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A dynamic leaf gas-exchange strategy is conserved in woody plants under changing ambient CO_2: evidence from carbon isotope discrimination in paleo and CO_2 enrichment studies

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Abstract

Rising atmospheric [CO_2], c_a, is expected to affect stomatal regulation of leaf gas-exchange of woody plants, thus influencing energy fluxes as well as carbon (C), water, and nutrient cycling of forests. Researchers have proposed various strategies for stomatal regulation of leaf gas-exchange that include maintaining a constant leaf internal [CO_2], c_i, a constant drawdown in CO_2 ($c_a - c_i$), and a constant c_i/c_a. These strategies can result in drastically different consequences for leaf gas-exchange. The accuracy of Earth systems models depends in part on assumptions about generalizable patterns in leaf gas-exchange responses to varying c_a. The concept of optimal stomatal behavior, exemplified

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by woody plants shifting along a continuum of these strategies, provides a unifying framework for understanding leaf gas-exchange responses to c_a. To assess leaf gas-exchange regulation strategies, we analyzed patterns in c_i inferred from studies reporting C stable isotope ratios ($\delta^{13}C$) or photosynthetic discrimination (Δ) in woody angiosperms and gymnosperms that grew across a range of c_a spanning at least 100 ppm. Our results suggest that much of the c_a-induced changes in c_i/c_a occurred across c_a spanning 200 to 400 ppm. These patterns imply that $c_a - c_i$ will eventually approach a constant level at high c_a because assimilation rates will reach a maximum and stomatal conductance of each species should be constrained to some minimum level. These analyses are not consistent with canalization toward any single strategy, particularly maintaining a constant c_i. Rather, the results are consistent with the existence of a broadly conserved pattern of stomatal optimization in woody angiosperms and gymnosperms. This results in trees being profligate water users at low c_a, when additional water loss is small for each unit of C gain, and increasingly water-conservative at high c_a, when photosystems are saturated and water loss is large for each unit C gain.

Keywords: angiosperm, carbon dioxide, free-air CO_2 enrichment, gymnosperm, optimal stomatal behavior, photosynthesis, stomatal conductance, water use efficiency

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Introduction

Atmospheric CO_2 concentration (c_a) is presently more than twofold greater than it was during the Last Glacial Maximum (IPCC, 2013). The additional twofold increase projected over the next 100 years is expected to increase net photosynthetic assimilation rates (A) as well as reduce stomatal conductance (g_s) of most C_3 plant species (Ainsworth & Long, 2005; Lammertsma *et al*., 2011; Norby & Zak, 2011; Warren *et al*., 2011; Bader *et al*., 2013; Franks *et al*., 2013). Hence, these shifts in leaf gas-exchange will affect the energy balance and coupled C, water, and nutrient cycling of forests worldwide. How much leaf gas-exchange will be impacted by further increases in c_a will depend greatly on the degree to which woody plants are evolutionarily canalized toward maximizing C gain or avoiding drought stress.

Carbon assimilation (A) is related to stomatal conductance to CO_2 (g_s) through Fick's law (Farquhar *et al*., 1989):

$$A = g_s(c_a - c_i), \tag{1}$$

$$A = g_s\left(1 - \frac{c_i}{c_a}\right), \tag{2}$$

where c_i is the leaf intercellular space CO_2 concentration. The ratio c_i/c_a is homeostatic across a wide range of g_s (Norman, 1982), indicating that most variation in A within a given leaf results from variation in g_s. The close association of A and g_s also holds across a large number of species and life forms (Körner *et al*., 1979). Ehleringer (1993) first proposed that C stable isotope discrimination (Δ) could be used to identify 'metabolic set points' for leaf gas-exchange activity within or among taxa (i.e. in a constant c_a environment) because Δ can be used to infer the long-term, integrated records of c_i, $c_a - c_i$ and c_i/c_a. Shortly thereafter, Ehleringer & Cerling (1995) reviewed early studies of how Δ and c_i/c_a changed across a range of c_a, concluding that although no primary response to c_a was yet detectable, future studies would lead to a greater understanding of compensatory changes to gas exchange metabolism in plants. Subsequent studies of woody plants have employed the broader concept of a metabolic set point put forward by Ehleringer (1993), and have often assessed variability in Δ across a range of c_a as a means to characterize woody plants as having one of three homeostatic gas-exchange regulation strategies: constant c_i, constant $c_a - c_i$ and constant c_i/c_a (Marshall & Monserud, 1996; Saurer *et al*., 2004; Frank *et al*., 2015). These strategies can yield similar results for C gain and tree growth under a quasi-stable c_a regime; however, across large shifts in c_a, they can imply very different priorities for leaf gas-exchange. For example, according to Eqn (1) if c_a increases, a constant c_i (Fig. 1; green line) would demand a dramatic increase in A, or decrease in g_s, or both whereas a constant $c_a - c_i$ (Fig. 1; purple line) would require smaller changes in A, g_s or both. Intermediate between these endpoints are constant c_i/c_a strategies (Fig. 1; dark and light blue lines).

Empirical support for woody plants maintaining a constant c_i as c_a increases is sparse. Only one study, based on eddy covariance estimation c_i of across a narrow range of c_a in 21 forests, suggested constancy of c_i with increasing c_a. (Keenan *et al*., 2013). If this leaf gas-exchange strategy were sustained, c_i/c_a would decrease and water use efficiency of forests would see massive increases as c_a rises. A leaf gas-exchange strategy that appears to result in a constant $c_a - c_i$ has been demonstrated for some species (Marshall & Monserud, 1996; Marshall & Linder, 2013), whereas most species and growing environments examined to date have exhib-

