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Stomatal density and stomatal index as indicators of paleoatmospheric CO_2 concentration

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Abstract

A growing number of studies use the plant species-specific inverse relationship between atmospheric CO_2 concentration and stomatal density (SD) or stomatal index (SI) as a proxy for paleo-CO_2 levels. A total of 285 previously published SD and 145 SI responses to variable CO_2 concentrations from a pool of 176 C_3 plant species are analyzed here to test the reliability of this method. The percentage of responses inversely responding to CO_2 rises from 40 and 36% (for SD and SI, respectively) in experimental studies to 88 and 94% (for SD and SI, respectively) in fossil studies. The inconsistent experimental responses verify previous concerns involving this method, however the high percentage of fossil responses showing an inverse relationship clearly validates the method when applied over time scales of similar length. Furthermore, for all groups of observations, a positive relationship between CO_2 and SD/SI is found in only $\leq$12% of cases. Thus, CO_2 appears to inversely affect stomatal initiation, although the mechanism may involve genetic adaptation and therefore is often not clearly expressed under short CO_2 exposure times.

Experimental responses of SD and SI based on open-top chambers (OTCs) inversely relate to CO_2 less often than greenhouse-based responses ($P < 0.01$ for both SD and SI), and should be avoided when experimental responses are required for CO_2 reconstructions. In the combined data set, hypostomatous species follow the inverse relationship more often than amphistomatous species (56 vs. 44% for SD; 69 vs. 32% for SI; $P < 0.03$ for both comparisons). Both the SD and SI of fossil responses are equally likely to inversely relate to CO_2 when exposed to elevated versus subambient CO_2 concentrations (relative to today). This result casts doubt on previous claims that stomata cannot respond to CO_2 concentrations above present-day levels. Although the proportion of SD and SI responses inversely relating to CO_2 are similar, SD is more strongly affected by various environmental stresses, and thus SI-based CO_2 reconstructions are probably more accurate.

Keywords: carbon dioxide; stomatal frequency; paleoatmosphere; paleoclimatology; leaf anatomy; cuticles

1. Introduction

The increase in atmospheric CO_2 concentration since industrialization (Friedli et al., 1986; Keeling et al., 1995) and the predicted continued increase into the near future (Houghton et al., 1995) forces the need to understand how the biosphere operates under elevated (relative to pre-industrial) CO_2 levels. The geologic record affords a wealth of such information. Fundamental to the use of the geologic record, however, is a reliable estimate of CO_2 concentration throughout the intervals of interest. The results of a computer-based model for the Phanerozoic (Berner, 1994; see Fig. 1), based on rates of Ca–Mg silicate weathering and burial as carbonates, weathering and

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burial of organic carbon, CO_2 degassing, vascular land plant evolution, and solar radiation, have gained considerable use (e.g. Retallack, 1997; Kump et al., 1999). Proxy data are still crucial, however, for both testing and refining this model. Currently used proxies include $\delta^{13}C$ from pedogenic carbonates (Cerling, 1991, 1992; Mora et al., 1991, 1996; Ekart et al., 1999), $\delta^{13}C$ from trace carbonates contained within goethite (Yapp and Poths, 1992, 1996), $\delta^{13}C$ from phytoplankton (Freeman and Hayes, 1992; Pagani et al., 1999a,b), and $\delta^{11}B$ from planktonic foraminifera (Pearson and Palmer, 1999). To a first approximation, these proxies largely support the model of Berner (1994) (Fig. 1). A discrepancy exists during the late Carboniferous and early Permian between the pedogenic carbonate-derived data of Ekart et al. (1999) and the model of Berner (1994). However, this discrepancy disappears if the $\delta^{13}C$ values for marine carbonates of Popp et al. (1986) are used during this time interval instead of those of Veizer et al. (1999) in calculating CO_2 from the data of Ekart et al. (1999) (Berner, R.A., unpublished data; see Fig. 1b).

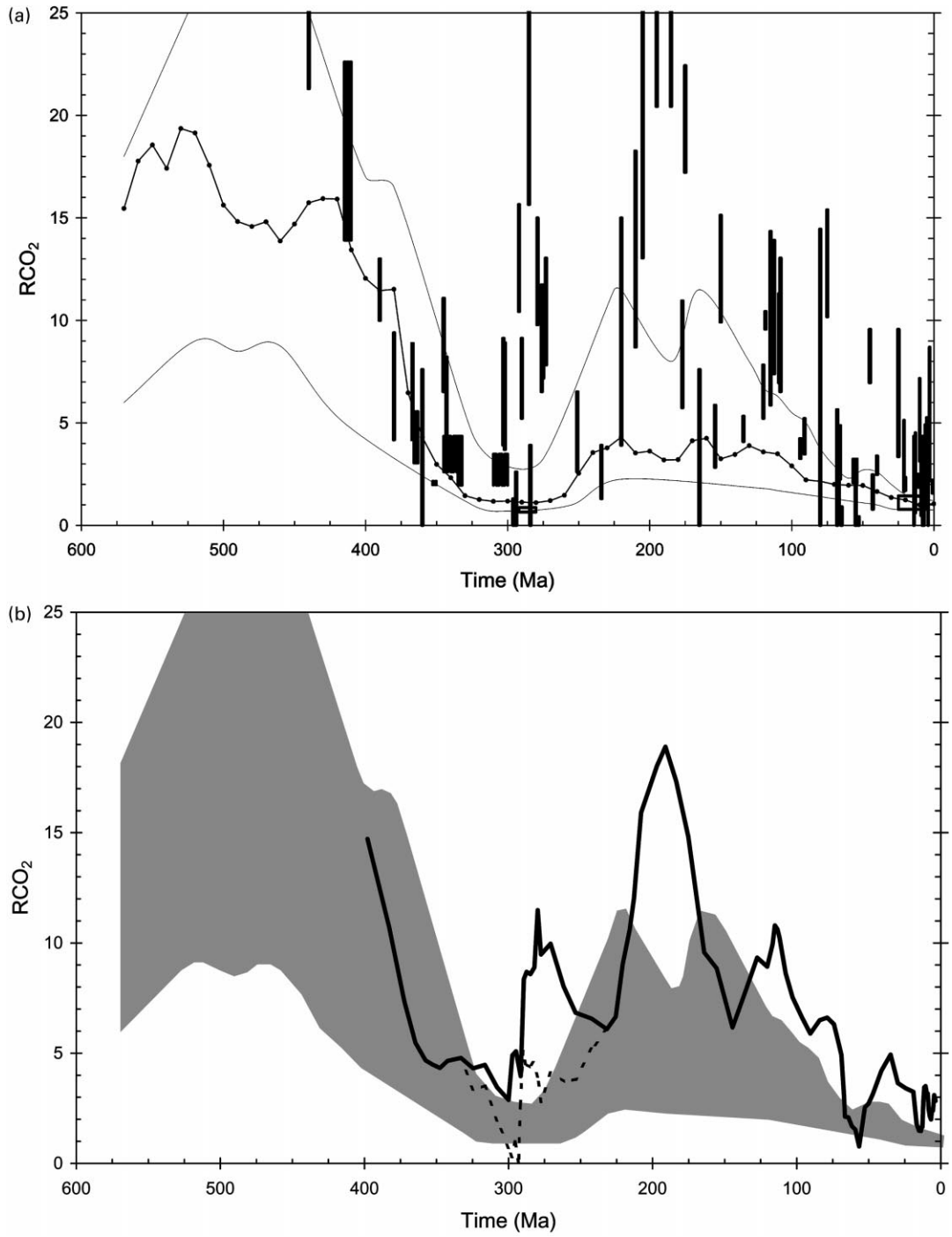
Another emerging proxy relies on the plant species-specific inverse relationship between atmospheric CO_2 concentration and stomatal density and/or stomatal index. Concerns have been raised regarding this method's reliability (Körner, 1988; Poole et al., 1996), and it is the purpose of this paper to address these concerns via an extensive analysis of the literature. Analysis includes stomatal responses from fossil observations as well as short-term (experimental, natural CO_2 springs, altitudinal transects, and herbaria) observations, as responses from the latter category are often used to generate standard curves for estimating CO_2 from fossil observations (van der Burgh et al., 1993; Beerling et al., 1995; Kürschner, 1996; Kürschner et al., 1996; Rundgren and Beerling, 1999; Wagner et al., 1999). Specifically, the utility of stomatal indices will be examined, an approach not analyzed in previous reviews (Beerling and Chaloner, 1992, 1994; Woodward and Kelly, 1995).

2. Mechanism controlling stomatal density

Stomata are pores on leaf surfaces through which plants exchange CO_2, water vapor, and other constituents with the atmosphere. They form early in leaf development, and typically mature by the time the leaf reaches 10–60% of its final leaf size (Tichá, 1982). Thus, the timing for the mechanism(s) of stomatal initiation lies early in leaf ontogeny (Gay and Hurd, 1975; Schoch et al., 1980). Currently, no mechanism or combination of mechanisms adequately explains the expression of stomatal initiation, although genetic work may provide insights in the near future (e.g. Berger and Altmann, 2000). Proposed mechanisms include irradiance (Gay and Hurd, 1975; Schoch et al., 1980), humidity (Salisbury, 1927), and pCO_2 (Woodward, 1986; Beerling and Chaloner, 1992; Woodward and Kelly, 1995; Beerling and Woodward, 1996).

