost proportional increase in dark respiration (r^2=0.81, p<0.001) with increasing
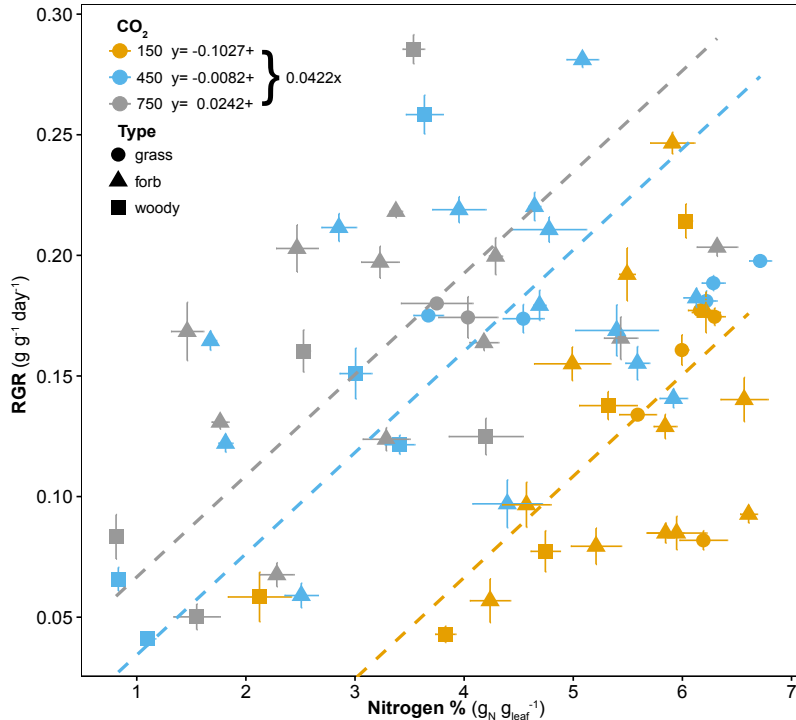

Figure 4.4. Relationship between leaf nitrogen content (g N· g leaf[-1]) and relative growth rate (RGR) at past low (160 ppm), current ambient (450 ppm) and future high (750 ppm) CO_2. Points indicate species mean RGR and N content with SE with different symbols noting different types and different colours for the different CO_2 treatments; circles: forb species, triangles: grass species, squares: woody species. Lines represent the standard major axis regression of nitrogen % and RGR, which showed no significant difference in slope but did show significant difference in elevation.

SLA (**Fig. 4.3c**). Energy demanding leaf tissue appeared to have remained stable whereas less demanding tissue was reduced as evidenced by the large increase in SLA and only slight decrease in respiration per area (**Fig. 4.1b**).

At high CO_2 plant photosynthesis per area was increased (**Fig. 4.1a**). At the same time however SLA was decreased for most species resulting in no net change in A_{mass} across all species at high CO_2, though there was strong variation between species. The relationship between shifts in SLA and A_{mass} at high CO_2 was such that species that had a greater reduction in SLA (thicker or denser leaves) also had a greater reduction in A_{mass} (r^2=0.52, p<0.01). However, across species increased carbon availability appears to offset the relationship in

such a manner that plants can afford to decrease their SLA while maintaining a similar A_{mass} as at ambient CO_2 (**Fig. 4.3b**). Interestingly the decrease in SLA at higher CO_2 appears to not be the result of increased energy demanding tissue as there is no clear relationship between decreases in SLA and respiration per unit mass (**Fig. 4.3d**).

Physiological traits and growth

Nitrogen content per unit mass remained positively coupled to RGR at all three CO_2 levels (**Fig. 4.4**). When analysed together using SMA regression we found no significant difference in the slope of the relationship between nitrogen content (N_g, g_n g_{leaf}^{-1}) and relative growth rate (RGR, g g^{-1} day^{-1}) with different growth CO_2. Rather the intercept of the relationship was significantly altered by CO_2 ($p<0.001$). Pairwise comparison among CO_2 treatments showed that the elevation at ambient and high CO_2 were not significantly different but that the elevation at low CO_2 was significantly lower than at ambient and high CO_2 ($p<0.001$). Thus regardless of CO_2 concentration species interspecific variation in growth rate was related to variation in nitrogen content. CO_2 starvation offset this relationship across species reducing growth rate at all nitrogen levels and requiring a greater investment in nitrogen to maintain growth rates.