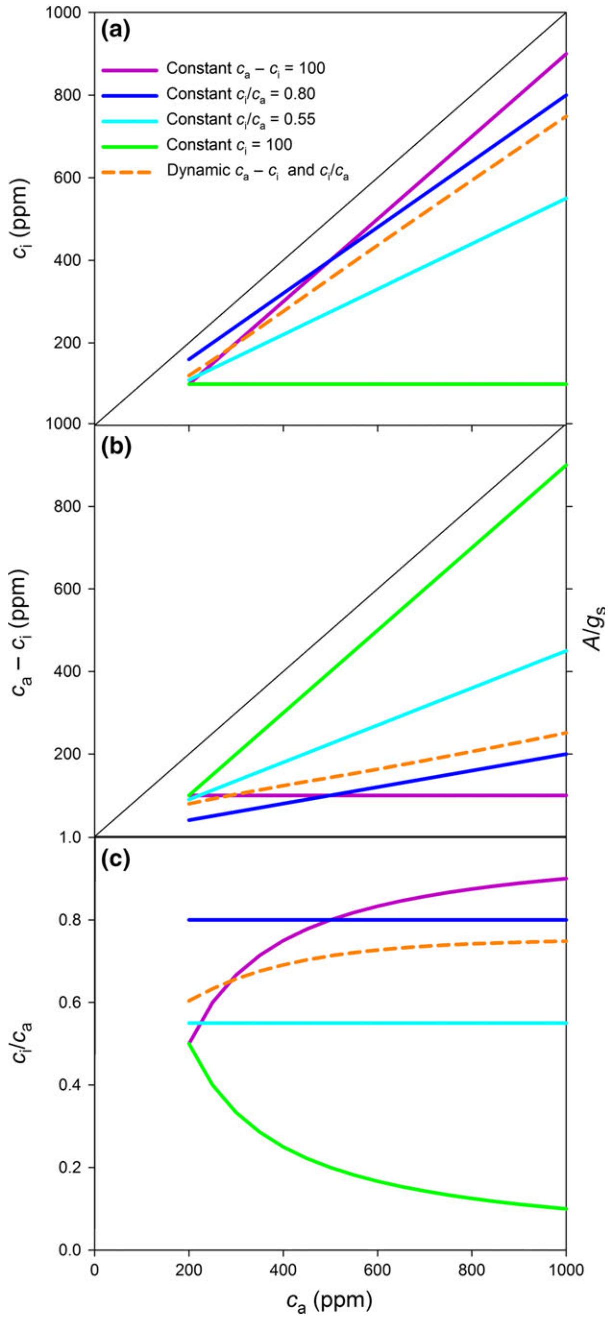

Fig. 1 Selected leaf gas-exchange regulation strategies plotted as leaf internal [CO_2] (c_i) vs. ambient [CO_2] (c_a) (Panel a), $c_a - c_i$ (Panel b) and c_i/c_a (Panel c). The thin solid line represents 1 : 1 scaling. The second y-axis representing variation in A/g_s in Panel (b) follows from Eqn (1), where $A/g_s = c_a - c_i$.

ited responses that were most consistent with a constant c_i/c_a (Wong *et al.*, 1979; Saurer *et al.*, 2004; Ward *et al.*, 2005; Bonal *et al.*, 2011; Franks *et al.*, 2013). A previous meta-analysis of free air CO_2 enrichment (eCO_2) experiments from C_3 and C_4 grasses, crop species and only four woody plants found no significant alteration of c_i/c_a (Ainsworth & Long, 2005), which supports the notion that stomatal regulation may result in species-specific, homeostasis of c_i/c_a values (c.f. Fig. 1; blue lines). Leaf gas-exchange of trees might respond differently to varying c_a than in other growth forms because their leaves are displayed at greater heights, leading to reduced c_i via height-related reductions in water potentials and thus sufficient turgor to maintain opening of stomata (Woodruff *et al.*, 2010). In conifers, for example, a constant $c_a - c_i$ was observed after controlling for such a height effect, but a constant c_i/c_a was observed when height was not considered (Marshall & Monserud, 1996; Monserud & Marshall, 2001). As Ehleringer & Cerling (1995) noted, and the array of responses reviewed above suggests, important differences exist among species and environmental settings. This calls for additional analyses that integrate results from both paleo and CO_2 enrichment (eCO_2) field studies to yield a better understanding of overall strategies for leaf gas-exchange regulation in response to changing c_a.

Alternatives to homeostatic leaf gas-exchange strategies can occur when both c_i/c_a and $c_a - c_i$ show nonlinear behavior across a wide range of c_a (Fig. 1; dashed orange line). This strategy of leaf gas-exchange regulation would be expected to occur following the hypothesis that plants optimize their anatomy and physiological function 'so that the total loss of water during a day is a minimum for the total amount of C taken up' (Cowan & Farquhar, 1977). Minimizing the ratio of rate changes in transpiration to assimilation (dE/dA) suggests stomata should simultaneously maximize C gain and minimize water loss. To be conserved as a strategy, stomatal optimization should operate at time-scales longer than a single day. Indeed, Cowan (1982) built on previous theory to show that, over time periods relevant to the development of significant soil moisture deficits, plants should converge on an optimal level dE/dA that depends on growth rates, mortality rates and competition for water. The same argument should hold for longer-term variation in c_a as it influences dE/dA. Empirical models of stomatal regulation have been recently unified with the Cowan & Farquhar (1977) concept of optimal stomatal behavior, including stomatal responses to c_a (Medlyn *et al.*, 2011; Héroult *et al.*, 2013; Lin *et al.*, 2015). This work has demonstrated that, across a wide range of species, g_s can be closely predicted as follows:

$$g_s \cong g_0 + \left(1 + \frac{g_1}{\sqrt{D}}\right)\frac{A}{c_a}, \qquad (3)$$

where g_0 is the stomatal conductance when A is zero, g_1 is a fitted slope parameter representing stomatal optimization and D is water vapor pressure deficit. For C_3

species, both theory and empirical evidence indicate that at low c_a (as it constrains c_i), A is limited by the carboxylation rate of Ribulose-bisphosphate carboxylase/oxygenase (Rubisco) and will rise steadily with c_a and then reach an asymptote as c_a passes 400 ppm and approaches 1000 ppm, corresponding to A being limited by the amount of Ribulose-1, 5-bisphosphate (Farquhar *et al.*, 1980; Wullschleger, 1993; Long & Bernacchi, 2003). The near-asymptotic phase in A at high c_a would result from photosynthesis being saturated as leaf N concentrations are diluted by CO_2-induced growth and/or as N availability becomes increasingly limited. Likewise, at high c_a, g_s may eventually reach a species-specific minimum. In combination these effects will cause the increases in $c_a - c_i$ and c_i/c_a to slow and eventually approach asymptotic values (Fig. 1; dashed orange line). Consequently, as suggested by both theory and empirical evidence, increases in c_a should result in woody plants regulating leaf gas-exchange along a continuum of $c_a - c_i$ and c_i/c_a that minimizes water loss for a given amount of C gain and therefore increasingly minimizes the likelihood of exposure to drought stress.

Plant tissue Δ can be used to calculate c_i (see Materials and methods), thereby shedding light on leaf gas-exchange strategies integrated across entire growing seasons (Francey & Farquhar, 1982; Ehleringer, 1993; Marshall & Zhang, 1994; Dawson *et al.*, 2002; McCarroll & Loader, 2004; Brooks & Coulombe, 2009). Some studies have exploited this approach to foster a broader synthesis of leaf gas-exchange responses to CO_2 (Ainsworth & Long, 2005; Battipaglia *et al.*, 2013; Becklin *et al.*, 2014), but most research has been conducted on a species by species basis. Here, we expand the scope of inference by examining Δ values in leaves and wood of many taxa growing in native soil over an evolutionary significant range of c_a from paleo to eCO2 conditions (Table 1). To our knowledge, this constitutes the first attempt to investigate *in situ* plant responses spanning c_a of approximately 200 to 380 ppm for paleo and modern studies and 370 to 700 ppm for eCO_2 experiments. Our particular approach was designed to evaluate the hypothesis of whether woody plants primarily regulate leaf gas-exchange toward any of three homeostatic strategies (constant c_i, $c_a - c_i$, or c_i/c_a), or whether they shift along a continuum of c_i, $c_a - c_i$, and c_i/c_a values that are consistent with minimizing water loss per unit C gain at low c_a, and an enhanced avoidance of drought stress at high c_a.

