Chapter 3

Winners Always Win: Growth of a Wide Range of Plant Species from Low to Future High CO$_2$

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Abstract

Evolutionary adaptation to variation in resource supply has resulted in plant strategies that are based on trade-offs in functional traits. Here we investigate, for the first time across multiple species, if such trade-offs are also apparent in growth and morphology responses to past low, current ambient and future high CO_2 concentrations. We grew freshly germinated seedlings of up to 28 C_3 species (16 forbs, 6 woody and 6 grasses) in climate chambers at 160 ppm, 450 ppm and 750 ppm CO_2. We determined biomass, allocation, specific leaf area (SLA), leaf area ratio (LAR) and relative growth rate (RGR), thereby doubling the available data on these plant responses to low CO_2. High CO_2 increased RGR by 8%; low CO_2 decreased RGR by 23%. Fast growers at ambient CO_2 had the greatest reduction in RGR at low CO_2 as they lost the benefits of a fast-growth morphology (decoupling of RGR and LAR). Despite these shifts species ranking on biomass and RGR was unaffected by CO_2. Winners continued to win, regardless of CO_2. Unlike for other plant resources we found no trade-offs in morphological and growth responses to CO_2 variation, changes in morphological traits were unrelated to changes in growth at low or high CO_2. Thus, changes in physiology may be more important than morphological changes in response to CO_2 variation.

Introduction

From slow-growing cypresses to prolific kudzu vines, plants employ a wide variety of different growth strategies depending on environmental resource availability (Bloom & Mooney 1985). Plant growth not only depends on external resources such as light, carbon dioxide, water and nutrients but also on plant morphology and photosynthetic capacity and their underlying traits. Due to constraints and trade-offs in evolution of plant functioning, no single plant species has solutions to cope with more than a limited fraction of the environmental variation in space and time. Trait combinations that result in high growth rates in one environment may preclude good performance in another environment. Such trade-offs are widespread in the plant kingdom and for light, nutrients and water they have been analysed in great detail (e.g. Aerts & Chapin 2000, Diaz *et al.* 2004, Wright *et al.* 2004, Freschet *et al.* 2010, Reich 2014). These trade-offs underpin the current understanding of plant strategy theory (Grime 2006). Given the trade-offs observed for other growth-related resources, it seems logical to assume that they must be present for CO_2 as well, as indicated previously by plant responses to high CO_2 concentrations as predicted for the latter part of this century (Poorter & Navas 2003). However such trade-offs have not been analysed for a substantial set of species over the whole range from Pleistocene via ambient to future CO_2 concentrations.

Carbon dioxide is special as it shows little spatial variation. All over the globe, CO_2 concentrations in open vegetation show only limited variation with season, latitude, and elevation (Peters *et al.* 2007). However, currently plants worldwide are faced with rapidly increasing CO_2 concentrations: CO_2 will double from the current 400 ppm to 700-800 ppm by the end of this century (Collins *et al.* 2013). At an evolutionary timescale the variation in CO_2 has been even larger, ranging from 3000 ppm in the Devonian down to 180 ppm during the Pleistocene Ice Age (Royer 2006, Hönisch *et al.* 2009). It is only after the Industrial Revolution that CO_2 levels started to rise from 280ppm to the 400 ppm we have today.

Thus compared to the current situation, recent plant evolution has been at a low level of atmospheric CO_2.

Plant responses to environmental factors are often treated categorically using the plant functional type (PFT) concept (Chapin *et al.* 1996), which groups plant species by their similar adaptations and responses to certain environmental factors. As a result of different growth form strategies and physiological mechanisms plants could show contrasting responses to shifts in CO_2 concentration. For example woody plant species invest a lot of their biomass in non-photosynthetic stem tissue (Poorter *et al.* 2012b), so in early stages of development at low CO_2 they might be outcompeted by grasses or forbs that can invest more in carbon acquiring leaf tissue (Bond & Midgley 2012). However, at high CO_2 their observed greater biomass accumulation in perennial tissues (Ainsworth & Long 2005) might lead them to outcompete grasses and forbs. Differences in direction and magnitude of trait responses to CO_2 between plant functional types could thus lead to shifts in competitive interactions.

In terms of carbon capture, the relative growth rate (RGR, g g^{-1} d^{-1}) of plants depends on two aspects: leafiness and physiology. This is encapsulated in the equation RGR=LAR*ULR (Evans 1972), in which relative growth rate (RGR) is dependent on leaf area ratio (LAR, m^2 leaf per g plant) and unit leaf rate (ULR, g plant grown per m^2 leaf per day). Differences in carbon capture may be driven by either: the chemical and physiological traits underlying ULR, tissue carbon content and photosynthesis rate; or the allocation and morphology traits underlying LAR, leaf mass fraction (LMF, leaf mass per unit plant mass) and specific leaf area (SLA, leaf area per unit dry mass) (Lambers & Poorter 1992). At low CO_2 a high SLA might be advantageous because of reduced diffusive resistance in the leaf (Loreto *et al.* 1992, Medlyn *et al.* 2011) and serve to increase the area available for photosynthesis at a lower carbon cost to biomass. A higher biomass allocation to leaves (LMF) serves to take up more of the most limiting resource, carbon, required for optimal growth (Bloom & Mooney 1985). At high CO_2 these traits show less return upon investment due to increased CO_2 availability. With an abundant availability of carbon other factors determining growth such as nutrient uptake rate and light availability can become more limiting (Poorter & Pérez-Soba 2001, Lewis *et al.* 2010). Physiologically, at low CO_2 photosynthetic rates are limited by RuBisCO carboxylation rate and thus more nitrogen invested in the photosynthetic machinery would increase carbon gain per unit of time (Sage & Coleman 2001, Ripley *et al.* 2013). Again, at high CO_2 that high nitrogen investment is less beneficial and nitrogen could be used elsewhere, for instance to speed up RuBP regeneration (Makino *et al.* 2000). Trade-offs in plant design thus lead to different patterns of carbon capture and processing at low versus high CO_2. But how do different plant species and PFTs vary in their traits and associated growth performance across the whole range from low to high CO_2 while obeying such tradeoffs?

