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Reading a CO_2 signal from fossil stomata

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Summary

The inverse relationship between atmospheric CO_2 and the stomatal index (proportion of epidermal cells that are stomata) of vascular land plant leaves has led to the use of fossil plant cuticles for determining ancient levels of CO_2. In contemporary plants the stomatal index repeatedly shows a lower sensitivity atmospheric CO_2 levels above 340 ppm in the short term. These observations demonstrate that the phenotypic response is nonlinear and may place constraints on estimating higher-than-present palaeo-CO_2 levels in this way. We review a range of evidence to investigate the nature of this nonlinearity. Our new data, from fossil *Ginkgo* cuticles, suggest that the genotypic response of fossil *Ginkgo* closely tracks the phenotypic response seen in CO_2 enrichment experiments. Reconstructed atmospheric CO_2 values from fossil *Ginkgo* cuticles compare well with the stomatal ratio method of obtaining a quantitative CO_2 signal from extinct fossil plants, and independent geochemical modelling studies of the long-term carbon cycle. Although there is self-consistency between palaeobiological and geochemical CO_2 estimates, it should be recognized that the nonlinear response is a limitation of the stomatal approach to estimating high palaeo-CO_2 levels.

Introduction

With remarkable perception, Arrhenius (1896) postulated that past variations in atmospheric CO_2 were responsible for the major changes in climatic conditions recorded by rocks and fossils, following his quantification of the greenhouse effect by CO_2 molecules (i.e. absorption of outgoing long-wave radiation from the Earth's surface). The proposal was formulated in further detail by Chamberlin (1898) who considered at length the regulation of CO_2 on geological time-scales in relation to source–sink behaviour. This fundamental concept underpins much of palaeoclimatalogy and identified early on the critical requirement for determining historical changes in the concentration of CO_2 in the atmosphere. For the late Quaternary (past 400 000 yr), this objective has been well achieved through studies of ice-core records of atmospheric CO_2 (Petit *et al.*, 1999) particularly in Antarctica where artefacts associated with chemical reactions in the ice are less likely to affect measured CO_2 levels (Fischer *et al.*, 1999; Monnin *et al.*, 2001). Coupled with isotopic measurements on the ice, analyses of ice cores have shown that CO_2 oscillated between 180 and 280 ppm in 100 000 years cycles, in phase with changes in temperature (Petit *et al.*, 1999; Shackleton, 2000). An interesting feature of the high resolution analyses of the millennial palaeoclimate records is that a change in air temperature can apparently occur quite rapidly without changes in CO_2, whereas the converse has not yet been seen to occur (Falkowski *et al.*, 2000).

For back in time, in the pre-Quaternary, alternative approaches are required for determining the past history of atmospheric CO_2 (Royer *et al.*, 2001a) because the world's oldest ice sheets only date back *c.* 500 000 yr. The approaches can be divided into two groups. One group is based on geochemical modelling of the carbon cycle at multimillion year time-scales and invokes volcanism and metamorphism to supply CO_2, and tectonic uplift and silicate rock weathering, accelerated by the biota, to remove it (Berner, 1994, 1997; Tajika, 1998; Berner & Kothavala, 2001; Wallmann, 2001). The other group is the proxy (indirect) geochemical and palaeobiological indicators of CO_2. Comparison of predicted CO_2 variations from carbon cycle modelling and the various proxies shows a good first-order agreement over the past 550 Myr (Crowley & Berner, 2001; Royer *et al.*, 2001a) with episodes of low CO_2 coinciding with evidence for continental glaciation (Crowley & Berner, 2001). In addition, calculated reductions in CO_2-related radiative forcing, as CO_2 levels declined from the Cretaceous onwards, correlate well with changes in oceanic temperature inferred from the deep-sea oxygen isotope ratios of foraminifera. This close correspondence suggests that, at least at a very coarse level, our confidence in the relationship between atmospheric CO_2 and global temperatures remains intact, over the 100 yr since it was proposed (Arrhenius, 1896; Chamberlin, 1898).

Most recently, a long-term (550 Myr) reconstruction of tropical sea surface temperatures (SSTs) has cast doubt on the link between climate and CO_2 at certain times during the Phanerozoic (Veizer *et al.*, 2000). Reconstructing SSTs from the oxygen isotope composition of tropical marine fossil organisms, Veizer *et al.* (2000) identified anomalously low values during the Mesozoic, a time when geochemical models and proxy evidence indicate atmospheric CO_2 concentrations were up to sixfold higher than they are now (Berner, 1997; Crowley & Berner, 2001; Royer *et al.*, 2001a). Radiative forcing by such palaeo-CO_2 concentrations is calculated to have been sufficient to raise global temperatures by 4–8°C, in agreement with the lack of evidence for substantial ice sheets at this time (Crowley & Berner, 2001). Reconciling these differences remains a major task and prompts the need to critically re-examine not only the interpretation of the oxygen isotopic measurements themselves but also the different approaches for reconstructing CO_2 in deep time.

Stomata as indicators of palaeo-CO_2 levels

In the context of the present Stomata 2001 meeting, this review focuses on the stomatal approach (Beerling & Chaloner, 1994; McElwain *et al.*, 1999; Rundgren & Beerling, 1999; Royer *et al.*, 2001b) to estimating palaeo-CO_2 levels using fossilized leaves of land plants. However, it should be recognized that three geochemical palaeo-CO_2 proxies based on the carbon isotopic composition of fossil soils (Cerling, 1991, 1992; Ekart *et al.*, 1999) and phytoplankton (Freeman & Hayes, 1992; Pagani *et al.*, 1999), and the boron isotope composition of planktonic foraminifera (Pearson & Palmer, 2000) exist in addition to this palaeobotanical one. The potential to detect changes in atmospheric CO_2 from fossil stomata derives from the original observations of Woodward (1987), who demonstrated that both stomatal density (number of stomata per unit area of leaf) and stomatal index (percentage of leaf epidermal cells that are stomata) were inversely related to atmospheric CO_2 level during leaf development. Although both density and index respond to CO_2, stomatal index is rather insensitive to changes in soil moisture supply, atmospheric humidity and temperature (Beerling, 1999) making it a more suitable indicator of palaeo-CO_2 changes. By comparison, stomatal density is quite susceptible to fluctuations in the growing environment, being directly related to leaf expansion, with the consequence that it is a less reliable indictor of past CO_2 levels. This review therefore considers only the use of stomatal index as a CO_2 indicator.