Materials and methods

We gleaned $\delta^{13}C$ and/or Δ values from leaves and wood in the published literature that included trees growing under low c_a (paleo), modern ambient c_a, and high c_a associated with eCO_2. Data from tables were utilized directly and data from figures were digitized by using ImageJ software (http://imagej.nih.gov/ij/). In some cases, the authors were contacted to obtain raw published and unpublished data (Table 1). Any $\delta^{13}C$ data from nonphotosynthetic tissues were corrected to that expected from leaves by subtracting 1.9‰ (Badeck *et al.*, 2005). Data set characteristics and sources are listed in Table 1.

Carbon isotope discrimination and estimation of c_i

For all studies surveyed, $\delta^{13}C$ data were expressed relative to the VPDB standard in ‰. For studies reporting $\delta^{13}C$, values were converted to Δ, following Farquhar (1983):

$$\Delta = \frac{\delta^{13}C_{air} - \delta^{13}C_{plant}}{1 + \delta^{13}C_{plant}}. \quad (4)$$

Δ values were then converted into c_i/c_a ratios following Farquhar *et al.* (1989):

$$\Delta = a + (b - a)\left(\frac{c_i}{c_a}\right), \quad (5)$$

where a is the fractionation from diffusion through the stomata (4.4‰), and b is the fractionation due to carboxylation by Rubisco (~27‰). $\delta^{13}C_{plant}$ is ultimately related to chloroplast [CO_2], but without detailed knowledge of how mesophyll conductance (g_m) may respond to changes in c_a across diverse taxa and growing conditions, the use of c_i is the only feasible approximation for this type of study (Cernusak *et al.*, 2013; Franks *et al.*, 2013). Equation (5) can be rearranged to calculate c_i if c_a and $\delta\ ^{13}C_{air}$ at the time of C fixation are reasonably well-constrained. For eCO_2 studies, published c_a and $\delta^{13}C_{air}$ values were used. For each eCO_2 level, the ambient c_a and $\delta^{13}C_{air}$ as well as the targeted CO_2 enrichment rate and the fossil fuel-sourced $\delta^{13}C_{air}$ signal together determined c_a and $\delta^{13}C_{air}$ taken up by the trees. For other paleo and modern studies, we obtained annual values of c_a and $\delta\ ^{13}C_{air}$ from 1850 to 2003 from McCarroll & Loader (2004). For 2004 to 2012 we used the records from Mauna Loa, Hawaii (http://cdiac.ornl.gov/). Values prior to 1850 were estimated from a Loess smoothing curve fitted to paleo c_a and/or $\delta\ ^{13}C_{air}$ estimates from ice cores (Leuenberger *et al.*, 1992; Indermühle *et al.*, 1998; Smith *et al.*, 1999; Elsig *et al.*, 2009). Radiocarbon dates from published paleo vegetation and ice core studies were used (i.e. not recalibrated). This likely introduced additional variation in the results but it should be very small compared to the responses obtained.

Modeling of tree-ring ^{13}C signals in CO_2 enrichment experiments

Step changes in c_a and isotopic depletion of $\delta^{13}C_{air}$ during eCO_2 could provide a powerful tracer for newly assimilated C. However, the appearance of the $\delta^{13}C$ tracer can apparently lag eCO_2 treatment when a proportion of stem growth is derived from C fixed in previous years or when environmental

Table 1 Characteristics of data used in analyses of leaf gas-exchange regulation strategies inferred from plant $\delta^{13}C$

Taxa	Data type	Min [CO_2]	Max [CO_2]	Slope of c_i vs. c_a	Intercept of c_i vs. c_a	Notes and data sources
Abies concolor	Holocene	266	388	1.26	−180.5	12
Juniperus coahuilensis	Holocene	264	388	0.59	−25.7	34, 36
Juniperus coahuilensis	Transition	238	361	0.57	−17.3	34, 36
Juniperus coahuilensis	Glacial	186	361	0.6	−29.4	34, 36
Juniperus communis	Glacial	238	388	0.71	−30.8	12
Juniperus monosperma	Holocene	262	361	0.67	−38.7	16, 17, 34, 36
Juniperus osteosperma	Holocene	266	388	0.57	−8.1	12, 16, 17, 34, 36, 40
Juniperus spp.	Glacial	190	351	0.83	−28.5	39
Nothofagus solandri	Glacial	193	363	0.86	−43.1	1, 33
Nothofagus solandri	Glacial	193	363	1.03	−72.4	2, 33
Phyllocladus alpinus	Glacial	190	370	0.63	12.7	32
Picea glauca	Holocene	259	369	1	−113.3	19, 30
Picea glauca	Transition	238	369	0.77	−30.1	19, 30
Picea glauca	Glacial	187	369	0.76	−25.7	19, 30
Pinus edulis	Holocene	260	388	0.74	−68.9	18, 23, 24
Pinus edulis	Transition	228	369	0.68	−47.3	18, 23, 24
Pinus edulis	Glacial	186	369	0.63	−29.8	18, 23, 24
Pinus flexilis	Holocene	260	388	0.84	−70.9	12, 23, 35
Pinus flexilis	Transition	228	388	0.88	−83.8	12, 23, 35
Pinus flexilis	Glacial	186	388	0.84	−69.4	12, 23, 35
Pinus longaeva	Glacial	192	388	0.86	−88.6	12
Pinus monophylla	Holocene	192	388	0.78	0.8	12, 16, 34
Pinus ponderosa	Holocene	266	388	0.89	−79.6	12
Pinus sylvestris	Holocene	260	354	0.81	2.4	14
Pinus sylvestris	Transition	261	363	0.55	69.8	11, 22, 28
Pseudotsuga menziessii	Glacial	211	370	0.7	4.9	1, 8, 15, 25
Quercus macrocarpa	Holocene	259	380	0.86	−14.79	37, 38
Quercus macrocarpa	Transition	238	380	0.86	−14.46	37, 38
Salix herbacea	Holocene	259	350	1.03	−97.2	13, 31
Salix herbacea	Transition	238	350	0.98	−80.8	13, 31
Salix herbacea	Glacial	190	350	0.98	−83	13, 31
Acer saccharum	eCO_2	372	552	0.92	−50.4	29
Alnus glutinosa	eCO_2	382	580	0.77	12.4	27
Betula papyrifera	eCO_2	372	552	1.22	−153.5	29
Betula pendula	eCO_2	382	580	0.79	14.5	27
Fagus sylvatica	eCO_2	361	572	0.34	161	9
Fagus sylvatica	eCO_2	377	580	0.77	22.8	27
Liquidambar styraciflua	eCO_2	351	552	0.87	−42	10
Liquidambar styraciflua	eCO_2	357	575	1	−87.3	3, 10
Picea abies	eCO_2	359	700	1.08	−113.4	20
Picea abies	eCO_2	359	700	0.89	−35.3	4, 20
Picea abies	eCO_2	377	524	0.66	24.7	21
Pinus taeda	eCO_2	343	591	0.32	95	10
Pinus taeda	eCO_2	343	591	0.25	120.2	4, 10, 41
Populus alba	eCO_2	370	578	1.06	−109.1	10
Populus nigra	eCO_2	370	578	0.9	−30.6	10
Populus tremuloides	eCO_2	372	552	0.95	−59.6	29
Populus tremuloides	eCO_2	372	552	0.95	−64.8	5, 29
Populus tremuloides	eCO_2	372	552	1.05	−107.4	6, 29
Populus x euramericana	eCO_2	370	578	0.82	12.8	10

conditions from previous years affect the structure of leaves, xylem, and plant canopies (Van de Water *et al.*, 1994; Monserud & Marshall, 2001; Reid *et al.*, 2003; Lammertsma *et al.*, 2011; Franks *et al.*, 2013; Tor-ngern *et al.*, 2015). For example, angiosperm trees in a mature, closed-canopied temperate forest exposed to eCO_2 showed significant lagging responses