While there is ample data on plant species response to elevated CO_2 (Poorter & Navas 2003, Ainsworth & Rogers 2007, Norby & Zak 2011), far less is known on plant responses to low CO_2 (reviewed in Gerhart & Ward 2010, Temme *et al.* 2013). A previous analysis of literature data revealed that the response of plant species to low and high CO_2 is opposite both in magnitude and direction and that plant trait adjustments to low CO_2 are far greater than to high CO_2 (Temme *et al.* 2013). Which has consequently greater effects on C and N cycling (Gill *et al.* 2002) at low CO_2. At high CO_2 only moderate increases in biomass are

found (perhaps due to the saturating nature of CO_2 capture) with a small decrease of SLA and LAR (Poorter & Navas 2003, Ainsworth & Rogers 2007, Norby & Zak 2011). Thus, at increased CO_2 concentrations plant morphological and growth responses (lower SLA, higher RGR) move away from the trait values that, at the interspecific levels, are associated with fast growth (higher SLA = higher RGR), possibly due to a disproportionate increase in photosynthesis per unit leaf area. The very limited experimental results so far have shown that at low CO_2 representing the Pleistocene Ice ages a whole suite of traits is drastically altered compared to ambient CO_2. Morphological traits are strongly adjusted in response to low CO_2. Thinner and less dense leaves (Smith *et al.* 2012) lead to a much higher SLA. Combined with an increase in LMF this results in a higher LAR (Gerhart & Ward 2010, Temme *et al.* 2013). Plant morphological traits at low CO_2 are thus adjusted towards the trait spectrum of today's fast growers. However, despite these substantial phenotypic responses resource starvation is such that there is nevertheless a strong reduction in biomass, amounting to up to 90% for some species (Temme *et al.* 2013). Trait shifts thus may ameliorate some of the effects of low CO_2 but are insufficient to entirely compensate for the diminished concentration of the resource.

Current knowledge makes it difficult to determine how the relationships between leaf morphology, plant allocation and growth rate have changed from past to present atmospheric CO_2 concentrations, and how these relationships compare to the responses of today's plant species to future CO_2 concentrations. Thus our study strives for generality and addresses responses of morphological and allocation traits and their links to growth performance from low to high atmospheric CO_2 concentrations for a wide range of species in the same experiment. It will also shed light on the question whether, among diverse species, the winners in terms of growth performance at current CO_2 would still be the winners at low or high CO_2.

Thus, our study had the following research questions: (1) Do species-specific responses in relative growth rate at low and high CO_2 as compared to ambient CO_2 affect the ranking of species in relative growth rate? To put it differently: are the winners in today's atmosphere also the winners at low and at high atmospheric CO_2? (2) Which Plant Functional Types (woody, grass, forb) lose or will gain the most in terms of growth rate at low and high CO_2, respectively? (3) How are changes in relative growth rate related to changes in underlying allocation and morphological traits?

To that end, we performed an experiment to quantify variation in growth rate and morphological and allocation traits among 28 different species belonging to a wide variety of C_3 Plant Functional types in walk-in climate chambers at a wide range of CO_2 concentrations, 160 ppm, 450 ppm and 750 ppm CO_2.

Materials and methods

Species
To determine the response of a variety of plant growth forms to variation in CO_2 concentration, we obtained seeds from a wide range of temperate (and partly subtropical) woody, forb and grass C_3 species. These had been field collected in Sheffield, UK and the

Chongqing region, SW China as well as supplied by B&T World Seeds, Sheffield Seed co., USA and Kruythoeck Seeds, Netherlands. The seeds were set out to germinate by placing them on either wet sand or wet tissue paper. Special pre-treatment of seeds (scarification, soaking, hot/cold shock) was carried out according to supplier instructions and the authors' experience. This resulted in successful germination of 28 different species (**Appendix table 2.1**) which consisted of 6 woody species (2 trees, 4 shrubs), 16 forb species, and 6 grasses. Shortly after germination individual plants were transferred to experimental CO_2 conditions at the Phytotron labs at Utrecht University, The Netherlands. The growth experiment was spread out over the period October-2012 – October-2013, during which batches of different species were sequentially screened in the standardized environmental regimes.

Growth conditions

We used three separate custom-built walk-in climate rooms (Reftech B.V., Sassenheim) in which we maintained three CO_2 levels: low, ambient and high. These levels broadly (±50 ppm) represented the large range from Pleistocene past to future high CO_2. The low CO_2 concentration of 160 ppm (peaking to 180 ppm when handling the plants inside the chamber) was achieved by scrubbing CO_2 from ambient air ventilating the room by using a molecular sieve (PG 1500L, CMC Instruments GmbH, Eschborn). The ambient level (450 ppm) was slightly higher than outside air due to elevated levels inside the office building. The high level (750 ppm) was achieved by adding fossil fuel derived CO_2 from high pressure tanks to ambient air in the climate room. CO_2 levels inside the chambers were digitally monitored (GMP343, Vaisala GmbH, Bonn) and scrubber or valve capacity adjusted accordingly. Low levels of CO_2 while handling plants were maintained by using a gas mask to capture exhaled breath in a large airtight bag.

Growth conditions were ~350 μmol light, 18°C night/21°C day temperature, 10h photo-period and 70% relative air humidity. 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 start to grow. Pots were watered thrice daily up to field capacity using an automated watering system supplying water from below. To prevent nutrient limitation during the experiment, nutrients were added three times per week with 50 ml full Hoagland solution (6mM KNO_3, 4mM $Ca(NO_3)$, 2mM $NH_4H_2PO_4$, 1μm KCl, 25μm H_3BO_3, 2μm $MnSO_4$, 2μm $ZnSO_4$, 0.1μm $CuSO_4$, 0.1 μm $(NH_4)_6Mo_7O_{24}$, 20μm Fe(Na)EDTA). To prevent damage from excess nutrients to young plants, freshly germinated individuals were supplied with an increasing concentration starting with 25% nutrients after germination to full Hoagland at the onset of the first leaf and subsequent growth period.

Shortly after seed burst and germination at ambient CO_2 seedlings were transferred to the CO_2 chambers in 400ml plastic pots containing coarse sand. Because of the small size the seedlings were expected to obtain during the duration of the experiment pot size was assumed to be sufficient to maintain less than 2 g plant L^{-1} soil to avoid pot size effects (Poorter *et al.* 2012a). Although we did not observe any strong symptoms of pot-boundness we cannot entirely exclude such effect in the largest plants (see Discussion). We tried to standardize the period of the exponential growth phase based on the ontogenetic phase of the plants at the start of this period. After expansion of the first leaf (as in Cornelissen *et al.* 1996) a representative subset of each species (4-8 individuals depending on germination

success) was harvested and oven dried at 70°C for 48 hr. as a baseline biomass measure of the total set of individuals. Subsequently plants were grown for three more weeks after which the remaining ±7 individuals were harvested. Due to the small plant size and young age, growth during these three weeks was assumed to be in the exponential phase (Grime & Hunt 1975). By using the baseline biomass and final biomass we could calculate Relative Growth Rate (Hoffmann & Poorter 2002). Because of space constraints species were staggered in 6 batches of species where the experimental regime in the climate chambers was held constant and continuously monitored.