Discussion

In this study we sought to understand how plants' physiological traits are affected by CO_2 availability going from being carbon starved at a level representative of the Pleistocene past to plentiful carbon (750 ppm) representative of the end of the present century. In a previous study we found that plants' morphological traits were greatly affected by CO_2 but that this variation was decoupled from interspecific differences in growth rate (Temme *et al.* 2015). In contrast, we found in this study that interspecific variation in growth rate at past, present and future CO_2 can be explained satisfactorily by responses of plant physiological traits. The great adjustment we found in morphological traits such as specific leaf area serves to offset changes in photosynthesis due to carbon availability by reducing the carbon cost for growth at low CO_2 while increased photosynthesis at higher CO_2 allows for more robust (lower SLA) leaves at high CO_2. Our multispecies findings, together with previous studies on small species numbers, demonstrate that, going from past low CO_2 to future high CO_2 plants *generally* reduce both their specific leaf area and their leaf nitrogen content. This strongly suggests that increasing CO_2 has allowed plant species to grow faster while at the same time adopting a more resource conservative ("slow") strategy on the leaf economic spectrum, as expressed by lower nitrogen content, higher SLA and lower photosynthesis per unit mass.

Plant physiological traits and CO_2

Plant physiological traits were strongly affected by CO_2. Across 17 species we found that low CO_2 decreased photosynthesis and respiration rates but stomatal conductance remained surprisingly unchanged. High CO_2 increased photosynthesis and respiration rates and, contrasting to low CO_2, stomatal conductance was reduced, albeit modestly. Intrinsic water use efficiency thus followed the response of photosynthesis and respiration being lower at low

CO_2 and higher at high CO_2. Combined with results from previous experiments showing that specific leaf area is greatly increased at low CO_2 (i.e. thinner or less dense leaves) and reduced at high CO_2 (thicker or denser leaves) (Temme *et al.* 2015), the results for respiration and iWUE provide an interesting picture. At low CO_2 the carbon cost for growth is reduced (lower respiration, more leaf area per unit mass) next to a greater water cost (lower iWUE). In high CO_2 photosynthesis the carbon cost for growth is increased (greater respiration, less leaf area unit mass) yet the water cost is reduced (higher iWUE). Plant nitrogen content per mass was greatly increased at low CO_2 and reduced at high CO_2. In combination with changes in photosynthesis this resulted in photosynthetic nitrogen use efficiency moving in tandem with CO_2 availability. It should be noted however that nitrogen content per area was not affected by CO_2 (**Appendix 3.4**).

Interestingly and contrary to expectations from our meta-analysis (Temme *et al.* 2013), we found no change in stomatal conductance (g_s) from ambient to low CO_2. Also at the short time scale of our experiment, i.e. within a single generation, species showed no plasticity in stomatal size and density. As the species measured for stomatal traits were relatively fast growing forbs, it is more likely they would respond plastically by regulating stomatal aperture than developmentally by changing stomatal frequency to CO_2 (Haworth, Elliott-Kingston & McElwain 2013). Only in variable environments (e.g. drought-prone), where fine control of stomata is necessary, might there be stronger selection on stomatal size and densities resulting in lower numbers of bigger stomata (Raven 2014).

The striking lack of responsiveness of stomata to our CO_2 treatments points to limitations in generalizing our data to past conditions due to differences in adaptive (i.e. evolutionary) versus plastic response to CO_2. As we grew plants from seed and harvested them in the seedling stage, only plastic responses to CO_2 were measured. We did not allow our plant species the time to adjust evolutionarily to CO_2 over multiple generations. Generally there is a clear relationship between CO_2 and number and size of stomata (Woodward 1987, Royer 2001). Already over a comparatively short 150 year period a decreasing number of stomata were found across most species in a Florida bog (Lammertsma *et al.* 2011). Contrastingly conifer needles from packrat middens gathered during the last glacial maximum showed no strong increase in stomatal density but did show a large increase in nitrogen content as we found as well (Becklin *et al.* 2014). We know from the very few available studies that CO_2 stress can act as a selective agent in *Arabidopsis* (Ward *et al.* 2000) and *Acer rubrum* (Mohan, Clark & Schlesinger 2004). The extent and rate at which plants adapt to CO_2 as a selective agent, and how this relates to phenotypic responsiveness to CO_2 in the present, is an area warranting in-depth research (Medlyn & McMurtrie 2005).