Table 1 (continued)

Taxa	Data type	Min [CO_2]	Max [CO_2]	Slope of c_i vs. c_a	Intercept of c_i vs. c_a	Notes and data sources
Quercus ilex	eCO_2	352	569	1.03	−91.2	7, 26
Quercus petraea	eCO_2	361	581	0.77	1.2	9
Tilia platyphyllos	eCO_2	361	595	0.47	105.7	9

Slopes and intercept values are from linear regressions fit to c_i and c_a. All data assume mixing among old and new C pools within CO_2 enrichment studies (see Materials and methods and Figs 2 and 3). The 'Data type' column gives the age class for the oldest period represented for paleo studies while CO_2 enrichment studies are listed as eCO_2.

[1]Shade-leaf morphology, [2]sunlit-leaf morphology, [3]grew in understory with *Pinus* overstory, [4]nitrogen-fertilized, [5]growing with *Betula papyrifera*, [6]growing with *Acer saccharum*, [7]spatial gradient in c_a near CO_2 vent, [8]Anderson *et al.* (2008), [9]Bader *et al.* (2013), [10]Battipaglia *et al.* (2013), [11]Becker *et al.* (1991), [12]Becklin *et al.* (2014), [13]Beerling *et al.* (1993), [14]Boettger *et al.* (2003), [15]Brooks & Mitchell (2011), [16]DeLucia & Schlesinger (1991), [17]Leavitt & Long (1982), [18]Leavitt & Long (1983), [19]Leavitt *et al.* (2006), [20]Marshall & Linder (2013), [21]Mildner *et al.* (2014), [22]Palmroth *et al.* (1999), [23]Pedicino *et al.* (2002), [24]Pendall *et al.* (1999), [25]Saffell *et al.* (2014), [26]Saurer *et al.* (2003), [27]A.R. Smith, unpublished data, [28]Szczepanek *et al.* (2006), [29]A.F. Talhelm & K.S. Pregitzer, unpublished data, [30]Tardif *et al.* (2008), [31]Turney *et al.* (1997), [32]Turney *et al.* (1999), [33]Turney *et al.* (2002), [34]P.K. Van de Water, J.L. Betancourt & S.W. Leavitt, unpublished data, [35]Van de Water *et al.* (1994), [36]Van de Water *et al.* (2002), [37]Voelker *et al.* (2014), [38]S.L. Voelker, J.R. Brooks, F.C. Meinzer, R.P. Guyette & M.C. Stambaugh, unpublished data, [39]Ward *et al.* (2005), [40]Williams & Ehleringer (1996), [41]L. Wingate, D. Bert, H.J. Plumpton, J-C. Domec & J. Ogée, unpublished data.

despite these species being deciduous, thus all leaves were formed during elevated c_a conditions (Keel *et al*., 2006). A different study of an evergreen gymnosperm species in an open-canopied boreal forest, however, found no lagging response, indicative of little if any lagged response (Marshall & Linder, 2013). For more accurate interpretations of tree-ring Δ and c_i responses to step changes in c_a, we employ a flexible approach to account for lagging responses. To do this we empirically fitted functions to c_a and $\delta^{13}C_{air}$ before calculating Δ and c_i from eCO_2 studies. Interannual values of c_a were always used in these analyses. For some studies only an average value of $\delta^{13}C_{air}$ was measured and used to calculate Δ, but when measurements were available, we used interannual variation in $\delta^{13}C_{air}$. This approach assumes the lagged response was due to variation in the amount of stored non-structural C (i.e. old C) used for stem radial growth among species and over time since the step change in c_a. Although this approach does not address the various responsible mechanisms, it should adequately account for the observed lags in Δ responses. These processes were represented by a function F, where F represents the weighted fraction of newly assimilated C (i.e. new C), expressed as a function of time after the step change in c_a. Hence, $1 - F$ represents the fraction of old C utilized for growth. For this model, old C was defined as having c_a and $\delta^{13}C_{air}$ signals fixed equally across the three years prior to the step change in CO_2 fumigation. These functions F were fitted to maximize the explained variance (R^2) of c_i (estimated from $\delta^{13}C$) plotted vs. c_a (Fig. 2). For the 'web-free-air CO_2 enrichment' (FACE) data of Bader *et al.* (2013) we also included post-treatment data by assuming stored C had the treatment c_a and $\delta^{13}C_{air}$ signals and new C had the ambient signature. Additionally, for both web-FACE data sets (Bader *et al*., 2013; Mildner *et al*., 2014), we adjusted each c_a and $\delta^{13}C_{air}$ signal to include 5% ambient signals during treatment years, because the subcanopy zone was not CO_2-enriched (Keel *et al*., 2006). These effects were modeled in reverse for post-treatment data from Bader *et al.* (2013) to reflect an assumption that air depleted in ^{13}C would have been slowly released from soils and fixed by the lower canopy.

For most species it was found that an exponential model fitted the data best using the function $F = 1 - e^{-ax}$, where x is the number of years after the step change in ambient [CO_2] and a is a parameter defining the curvature (Fig. 2). One notable exception was understory *Liquidambar styraciflua* growing at the Duke FACE site, for which a sigmoidal function was fitted as $F = a/(a + e^{(-(x - b)/c)})$ where parameters a, b, and c were 1.0, 5.0 and 0.7 (Fig. 2). The other exception was Marshall & Linder (2013), where boreal *Picea abies* showed no evidence of using old C for stem growth or CO_2-induced modifications to structural characteristics of the trees that could have induced lagged Δ responses (Fig. 2). For statistical analyses (see below) we only analyzed data from the scenario that assumed that tree-ring $\delta^{13}C$ from eCO_2 studies reflected lagging effects of low c_a during pretreatment conditions and/or incorporation of old C.

Statistical analyses

For eCO_2 studies including N fertilization, the CO_2 and CO_2 + N treatments were considered separately. Data sets from eCO_2 experiments were also considered separately in the two cases where the same species was sampled, but at different locations and under different climatic and soil conditions. For paleo studies, data were grouped according to calendar ages, from calibrated ^{14}C ages reported for each study: glacial (older than 14 700 years BP), transition (14 700–11 500 years BP) and Holocene (younger than 11 500 years BP). To establish slopes of c_i vs. c_a, each age group from a species was compared to the same modern c_i and c_a data. Like data from eCO_2 experiments, paleo studies of the same species but differing by region were treated as separate data sets. For one paleo study, sunlit and shaded leaves were identified by their morphological characteristics and these were treated separately. Details for each data set are given in Table 1.