Final harvest
At final harvest plants were washed to remove sand from roots, and fresh weight (weighed to the nearest mg) was measured for above and belowground plant parts. Images were taken to illustrate effects of CO_2 on plant size (**Fig. 3.1**). Leaf area was measured by scanning (Canon LiDe 110 at 300dpi) a representative full-grown leaf for SLA (m^2_{leaf} g^{-1}_{leaf} dry weight) measurements. Leaf area (m^2) was then determined by pixel counting using ImageJ version 1.47. Fresh plant material was oven dried at 70 °C for 48 hours and weighed. After drying leaves were removed from stems and stems were weighed to calculate leaf and stem mass fraction. Plant Leaf Area Ratio (LAR, m^2_{leaf} g^{-1}_{plant}) was then calculated by multiplying SLA with leaf mass fraction.

Statistics
Due to difficulties in germinating enough seedlings to do a representative baseline harvest RGR of three species *(Buddleja davidii, Clinopodium chinense, Stellaria media)* could not be determined. Two episodes where the low CO_2 and high CO_2 chamber were unavailable due to emergency maintenance led to 25 species in the low CO_2 treatment, 22 species in the high CO_2 treatment and 19 species with all three treatments.

Given the limited number of species analysed for low to high CO_2 response (Gerhart & Ward 2010, Temme *et al.* 2013) we felt that a higher number of species would further our understanding of plant responses to CO_2 more than an in depth look at a limited number of species with more chamber replicates. As this entails some danger of pseudoreplication we tested the robustness of our approach by (1) measuring a single species at multiple time intervals; and (2) comparing the results of multiple batches of different species at different times. Data on RGR and SLA for *Avena sativa* grown in different batches with substantial time intervals showed a consistent response to CO_2 and supports the robustness of the treatment in the climate chambers (**Appendix 2.1**); only one batch was used in this study. While different species grown during different batches show moderately different responses to CO_2 the overall effect of low or high CO_2 was comparable between species and batches (**Appendix 2.2**). We are thus confident that a repeat of the experiment with a different "random draw" of species would lead to similar conclusions.

Results were analysed using R (version 3.0.1, R Core Team, Vienna, Austria) and RStudio (version 0.98, RStudio, Inc., Boston, MA, USA). Changes in the interspecific ranking based on RGR were analysed non-parametrically by determining species rank on relative growth rate (RGR) at low, ambient and high CO_2. Rank changes were then tested pairwise between

CO_2 treatments in a paired-Wilcoxon-signed-rank test with Bonferroni correction. 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 analysing CO_2 effect on trait shifts. The difference in ln-transformed trait level from ln-transformed ambient level was then the relative shift in trait level via $e^{\text{ln transformed difference}}-1$. 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). To determine if the reduction and stimulation in growth and biomass was related to trait values at ambient CO_2 or shifts in trait value towards low or high CO_2 we performed a stepwise model selection procedure selecting models based on AIC using the MASS package (version 7.3). The initial model to determine if trait values at ambient CO_2 were related to RGR and biomass differences included RGR or biomass at ambient CO_2 and root mass fraction (RMF), leaf mass fraction (LMF) and leaf area ratio (LAR) at ambient CO_2. The initial model to test the relationship between the difference in RGR and shifts in trait level included the shifts in RMF, LMF and LAR. The relationship between RGR and LAR at all three CO_2 levels was determined using ordinary least squares regression as we viewed LAR as a predictor of RGR.

Results

Plants responded strongly to the low and high CO_2 treatments. The photos of figure 3.1 illustrate the large effect of CO_2 on plant size. In general, plants at low CO_2 were tiny compared to ambient CO_2 and as expected plants were stimulated by elevated CO_2. While different species showed moderately different responses, species grown in different batches in different periods showed comparable responses to CO_2 (**Appendix 2.2**).