Numerous studies have attempted to exploit the stomatal responses of leaves to CO_2 by using the fossil record of plant cuticles to determine palaeo-CO_2 levels (reviewed in Royer *et al.*, 2001a), with several extending the time-scale back beyond 300 Myr (McElwain & Chaloner, 1995; McElwain, 1998; Retallack, 2001). Perhaps the strongest evidence yet that an atmospheric CO_2 signal can genuinely be retrieved in this way comes from the work of Rundgren & Beerling (1999). These authors produced a high-resolution record of atmospheric CO_2 changes spanning the past 9000 yr using a transfer function and measurements of stomatal index made on a radiocarbon dated sequence of fossil *Salix herbacea* leaves from Swedish lake sediments (Fig. 1a). The resulting reconstruction displayed a remarkable similarity to the CO_2 record derived from the Taylor Dome Antarctic ice core study (Fig. 1b) (Indermühle *et al.*, 1999). Both approaches showed a gradual increase in atmospheric CO_2 during the Holocene with an oscillation around 500 radiocarbon years before present (Fig. 1). Together, these CO_2 records indicate that the global carbon cycle has not apparently been in steady-state over Holocene, a time of relative climatic stability compared with the last glacial period (Ditlevsen *et al.*, 1996).

A critical area of uncertainty in the use of fossil stomata in this way is the nonlinear response of stomatal index to atmospheric CO_2 concentrations above present-day levels (Woodward, 1987; Woodward & Bazzaz, 1988; Beerling & Chaloner, 1993; Royer *et al.*, 2001b). This effect undermines the ability of the technique to quantitatively reconstruct high palaeo-CO_2 levels during the early Tertiary and Mesozoic, the very times when there is a major discrepancy between low latitude SSTs (Veizer *et al.*, 2000) and the CO_2 history of the atmosphere (Crowley & Berner, 2001). Therefore, this review focuses on the nature of this apparent 'ceiling' of response by examining evidence from a range of different experiments, natural CO_2 settings and fossil materials. From these analyses, we explore the potential to develop an alternative transfer

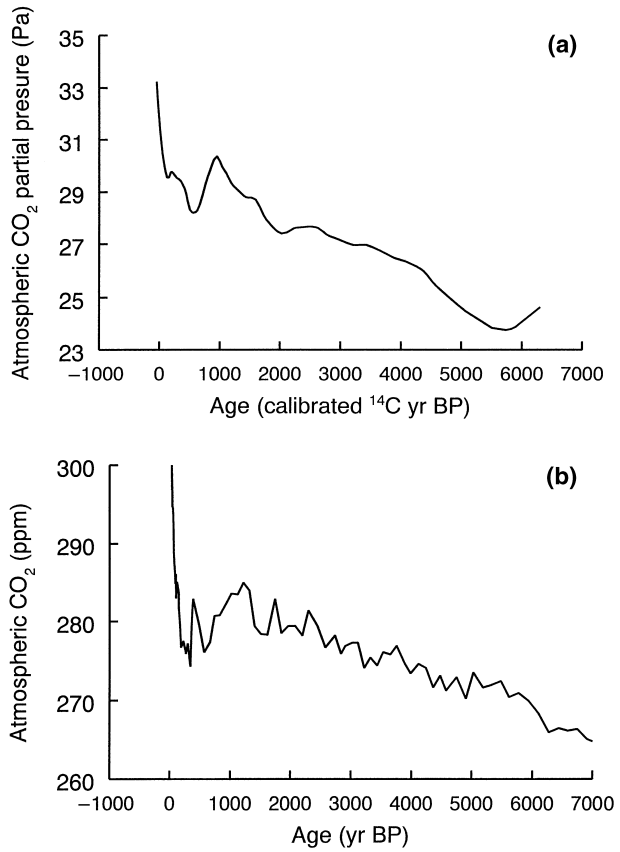

Fig. 1 Atmospheric CO_2 trends over the past 7000 yr of the Holocene (a) reconstructed from fossil leaves in Swedish lake sediments at 999 m above sea level and (b) from the Taylor Dome, Antarctic, ice core. Note that the reconstructed CO_2 partial pressures in (a) have not been converted to concentration because this assumes that total atmospheric pressure has not changed over the Holocene, an assumption difficult to test. Redrawn from Rundgren & Beerling (1999).

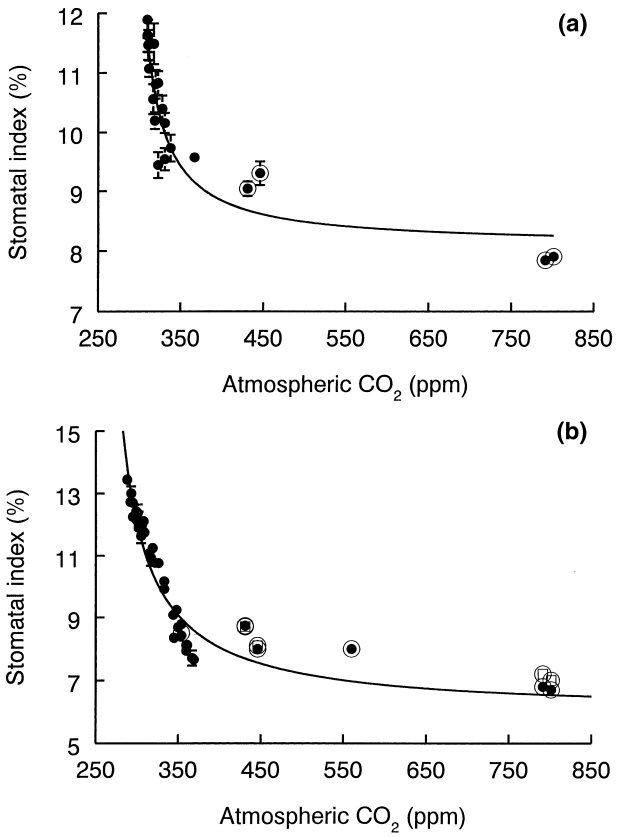

Fig. 2 Responses of stomatal index of (a) *Metasequoia glyptostroboides* and (b) *Ginkgo biloba* to atmospheric CO_2 changes as determined from herbarium leaves (closed circles) and experiments (open circles with solid centres). In (b) open squares denote results from plants that were 5 yr old at the start of the experiment. Redrawn from Royer *et al*. (2001b) with new data for the second year of treatment. The fitted curve in (a) is given by Royer *et al*. (2001b), in (b) it corresponds to Eqn 2a in Table 3.