A common theory for why CO_2 should (partially) control stomatal initiation is as follows (e.g. Woodward, 1987). Water vapor and CO_2 constitute the two main fluxes across the leaf epidermis. It is generally advantageous for plants to conserve water loss while maximizing CO_2 uptake, two typically antithetical processes. As CO_2 rises for a given water budget, for example, a plant can 'afford' to reduce its stomatal conductance without suffering a reduction in carbon assimilation rates. Two main pathways driving this response are smaller stomatal pores (Bettarini et al., 1998) and a reduction in stomatal numbers

Fig. 1. Atmospheric CO_2 versus time for the Phanerozoic. RCO_2 = ratio of mass of paleo-CO_2 to time-averaged pre-industrial value (230 ppmV, the mean CO_2 over at least the last 400 k.y. (Petit et al., 1999)). The centerline joining filled circles (10 m.y. time steps) represents the best estimate from the model of Berner (1994, 1998). The two straddling lines represent error estimates based on sensitivity analyses. Boxes in (a) represent 91 non-stomatal-based proxy estimates of varying RCO_2 resolution (data from Suchecky et al., 1988; Platt, 1989; Cerling, 1991, 1992; Freeman and Hayes, 1992; Koch et al., 1992; Muchez et al., 1993; Sinha and Stott, 1994; Andrews et al., 1995; Ghosh et al., 1995; Mora et al., 1996; Yapp and Poths, 1996; Ekart et al., 1999; Elick et al., 1999; Lee, 1999; Lee and Hisada, 1999; Pagani et al., 1999a, 1999b; Pearson and Palmer, 1999). The heavy line in (b) is a five-point running average of the mean RCO_2 of every box in (a). This approach smoothes short-term CO_2 fluctuations and is more directly comparable with the model of Berner (1994, 1998). The dashed line in (b) is a five-point running average incorporating a recalculation of Ekart et al. (1999) data during the late Carboniferous and early Permian using the marine carbonate $\delta^{13}C$ data of Popp et al. (1986) (see text for details).


(a)
RCO2
Time (Ma)
(b)
RCO2
Time (Ma)

(Woodward, 1987). Conversely, a drop in CO_2 requires an increase in stomatal conductance to maintain assimilation rates, but at the cost of increased water loss.

2.1. Stomatal index

Stomatal density (SD) is a function of both the number of stomata plus the size of the epidermal cells. Thus, SD is affected both by the initiation of stomata and the expansion of epidermal cells. This expansion is a function of many variables (e.g. light, temperature, water status, position of leaf on crown, and intra-leaf position), and can overprint the signal reflective of stomatal initiation. As it turns out, CO_2 plays a stronger role in stomatal initiation than in epidermal cell expansion (this is discussed in detail below). Salisbury (1927) introduced the concept of stomatal index (SI), which normalizes for the effects of this expansion (i.e. density of epidermal cells). It is defined as:

$$\mathrm{SI}(\%) = \frac{\text{stomatal density}}{\text{stomatal density} + \text{epidermal cell density}} \times 100$$

where stomata consist of the stomatal pore and two flanking guard cells.

2.2. C_4 plants

The fundamental photosynthetic differences between C_3 and C_4 plants have consequences for stomatal-based CO_2 reconstructions. Carbon in C_3 plants is fixed within the spongy and palisade mesophyll where CO_2 concentrations (c_i) are approximately 70% of the atmospheric value. As atmospheric CO_2 fluctuates, so too does c_i to maintain this ~0.7 ratio (Polley et al., 1993; Ehleringer and Cerling, 1995; Beerling, 1996; Bettarini et al., 1997). Thus the stomatal pore area is sensitive to changing atmospheric CO_2 levels. C_4 plants, in contrast, fix carbon within their bundle sheath cells. The endodermis enclosing these bundle sheath cells is highly impervious to CO_2, and consequently CO_2 concentrations within these cells can reach 1000–2000 ppmV (Lambers et al., 1998). One would therefore anticipate, based on the proposed mechanism between CO_2 and stomatal initiation discussed above, that even moderate changes in atmospheric CO_2 have little influence on stomatal pore area and, by extension, SD and SI (Raven and Ramsden, 1988). Of the nine responses derived from C_4 plants documented here, only one inversely responds to CO_2 (see Appendix A1). This marked insensitivity in C_4 plants lends indirect support for the proposed mechanism. Because of the above physiological reasons, none of the analyses considered here include responses from C_4 plants.

3. Stomatal density and stomatal index as CO_2 indicators

A database consisting of 285 SD responses and 145 SI responses to variable CO_2 concentrations was compiled to elucidate salient patterns (Appendices A1–3). 176 species are represented. This database is an expansion of previous reviews (Beerling and Chaloner, 1994; Woodward and Kelly, 1995) and includes, for the first time, stomatal indices.

Each response was first placed in one of three categories: experimental, subfossil, and fossil. Experimental responses stem from experimentally controlled CO_2 environments, typically in greenhouses, which last from 14 days to five years in length. For studies that measured SD and/or SI at several different times and/or CO_2 levels, typically only the response corresponding to the longest exposure time and highest CO_2 level was used. Most subfossil responses stem from dated herbarium specimens (from the last 240 years), where corresponding CO_2 concentrations are known from ice core data (Neftel et al., 1985; Friedli et al., 1986). Data from altitudinal transects and natural CO_2 springs are also placed in the subfossil category, as this category represents the closest match in terms of CO_2 exposure time. Finally, fossil responses consist of well-dated fossil material. Methods for obtaining reference CO_2 concentrations for the fossil responses are discussed below.

Each response was assigned as either increasing ($P < 0.05$), decreasing ($P < 0.05$), or remaining the same ($P > 0.05$) relative to controls. Where P-values were not reported, a test for overlapping standard deviations was used, which typically yields a conservative estimate for statistical significance (relative to the $\alpha = 0.05$ level).

Table 1
Statistical summary of stomatal responses to changing CO_2 concentrations

	Experimental				Subfossil				Fossil				Combined			
	SD[a]		SI[b]		SD		SI		SD		SI		SD		SI	
	%[c]	(*n*)	%	(*n*)	%	(*n*)	%	(*n*)	%	(*n*)	%	(*n*)	%	(*n*)	%	(*n*)
Total	40	(127)	36	(74)	50	(133)	34	(35)	88	(25)	94	(36)	49	(285)	50	(145)
Elevated CO_2[d]	39	(109)	29	(65)	–	–	–	–	100	(13)	96	(24)	45	(232)	41	(116)
Subambient CO_2	50	(18)	89	(9)	–	–	–	–	89	(9)	89	(9)	75	(40)	88	(25)
Opposite response[e]	9	(127)	4	(74)	11	(133)	9	(35)	12	(25)	3	(36)	11	(285)	5	(145)
Hypostomatous[f]	59	(27)	65	(17)	50	(80)	38	(24)	–	–	–	–	56	(121)	69	(70)
Amphistomatous[g]	36	(90)	27	(55)	49	(49)	25	(8)	–	–	–	–	44	(149)	32	(71)
Abaxial	40	(45)	24	(29)	41	(22)	–	–	–	–	–	–	41	(68)	21	(33)
Adaxial	29	(42)	31	(26)	55	(22)	–	–	–	–	–	–	38	(65)	33	(30)
Experiments using OTCs[h]	13	(31)	13	(24)	–	–	–	–	–	–	–	–	–	–	–	–
Experiments using greenhouses	48	(95)	48	(50)	–	–	–	–	–	–	–	–	–	–	–	–
Herbarium studies only	–[j]	–	–	–	57	(93)	89	(9)	–	–	–	–	–	–	–	–
Repeated species[i]	–	–	–	–	–	–	–	–	–	–	–	–	57	(28)	55	(11)

[a] Stomatal density.
[b] Stomatal index.
[c] Percentage of responses inversely correlating with CO_2.
[d] CO_2 concentrations are higher than controls.
[e] Percentage of responses positively correlating with CO_2.
[f] Leaves with stomata only on abaxial (lower) side.
[g] Leaves with stomata on both surfaces.
[h] OTC = open-top chamber; typically cone-shaped with an open top.
[i] For species with multiple responses with ≥1 inversely correlating with CO_2, percentage that consistently inversely correlate.
[j] Not applicable or sample size too small for meaningful comparison.

3.1. Experimental responses

127 SD and 74 SI responses from a pool of 68 species are represented here. For SD, 40% of the experimental responses inversely respond (at the $\alpha = 0.05$ level) to CO_2; the proportion for stomatal indices is similar (36%) (Table 1).