Species ranking on RGR and biomass
Species varied over 6-fold in their relative growth rates with the woody gymnosperm *Picea sitchensis* growing the slowest regardless of CO_2 concentration and the forb *Rumex acetosella* (missing at high CO_2) and semi-woody scrambler *Solanum dulcamara* growing fastest at low, ambient and high CO_2 (**Fig. 3.2**). Figure 3.2 shows that at low and high CO_2 there were only minor shifts in the ranking of species on relative growth rates as compared to the ranking at ambient CO_2: fast growers tended to grow relatively fast and slow growers grew relatively slowly irrespective of CO_2 treatment. This was confirmed by pairwise-Wilcoxon-signed-rank tests which showed no significant changes in species ranking on RGR between low, ambient and high CO_2. Averaged over all three PFTs RGR was reduced by 23.4%±4.7 ($p<0.001$) at low CO_2 (**Fig. 3.3b**). However, likely due to large variation among species and small sample size the separate response for grass and woody species was not significant. At high CO_2 RGR increased on average by 7.8%±2.5 ($p<0.01$) though grass species did not increase their RGR (**Fig. 3.3b**).
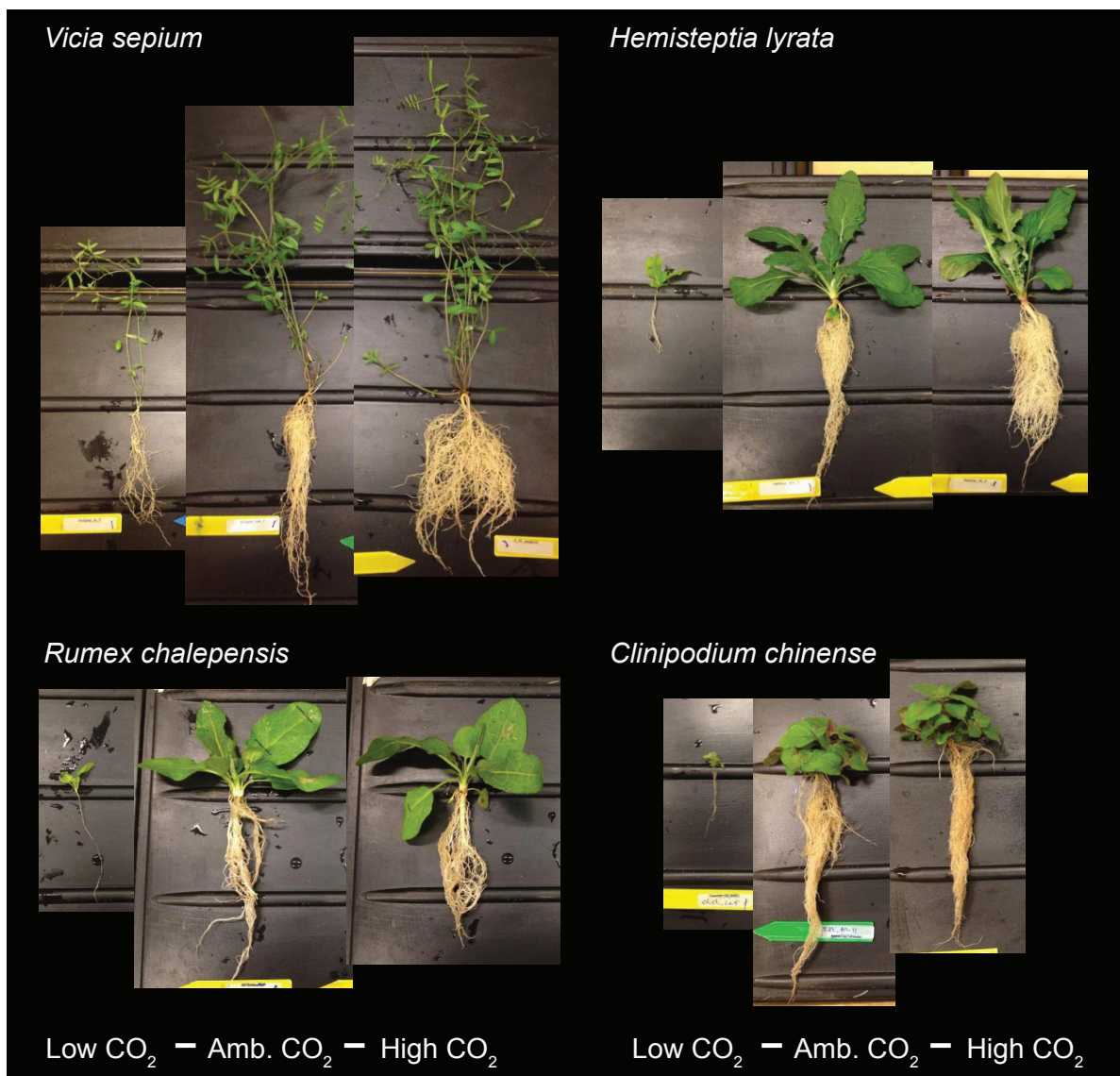
Figure 3.1 Plants grown at low, ambient and high CO_2. Images illustrate the response of four plant species, *Vicia sepium, Hemisteptia lyrata, Rumex chalepensis* and *Clinopodium chinense* to growth at 160 ppm, 450 ppm, 750 ppm CO_2.
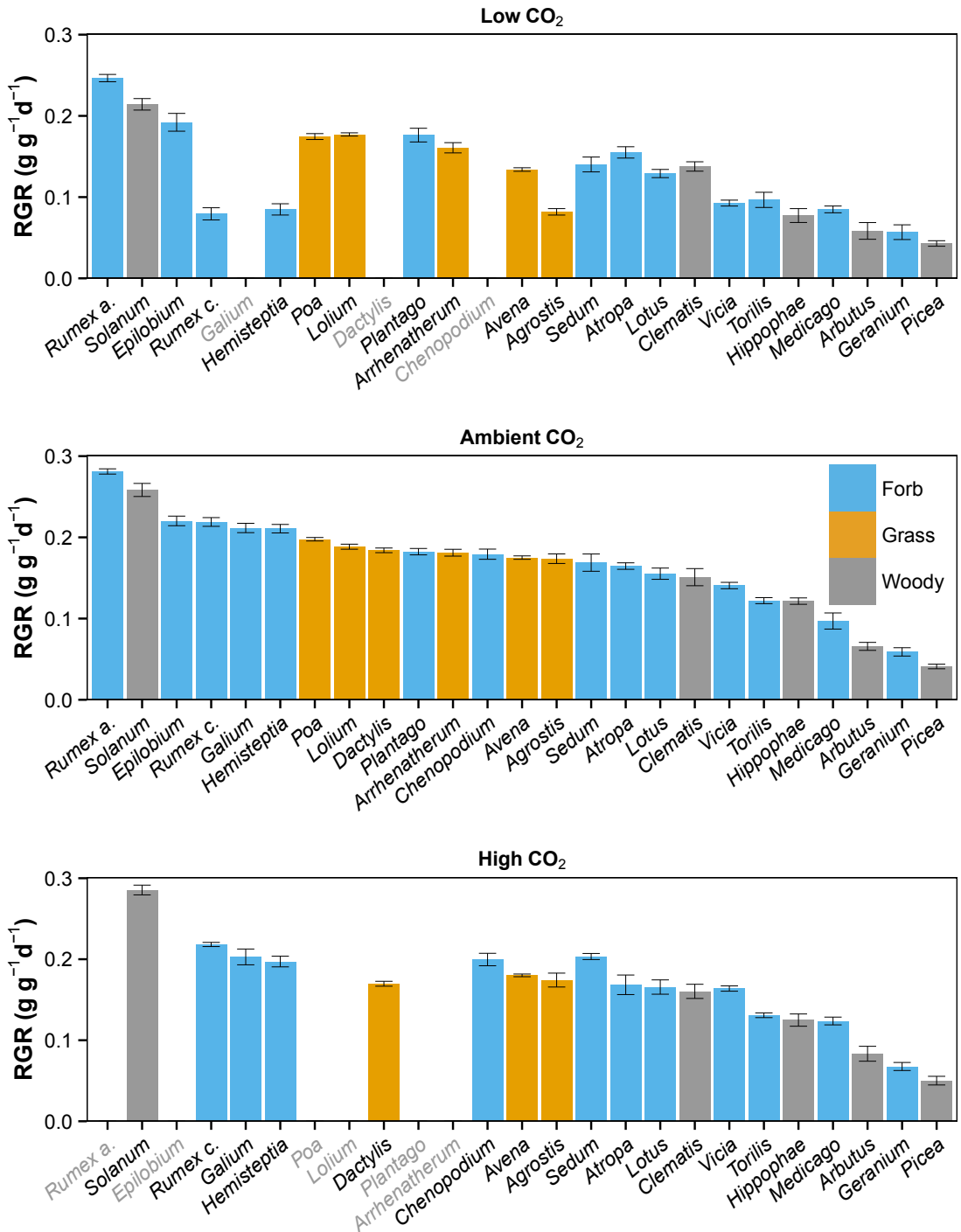