function for calibrating fossil cuticle records of *Ginkgo* stomatal index and its consequences for reconstructing palaeo-CO_2 levels for part of the Tertiary (Royer *et al*., 2001b). We also take the opportunity to use the updated observational datasets of *Ginkgo* to test earlier suggested calibration functions for reconstructing palaeo-CO_2 levels from the stomatal characteristics of extinct plants (McElwain & Chaloner, 1995; Chaloner & McElwain, 1997; McElwain, 1998). To independently test the various CO_2 reconstructions, the results are compared against CO_2 estimates from palaeosols (Ekart *et al*., 1999) and predictions from models of the long-term global carbon cycle (Tajika, 1998; Berner & Kothavala, 2001; Wallmann, 2001).

The nonlinear response of stomatal index to atmospheric CO_2

Historically, the nonlinear nature of the response was slow to emerge. The first experiments addressing the effects of CO_2 on leaf stomatal index were conducted in an atmosphere enriched with CO_2 up to 1000 ppm (Madsen, 1973; Thomas & Harvey, 1983). Those experiments hinted at a possible low sensitivity of stomatal index because only one of the two species investigated (*Lycopersicon esculentum*) (Madsen, 1973) showed a decline. It was not until experiments with subambient CO_2 concentrations were undertaken, and observations made on herbarium materials collected over the last two centuries of CO_2 increase (Woodward, 1987; Woodward & Bazzaz, 1988), that the differential sensitivity of stomatal index to CO_2 became apparent. In an extensive review of experimental studies (CO_2 exposure time ranged from 2 weeks to 5 years), stomatal index responded to elevated CO_2 in 29% of the cases (n = 65 studies) whereas to subambient CO_2 it responded in 50% of the cases (n = 18 studies) (Royer, 2001). The striking loss of CO_2 sensitivity of stomatal index at CO_2 concentrations above 350 ppm is well demonstrated by combining detailed analyses of historical collections of herbarium leaves with results from controlled environment growth experiments for two ancient plant taxa (*Metasequoia glyptostroboides* and *Ginkgo biloba*) (Fig. 2) (Royer *et al*., 2001b). The linear portions

ANOVA comparison	CO_2 treatment	
	Ambient (440 p.p.m)	Elevated (800 ppm)
Treatment year 1 Younger vs older plants	$F_{1,70}$, $P = 0.94$	$F_{1,67}$, $P = 0.20$
Treatment year 2 Younger vs older plants	$F_{1,46}$, $P = 0.60$	$F_{1,46}$, $P = 0.20$
Older plants Year 1 vs year 2 responses	$F_{1,70}$, $P = 0.04$	$F_{1,70}$, $P = 0.44$
Younger plants Year 1 vs year 2 responses	$F_{1,46}$, $P = 0.02$	$F_{1,43}$, $P = 0.57$

Table 1 Results of analysis of variance (ANOVA) testing for CO_2 effects on the stomatal index of *Ginkgo* leaves in relation to plant age and duration of exposure

F-values are given with the degrees of freedom for the CO_2 treatment and the residual effects, respectively. Younger plants and older plants were 1-yr-old and 5-yr-old-saplings, respectively, during the first year of treatment.

of the curves show that *Metasequoia* and *Gingko* reduced their leaf stomatal index by approximately 50% and 30%, respectively, as the CO_2 concentration rose from 280 to 300 ppm at the onset of preindustrial era to the more recent range of 340–360 ppm Above this CO_2 threshold, the response was less steep, with a 15–10% drop between 400 ppm and 800 ppm. CO_2 shown by plants in experiments (Royer *et al.*, 2001b).

One potentially important factor influencing the response of stomatal index to high CO_2 levels is the age of the plants themselves (Tichá, 1982). To test for this possibility, we compared the stomatal index responses of *Ginkgo biloba* saplings of different ages (1 yr and 5 yr old at the start of the experiment) to CO_2 enrichment under the same polar light (high latitude, 69° N) regime (Beerling & Osborne, 2002). In the case of the 5-yr-old plants, this material had a history of exposure to elevated CO_2 (560 ppm) in the previous 4 yr (Beerling *et al.*, 1998). Analysis of variance (Table 1) indicated no significant differences between the stomatal responses of the two groups of saplings differing in age (Fig. 3). There was, however, a significant reduction in the stomatal indices of control *Ginkgo* plants, but not *Metasequoia* during the second year of treatment (Table 1, Fig. 3). These results suggest that for *Ginkgo* at least, the age of the plant may not play a major role in determining the extent to which stomatal index is reduced under high CO_2.

An interesting feature of the differential sensitivity of stomatal index to CO_2 is its greater variability at sub-ambient CO_2 levels compared with that observed at above ambient CO_2 levels (Fig. 4). Comparisons across different plant groups (temperate trees, grasses, herbs, shrubs and ancient woody plant taxa) indicate this seems to be quite a general trend (Fig. 4). A similar phenomenon, whereby plants exhibit

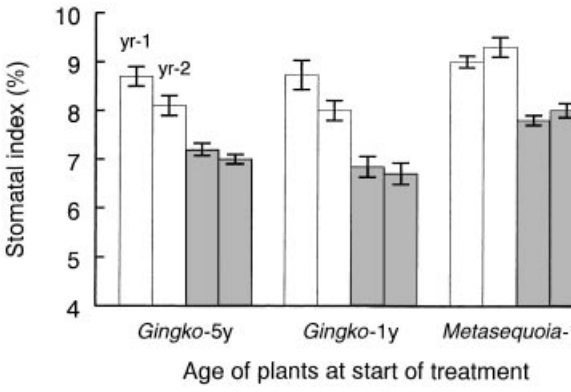

Fig. 3 Effects of plant age and duration of treatment on the response to stomatal index to atmospheric CO_2 enrichment. Open bars, ambient $CO_2 \approx 400$ ppm; shaded bars, elevated $CO_2 \approx 800$ ppm. All plants showed a significant ($P < 0.01$) reduction in stomatal index in response to growth in elevated CO_2. Plant age had no effect on the strength of the response, while duration of exposure influenced the control *Gingko* plants under ambient CO_2 conditions (see Table 1).