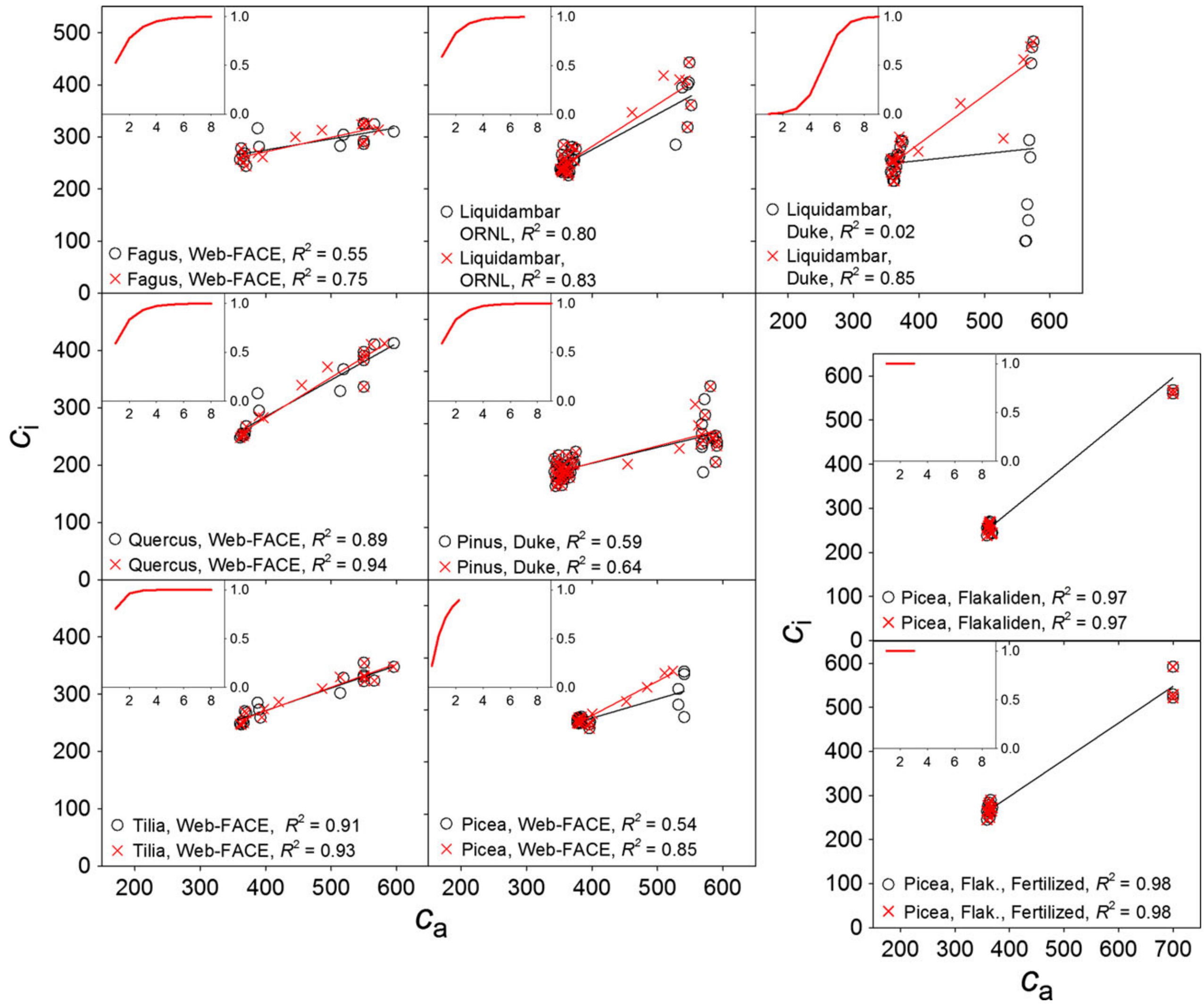

Fig. 2 Leaf internal $[CO_2]$ (c_i) plotted vs. ambient $[CO_2]$ (c_a) for free-air CO_2 enrichment (FACE) studies across two scenarios. The first or standard scenario (open circles) are the measured data, and assumes that tree-ring growth displayed no lagging effects on leaf, xylem and canopy structure associated with a previous c_a so that trees used only C assimilated during the current year (i.e. $F = 1$). The second scenario (red crosses) are the predicted c_i values, assuming that there were lagging effects on tree structure and that trees used some fraction (F) of current year C and some proportion of stored, old C ($1 - F$) that was fixed before the initiation of CO_2 fumigation, where F is represented on the Y-axis of the inset graphs and the X-axis are the years after the initiation of CO_2 fumigation. Note that F varies widely in magnitude and timing and that *Picea* growing at Flakaliden in northern Sweden (offset in lower right) displayed no evidence for use of old C for current year growth.

To compare among data sets that often differed in what was considered a treatment unit we used ordinary least squares linear regression to fit c_i, inferred from Δ, to c_a (SigmaPlot version 12.5; Systat Software Inc., San Jose, CA, USA). We used the linear regression-predicted c_i values for the maximum and minimum c_a represented by the data. More specifically, each data set was defined by two separate data points for all analyses. Mixed effects models (SAS version 9.2; SAS Institute, Inc., Cary, NC, USA) were used to determine if c_i/c_a and $c_a - c_i$ differed between angiosperms and gymnosperms (fixed effect) and if there was an interaction with c_a (specified as a random effect).

For linear regression analyses we plotted the raw c_i/c_a and $c_a - c_i$ values as they scaled with c_a as well as standardized values. To standardize values, we first calculated the change in c_i/c_a and $c_a - c_i$ predicted for the minimum and maximum c_a. To these data, we fitted a linear regression to all paleo studies. The absolute value of the linear regression-predicted change in c_i/c_a and $c_a - c_i$ at 190 ppm was added to each paleo datum, where 190 ppm represents an approximate average c_a for past glacial conditions. The same process was used for the eCO_2 data except the linear regression-predicted c_i/c_a and $c_a - c_i$ values at the average low c_a value for eCO2 studies of 372 ppm were standardized to be equal to the linear regression-predicted c_i/c_a and $c_a - c_i$ at the average high c_a value for paleo studies, which was also 372 ppm.

Results

Our meta-analyses of leaf-gas exchange responses to c_a in woody species are based on 31 paleo data sets and 22 eCO2 data sets spanning at least 100 ppm in c_a (Table 1). For estimating c_i responses to c_a, scenarios that either did or did not account for lagging responses of tree-ring Δ to step changes in c_a of eCO_2 experiments indicated that analyses using either scenario would give similar results (Fig. 3). Nevertheless, for simplicity, further analyses of responses to c_a use data only from the scenario in which the lagged responses and use of old C for growth were considered (see Materials and methods).