Figure 3.2 Plant species relative growth rate (RGR, $g \cdot g^{-1} \cdot d^{-1}$) ranking at 160 ppm, 450 ppm and 750 ppm CO_2. Species are ordered by RGR at 450 ppm CO_2. Light grey species names indicate species is missing at this CO_2 treatment. Light grey bars: grass species, medium grey bars: forb species, dark grey bars: woody species. Error bars denote SE.
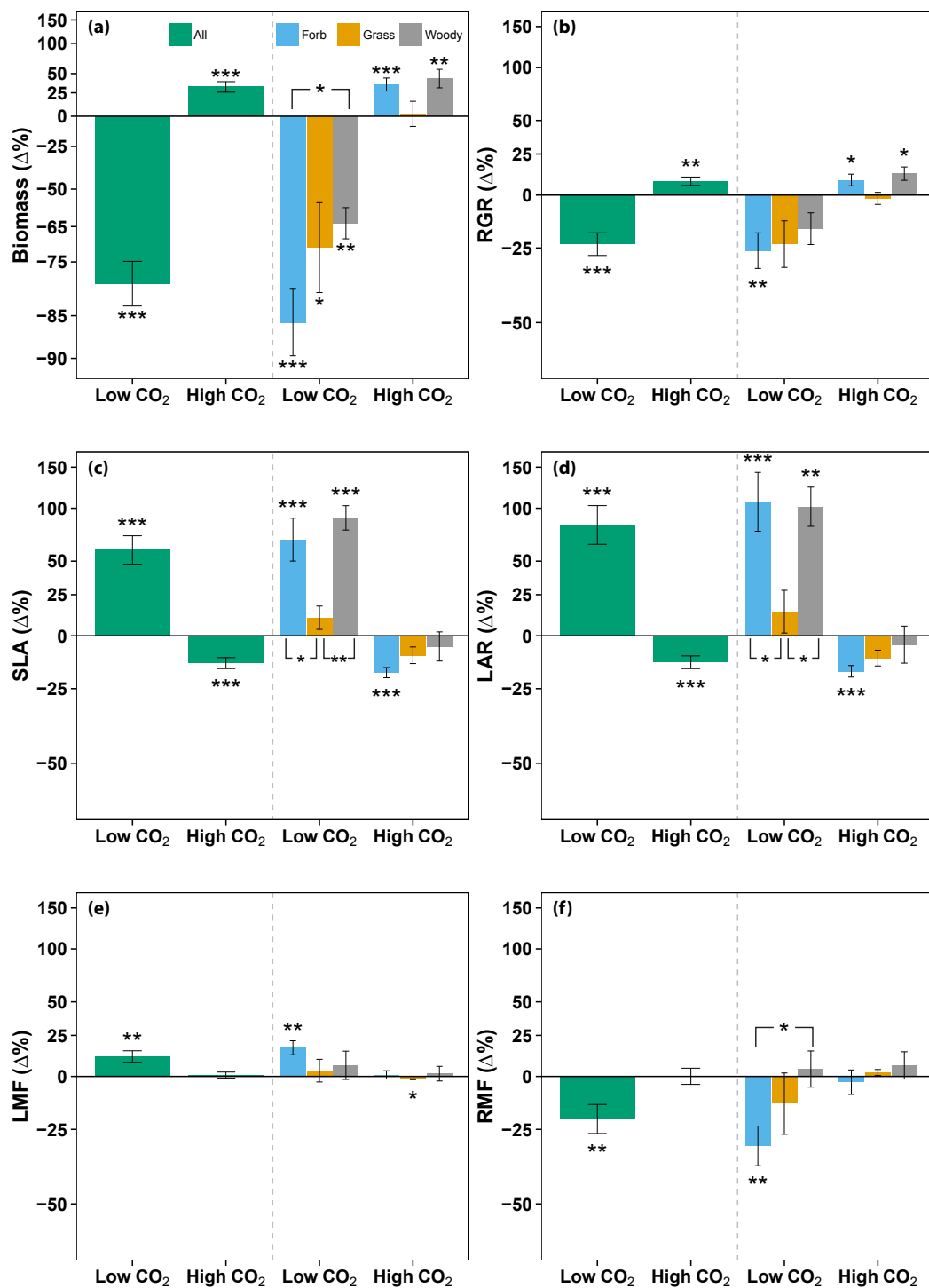
(left) **Figure 3.3** Relative shift in trait level at low or high CO_2 compared to ambient CO_2 for forb, grass and woody species. Bars indicate percentage shift in trait value at low CO_2 (160 ppm) and high (750 ppm) CO_2 compared to trait value at ambient (450ppm) CO_2. Axes are natural log transformed so that the size of the bars at a 50% decrease or a 100% increase is the same (reflecting a factor 2 adjustment). Left section: all species (25 low/22 high), Right section: medium grey bars: forb species (14 low/13 high), light grey bars: grass species (5 low/3 high), dark grey bars: woody species (6 low/6 high). Error bars give SE. *s near error bars indicate t-test significance from zero. *s opposite bars indicate significance of 2-sample t-test between linked types. *: p<0.05, **: p<0.01, ***: p<0.001. **(a)** Biomass, dry weight of plants (g). **(b)** Relative growth rate (RGR, $g \cdot g^{-1} \cdot d^{-1}$), two less forbs and one less woody species. **(c)** Specific leaf area (SLA, $m^2_{leaf} \cdot g^{-1}_{leaf}$). **(d)** Leaf area ratio (LAR, $m^2_{leaf} \cdot g^{-1}_{plant}$). **(e)** Leaf mass fraction (LMF, $g_{leaf} \cdot g^{-1}_{plant}$). **(f)** Root mass fraction (RMF, $g_{root} \cdot g^{-1}_{plant}$)