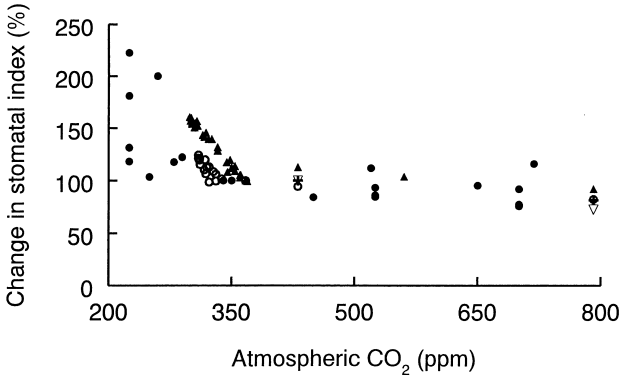

Fig. 4 Relative changes in stomatal index to CO_2 for a range of different plant groups, including data from modern tree, herb and shrub species (closed circles) (from Woodward & Bazzaz, 1988), and a range of more ancient so-called 'living fossil' taxa: open circles, *Metasequoia glyptostroboides*; closed triangles, *Ginkgo biloba*; plus signs, *Araucaria araucana*; open triangles, *Sequoia sempervirens*. Data for *M. glyptostroboides* and *G. biloba* are from Fig. 2b, data points for *A. auracana* and *S. sempervirens* at 440 ppm and 800 ppm CO_2 were obtained from an on-going CO_2 enrichment experiment (D.J. Beerling, unpublished).

wide variety of responses at low CO_2, that becomes lost at high CO_2, has been reported for herbaceous taxa (Tissue *et al.*, 1995; Ward & Strain, 1997). Such large differences in the variability of plants to different CO_2 levels may relate to the long-term history of CO_2 exposure and the potential for CO_2 to act as a selective agent. Ice-core records of atmospheric CO_2 indicate that plants experienced a preindustrial concentration of 280–300 ppm for the last 10 000 years (Neftel *et al.*, 1988; Indermühle *et al.*, 1999) and glacial–interglacial values of 180 ppm and 280 ppm for the last 400 000 Myr (Petit *et al.*, 1999), and possibly longer. These rather long timescales clearly indicate the possibility that plants have evolved and optimized a range of physiological, morphological and growth processes to low CO_2 levels.

Indeed, detailed analyses of the processes controlling photosynthesis (Lloyd *et al.*, 1995; Lloyd & Farquhar, 1996; Mitchell *et al.*, 2000) indicate the 'ghost' effects of this former lower-than-present CO_2 regime. Plants grown at CO_2 concentrations of 300–350 ppm, under saturating irradiance, generally show colimitation of photosynthetic CO_2 uptake by the capacity of Rubisco to catalyse CO_2 fixation in the photosynthetic carbon reduction cycle (carboxylation) and the capacity of the light-harvesting/electron-transport systems to regenerate Ribulose biphosphate (RuBP). This feature of modern plants with the C_3 photosynthetic pathway corresponds to an optimal distribution of chloroplastic nitrogen between RuBP carboxylation and RuBP regeneration. Under different growth conditions (e.g. lower irradiance), plants tend to adjust their nitrogen partitioning to maintain this colimitation in an effort to maximize carbon gain with respect to leaf nitrogen content. Experimental evidence, however, indicates that during exposure to elevated CO_2 concentrations, chloroplastic nitrogen allocation to the two control processes (RuBP carboxylation and regeneration) shows little change (Lloyd & Farquhar, 1996). On exposure to atmospheric CO_2 levels above 350 ppm, therefore, the control of photosynthesis may no longer be optimal in terms of nitrogen investment with respect to carbon gain. It is interesting to note that even the annual crop wheat, in which selection for high productivity is strongly directed, and which evolved only recently, has a photosynthetic system adapted to the preindustrial CO_2 concentration (Mitchell *et al.*, 2000). At the other extreme of plant longevity, analyses of CO_2 flux data from extensive field measurement campaigns in the Amazonian tropical rainforest indicate that these long-lived trees also exhibit a photosynthetic physiology and nitrogen investment adjusted for optimum carbon gain at a preindustrial CO_2 concentration of 270 ppm (Lloyd *et al.*, 1995).

These observations suggest that the high variability of plant responses to subambient CO_2 concentrations (Fig. 4) may directly reflect CO_2 selection and adaptation to Holocene CO_2 levels (260–290 ppm, Fig. 1). Alternatively, the rather restricted responses shown by plants to above-ambient CO_2 levels may reflect the limited potential for high CO_2 to act as a selective agent (Fig. 4). In multiple generation experiments with the annual *Arabidopsis thaliana*, Ward *et al.* (2000) reported that low CO_2 (200 ppm) acted as an effective selective agent for seed production whereas a high CO_2 concentration (700 ppm) failed to operate in this way. If current plant genotypes are strongly preadapted to preindustrial CO_2 levels, and take a long time to evolve, the stomatal responses observed in CO_2 enrichment experiments possibly reflect the lack of genetic variability arising from the short-term nature of the experiments (Beerling & Chaloner, 1993; Royer, 2001).