Plants exposed to subambient CO_2 are more likely to inversely respond than plants exposed to elevated CO_2 for both SD (50 vs. 39%; $P = 0.36$) and SI (89 vs. 29%; $P < 0.001$). These results support previous claims that plants more strongly express the CO_2–SD/SI inverse relationship when exposed to subambient versus elevated CO_2 concentrations (Woodward, 1987; Woodward and Bazzaz, 1988; Beerling and Chaloner, 1993a; Kürschner et al., 1997). A common explanation for this CO_2 'ceiling' phenomenon is that plants today have not experienced elevated CO_2 levels (350 + ppmV) for at least the entire Quaternary and possibly longer (Pagani et al., 1999a; Pearson and Palmer, 1999). Thus, for short time scales where only plant plasticity is tested, plants respond more favorably to CO_2 conditions which they most recently experienced, namely subambient concentrations (Woodward, 1988; Beerling and Chaloner, 1993a). The implication for stomatal-based CO_2 reconstructions is that experimental evidence based on elevated CO_2 treatments may not reflect the reliability of the method. Over 85% of the experimental responses analyzed here stem from elevated CO_2 treatments. Another related concern raised with experimental results is that CO_2 is shifted in one step in contrast to the smoother, longer-term trend in nature (Beerling and Chaloner, 1992; Kürschner et al., 1997).

An alternative explanation for the CO_2 ceiling is that while CO_2 is limiting for photosynthesis at CO_2 concentrations below present-day levels, it is not limiting at elevated levels. Therefore, for example, if CO_2 decreases in a subambient CO_2 regime

(where CO_2 is limiting for photosynthesis), a mechanism exists to increase stomatal pore area and, by extension, CO_2 uptake. The same may not be true at elevated CO_2 concentrations if CO_2 is not limiting for photosynthesis under such conditions (Wagner et al., 1996; Kürschner et al., 1998). Empirical data do not strongly support this alternative hypothesis. While assimilation rates generally decrease at subambient CO_2 levels (Polley et al., 1992; Robinson, 1994), they also typically increase in response to CO_2 concentrations of at least 700 ppmV (Long et al., 1996; Curtis and Wang, 1998). CO_2 therefore usually continues to limit photosynthesis in most plants above present-day CO_2 levels, even if the effects of this excess CO_2 are partially mediated by a reduction in photorespiration and enhancement in RuBP regeneration (the primary substrate used to fixed CO_2 in C_3 plants), and so only affect photosynthesis indirectly. Therefore, there is no reason to expect a CO_2 ceiling coincident with current CO_2 levels. It is likely, however, that the rate of change in assimilation rates is reduced at elevated CO_2 concentrations (Farquhar et al., 1980), which could reduce the sensitivity of SD and SI responses under such conditions.

Experimental manipulations are usually conducted in either enclosed greenhouses or open-top chambers (OTCs). Most OTCs have less control over humidity and temperature. Significant 'chamber effects' have been detected for stomatal parameters (Knapp et al., 1994; Apple et al., 2000), and results generated here support such claims. Plants in OTCs inversely respond to CO_2 in far fewer cases than greenhouse grown plants for both SD (13 vs. 48%; $P < 0.001$) and SI (13 vs. 48%; $P < 0.01$). Thus, it appears OTCs introduce confounding factors and should be avoided in SD/SI work.

Although the proportion of experimental responses inversely responding to CO_2 may appear low (40 and 36% for SD and SI, respectively), in part from the factors discussed above, it is important to note that the percentage of responses showing a positive relationship ($P < 0.05$) is very low (9 and 4% for SD and SI, respectively). Thus, the vast majority of plants either respond inversely to experimental exposure to CO_2 or do not respond at all.

3.2. Subfossil responses

133 SD and 35 SI responses from a pool of 95 species are represented here. For SD, 50% of the subfossil responses inversely relate (at the $\alpha = 0.05$ level) to CO_2. Thus, subfossil responses, which are based on longer exposure times, more often inversely relate to CO_2 than do experimental responses (50% vs. 40%; $P = 0.11$). For SI, only 34% of the responses show a significant inverse relationship, however the sample size is disproportionally small ($n = 35$) (Table 1).

As outlined above, three types of studies comprise the subfossil responses: altitudinal transects, natural CO_2 springs, and herbaria. If only herbarium responses are analyzed ($n = 93$ and $n = 9$ for SD and SI, respectively), the proportion showing an inverse response to CO_2 improves to 57 and 89%, respectively. Responses from altitudinal transects and natural CO_2 springs may therefore be of less value for paleo-CO_2 reconstructions. This dichotomy in response fidelity may be an expression of the CO_2 ceiling phenomenon discussed above. As CO_2 levels rose to current levels over the last 240 + years, the majority of plants (57 and 89% for SD and SI, respectively) responded with significant decreases in SD and/or SI. At higher CO_2 levels, however, as expressed near natural CO_2 springs, a smaller proportion of plants responded with lower SD (30%; $n = 30$) and/or SI (16%; $n = 25$). If, on the other hand, current CO_2 concentrations do *not* represent a true genetic ceiling for plants, than these data show that the majority of plants cannot adapt to CO_2 levels above today's within the special residence time near natural CO_2 springs (10^2–10^3 years?).

In accordance with the experimental responses, a very small proportion of the subfossil observations positively respond to CO_2 (11 and 9% for SD and SI, respectively). Most plants either inversely respond to CO_2 or do not respond at all. If CO_2 exerts any influence on stomatal initiation, it must be of an inverse behavior.

3.3. Fossil responses

25 SD and 36 SI responses from a pool of 28 species are represented here. For SD, 88% of the observations show an inverse relationship (at the $\alpha =$

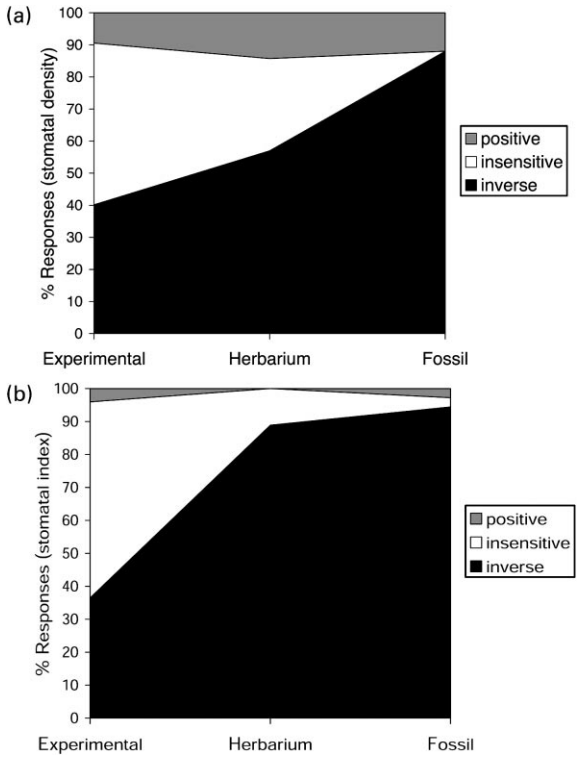


Fig. 2. The percentage of responses for (a) SD and (b) SI that inversely relate to CO_2 ('inverse'), show no significant change to CO_2 ('insensitive'), or respond positively to CO_2 ('positive') in each of three categories. Note that only herbarium responses compose the subfossil category.

0.05 level) to CO_2; for SI, the proportion is 94% (Table 1). Only 12 and 3% of the observations positively respond to CO_2 for SD and SI, respectively. Thus, the robustness of the SD/SI method improves with increased CO_2 exposure time (Fig. 2), supporting earlier hypotheses (Beerling and Chaloner, 1992, 1993a).

Qualitatively, the transition between dominance of stomatal response by plasticity within a given gene pool and genetic adaptation appears to occur for most plants between 10^2 and 10^3 years (i.e. intermediate between CO_2 exposure times typical for subfossil and fossil responses). This conclusion hinges on the assumption that CO_2 exerts a consistent genetic pressure on stomatal initiation, and given sufficient exposure time will overprint the smaller scale plastic responses (including changes in individual stomatal pore size). The fact that the increase in responses showing an inverse relationship to CO_2 as a function of exposure time comes at the expense of insensitive responses (Fig. 2) supports this assumption. 10^2 to 10^3 years is slightly longer than previous estimates (Beerling and Chaloner, 1993a), and should give rise to some caution in using experimental and subfossil responses in paleo-CO_2 reconstructions (i.e. comparing responses due mainly to plasticity versus genetic adaptation).