As with RGR there were only minor shifts in the ranking of plant biomass at the end of the experimental period (**Appendix 2.3**). Low CO_2 reduced biomass on average by 79.7%±4.3 (p<0.001) and high CO_2 increased biomass by 32.2%±6.7 (p<0.001) (**Fig. 3.3a**) but again there were no significant changes in species ranking.

While the ranking of species for RGR and biomass was not significantly altered by CO_2 we did find that, in general, species with a higher RGR at ambient CO_2 had a stronger reduction in RGR with low CO_2 (**Fig. 3.4a**). As the ranking of species on RGR remained similar across CO_2 levels and fast growers at ambient were more affected by low CO_2 this had the effect that in the community of species the difference in RGR between the top ranked and bottom ranked species was reduced at low CO_2 (**Appendix 2.4**). These RGR differences led to absolute biomass loss at low CO_2 compared to ambient being highest for large species (**Fig. 3.4b**) while relative biomass loss was overall not significantly different between species, though the largest woody species had a greater reduction then the smaller species (**Appendix 2.5**). No such results were found at high CO_2 however (**Appendix 2.6**).

Differences between plant types

Plant types showed only limited differences in their responses to CO_2, only at the stress of low CO_2 did we find significant differences in trait adjustment between types (**Fig. 3.3**). Biomass loss was different between forbs and woody species with forbs having a 85.9%±4.4 reduction in biomass and woody species only a 63.9%±5.5 reduction. Between woody and forb species the adjustment in Root Mass Fraction (RMF) was significantly different as well (**Fig. 3.3f**) with woody species not adjusting RMF but forbs decreasing RMF by 16.9%±4.4 (p<0.01). Grass species had a markedly different response in Specific Leaf Area (SLA) and Leaf Area Ratio (LAR). On average, relative to ambient CO_2, SLA increased strongly at low CO_2 by 59.4%±12.4 (p<0.001) and decreased modestly by 13.8%±2.6 at high CO_2 (p<0.001). However when viewed separately at low CO_2 woody and forb species had a very large increase in SLA (68.6% and 89.8%, respectively) whereas grass species did not significantly increase their SLA (**Fig. 3.3c**). At high CO_2 forb species decreased SLA by 18.2%±2.3 (p<0.001), grass and woody species however did not significantly reduce SLA. For Leaf Area Ratio (LAR) a similar result was found (**Fig. 3.3d**) with grass species not significantly increasing LAR but a large increase in LAR for forbs (107.1%±33.3) and woody species (101.7±21.6) was found at low CO_2. At high CO_2 only forbs showed a significant decrease in LAR (17.6%±2.6).
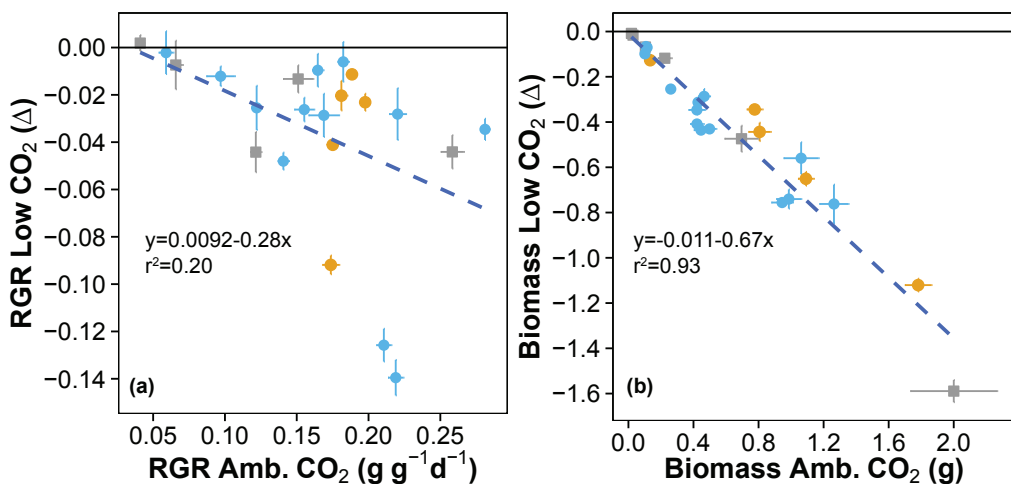
Figure 3.4 Difference in growth rate and plant biomass at past low (160 ppm) CO_2 compared to current ambient (450 ppm) CO_2. **(a)** Relative Growth Rate difference at low CO_2 shows a negative relationship (black line) to growth rate at ambient CO_2, species that grow fast at 450ppm CO_2 are more reduced in growth rate than slow growing species. R^2=0.20, $p<0.05$. **(b)** Biomass difference at low CO_2 shows a negative relationship with biomass at ambient CO_2 (black line), i.e. bigger species lose more biomass at low CO_2. R^2=0.93, $p<0.001$. Blue circles: forb species, orange circles: grass species, grey squares: woody species. Error bars give SE