Evidence from plants exposed to high CO_2 in the long-term

It follows from this discussion that assessment of the evolutionary (genotypic) response of stomatal index to high CO_2 requires observations encompassing an appropriately long duration of exposure. One approach to dealing with this issue has been to use plants growing naturally in the vicinity of CO_2-enriched geothermal springs (Raschi *et al.*, 1997), with the notion that the vegetation has grown in a high CO_2 environment in the long-term (decadal or longer) and therefore exhibits an adaptive (rather than acclimatory) response. Bettarini *et al.* (1998) compared the stomatal indices of 17 species of grasses, herbs and trees growing in an Italian high-CO_2 spring (Bossoleto) with the same species at control sites with similar soils and climate but without elevated CO_2. Historical records indicate that CO_2 emissions at Bossoleto have occurred for the past two centuries. Taken at face value, the results (Fig. 5) show rather little consistent change in the stomatal indices of the two groups of plants suggesting that with this extended level of exposure to elevated CO_2, an apparent ceiling to above-ambient CO_2

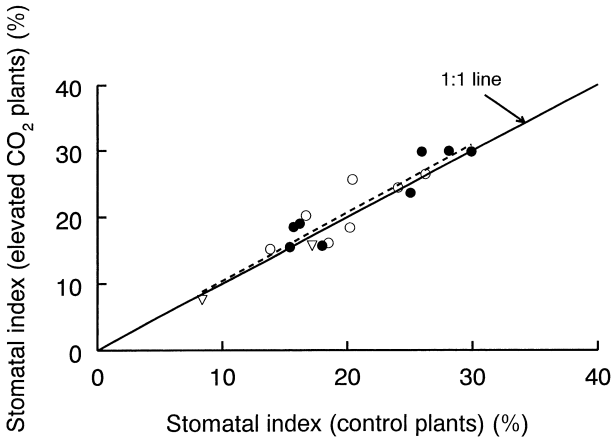

Fig. 5 Response to stomatal index of 17 species of trees (triangles), herbs (open circles) and grasses (closed circles) to CO_2 enrichment in the long term, as determined from the Bossoleto high-CO_2 spring in Italy (data from Bettarini *et al.*, 1998). The solid line indicates the 1 : 1, the dashed line indicates the fitted regression to the data, with a slope not significantly different from unity.

levels remains. No particular differences in the responsiveness between life forms (trees, herbs and grasses) with different generation times were observed (Fig. 5). An exception to this trend is the remarkable reduction in stomatal index (73–85%) of a subtropical herb and a tree species from cold CO_2 springs in Venezuela (Fernandez *et al.*, 1998). In this example the plants experienced very high CO_2 concentrations (27 000–35 000 ppm) for an unknown duration.

Observations from plants at Bossoleto appear to support the responses of plants shown in CO_2 enrichment experiments (Figs 2 and 3), but there are difficulties associated with using high-CO_2 springs in this manner. In particular, convective activity during the day can disturb the boundary layer at the sites, resulting in greater mixing and dilution of the CO_2 emissions and, as plants increase in height, the different organs are exposed to different degrees of CO_2 enrichment. There is also a need to recognize that the sites are usually not genetically isolated, so that cross-fertilization (and dilution of any CO_2 selection) may take place from plants outside the springs. Moreover, exposure to elevated CO_2 for two centuries represents one or possibly two generations for trees, with little potential for an adaptive response to be expressed.

From observations in the Amazon (Lloyd *et al.*, 1995), it seems that even 10 000 years might not be sufficient time to allow natural selection to operate during an altered CO_2 regime. Given this clue about the length of time required for adaptation to high CO_2 to occur, we sought to assess the response of stomatal index to atmospheric CO_2 of *Ginkgo* on a multimillion year time-scale. To achieve this aim, we identified sites in which fossil *Ginkgo* cuticles were well preserved and reasonably abundant (Royer *et al.*, 2001b) and which had pedogenic carbonate isotope ($\delta^{13}C$) data (Koch *et al.*, 1992, 1995) from geographically and stratigraphically nearby sites. Stratigraphically, all of the palaesol sites were within 15 m (*c.* 30 000 yr) of the cuticle-bearing sites and allowed us to generate independent atmospheric CO_2 estimates using the palaeosol CO_2 barometer (Cerling, 1991, 1992). In total, six early Palaeogene North American sites were identified (Table 2) fulfilling these criteria and which gave CO_2 estimates within the range of our training set; all had reasonably good age control (Wing *et al.*, 2000). For all sites, we used fossils of *Ginkgo adiantoides*, a taxon with close morphologically similarity to *Ginkgo biloba* (Tralau, 1968).

At each site, atmospheric CO_2 was calculated from the carbon isotope composition of pedogenic carbonates (δ_{cc}) and *Ginkgo* cuticles (δ_p) using the diffusion-reaction model of Cerling (1991, 1992). According to the model, these quantities can be used to estimate atmospheric CO_2 concentration (C_a in ppm) from:

$$C_a = S(z) \times \frac{\delta - 1.0044 \times \delta_\phi - 4.4}{\delta_a - \delta_s} \qquad \text{Eqn 1}$$

where $S(z)$, is the concentration of CO_2 contributed by biological respiration (typically 5000 ppm for well-drained arid–semiarid soils); δ_a, is the isotopic composition of atmospheric CO_2, taken from marine carbonate records with a 7‰ negative offset (–6.5‰ for the late Palaeocene-early Eocene); and δ_φ, the isotopic composition of soil respired CO_2 assumed to equal δ_p (Table 2). Soil carbonate isotopic composition (δ_{cc}) is assumed, under equilibrium conditions, to equal the isotopic composition of soil CO_2 (δ_s) with a temperature-dependent fractionation (*c.* +10‰ at 25°C). It should be emphasized that the error terms for the CO_2 estimates are large (Table 2) and were derived, according to convention, by varying $S(z)$ between 3000 and 7000 ppm (Royer *et al.*, 2001a).

The large error term in the CO_2 estimates, especially at higher CO_2 values, introduces some uncertainty in our attempt at determining the long-term response of *Ginkgo* stomatal index to CO_2. For the range of sites investigated, atmospheric CO_2 varied between 200 ppm and 985 ppm (Table 2).