The fossil data cast doubt on the notion that stomata cannot respond to CO_2 concentrations above present-day levels. The proportion of fossil responses showing an inverse relationship based on subambient CO_2 exposure are nearly equal to those fossil observations based on elevated CO_2 exposure for both SD (89 and 100%, respectively) and SI (89 and 96%, respectively), although sample sizes are fairly small (Table 1). Some groups of plants respond to CO_2 levels of at least 2700 ppmV (McElwain and Chaloner, 1995; Appendix C). This result does not discount, however, that stomatal parameters may be less *sensitive* at elevated than at subambient (relative to today) CO_2 levels. The CO_2 ceiling observed in experimental responses therefore appear to stem from the short-term inability of plants to respond to elevated CO_2, not a long-term genetic limit. Interestingly, Woodward (1988) noted that plants with short generation times (e.g. annuals) are often capable of decreasing their stomatal densities when experimentally exposed to elevated CO_2 levels (for $\geq$1 year), probably because of their quicker genetic adaptation rates (Woodward, 1988). This suggests that the exposure time required to mitigate the CO_2 ceiling may not be much beyond typical experimental exposure times, and in fact may not exist at all for some plants.

Caution is urged with regard to several features concerning the fossil responses. First, in several studies stomatal comparisons between fossil and modern plants were made with two separate but ecologically equivalent sets of species (McElwain and Chaloner, 1995, 1996; McElwain, 1998; McElwain et al., 1999). In addition to the long-term influence of CO_2 on SD and SI for a given species, it has also been shown, for example, that high CO_2 selects for groups of plants with lower mean stomatal densities/indices (Beerling, 1996; Beerling and Woodward, 1997) (Fig. 3). Thus, it is not particularly surprising that stomatal densities and indices from times of high

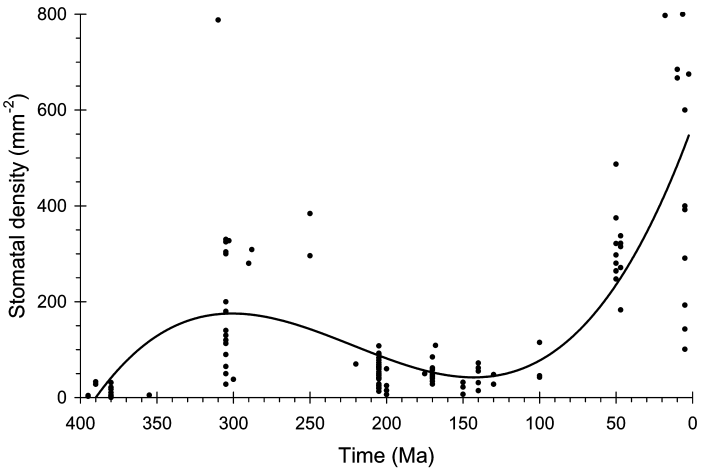


Fig. 3. SD versus time for the Phanerozoic. Redrawn from Beerling and Woodward (1997), with additional data plotted from McElwain and Chaloner (1996), Edwards et al. (1998), McElwain (1998), Cleal et al. (1999) and McElwain et al. (1999). Regression is a third order polynomial ($r^2 = 0.57$; $n = 132$). Compare trend with Fig. 1.

CO_2 are lower than for ecologically equivalent modern species. Ideally, these two effects should be kept separate.

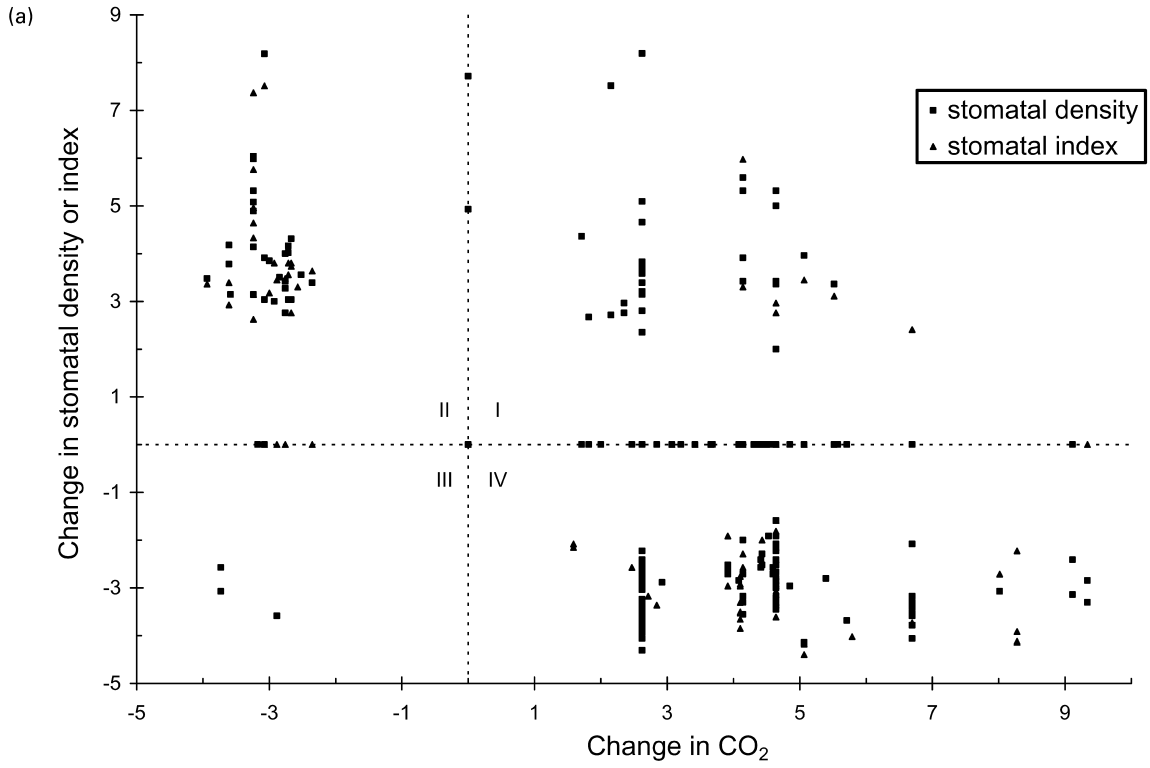
Second, estimates of CO_2 for the fossil responses are invariably not as accurate as those estimates for experimental and subfossil responses. Ice core derived data are used for the last 150 k.y., and the model of Berner (1994) or other proxy data are most often used for pre-ice core responses. In particular, estimates from Berner's curve are highly approximate due to its sizable error envelope and coarse 10 m.y. time resolution (see Fig. 1); brief but large CO_2 excursions discernable with the various proxy methods are probably too temporally constrained to influence Berner's model (Montañez et al., 1999). In cases where experimental and subfossil responses are used to generate a standard curve upon which CO_2 concentrations are directly calculated from fossil responses, ice core data (Beerling et al., 1995; Wagner et al., 1999; Rundgren and Beerling, 1999) or the presence of temperature excursions (van der Burgh et al., 1993; Kürschner, 1996; Kürschner et al., 1996) are used to corroborate the stomatal-based estimates.

3.4. Combined data set

Based on the combination of the above three categories, both SD and SI inversely correlate with CO_2 ca. 50% of the time ($n = 285$ and 145, respectively) (Table 1). Very rarely do the responses positively correlate with CO_2 (11 and 5% for SD and SI, respectively). For species that have been analyzed repeatedly by different researchers, those that inversely respond to CO_2 tend always to respond in such a way (57% ($n = 28$) and 55% ($n = 11$) for SD and SI, respectively). Woodward and Kelly (1995) reported a similar behavior, where 76% of their sensitive species consistently responded.

Thus, although response times differ (see above and Fig. 2), CO_2 is highly negatively correlated with stomatal initiation. A scatterplot of all data shows an overall inverse relationship between SD/SI and CO_2 (Fig. 4a). Although the overall regression is not robust ($r^2 = 0.26$; $n = 420$), this principally stems from equivocal experimental and natural CO_2 spring data. The fossil data, when regressed independently, yield an r^2 of 0.68 ($n = 59$) (Fig. 4b). Given the species-specific and

Fig. 4. (a) Scatterplot of all data ($r^2 = 0.26$; $n = 420$) showing the cube root transform of percentage change in SD and SI in response to percentage change in atmospheric CO_2 concentration. Responses in quadrants II and IV inversely relate to CO_2 while responses in quadrants I and III positively relate. (b) Similar scatterplot for fossil data only. Regression equation of untransformed data: $y = 112.43\exp(-0.0026x) - 100$. ($r^2 = 0.68$; $n = 59$).