Morphology and growth

In general, plant morphological traits were poor predictors of growth response to CO_2. None of the morphological traits (RMF, LMF, LAR) was significantly related to differences in growth rate or biomass at low CO_2. However, species with the highest growth rate or largest biomass at ambient CO_2 did show the strongest absolute reduction at low CO_2 (**Fig. 3.4**). Shifts in trait value of RMF, LMF and LAR were also not significantly related to differences in RGR or biomass at low CO_2. Similarly for high CO_2, none of the trait shifts or trait values at ambient CO_2 was significantly related to stimulation of growth or biomass at high CO_2. This is possibly due to a changed relationship between LAR and RGR at low CO_2 (**Fig. 3.5**). At ambient CO_2 and high CO_2 there was a positive relationship between LAR and RGR (r^2=0.38 & r^2=0.30 respectively, $p<0.01$). However, at low CO_2 (**Fig. 3.5a**) RGR decreased despite a strong increase in LAR thereby decoupling the generally observed positive relation between RGR and LAR. The LAR-RGR relationships were determined more strongly by SLA than by LMF (**Appendix 2.7**).
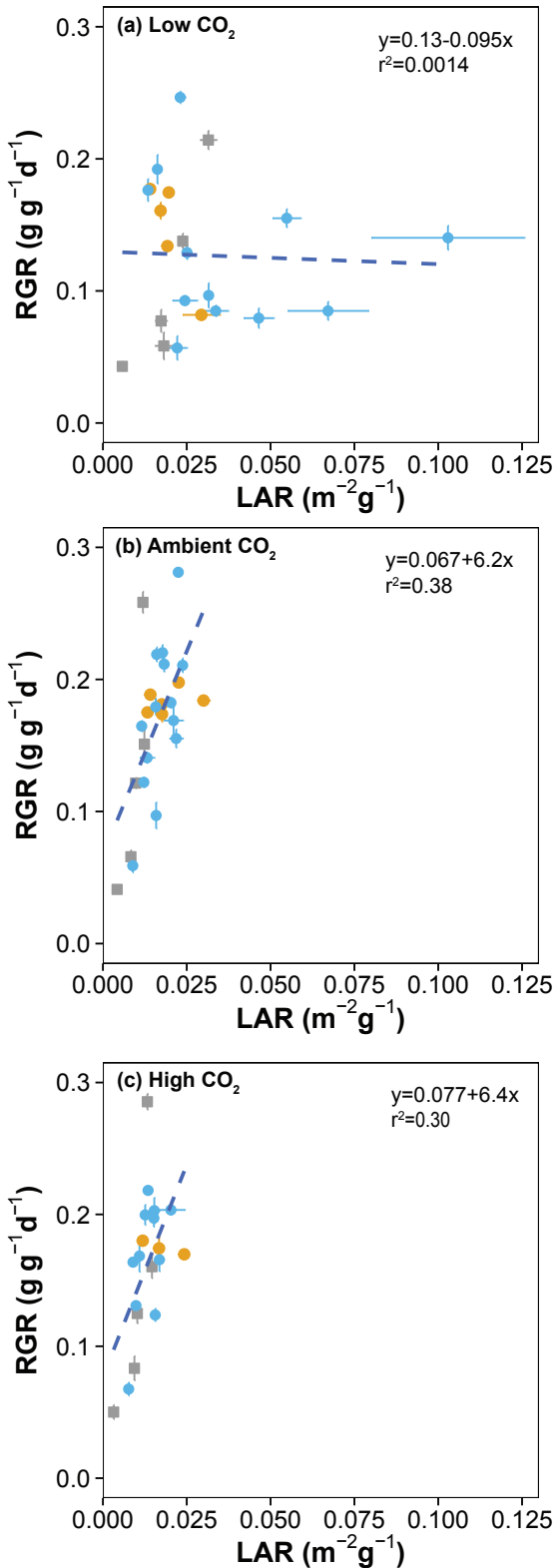
Figure 3.5 Relationship between leaf area ratio (LAR) and relative growth rate (RGR) at past low (160 ppm), current ambient (450 ppm) and future high (750 ppm) CO_2. The relationship between LAR and RGR is positive at ambient **(b)** and high **(c)** CO_2 ($p<0.05$, $R^2=0.38$ & $R^2=0.3$ respectively). At low CO_2 **(a)** no significant relation is found. Points indicate species mean RGR and LAR with SE; blue circles: forb species, orange circles: grass species, grey squares: woody species.

Discussion

This study is novel in that we investigated 19 plant species belonging to different functional types in their performance across the whole range of Pleistocene low, via ambient to future high CO_2 levels, and 25 species for their performance at low CO_2 thereby doubling the available data on plants' low CO_2 response (Temme *et al.* 2013). This approach has enabled us to make an experimental analysis of growth responses of plants to variation in CO_2 and how these are related to changes in morphological traits. We found that, while relative growth rate (RGR) and plant biomass were strongly affected by both low and high CO_2, the ranking of species for RGR and biomass was not affected. Thus, we did not find a classical "trade-off" by which species with faster growth in response to low CO_2 compared to other species would have grown relatively slowly at high CO_2, and *vice versa*. This could be because while CO_2 concentration can act as a selective force (Ward *et al.* 2000, Mohan *et al.* 2004), in open vegetation there is little spatial variation in CO_2. As such, unlike for all other plant resources (see Aerts & Chapin 2000), there cannot be selection at any given time for high and low CO_2 specialists. The morphological explanation for this lack of trade-off might be that, contrary to what we expected, changes in RGR were unrelated to changes in leaf morphology (SLA) and allocation (LMF, RMF), and thereby to changes in leaf area ratio. Indeed the well-established positive relationship between LAR and RGR, as seen at ambient and high CO_2, broke down entirely at low CO_2.

Species rankings on RGR unaffected by CO_2

In general in plant strategy theory, there are trade-offs in species performance across resource supply gradients. For example species that perform well at high nitrogen supply are poor performers at N-limited growth conditions (Aerts & Chapin 2000) and we expected a similar pattern for CO_2. However with some exceptions species ranking remained similar (**Fig. 3.2**). These exceptions are *Rumex chalepensis, Hemisteptia lyrata* and *Agrostis capillaris* which dropped considerably in RGR ranking at low CO_2. Still, the fast growers at ambient CO_2 are generally also the fast growers at low and high CO_2 (**Fig. 3.2**). Although the ranking on growth remained the same, it is the fast growers at ambient CO_2 that suffer from a stronger reduction in relative growth rate at low CO_2. In terms of *absolute* biomass loss this pattern is even clearer as there is a strong connection between plant biomass at ambient CO_2 and biomass reduction at low CO_2 (**Fig. 3.4**). Interestingly, the *relative* biomass loss is not significantly related to plant biomass. Larger plants in general have a similar percentage of biomass reduction at low CO_2 as smaller plants (**Appendix 2.5**). This poses the question whether there are clearer winners and losers in interspecific competition now than in the Pleistocene past due to increased differences in growth rate between species.

The interactive effect of CO_2 with other resources and environmental factors does however modulate different plants species response to CO_2. Limiting N and P supply changes plants response to increasing CO_2 (Grunzweig & Korner 2003, Lewis *et al.* 2010, Ripley *et al.* 2013) as does water supply (Ward *et al.* 1999, Medeiros & Ward 2013) and temperature (Cowling & Sage 1998, Ward *et al.* 2008). Indeed, species response to environmental change since the LGM had a greater effect on conifer stand community composition then species response to CO_2 increase (Becklin *et al.* 2014). This shows that while our results provide potential shifts in species relative competitive ability due to CO_2, understanding how changes

in resources and the environment interact with CO_2 is important for understanding shifts in community composition since the last glacial maximum (LGM). Furthermore in dense canopies a vertical gradient in CO_2 can occur with elevated CO_2 close to the soil (Medina *et al.* 1986) and depleted (down to 280-300) in the canopy during peak photosynthesis times (Bazzaz & Williams 1991). It could be that for species that occur only in those zones there might be selection for high and low CO_2 specialists.