Table 2 Fossil *Ginkgo* cuticle stomatal index values and corresponding independent estimates of atmospheric CO_2 calculated from soil carbonate and *Ginkgo* cuticle ($\delta^{13}C$ values using the diffusion-reaction model (Cerling, 1991, 1992)

Plant materials						Palaeosols			
Site[1]	Depth[2] (m)	n leaves	Stomatal[1] index	($\delta^{13}C$ (‰)	age[4] (Myr ago)	Site[3]	depth[2] (m)	($\delta^{13}C$ (‰)	Atmospheric CO_2 concentration (ppm)[5]
LJH 7132	~935	5	8.8	–23.9	56.4	SC 85/185	940	–8.0	697 ± 280
SLW 9411	~1355	8	11.5	–23.8	55.6	SC 118U	1325	–8.7	329 ± 132
SLW 9434	~1460	7	12.2	–22.5	55.4	SC 22	1460	–7.7	223 ± 90
SLW 9715	1470	12	8.2	–24.2	55.3	SC 22	1460	–7.7	985 ± 394
SLW 9812	1570	22	8.5	–24.5	55.1	SC 4	1570	–8.4	760 ± 303
SLW H	~2320	9	10.2	–26.9	53.5	YPM 320	~2320	–12.0	200 ± 80

[1]Stomatal index data and site details from Royer *et al*. (2001b). [2]Depth in section from Polecat Bench/Clarks Fork Basin, where the Cretaceous–Tertiary boundary 65 Myr ago = 0 m. Values preceded by ~ were converted from the Elk Creek section. [3]From Koch *et al*. (1995), except YPM 320 which is from Royer *et al*. (2001b). [4]From the Age Model 2 of Wing *et al*. (2000). [5]Calculated using the palaeosol pCO_2 model of Cerling (1991, 1992); error estimates were obtained by varying $S(z)$ between 3000 and 7000 ppm.

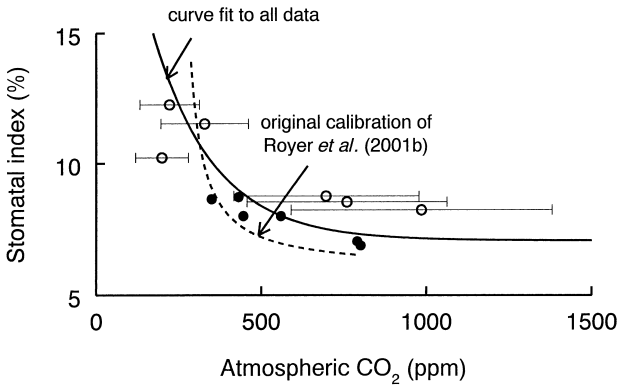

Fig. 6 Comparison of the response of leaf stomatal index determined from fossil *Ginkgo* cuticles over 3 Myr to CO_2 concentrations estimated from palaeosol carbonates (see Table 2) with data from herbarium leaves and experiments (from Fig. 2b). The fitted solid curve is that given in Fig. 2(b), the dashed curve is fitted to the entire dataset (Eqn 3a in Table 3).

Nevertheless, within this uncertainty, the new dataset shows an inverse relationship between the stomatal index and the estimated concentration of atmospheric CO_2 under which they grew (Fig. 6). The two end-member stomatal index–CO_2 concentrations are separated by 2 Myr (Table 2), whilst the entire dataset spans 3 Myr. The fossils therefore extend the duration of exposure seen in high CO_2 springs by a factor of *c.* 10^4. From this standpoint, the dataset encompasses a sufficient time with which to observe a genotypic response (i.e. adaptive) to the different levels of CO_2 (Beerling & Chaloner, 1993).

It emerges that the response of fossil leaf stomatal index to CO_2 fits into the existing calibration dataset based on leaves from herbarium sheets and experiments (Fig. 6). This seems to indicate that the short-term phenotypic CO_2 response seen in experiments realistically reflects the sensitivity of the genotypic response. The implication here is that the nonlinear nature of the stomatal index response to CO_2 is real and likely to limit reconstruction of high (> 600 ppm) palaeo-CO_2 levels. However, at CO_2 concentrations below 300 ppm two fossil plant stomatal indices are lower than expected (Fig. 6), although determining whether this effect is real is hampered by the relative paucity of observations. Identification of a CO_2-regulated gene controlling stomatal development (Gray *et al.*, 2000) provides a genetic basis for the action of CO_2, and it is possible that this underpins both phenotypic and genotypic stomatal responses.

Calibrating a CO_2 signal from the stomata of extinct plants

All observations to date suggest the responses of the stomatal index of vascular land plant leaves with C_3 photosynthesis to atmospheric CO_2 is species-specific (Royer, 2001). Plants growing in the same location and exposed to the same changes in CO_2 will therefore show different degrees of responsiveness. In consequence, there is a clear need to study fossil materials with close modern analogues. However, for times when fossils represent extinct plants, some other approach is required to discover if those fossils carry a CO_2 signal (McElwain & Chaloner, 1995; Chaloner & McElwain, 1997). In the Palaeozoic, this assumes that CO_2 overrides other selection pressures involved in the evolution of stomata (Edwards, 1998; Beerling *et al.*, 2001; Raven & Edwards, 2001). Stomatal measurements on Devonian and Carboniferous plant fossils provide some evidence supporting this assumption by revealing a large (two-orders of magnitude) increase in stomatal density during the 90% drop in atmospheric CO_2 over this time (McElwain & Chaloner, 1995), indicating the potential for long-extinct plants to record palaeo-CO_2 change.

One technique for obtaining semi-quantitative CO_2 estimates is the stomatal ratio (SR), defined as the stomatal index of a nearest living morphological or ecological equivalent (or both) to the fossil plant under consideration, divided by stomatal index of the fossil plants. SR values are related to the ratio of atmospheric CO_2 in the past relative to the preindustrial (or the time when the nearest living equivalent materials were collected) (RCO_2) (Chaloner & McElwain, 1997; McElwain, 1998). For Carboniferous (*Swillingtonia denticulata*) and Permian (*Lebachia frondosa*) conifers, calibration against Berner's (1994) model predictions of CO_2 at those times gave $1SR = 2RCO_2$ (Eqn 4a, Table 3). A later analysis suggested a shift in the calibration so that $1SR = 1RCO_2$ (Eqn 5a, Table 3), given that the stomatal index of nearest living equivalents reflects a current or near-current atmospheric CO_2 level (McElwain, 1998).

In the context of the present review, it is of interest to compare the SR approach for elucidating palaeo-CO_2 trends with the more quantitative calibration functions (Table 3). This has been achieved following some simple manipulation of the equations (Table 3), to predict the response of the stomatal index of *Ginkgo* to atmospheric CO_2 change, for comparison with the two nonlinear functions derived from observations (Fig. 6). This comparison provides the first direct test of the SR approach to assess whether the degree of responsiveness set by these functions is realistic and appropriate for palaeo-CO_2 reconstructions.