Leaf traits and changes in physiology
The unchanged nitrogen content per area with a higher SLA suggests that the same amount of protein, including RuBisCO, is now embedded in less leaf mass. Consequently, while photosynthesis per area is reduced, photosynthetic gain per unit mass is less affected, thereby somewhat counteracting strong reductions in photosynthetic rate. Thus the return on invested C in leaf is diminished less for species that increase their SLA more. However, from a mechanical and defensive point of view there are substantial costs involved in having such a high SLA leaf: they should be mechanically weak, highly palatable and prone to

wilting (Poorter *et al.* 2009). Increasing CO_2 leads to increased photosynthetic rates which thus allows for a reduction in SLA with the additional benefit of a mechanically stronger and less palatable leaf with a longer life span. We recommend further study to directly test for leaf toughness, palatability and lifespan (Pérez-Harguindeguy *et al.* 2003) of leaves of different species grown at CO_2 levels ranging from low to high.

For herbivores this nitrogen dilution with increasing CO_2 may have had a large effect going from glacial to present levels (Cowling 2001) and may have an effect with CO_2 increasing in the future (Bazzaz 1990). Per gram of foliage consumed (at least for herbs and woody species) in a low CO_2 atmosphere a lot more nitrogen is consumed. Thus, while leaf quantity may have been lower due to reduced growth in low CO_2 (Temme *et al.* 2015), food quality may have been substantially higher than from leaves at ambient CO_2. Altogether, as CO_2 increases from past to future levels it appears food quality decreases but food quantity increases.

CO_2, the Leaf Economic Spectrum and relative growth rate

The Leaf Economic Spectrum (LES) is a set of coordinated traits along the slow-fast growth axis. In deep shade species with slow traits that conserve energy (low respiratory losses and slow leaf turnover) are successful (Baltzer & Thomas 2007, Lusk *et al.* 2008) (Baltzer & Thomas 2007, Lusk et al. 2011). In drought-prone habitats species that conserve water and have relatively large root systems are successful (Poorter *et al.* 2012b). Similarly in environments of low nutrient availability species adopt slow strategies (Grime & Hunt 1975, Aerts & Chapin 2000). Our multi-species experiment has shown that, unlike other resources, CO_2 causes traits (SLA, N concentration, A_{weight}) to move in the opposite direction within given species, pushing plants towards the resource acquisitive ("fast") side at resource-poor conditions in terms of CO_2 and towards the resource-conservative ("slow") side at resource-rich conditions (i.e. higher CO_2).

Thus CO_2 serves to provide a negative feedback to the commonly observed positive relationships of the LES with RGR (Lambers & Poorter 1992, Cornelissen *et al.* 1997, Reich 2014). The question remains whether the envelope of trait levels is moved in its entirety or whether CO_2 adjusts only the boundaries of viable strategies. The mechanical limits precluding further increases in SLA at low CO_2 (Milla & Reich 2007) seem to suggest that at low CO_2 species' possible range of trait values is constrained. Moreover, the results of this study show that the relationship between N content and RGR has a common slope shifted in elevation (higher RGR and lower N) due to CO_2 increase. Thus the increase in photosynthetic rate due to higher carbon availability makes even more resource-conservative traits in terms of nutrient and water economy a viable possibility.

If increases in CO_2 do lead to slower strategies, has this led and will this lead to changing inter- and intraspecific competition? Individual plants can survive at poorer conditions with "slower" traits. As increased CO_2 make slower traits a viable strategy, are specialists already exhibiting slow traits now crowded out by generalists encroaching on their range? Are the specialists now capable of expanding to even poorer conditions due to even slower traits? Thus while the ranking of species for traits and RGR remains broadly the same regardless of CO_2 (Albert *et al.* 2011, Temme *et al.* 2015), the winners and losers at the edges of a

resource niche could shift (Alberto *et al.* 2013) due to changes in economically viable leaf strategies. Shifts in community composition can then lead to changes in the carbon cycling in a broad area. With carbon storage in soils and plants becoming increasingly important for climate change models (McMahon *et al.* 2011) understanding the impact of plant trait adjustments due to CO_2 increase on community composition becomes increasingly important.

Conclusion

CO_2 has strong effects on plant physiology with photosynthesis and respiration closely following CO_2 availability. Across all 28 species measured we found a concerted response to CO_2 where plants adjust their leaf morphology by thinning the leaf and incorporating less mass per area but keeping the nitrogen content per area constant. From a resource economics perspective plants thus adjust the cost for growth towards the availability of carbon and the rate of assimilation: lower respiration at low CO_2 and a lower carbon cost to grow a unit leaf area. At higher CO_2 the carbon costs increase due to increased respiration and higher leaf mass per area. Increasing CO_2 from past low to future high serves to make leaves more robust along the leaf economic spectrum across all species. CO_2 starvation, which has been common for the past 10 million years, forces plants to adopt more resource-acquisitive trait levels at high nutrients and water conditions. The question how plant response to CO_2 interacts with other limiting conditions is an area warranting further research.