(a)
stomatal density
stomatal index
Change in stomatal density or index
Change in CO_2
II
I
III
IV
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7
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-1
-3
-5
-5
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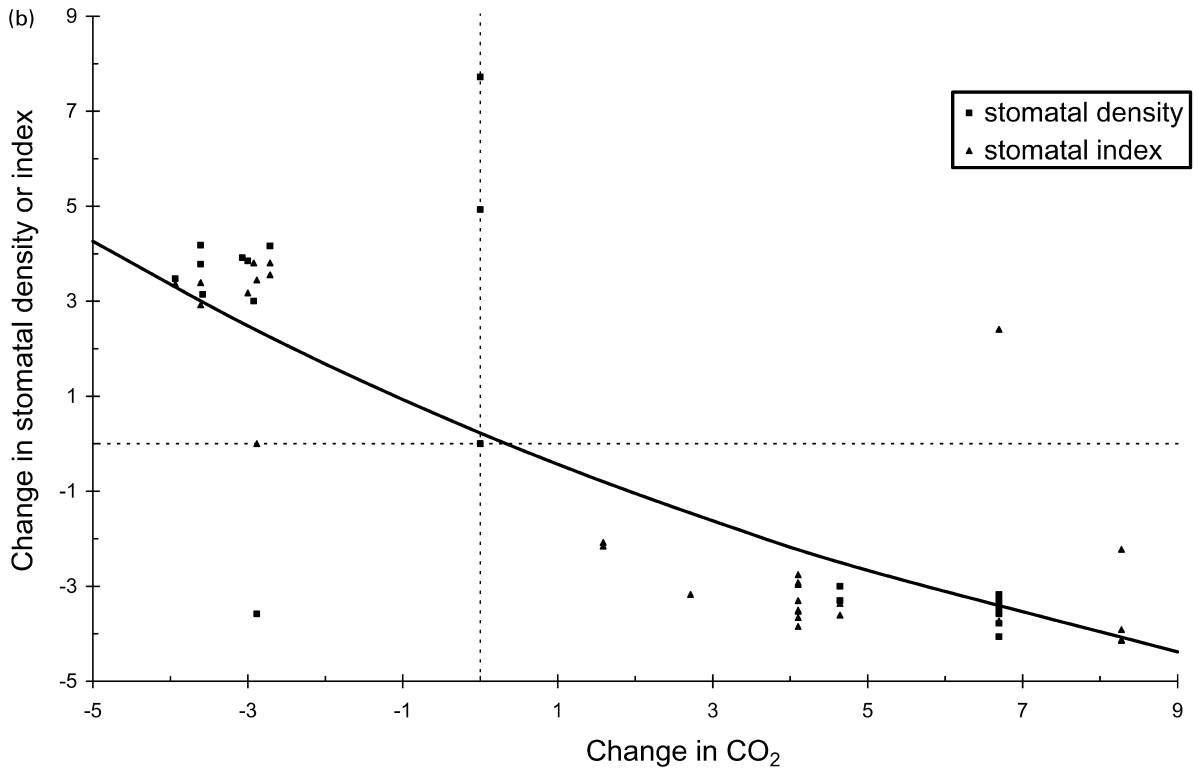
9


(b)
stomatal density
stomatal index
Change in stomatal density or index
Change in CO_2
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7
9

probable multi-mechanistic nature of the relationship, this regression is surprisingly robust.

Curiously, based solely on the combined results, it appears SD is equally reliable as SI as a CO_2 indicator (Table 1). The implications are tempting, as epidermal cells are often difficult to resolve in fossil material (Beerling et al., 1991; McElwain and Chaloner, 1996). This issue is discussed in the section below.

Most vascular land plants have stomata on either both surfaces (amphistomatous) or only the abaxial (lower) surface (hypostomatous). Woodward and Kelly (1995) reported no strong differences in responses between the two leaf types, although in experimental responses amphistomatous species appeared more likely to inversely relate to CO_2. Results here indicate hypostomatous species more often inversely respond to CO_2 for both SD (56 vs. 44%; $P < 0.03$) and SI (69 vs. 32%; $P < 0.001$). For amphistomatous species, neither the abaxial nor adaxial (upper) surface yield statistically different responses (Table 1).

4. Potential confounding factors

CO_2 is likely not the sole factor determining stomatal density and stomatal index. As discussed above, SD is sensitive to both stomatal initiation and epidermal cell expansion, while SI is sensitive only to stomatal initiation. The influence of natural variability, water stress, irradiance, temperature and other factors on stomatal parameters are briefly discussed below. More thorough reviews are given by Salisbury (1927), Tichá (1982) and Woodward and Kelly (1995).

4.1. Natural variability

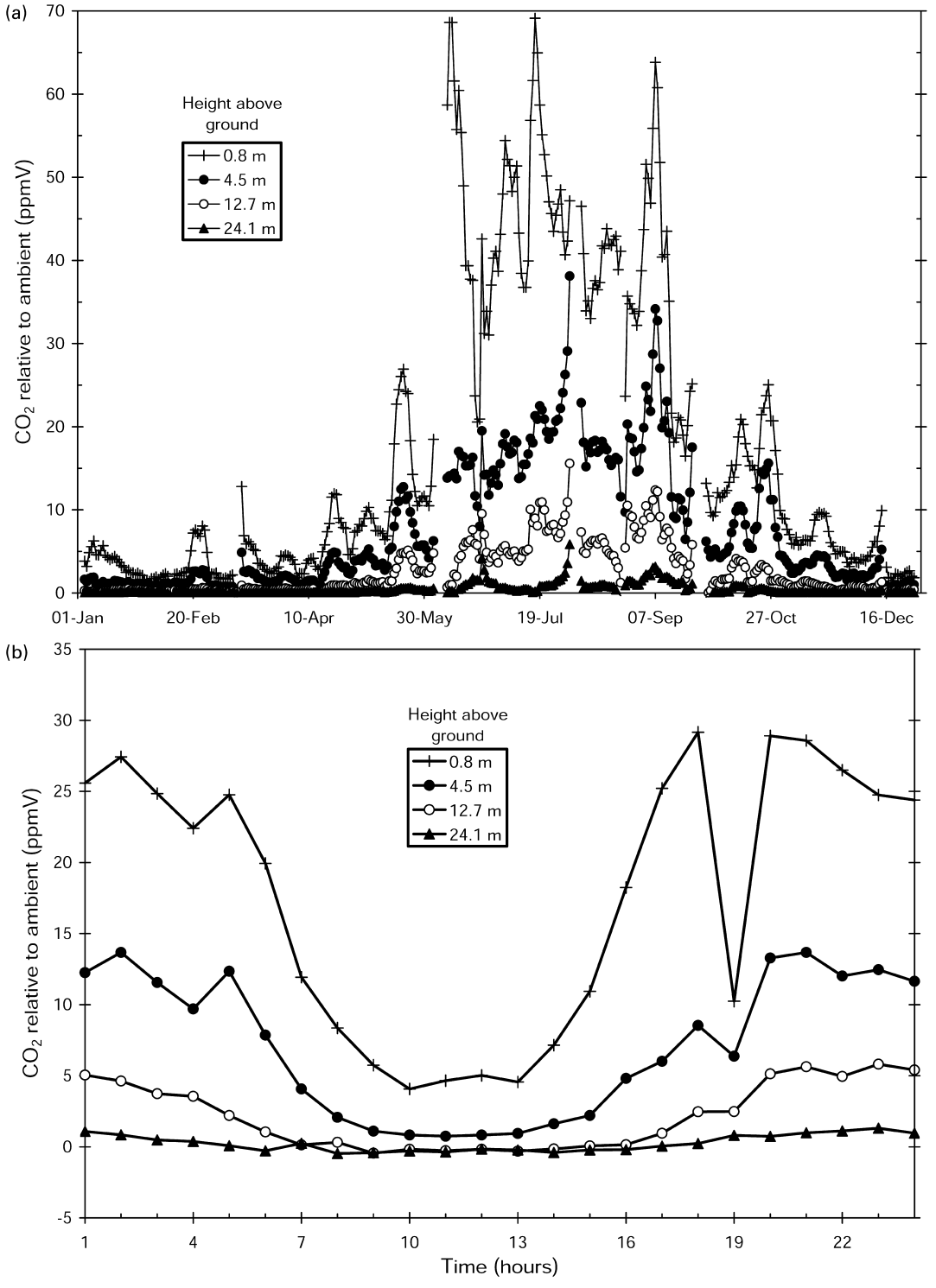
In general, stomatal density increases from leaf base to tip (Salisbury, 1927; Sharma and Dunn, 1968, 1969; Tichá, 1982; Smith et al., 1989; Ferris et al., 1996; Zacchini et al., 1997; Stancato et al., 1999). SD also often increases from leaf midrib to margin (Salisbury, 1927; Sharma and Dunn, 1968; Smith et al., 1989), although sometimes the differences are not significant (Sharma and Dunn, 1969; Tichá 1982). In contrast, very little intra-leaf variation in SI is present (Salisbury, 1927; Rowson, 1946; Sharma and Dunn, 1968, 1969; Rahim and Fordham, 1991), although Poole et al. (1996) found significant intra-leaf variation in *Alnus glutinosa*. For amphistomatous species, the distribution of stomata are generally more uniform on the abaxial surface (Rowson, 1946; Sharma and Dunn, 1968, 1969), and so for all species typically the mid-lamina of the abaxial surface yields the least variation.