The calibration of $1SR = 2RCO_2$ shows rather large discrepancies between predictions and observations (Fig. 7). Calculated using the stomatal ratio relationships, it emerges that over the CO_2 range 300–800 ppm, the $1SR = 1RCO_2$ calibration gives an approximate fit to the observations (Fig. 7). All of the various approaches converge in their predicted responses of *Ginkgo* stomatal index to high atmospheric CO_2 concentrations (Fig. 7b). At CO_2 concentrations below 600 ppm, however, the $1SR = 2RCO_2$ calibration diverges markedly from the others.

Table 3 Equations describing the response of the stomatal index (*SI*) of *Ginkgo* leaves to atmospheric CO_2 and their inverse solutions for predicting CO_2 from stomatal index

Equation		Derivation
2a	$SI = \frac{(C_a - 194.4)}{(0.16784 \times C_a) - 41.6}$	From observations on herbarium leaves and experiments (Royer *et al.*, 2001a) (Fig. 2b).
2b	$C_a = \frac{(52 \times SI - 243)}{1049 \times SI - 6250} \times 5000$	Inverse prediction of Eqn 2a.
3a	$SI = 7.085 + 20.73 \times \exp(-0.005538 \times C_a)$	From entire set of observations on herbarium leaves, experiments and fossil cuticles and CO_2 estimates from palaeosols (Fig. 6, Table 2).
3b	$C_a = -180.57 \times \ln(0.048 \times SI - 0.3418)$	Inverse prediction of Eqn 3a.
4a	$\frac{SI(m)}{SI(f)} = \frac{C_a(past) \times 0.5}{C_a(present)}$	Relates the stomatal index of a fossil plant, *SI*(*f*), to its modern nearest ecological equivalent, *SI*(*m*) (Chaloner & McElwain, 1997). C_a(present) and C_a(past) represent atmospheric CO_2 concentrations during the preindustrial (300 ppm) and at some time in the past, respectively.
4b	$SI = SI(m) \times 2 \times \frac{C_a(present)}{C_a}$	Predicts the response of stomatal index of *Ginkgo* to CO_2 change by solving Eqn 4a for *SI*, where *SI*(*m*) = 11.33 (Fig. 7).
4c	$C_a = \frac{SI(m)}{SI(f)} \times C_a(present) \times 2$	Inverse prediction of Eqn 4b.
5a	$\frac{SI(m)}{SI(f)} = \frac{C_a(past)}{C_a(present)}$	Second formulation of Eqn 4a, but calibrated assuming *SI*(*m*) reflects a near-present day atmospheric CO_2 level (McElwain, 1998).
5b	$SI = SI(m) \times \frac{C_a(present)}{C_a}$	Predicts the response of stomatal index of *Ginkgo* to CO_2 change by solving Eqn 5a for *SI*, with *SI*(*m*) = 11.33 (Fig. 7).
5c	$C_a = \frac{SI(m)}{SI(f)} \times C_a(present)$	Inverse prediction of Eqn 5b.

C_a = atmospheric CO_2 concentration (ppm).

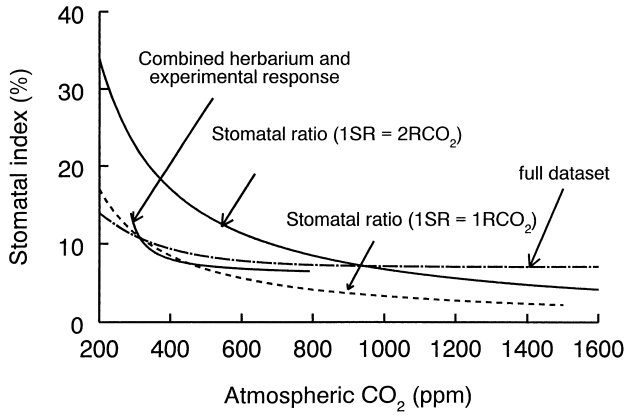

Fig. 7 Comparison of the responses of stomatal index to atmospheric CO_2 predicted by the two non-linear calibration functions derived in Figs 2b and 6, with the stomatal ratio approaches (Eqns 4b and 4b, Table 3).

Stomata and palaeo-CO_2 levels during the early Tertiary

We next compare the effects of the two different nonlinear transfer functions (Fig. 6, Table 3), and the stomatal ratio approach, on reconstructed atmospheric CO_2 levels with fossil cuticles. We focus on 18 sites in western North American, dating to between 58.5 Myr and 53.4 Myr ago, with well-replicated stomatal index counts from fossil *Ginkgo adiantoides* cuticles (Royer *et al.*, 2001b). Calibration of the fossil *Ginkgo* stomatal records using the nonlinear function derived from observations on herbarium leaves and experiments (Eqn 2, Table 3) yields atmospheric CO_2 concentrations of between 300 ppm and 450 ppm during the Palaeocene and early Eocene (Fig. 8a) (Royer *et al.*, 2001b). These estimates are towards the lower end of the CO_2 range predicted by geochemical models (Tajika, 1998; Berner & Kothavala, 2001; Wallmann, 2001) and calculated from palaeosols (Ekart *et al.*, 1999). Estimates from boron isotopes are very much higher, in the range 1000–4000 ppm (Pearson & Palmer, 2000), and not generally consistent with any other evidence (Royer *et al.*, 2001a,b). However, this CO_2 proxy may be compromised by the varying global riverine input influencing the marine boron isotopic budget in a manner previously unrealized (Lemarchand *et al.*, 2000). When the fossil *Ginkgo* stomatal index records are calibrated with the second nonlinear function (Eqn 3, Table 3), the resulting palaeo-CO_2 estimates are generally rather close to those obtained previously (Fig. 8b).