A strong interactive effect between c_a and lineage was found for c_i/c_a (i.e. gymnosperm vs. angiosperm) (ANOVA, $F = 9.23$, df = 102, $P < 0.001$). Angiosperms had a significantly higher c_i/c_a, by 0.124, compared to gymnosperms (least squares means, $t = 6.29$, $P < 0.001$), confirming previously reported trends in Δ across a wide array of modern tree species (Marshall & Zhang, 1994; Diefendorf *et al.*, 2010). Positive and significant correlations between c_i/c_a and c_a existed for angiosperms (Fig. 4a; $c_i/c_a = 0.00031c_a + 0.649$, $R^2 = 0.26$, $P < 0.001$) and gymnosperms (Fig. 4a; $c_i/c_a = 0.00038c_a + 0.502$, $R^2 = 0.11$, $P < 0.001$) despite species and environments contributing much of the variation to these comparisons.

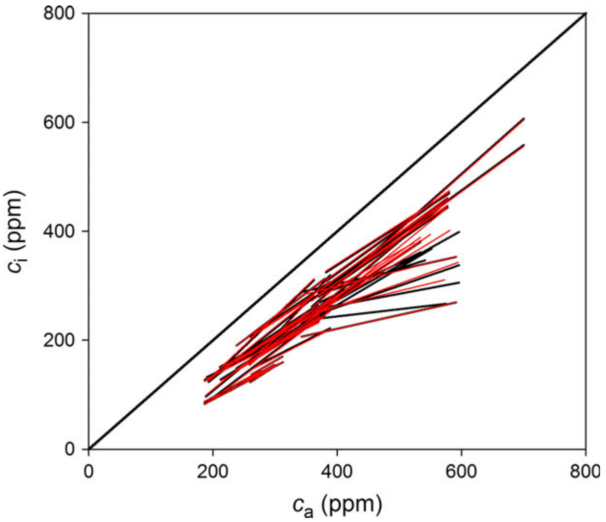

Fig. 3 Leaf internal $[CO_2]$ (c_i) plotted vs. ambient $[CO_2]$ (c_a) for paleo-to modern and CO_2 enrichment studies for two scenarios. The standard scenario, assuming no lagging effects of previous c_a (i.e. no old C-utilization) in CO_2 enrichment studies is shown as black lines. The scenario assuming that there were lagging effects of previous c_a (i.e. old C-utilization) in eCO_2 studies uses predicted c_i values and is shown as thin red lines. All subsequent analyses used the latter scenario, assuming a mixture of old and new C-utilization. The central black line indicates the 1 : 1 scaling.

A strong interactive effect between c_a and lineage also was found for $c_a - c_i$ (ANOVA, $F = 14.60$, df = 102, $P < 0.001$). Angiosperm $c_a - c_i$ was significantly lower by about 45.5 ppm compared to gymnosperms (least square means, $t = -5.69$, $P < 0.001$), as driven by the generally higher stomatal conductance of the former. Angiosperms exhibited a significant increase in $c_a - c_i$ with c_a (Fig. 4b; $c_a - c_i = 0.119c_a + 37.55$, $R^2 = 0.21$, $P < 0.001$), as did gymnosperms (Fig. 4b; $c_a - c_i = 0.220c_a + 46.37$, $R^2 = 0.25$, $P < 0.001$), indicating that for every 10 ppm increase in c_a, the diffusion gradient between the atmosphere and the leaf intercellular spaces increased between 1.2 to 2.2 ppm.

The c_i/c_a and $c_a - c_i$ data were then recast as differences from that observed at a c_a equal to 190 ppm, an approximate average of the minimum c_a in our data set, which occurred during the previous glacial period. This standardization (see Materials and methods) accounted for offsets in average c_i/c_a or $c_a - c_i$ among species, lineages or any potential bias in environmental conditions among lineages or among paleo vs. eCO_2 studies. The data also were combined across all taxa because of the similarity among angiosperms gymnosperms in their trends in c_i/c_a and $c_a - c_i$ as they scaled with c_a. These differences in c_i/c_a and $c_a - c_i$ were significantly correlated with c_a, (Fig. 4c, d; c_i/c_a difference $= -0.3974 + 0.5163(e^{(-0.0076c_a)})$, $R^2 = 0.43$, $P < 0.001$, $c_a - c_i$ difference $= -49.0039 + 306.1990(e^{(-0.0009c_a)})$, $R^2 = 0.57$, $P < 0.001$). A linear fit to $c_a - c_i$ differences, as they scaled with c_a, described slightly more of the variation in the data compared to the relationship given above. However, a linear response of $c_a - c_i$ differences cannot be reconciled with the nonlinear and saturating response of c_i/c_a difference scaling with c_a.

Discussion

Leaf gas-exchange regulation plays a pivotal role in determining canopy to atmospheric fluxes of CO_2 and water as well as energy balance and biogeochemical cycling. To accurately predict these basic ecosystem properties, robust understanding of how leaf gas-exchange will respond to rising c_a is essential. Three homeostatic leaf gas-exchange regulation strategies have been posited for C_3 plants including a constant c_i, $c_a - c_i$ or c_i/c_a. Altogether, our analyses provide powerful evidence that angiosperms and gymnosperms share a common response to increasing c_a, but one that does not strictly follow any of the three proposed homeostatic leaf gas-exchange responses. If one type of