Stomatal density also increases from the basal to distal regions of the plant (Salisbury, 1927; Gay and Hurd, 1975; Tichá, 1982; Oberbauer and Strain, 1986; Zacchini et al., 1997), primarily as a consequence of decreased water potential. Decreased water potential stimulates xerophytic traits, which include smaller epidermal cells, which in turn promote closer packing of stomata, and thus increased SD. Little effect is reported for SI (Rowson, 1946), although evergreen species may exhibit a significant gradient (Kürschner, W.M., personal communication, 2000). Conflated with this trend are the differences between sun and shade leaves. Again, SD is consistently higher in sun leaves while SI values remain conservative (Salisbury, 1927; Poole et al., 1996; Kürschner, 1997; Wagner, 1998) with the exception of the study of Poole et al. (1996), who found a small 7% decline in SI for shade versus sun leaves. For fossil studies, since sun leaves in allochthonous assemblages are preferentially preserved (Spicer, 1981), this issue is often not a concern even for SD-based work. For example, Kürschner (1997) observed that 90% of his Miocene *Quercus pseudocastanea* leaves were sun morphotypes.

4.2. Water stress

In general, water stress correlates with increased SD (Salisbury, 1927; Sharma and Dunn, 1968, 1969; Tichá, 1982; Abrams, 1994; Estiarte et al., 1994;

Fig. 5. (a) CO_2 relative to ambient concentrations for four heights within a tree canopy in 1996. Canopy height is ca. 24 m. Ordinate represents seven day running average of daily averages of hourly measurements at each height ($n = 5311$ for each height). Measurements at 29.0 m height taken as ambient value (mean for time interval at this height = 370 ppmV). (b) Diurnal trend of CO_2 relative to ambient concentrations (data from 9 April–13 July 1996). Ordinate represents mean for each hour at each height ($n = 1388$ for each height). Standard errors approximate size of symbols. Raw data used with permission of S. Wofsy.


(a)
Height above ground
0.8 m
4.5 m
12.7 m
24.1 m
CO2 relative to ambient (ppmV)
70
60
50
40
30
20
10
0
01-Jan
20-Feb
10-Apr
30-May
19-Jul
07-Sep
27-Oct
16-Dec
(b)
Height above ground
0.8 m
4.5 m
12.7 m
24.1 m
CO2 relative to ambient (ppmV)
35
30
25
20
15
10
5
0
-5
1
4
7
10
13
16
19
22
Time (hours)

Clifford et al., 1995; Heckenberger et al., 1998; Pääkkönen et al., 1998). Some studies, however, report no response (Estiarte et al., 1994; Dixon et al., 1995; Pritchard et al., 1998; Centritto et al., 1999). No studies report a decrease. SI consistently appears insensitive to water stress (Salisbury, 1927; Sharma and Dunn, 1968, 1969; Estiarte et al., 1994; Clifford et al., 1995).

Salisbury (1927) proposed humidity as a mechanism for controlling stomatal initiation, and thus SI. Increased humidity slightly increased SI ($P > 0.05$) for *Scilla nutans*, however, Tichá (1982) concluded that humidity may actually reduce stomatal index. Sharma and Dunn (1968, 1969) found no effect. Thus, the current data are equivocal.

4.3. Irradiance

Not surprisingly, light intensity usually positively correlates with SD (Sharma and Dunn, 1968, 1969; Gay and Hurd, 1975; Tichá, 1982; Oberbauer and Strain, 1986; Solárová and Pospíšilová, 1988; Stewart and Hoddinott, 1993; Ashton and Berlyn, 1994; Furukawa, 1997; Zacchini et al., 1997). This response is driven (partially) by enhanced water stress. Light intensity may also positively affect SI (Sharma and Dunn, 1968, 1969; Furukawa, 1997), although some report no response (Salisbury, 1927; Sharma and Dunn, 1968, 1969) and Rahim and Fordham (1991) observed a small decrease with increasing irradiance. In the case of Sharma and Dunn (1968, 1969), they speculated that the low irradiance levels required to depress SI could not sustain plants in a competitive environment.

In experimental manipulations, photoperiod strongly affects both SD and SI (Schoch et al., 1980; Zacchini et al., 1997). Schoch et al. (1980) observed that even one day of low irradiance levels during the critical period of stomatal initiation could affect SD and SI. Given that SI is typically conservative in deciduous species within a given crown, it is possible the effects of photoperiod on SI observed in experiments are minimal in nature.

4.4. Temperature

Temperature appears positively correlated with SD (Ferris et al., 1996; Reddy et al., 1998; Wagner, 1998; but see Apple et al., 2000), a likely consequence of enhanced water stress. Temperature may also affect SI (Ferris et al., 1996; Wagner, 1998), suggesting an influence on stomatal initiation. Reddy et al. (1998), however, found no response. The influence of temperature on stomatal initiation may be inconsequential, though, as most plants partially normalize for fluctuating temperatures by adjusting the timing of leaf development, and so the temperature at which stomata form remains fairly constant (Wagner, 1998).

4.5. Canopy CO_2 gradient

If CO_2 concentrations within canopies deviate significantly from ambient concentrations, CO_2 estimates based on stomatal parameters could be skewed. Empirical evidence, however, does not suggest such large deviations. Hourly measurements of CO_2 at eight different heights (0.3, 0.8, 4.5, 7.5, 12.7, 18.3, 24.1 and 29.0 m above the ground surface) have been recorded for several consecutive years from an atmospheric tower in the Harvard Forest (data available at http://www-as.harvard.edu/chemistry/hf/profile/profile.html). This forest, in north central Massachusetts, USA, consists of mixed hardwoods and conifers. As shown in Fig. 5a, above 4.5 m, where the bulk of leaves from mature trees form, canopy CO_2 levels are virtually indistinguishable from ambient levels. Furthermore, all deviations diminish during the middle of the day (Fig. 5b), a period when cell respiration and division is highest. Thus, CO_2 gradients within canopies are likely not strong enough to influence stomatal initiation rates.

4.6. Paleotaxonomy

Paleobotanical species identification via morphological comparison with modern representatives is often tenuous, particularly with pre-Neogene material. There are methods, however, to bolster confidence in such morphologically based species identification. These include comparing the sedimentological and ecological contexts with the proposed extant representative. For example, if a strictly swamp margin fossil species is morphologically identical to a modern representative that is also restricted to swamp margins, then one can be more confident that the two are identical species. Independent of species identification, however, it is also possible that a single species may develop, for example, different stomatal

behaviors through time. This, in turn, could affect paleo-CO_2 reconstructions. One way to circumvent this problem is through the study of the species' closest extant sister group (e.g. de Queiroz and Gauthier, 1990). If the stomata in both extant species show a similar response to CO_2, then it can be assumed that this stomatal behavior in both species is conservative in time back to at least when the species branched.

4.7. Other potential confounding factors

Through the comparison of 100 species, neither growth form (woodiness vs. non-woodiness; trees vs. shrubs), habitat (cool vs. warm), nor taxonomic relatedness strongly correlated with SD (Woodward and Kelly, 1995). Habitat has also been found not to affect SI (Rowson, 1946). Analysis of the data set presented here shows that for genera represented by >1 species, only 19% ($n = 16$) and 14% ($n = 7$) of these genera respond in a consistent fashion to CO_2 (i.e. positive, negative, or insensitive) for SD and SI, respectively. These results provide further support for the taxonomic independence of stomatal responses to CO_2.

An increase in ploidy level is associated with lower SD (Rowson, 1946; Mishra, 1997). No clear trend is found in SI (based on two studies), with Rowson (1946) reporting a decrease and Mishra (1997) no change. Given the widespread variability of ploidy levels in the fossil record (Masterson, 1994), this may have important consequences for stomatal-based CO_2 reconstructions.

Elevated levels of ozone increase SD in *Betula pendula* (Frey et al., 1996; Pääkkönen et al., 1998), *Fraxinus excelsior* (Wiltshire et al., 1996) and *Olea europaea* (Minnocci et al., 1999). Effects on SI were not reported.

Although largely untested, atmospheric oxygen may influence SD and SI. Elevated O_2/CO_2 ratios increase photorespiration in C_3 plants, suppressing CO_2 assimilation rates. One pathway to mediate this decline is increasing SD and/or SI. Experimental work on *Hedera helix* and *Betula pubescens* show slightly higher stomatal indices in a 35% versus 21% O_2 atmosphere (Beerling et al., 1998b). If correct, this factor may be particularly important during the Carboniferous and early Permian when O_2 concentrations are modeled to exceed 30% (Berner and Canfield, 1989; Berner et al., 2000).

5. Summary

Based on the data presented here, nearly all species appear responsive on the time scales inherent in most fossil CO_2 reconstructions ($>10^2$ years) (Fig. 2; Table 1). Only 40–50% of species are responsive over the time scales of experimental and subfossil studies ($\sim 10^{-2}$–10^2 years), and so those conducting studies requiring such responses must take care in choosing sensitive species (Appendices A and B). Another potential weakness in utilizing experimental and subfossil responses is that they are more reflective of plasticity within given gene pools, and may display different behaviors than their respective fossil responses (which are more reflective of genetic adaptation).