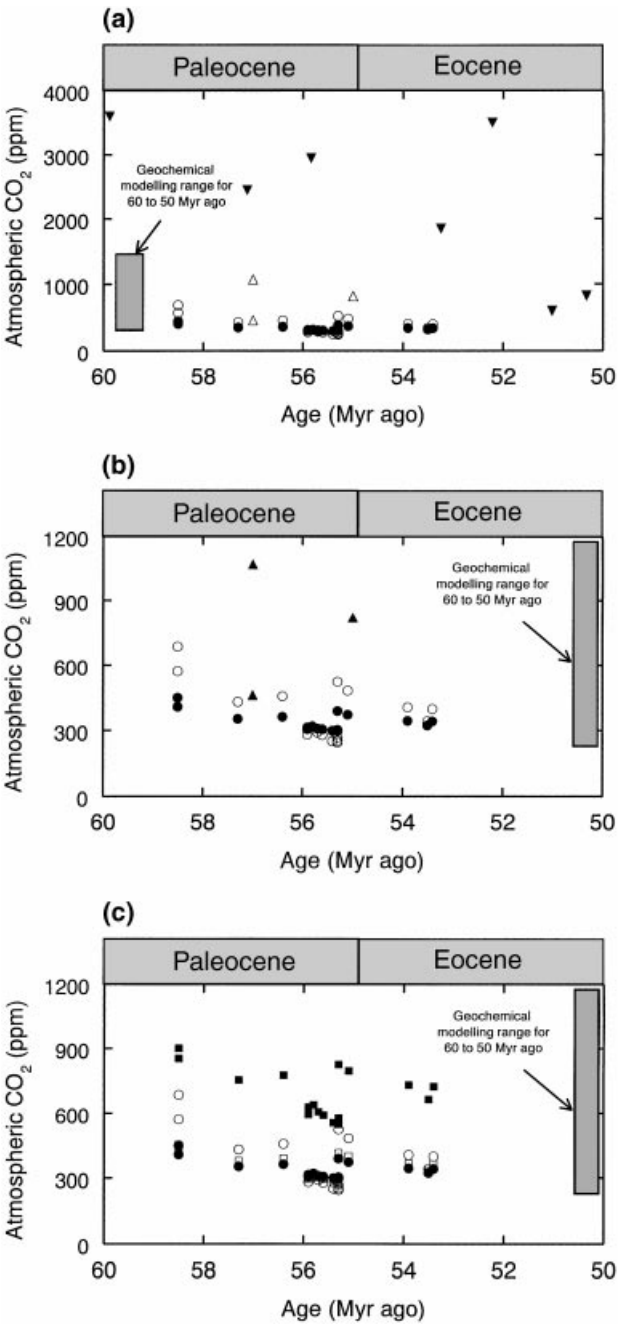

Fig. 8 Atmospheric CO_2 concentrations reconstructed for the early Tertiary from (a) the stomata index of fossil *Ginkgo* cuticles (Royer *et al.*, 2001b) calibrated with two non-linear functions fitted to observations (closed circles, S cal. 1 = Eqn 2b; open circles, S cal. 2 = Eqn 3b, Table 3). Also shown, for comparison, are CO_2 estimates from palaeosols (open triangles) (Ekart *et al.*, 1999), boron isotopes (closed triangles) (Pearson & Palmer, 2000) and the range for 50–60 Myr ago predicted by geochemical modelling of the long-term carbon cycle (Tajika, 1998; Berner & Kothavala, 2001; Wallmann, 2001). In (b) the stomatal and palaeosol data are displayed from (a), but with an expanded CO_2 axis to show the close similarity of CO_2 estimates from stomata using the two nonlinear transfer functions. (c) Comparison of reconstructed CO_2 levels from the stomatal ratio approach using two calibrations (open squares, SR1 = Eqn 5c; closed squares, SR^2 = Eqn 4c, Table 3) with those in (b).

We note that the stomatal ratio approach, calibrated to $1SR = 1RCO_2$, yields quantitative results that are very compatible with the other two sets of predictions (Fig. 8c). This suggests that even though SR responses are calibrated against fossil, rather than on modern plants, they nevertheless provide a useful technique for estimating palaeo-CO_2 levels. The alternative calibration gives CO_2 estimates higher than all three of the previous transfer functions considered here (Fig. 8c) but these, nevertheless, remain within the bounds suggested by geochemical modelling of the long-term carbon cycle. The principal drawback with the approach is that is not completely independent of carbon cycle model predictions (Royer *et al.*, 2001a).

The different palaeo-CO_2 reconstructions can usefully be considered in the context of independent palaeoclimate records to determine the contribution of atmospheric CO_2-related 'greenhouse' effect to the climate at the time. Deep-sea oxygen isotope records from a range of low- and high-latitude sites indicate that ocean temperatures were some 4°C warmer between 50 and 60 Myr ago (Shackleton & Boersma, 1981; Shackleton, 1986). A more recent compilation of global deep-sea isotope records suggests even warmer temperatures (8–12°C) for this interval (Zachos *et al.*, 2001). Near-surface terrestrial mean annual air temperatures (MATs) have been reconstructed from leaf margin analyses of western North American plant fossil assemblages at several sites in Wyoming, including the Bighorn basin (Wilf, 2000). During the late Palaeocene (59–55 Myr ago), MATs were reconstructed to be 10–16°C, while the early Eocene (55–50 Myr ago) was even warmer, with MATs between 15°C and 20°C. Against a modern MAT for that area of *c.* 7°C (Müller, 1982), the fossil plants clearly signal a time of extreme warmth between 60 Myr and 50 Myr ago, in agreement with the marine records (Zachos *et al.*, 2001).

Mean (± SE) reconstructed atmospheric CO_2 concentrations over the entire period encompassed by all 18 samples for the two nonlinear calibrations were 338 ± 10 ppm CO_2 (without fossil data) and 377 ± 30 ppm CO_2 (with fossil data), respectively. These compare with 343 ± 13 ppm CO_2 and 685 ± 27 ppm CO_2 for the $1SR = 1RCO_2$ and $1SR = 2RCO_2$ functions, respectively. Based on the logarithmic relationship between global temperature and atmospheric CO_2 concentrations (Kothavala *et al.*, 1999), the upper and lower atmospheric CO_2 concentrations of all approaches would raise the Earth's global mean temperature by between 0.5°C and 2.2°C, respectively, both being insufficient to account to the warm early Tertiary climate. Clearly, although the absolute values of CO_2 estimated from fossil stomata are sensitive to the type of calibration curve employed (Fig. 8), the key conclusion remains that other climate-forcing mechanisms must have operated 50–60 Myr ago. In particular, atmospheric CH_4 concentration, land surface albedo and ocean heat transport may all have played major, but not mutually exclusive, roles (Valdes, 2000).

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