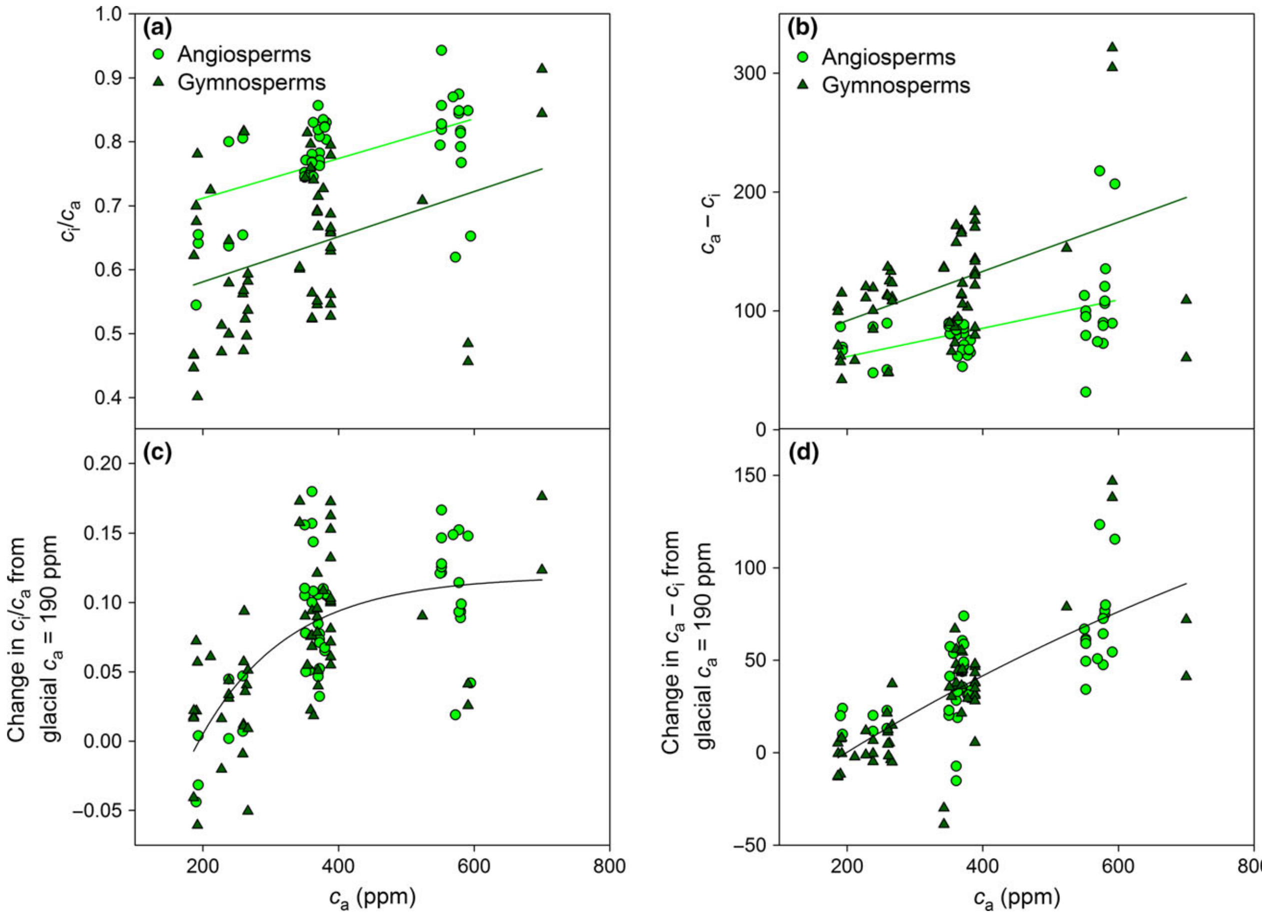

Fig. 4 Scaling with c_a for c_i/c_a or $c_a - c_i$ in angiosperms or gymnosperms (Panels a and b). Scaling with c_a for changes in c_i/c_a or $c_a - c_i$ set relative to a glacial c_a of 190 ppm with exponential relationships fit to data from angiosperms and gymnosperm together (Panels c and d). Plotted regression lines were significant ($P < 0.001$). Data from CO_2 enrichment studies assume a scenario whereby trees incorporate lagging effects of previous c_a and can utilize various proportions of new and old C for growth (see red crosses in Fig. 2 and thin red lines in Fig. 3).

homeostasis were predominately conserved across species, it would greatly simplify predictions of leaf gas-exchange responses to rising c_a. However, predictions of leaf gas-exchange are complicated by a number of factors including the biochemical constraints ultimately imposed by water and nutrient limitations that scale from the leaf to canopy levels, resulting in diminishing returns for A as c_a progresses ever higher (Oren *et al.*, 2001; Warren *et al.*, 2015). Theory and empirical research have helped elucidate controls over a number of these factors influencing leaf gas-exchange (Cowan & Farquhar, 1977; Cowan, 1982; Medlyn *et al.*, 2011). Through changes in parameter g_1 in Eqn (3), which will depend on the proportionality of A to c_a and associated changes in g_s, it can be predicted that leaf gas-exchange should not be regulated homeostatically with changing c_a, but maintain lower c_i/c_a and $c_a - c_i$ at low c_a and higher c_i/c_a and $c_a - c_i$ at progressively greater c_a (see dashed orange line in Fig. 1). Our data from individual species and analyses across species clearly reject the subhypothesis that woody plants primarily maintain a constant c_i across large changes in c_a. These results differ markedly from the constant c_i pattern described by (Keenan *et al.*, 2013), which further emphasizes the need for more research to explain why data streams from eddy flux monitoring sites suggest such a tremendous increase in water use efficiency (Medlyn & De Kauwe, 2013). The subhypotheses of a strategy of constant c_i/c_a, or $c_a - c_i$ also can be rejected when considering our analyses across species and a large range in c_a (Fig. 4). Rather, across c_a of 200–700 ppm, the evidence points toward leaf gas-exchange of woody plants having the capacity to respond dynamically to c_a by minimizing the increase in E for a given increase of A. At low c_a, woody plants employ a strategy using low gain ratios, termed λ by Cowan & Farquhar (1977), whereas at high c_a plants increase λ. This transition between strategies means that at low c_a, woody plants emphasize greater C gain by risking greater likelihood of incurring drought stress because an incremental increase in g_s leads to a relatively large increase in *A*. At high c_a, woody plants utilize a more conservative leaf gas exchange strategy that emphasizes drought avoidance because the photosynthetic machinery is closer to saturation and an incremental increase in g_s would lead to a relatively small increase in *A*.

Woody plants exhibit c_a-driven plasticity in above and below-ground functional traits and allocation that can contribute to changes in canopy characteristics and hydraulic architecture, which in turn contribute to apparently divergent leaf gas-exchange responses among individual species. However, an overall strategy of leaf gas-exchange response emerges when viewed across species and a large gradient in c_a (Fig. 4c, d). An example of this trait plasticity is the stimulated leaf area production by eCO_2 in angiosperm and gymnosperm trees in some studies (Tor-ngern *et al.*, 2015), but no detectable effect or reduced canopy leaf area in other studies of eCO_2 in other angiosperms and gymnosperms undergoing eCO_2 (Körner & Arnone, 1992; Hättenshwiler & Körner, 1996; Bader *et al.*, 2013). Likewise, many species display changes in stomatal size and density that are tied to variation in c_a but this effect has not been found in all species (Van de Water *et al.*, 1994; Reid *et al.*, 2003; Lammertsma *et al.*, 2011; Franks *et al.*, 2013; Becklin *et al.*, 2014). Five species of Pinaceae that grew during the last glacial or deglacial (i.e. glacial to interglacial transition) periods maintained relatively higher assimilation rates at low c_a by increasing leaf N (i.e. invested in greater leaf N and Rubisco), but did not modify stomatal density compared to modern trees of the same species and region (Becklin *et al.*, 2014). By contrast, the same study found two species in the Cupressaceae exhibited no change in leaf nitrogen compared to modern trees of the same species. These differences in trait plasticity may be related to *Pinus* and *Juniperus* being consistently near the isohydric and anisohydric ends of the spectrum of stomatal regulation responses to drought, respectively. In isohydric species, stomatal closure during soil drying avoids significant xylem embolism by keeping leaf water potential from dropping below a species-specific minimum value. In contrast, stomata of more embolism-resistant anisohydric species do not act to regulate minimum water potential at a specific value, but instead allow minimum water potential to decline as the soil dries (McDowell *et al.*, 2008; Brodribb *et al.*, 2014; Meinzer *et al.*, 2014; Garcia-Forner *et al.*, 2015). As a result, anisohydric species like *Juniperus* may not be under strong selective pressure to increase photosynthetic capacity under low c_a because they can often withstand additional drought stress induced by increasing g_s, whereas isohydric species like *Pinus* may be more likely to modify their gas-exchange strategy by increasing photosynthetic capacity at low c_a to maximize C uptake during wet periods to help avoid C starvation during drought stress.