SD and SI are both equally likely to inversely relate to CO_2. SD, however, is sensitive to factors affecting cell expansion such as water stress, temperature, and irradiation. SI, in contrast, is sensitive only to factors affecting cell initiation, of which CO_2 appears to be one factor. Thus, even if SD and SI show similar responses for a given species (e.g. both positive or negative), SI should yield more accurate CO_2 estimates.

5.1. Applications of method

Although experimental work has been carried out for many years, Woodward (1987) was the first to document the inverse CO_2–SD/SI relationship over longer time scales (200 years). Beerling et al. (1991, 1993) extended the applicability of the method to 140 k.y. with *Salix herbacea*, where stomatal densities showed a general inverse relationship with ice core reconstructed CO_2 concentrations. This method has also proven successful with 9.2 ka *Salix cinerea* (McElwain et al., 1995), 13 ka *Betula nana* (Beerling, 1993), and 28 ka *Pinus flexilis* (van de Water et al., 1994).

While the above studies validate the relationship over time scales useful for fossil studies, they do not generate independent estimates of paleo-CO_2. For this, fossil responses must be fitted to a standard curve based on experimental, subfossil, and fossil

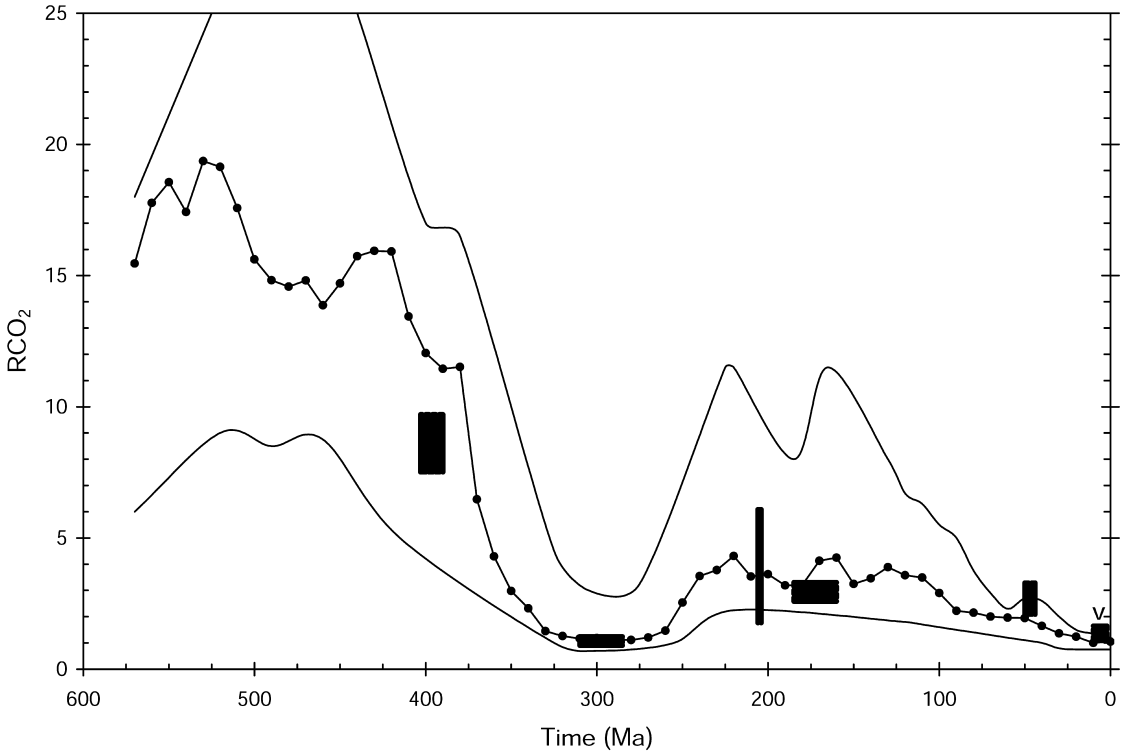


Fig. 6. Estimates of CO_2 for the Devonian, Carboniferous–Permian, Triassic, Jurassic, and Eocene (unmarked boxes) calculated from the stomatal ratio technique of McElwain and Chaloner (1995) superimposed over the CO_2 curve and corresponding error envelope of Berner (1994, 1998). Stomatal ratio scale calibrated to RCO_2 scale using a 1:1 correspondence; this is the 'Recent standard' of McElwain (1998). RCO_2 and stomatal ratio defined in Fig. 1 and text, respectively. Data from McElwain (1998) and McElwain et al. (1999). Estimates of CO_2 for the Miocene ("v") calculated from a herbaria-based training set. Data from van der Burgh et al. (1993) and Kürschner et al. (1996).

responses (from the last 400 k.y., for which ice core data exist; e.g. Petit et al., 1999) of the *same* species. This approach has been successful in the Holocene with *Salix herbacea* (Beerling and Chaloner, 1993a; Beerling et al., 1995; Rundgren and Beerling, 1999) and *Betula pubescens* and *B. pendula* (Wagner et al., 1999), and in the Miocene with *Quercus petraea* and *Betula subpubescens* (van der Burgh et al., 1993; Kürschner, 1996; Kürschner et al., 1996). While this approach produces the most accurate CO_2 reconstructions, it is limited by its requirement to compare identical species (or highly similar species within a genus; Wagner et al., 1999). There are, however, single species spanning most or all of the late Cretaceous and Tertiary (e.g. *Ginkgo adiantoides/biloba*, several members of Taxodiaceae), and so CO_2 estimates for this interval are possible.

One clear advantage of the stomatal method over other proxies and models is its high temporal resolution. The temporal resolution of late Quaternary fossil material often exceeds that of ice cores (Beerling et al., 1995; Wagner et al., 1999), and similar high resolution data have been used to document CO_2 excursions across the Allerød/Younger Dryas (Beerling et al., 1995) and Triassic/Jurassic (McElwain et al., 1999) boundaries. Another advantage over other proxies and models is its higher level of precision (compare Fig. 1a with Fig. 6).

Estimating pre-Cretaceous CO_2 levels proves more difficult. McElwain and Chaloner (1995) developed a technique comparing responses of fossil species to those of their Nearest Living Equivalents (NLEs). NLEs are defined ecologically, not taxonomically, and represent the ecologically closest living analog to the fossil species. SI ratios of the fossils:NLEs were calculated, and in order to estimate CO_2 the

Carboniferous:NLE stomatal ratio was normalized to the Phanerozoic CO_2 curve of Berner (1994), with the remainder of the ratios scaled accordingly in a linear fashion. Given that this method assumes a linear response and is not a true independent CO_2 indicator, reconstructed CO_2 concentrations from the Devonian, Carboniferous, Permian, and Jurassic all matched Berner's values remarkably well (McElwain and Chaloner, 1995, 1996). Later (McElwain, 1998), in order to reduce the method's dependence on Berner (1994), data (including new material from the Eocene) were plotted assuming a 1:1 correspondence between stomatal ratios and RCO_2 (RCO_2 = ratio of mass of paleo-CO_2 to pre-industrial value; see Fig. 1). Using this more independent technique, all but the Devonian material agreed well with the estimates of Berner (1994). Recent changes in Berner's model, however, have pushed back the sharp drop in Paleozoic CO_2 ~40 m.y. (Berner, 1998), resulting in closer agreement between the two methods for the Devonian (Fig. 6).

There is growing interest in quantifying Tertiary CO_2 concentrations (Cerling et al., 1997; Pagani et al., 1999a, 1999b; Pearson and Palmer, 1999), primarily fueled by the question of whether CO_2 and temperature are coupled during this interval. Estimates from stomatal indices have the potential to help resolve this question. As for pre-Cretaceous estimates, the less quantitative stomatal ratio method of McElwain and Chaloner (1995) remains the best option.

Acknowledgements

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Appendix A1

Experimental stomatal responses

Experiment length (days)	CO_2 levels (relative to controls[a])	Species used	Side of leaf	SD response	SI response	Source
?	↑ 300%	*Phaseolus vulgaris*	abaxial	* ↓ 9%	–	O'Leary and Knecht, 1981[b]
			adaxial	↔		
14	↑ 2067%	*Marsilea vestita*	abaxial	* ↓ 91%	–	Bristow and Looi, 1968[b]
			adaxial	↔	–	
	↑ ~10^5%	*Marsilea vestita*	abaxial	* ↓ 99%	–	
			adaxial	↔		
15	↑ 100%	*Populus euroamericana*	–	↑ 38%	↔	Gaudillère and Mousseau, 1989[b]
20	↑ 80%	*Phaseolus vulgaris*	abaxial	↔	↔	Ranasinghe and Taylor, 1996[b]
			adaxial	↔	↔	
21	↑ 86%	*Tradescantia* (*fluminensis*?)	abaxial	↔	↔	Boetsch et al., 1996[b]
21	↓ 29%	*Vaccinium myrtillus*	abaxial	↔	↔	Woodward, 1986[b]
			adaxial	* ↑ 548%	* ↑ 424%	
	↑ 29%	*Vaccinium myrtillus*	abaxial	↔	↔	
			adaxial	↔	↔	
21	↓ 34%	*Acer pseudoplatanus*	abaxial	* ↑ 220%	* ↑ 122%	Woodward and Bazzaz, 1988[b]
		Geum urbanum	abaxial	* ↑ 31%	* ↑ 18%	
			adaxial	* ↑ 214%	* ↑ 191%	
		Quercus robur	abaxial	* ↑ 131%	* ↑ 81%	
		Rhamnus catharticus	abaxial	* ↑ 117%	* ↑ 100%	
		Rumex crispus	abaxial	* ↑ 71%	* ↑ 31%	
			adaxial	* ↑ 150%	* ↑ 400%	