Based on the literature we expected that, at high CO_2, fast growing species would be stimulated more than slow growers (Poorter & Navas 2003). However, we found only minor stimulation of RGR and biomass and this was not related to growth rate or plant biomass at ambient CO_2. While strong pot boundness was not observed visually, we cannot exclude the possibility of pot size having played a small role in this. Large plants at ambient CO_2 were at or above the recommended limit of 2g L^{-1}, implying that pot size might have limited growth increase of the largest species at high CO_2 (Poorter *et al.* 2012a); see also **Fig. 3.2** and **Appendix 2.6**) although there is evidence of pot size not playing a large role in plants high CO_2 response (Kerstiens & Hawes 1994). Alternatively while at lower light levels the morphological traits assessed here better explain interspecific variation in plant performance (Evans & Poorter 2001) the relatively low light levels could be a factor in the limited growth response. In natural understory stands shade tolerant species were most stimulated by elevated CO_2 at low light conditions whereas less shade tolerant species showed no stimulation (Hattenschwiller & Korner 2000). Indeed the 6 heaviest species at ambient CO_2 that showed little biomass stimulation (**Appendix 2.3**) are not generally found in shady habitats. From a resource economics perspective the extent of CO_2 stimulation should be dependent on the availability of other resources (Bloom & Mooney 1985). However, the interaction with light has generally been found to be small (Poorter & Pérez-soba 2001).

No major differences in CO_2 response among Plant Functional Types
We found comparable trait responses in the three plant types. Over the whole range of CO_2 treatments only the response of SLA, LAR and Root Mass Fraction was significantly different between plant types. Forbs and woody species greatly increased SLA at low CO_2, possibly to reduce mesophyll resistance in the leaf (Loreto *et al.* 1992, Medlyn *et al.* 2011) or to produce more carbon acquiring leaf area at a lower biomass expense. Grass species, in contrast, showed no significant increase in SLA at low CO_2 but this did not lead to a greater reduction in biomass at low CO_2. Whether this means that grasses are less plastic in their SLA response and maintain growth rates through a different (for instance physiological) mechanism is unclear.

While not significantly different over the whole range of CO_2, at elevated CO_2 growth and biomass stimulation was greatest for woody species (cf. Curtis & Wang 1998), for which woody tissues may act as a powerful carbon sink reducing build-up of photosynthates and slow-down in photosynthetic rates in the leaves. In contrast grasses, which aboveground consist mostly of foliage, showed little to no stimulation. The greater stimulation of woody species as compared to grasses at high CO_2 suggests important ecological implications where seedlings of both types compete, for example after gap formation in a forest (Loik & Holl 2001) or after savannah fires (Kgope *et al.* 2010, Bond & Midgley 2012).

Morphological traits and trait plasticity are poor predictors of CO_2 response

While all species were reduced in their growth rate, some were more affected than others by low CO_2 and while the difference was smaller at high CO_2 there was variation in stimulation there as well (**Fig. 3.2**). Through stepwise regression we sought to identify the source of this variation. We found that morphological traits and shifts in them were poor predictors of shifts in RGR from ambient to low or high CO_2. Allocation patterns to leaves and roots and leaf area ratio were not related to shifts in growth rate. Species that grew faster at ambient and high CO_2 were more affected by low CO_2. From the relationship between LAR and RGR (**Fig. 3.5**) it can be seen that while a fast growth morphology (high LAR) is related to fast growth at ambient and high CO_2, surprisingly there was a decoupling of RGR and LAR at low CO_2. On average plant species greatly increased SLA at low CO_2, a trait generally associated with higher RGR (Poorter & Garnier 2007). However, this seems to have been insufficient to ameliorate the carbon starvation experienced at low CO_2.

This decoupling or RGR and LAR at low CO_2 seems to suggest that unit leaf rate (ULR, see Introduction) and underlying plant physiological traits are of greater importance in driving differences in growth rate at low CO_2. At low CO_2 plants appear to lose the benefits of a fast growth morphology which explains why fast growers are most affected by low CO_2. Both from paleo-data and from growth chamber studies we know that nitrogen content and photosynthetic rate are strongly affected by low CO_2 (Gerhart & Ward 2010, Temme *et al.* 2013, Becklin *et al.* 2014). Potentially plants' capacity to adjust these physiological traits might better explain differences in RGR and biomass at low CO_2.

From the past to the present

Plant species have not experienced the low CO_2 concentrations that occurred during the last glacial maximum for at least 17Ka (Hönisch *et al.* 2009) but will likely experience a doubling of CO_2 in the next 80 yrs. This is a short period for evolutionary change especially given the rapid rise from 280 ppm to current ~400 ppm CO_2 since the start of the industrial revolution. RuBisCO as the key enzyme in carbon uptake seems to be fine-tuned to 200ppm CO_2 (Zhu *et al.* 2004). While there is evidence that CO_2 can act as a strong selective agent in *Arabidopsis thaliana* (Ward *et al.* 2000) and *Acer rubrum* (Mohan *et al.* 2004) at low CO_2, it remains unclear how much plants have adapted to the higher CO_2 concentration of today through evolutionary changes or whether they are currently adjusting through plasticity in trait responses.

While perhaps plant species trait levels were different during low CO_2 episodes in the Pleistocene, we believe the direction and magnitude of change of current plants grown at low CO_2 to be representative of trait levels during the Pleistocene. Although it should be noted that different families do show different levels of response to global change since the LGM (Becklin *et al.* 2014). The potential ecological and environmental implications for plant growth and development during glacial times are interesting. The reduced belowground biomass due to a combination of slower plant growth and lower allocation to roots has impacted chemical weathering rates of soil during low CO_2 periods during the Pleistocene (Beerling *et al.* 2012). Reduced growth rates with thin, high SLA leaves will have made plants more susceptible to damage from herbivory and made the leaves more palatable to

herbivores (Pérez-Harguindeguy et al. 2003, Poorter *et al.* 2009), the reduction of which is linked to the extinction of the Pleistocene megafauna (Cowling 2001). Slow growth at low CO_2 was likely a limiting factor for the origin of agriculture as well (Sage 1995, Cunniff *et al.* 2008).

Plant growth and development are strongly affected by CO_2 concentration. Differences in traits between plants grown at today's CO_2 concentration and past Pleistocene low CO_2 were far greater than differences in traits between plants grown in today's atmosphere and future high CO_2 atmosphere. Plant growth at past low CO_2 concentration was strongly reduced with fast growing species being more affected by carbon starvation than slow growing species. This had the effect of diminishing RGR differences between fast and slow growers while the ranking of species for growth rate remained broadly similar. Moreover, the greater reduction in growth rate and biomass of fast growing species at low CO_2 is likely associated with the decoupling of more 'leafy' (higher SLA, higher LAR) morphology with faster growth. Differences in growth rate at carbon starvation could therefore be driven more by physiological differences. Understanding how physiological traits are affected by carbon starvation and carbon excess will shed more light on the interaction between morphology, physiology and growth from past low to future high CO_2.