Mesophyll conductance (g_m) is yet another trait that can differ greatly among species and environments and would have influenced the Δ and associated leaf gas-exchange responses we report here. Over the short-term, increases in c_i generally cause g_m to decrease but there has been no consistent response of g_m demonstrated in long-term eCO_2 studies (Singsaas *et al.*, 2003; Bernacchi *et al.*, 2005; Flexas *et al.*, 2007, 2012; Vrábl *et al.*, 2009; Crous *et al.*, 2013). Further clouding knowledge of g_m responses to c_a are ongoing debates about how various methods to estimate g_m may impose artifacts (Tholen *et al.*, 2012; Gu & Sun, 2014). Until there is consensus on whether or not long-term changes in c_a modify g_m and the potential for phylogenetic differences in this response, we cannot determine how a g_m response to c_a may have contributed bias, if any, to the trends in c_i/c_a and $c_a - c_i$, or the convergence of these same responses among angiosperms vs. gymnosperms (Fig. 4c, d).

Paleo studies and eCO_2 experiments both have their own limitations, and study designs in each can contribute to inaccuracies in assessing leaf gas-exchange responses. For eCO_2 experiments, step-changes in c_a can cause difficulty in ascertaining long-term, steady-state leaf gas-exchange responses from covarying effects such as tree height or from the lagging effects of the previous, low c_a environment. Inferences from paleo studies can be limited by unknown past climates in which it is difficult to ascertain what modern climate provides analogous conditions. However, this variability should have imparted little directional bias to the results reported here because conditions across the wide array of paleo vs. modern comparisons made here are unlikely to have shown a strong bias toward wetter or drier conditions over time. Therefore, although combining data across study types may have introduced two types of unique variability, the much more comprehensive overall data set made available by combining across studies should yield more robust inferences for leaf gas-exchange responses to c_a.

It is also possible that species sampled over thousands of years in the paleo studies had the opportunity to display both phenotypic and adaptive changes in traits controlling leaf gas-exchange regulation as c_a slowly changed, whereas eCO_2-induced changes in traits would be constrained to the range of phenotypic plasticity alone. Therefore, evolutionary processes could have contributed to the larger c_i/c_a response in paleo studies compared to eCO_2 experiments. If evolutionary processes could be added to the treatment effects of eCO_2 experiments, it is possible that the response of c_i/c_a to c_a (i.e. Fig. 4c) would have been less curvilinear and had a greater overall effect at high c_a. Nonetheless, c_a has been rising so quickly that evolutionary processes may have had little chance to impose significant effects on leaf gas-exchange considering that many trees have generation times that can exceed 100 years. As such, c_i/c_a and $c_a - c_i$ responses to c_a reported here (Fig. 4c, d) should be applicable to gas-exchange projected across the near-term as well as at longer term paleo scales.

Predicting that woody plants will maintain a constant c_i/c_a or a constant $c_a - c_i$ at very high c_a assumes that g_s will eventually be reduced in direct proportion to c_a as A becomes increasingly saturated. Selective pressures should result in these strategies being avoided because progressive reductions in g_s could eventually result from leaves developing very low stomatal densities, stomatal indices, or stomatal aperture sizes at high c_a (Reid *et al.*, 2003). This would cause lateral diffusion of CO_2 through the leaf mesophyll to become patchy and inefficient at meeting the demand within the chloroplasts distant from fewer or smaller stomatal pores, particularly under variable light conditions. Extremely low g_s could also increase the risk of thermal damage because, all else being equal, latent heat exchange would be reduced (Beerling & Berner, 2005). A recent study of Δ responses to eCO_2 in two herbaceous C_3 species has suggested that c_i/c_a could approach a value of one at very high c_a (Schubert & Jahren, 2012). Although this study investigated plant responses across c_a ranging up to 4000 ppm, there is no direct evidence of how c_i could increase at a faster rate than increases in c_a if c_a is the only environmental factor that were modified. Our analyses indicate, c_i/c_a and $c_a - c_i$ should approach an upper asymptote as conceptualized in Fig. 1 (i.e. dashed orange line) and shown in Fig. 4c, d.

The responses of woody plants to changes in c_a are complex and will take novel research approaches like the one employed here to accurately project responses of forests globally. Nonetheless, without a geo-engineered solution or human energy consumption shifting dramatically away from the current dependence on fossil fuels, a point will be reached at which c_a rises to levels that increasingly saturate A. This will be particularly important for species in which leaf nitrogen concentrations tend to decrease with increasing c_a (Becklin *et al.*, 2014), a phenomenon that was consistently observed across eCO_2 experiments (Feng *et al.*, 2015). Extremely high c_a conditions have not occurred over the last 2.588 million years of the Quaternary Period, but c_a has approached and even exceeded 1000 ppm for extended periods that span major shifts in the trajectories of global climate as well as major evolutionary advances in plants (Royer *et al.*, 2004; Beerling & Berner, 2005; Brodribb & Field, 2010; Diefendorf *et al.*, 2010; Kohn, 2010; Franks *et al.*, 2013). A return to these high c_a conditions would lead to important changes to biogeochemical cycling of most ecosystems (Schäfer *et al.*, 2002; Finzi *et al.*, 2007; Zak *et al.*, 2007; Drake *et al.*, 2011; Warren *et al.*, 2011; Bader *et al.*, 2013; De Kauwe *et al.*, 2013; Hungate *et al.*, 2013; Feng *et al.*, 2015; Tor-ngern *et al.*, 2015). Given the complex biogeochemical and species-specific responses of forests to rising c_a (Talhelm *et al.*, 2009; Smith *et al.*, 2013a,b) further research on a number of fronts will be necessary to improve regional projections of the impact of rising c_a on C storage and cycling within forests (De Kauwe *et al.*, 2014).

Our results indicate that woody plants do not demonstrate homeostatic leaf gas exchange responses to c_a by maintaining a constant c_i, $c_a - c_i$ or c_i/c_a. Rather, a dynamic leaf gas-exchange strategy is conserved across woody taxa that helps plants maximize C gain at low c_a and contributes to the avoidance of drought stress at high c_a (Fig. 4c, d). Within this overall trend we detected that leaf gas-exchange strategies were also characterized by strong interactive effects between c_a and angiosperm vs. gymnosperm lineages, suggesting additional research is needed to clarify whether there are important differences related to phylogeny or plant functional type and to help identify the functional traits and associated mechanisms involved. A second generation of eCO_2 experiments are either being constructed or are already operational, and this will help fill in some research gaps. However, if the approach developed here is to be revisited, more paired paleo vs. modern Δ studies from many more species are needed, particularly focused on angiosperms which were poorly represented at low c_a during glacial periods. Continued research efforts toward this end will make a strong contribution to constraining Earth systems modeling efforts that explicitly represent forest productivity, canopy-atmosphere interactions and associated biogeochemical cycling.

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