Appendix A1 (*continued*)

Experiment length (days)	CO_2 levels (relative to controls[a])	Species used	Side of leaf	SD response	SI response	Source
	↑ 100%	*Amaranthus retroflexus*	abaxial	* ↓ 35%	* ↓ 23%	
			adaxial	* ↓ 38%	* ↓ 26%	
		Ambrosia artemisiifolia	abaxial	* ↓ 11%	↔	
			adaxial	* ↓ 24%	* ↓ 25%	
		Setaria faberii	abaxial	↔	* ↓ 21%	
			adaxial	* ↓ 22%	* ↓ 21%	
2199–35	↑ 94%	*Lolium perenne*	adaxial	↔	–	Ryle and Stanley, 1992[b]
26	↑ 86%	*Lycopersicum esculentum*	abaxial	* ↓ 17%	↔	Madsen, 1973[b]
			adaxial	* ↓ 14%	↔	
	↑ 814%	*Lycopersicum esculentum*	abaxial	* ↓ 23%	↔	
			adaxial	* ↓ 36%	↔	
~28	↑ 33%	*Lolium temultentum*	adaxial	↔	–	Gay and Hauck, 1994[b]
28	↑ 100%	*Phaseolus vulgaris*	abaxial	↔	↔	Radoglou and Jarvis, 1992[c]
			adaxial	↔	↔	
~40	↑ 91%	*Raphanus raphanistrum*	abaxial	↔	↔	Case et al., 1998[b]
45	↑ 71%	*Anthyllis vulneraria*	abaxial	* ↓ 32%	* ↓ 17%	Ferris and Taylor, 1994[b]
			adaxial	↔	↔	
		Lotus corniculatus	abaxial	↑ 60%	↔	
			adaxial	↑ 40%	↔	
		Plantago media	abaxial	* ↓ 20%	↔	
			adaxial	* ↓ 36%	* ↓ 12%	
		Sanguisorba minor	abaxial	↑ 175%	↑ 36%	
			adaxial	↑ 150%	↑ 213%	
45	↑ 100%	*Vicia faba*	abaxial	↔	↔	Radoglou and Jarvis, 1993[c]
			adaxial	↔	↔	
45	↑ 168%	*Glycine max*	abaxial	↑ 38%	↔	Thomas and Harvey, 1983[c]
			adaxial	↔	↔	
		Liquidambar styraciflua	abaxial	↔	↑ 30%	
		Zea mays(C_4)	abaxial	↔	↔	
			adaxial	↔	↔	
~50	↑ 68%	*Anthyllis vulneraria*	abaxial	* ↓ 23%	↔	Bryant et al., 1998[c]
		Sanguisorba minor	abaxial	↔	↔	
		Bromopsis erecta	abaxial	↔	↔	
50	↓ ~32%	*Avena sativa*	abaxial	↔	–	Malone et al., 1993[c]
			adaxial	↔	–	
		Prosopis glandulso	abaxial	↔	–	
			adaxial	↔	–	
		Schizachyrium scoparium(C_4)	abaxial	↔	–	
		Triticum aestivum	abaxial	↔	–	
			adaxial	↔	–	
54	↑ 93%	*Boehmeria cylindrica*	–	↔	–	Woodward and Beerling, 1997[b]
56	↑ 100%	*Coleus blumei*	abaxial	* ↓ 9%	* ↓ 4%	Beerling and Woodward, 1995[b]
		Tropaeolum major	abaxial	* ↓ 4%	* ↓ 10%	
59	↑ 186%	*Pelargonium hortorum*	abaxial	↔	–	Kelly et al., 1991[b]
			adaxial	* ↓ 50%	–	
60	↓ 29%	*Salix herbacea*	combined	* ↑ 28%	–	Beerling et al., 1995[b]
	↑ 100%		combined	* ↓ 41%	–	
60	↑ 93%	*Ochroma lagopus*	abaxial	↔	–	Oberbauer et al., 1985[b]
			adaxial	↔	–	
63	↑ 157%	*Panicum tricanthum*	abaxial	* ↓ 22%	–	Tipping and Murray, 1999[b]
		Panicum antidotale(C_4)	abaxial	↑ 28%	–	

Appendix A1 (*continued*)

Experiment length (days)	CO_2 levels (relative to controls[a])	Species used	Side of leaf	SD response	SI response	Source
< 66	↑ 100%	*Gossypium hirsutum*	abaxial	↔	↔	Reddy et al., 1998[b]
			adaxial	↔	↔	
72	↑ 89%	*Lolium perenne*	abaxial	↑	* ↓	Ferris et al., 1996[c]
80	↑ 100%	*Betula pendula*	abaxial	↔	↔	Wagner, 1998[b]
90	↑ 100%	*Quercus ilex*	abaxial	* ↓ 27%	–	Paoletti et al., 1997[b]
90–120	↑ 100%	*Andropogon gerardii*(C_4)	abaxial	* ↓ 28%	–	Knapp et al., 1994[c]
			adaxial	↑ 75%	–	
		Salvia pitcheri	abaxial	↑ 40%	–	
			adaxial	↑ 125%	–	
92	↑ 100%	*Populus trichocarpa*	abaxial	↔	↔	Radoglou and Jarvis, 1990[b]
			adaxial	↔	↔	
93	52%	*Oryza sativa*	abaxial	↓ 29%	–	Rowland-Bamford et al., 1990[b]
			adaxial	↓ 17%	–	
	↑ 173%	*Oryza sativa*	abaxial	↔	–	
			adaxial	↔	–	
105	↑ 186%	*Pelargonium hortorum*	abaxial	↔	–	Kelly et al., 1991[b]
			adaxial	↔	–	
114	↑ 87%	*Arachis hypogaea*	abaxial	* ↓ 12%	↔	Clifford et al., 1995[b]
			adaxial	* ↓ 16%	* ↓ 8%	
120	↑ 757%	*Rhizophora mangle*	abaxial	* ↓ 14%	–	Beerling, 1994[b]
		Laguncularia racemosa	abaxial	* ↓ 31%	–	
		Musa apiculata	abaxial	↔	–	
			adaxial	↔	–	
120	↑ 100%	*Populus trichocarpa*	abaxial	* ↓ 19%	* ↓ 31%	Ceulemans et al., 1995[c]
			adaxial	↔	↔	
		Populus deltoides	abaxial	* ↓ 27%	* ↓ 36%	
			adaxial	* ↓ 33%	↔	
120	↑ 100%	*Quercus petraea*	abaxial	* ↓ 25%	* ↓ 14%	Kürschner et al., 1998[b]
123	↑ 93%	*Pentaclethra macroloba*	abaxial	* ↓ 7%	–	Oberbauer et al., 1985[b]
			adaxial	↔	–	
125	↑ 49%	*Triticum aestivum*	abaxial	↔	↔	Estiarte et al., 1994[c]
			adaxial	↔	↔	
135	↑ 100%	*Prunus avium*	abaxial	↔	–	Centritto et al., 1999[c]
150	↑ 100%	*Chlorophytum picturatum*	abaxial	* ↓ 7%	* ↓ 23%	Beerling and Woodward, 1995[b]
		Hedera helix	abaxial	* ↓ 10%	* ↓ 29%	
		Hypoestes variegata	abaxial	* ↓ 9%	* ↓ 6%	
217	↑ 100%	*Maranthes corymbosa*	abaxial	* ↓ 14%	–	Eamus et al., 1993[b]
~240	↑ 98%	*Picea sitchensis*	abaxial	↔	–	Barton and Jarvis, 1999[b]
270	↑ 114%	*Pinus banksiana*	–	↔	–	Stewart and Hoddinott, 1993[b]
300	↑ 97%	*Eucalyptus tetrodonta*	abaxial	* ↓ 20%	–	Berryman et al., 1994[b,c]
~365	↑ 71%	*Rumex obtusifolius*	abaxial	* ↓ 8%	–	Pearson et al., 1995[b]
			adaxial	↔	–	
~400	↑ 100%	*Rhizophora mangle*	abaxial	* ↓ 16%	↔	Farnsworth et al., 1996[b]
~425	↑ 71%	*Bromus erectus*	abaxial	↔	↔	Lauber and Körner, 1997[c]
			adaxial	↔	↔	
		Plantago media	abaxial	↔	↔	
			adaxial	↔	↔	
		Sanguisorba minor	abaxial	↔	↔	
570	↑ 100%	*Prunus avium*	abaxial	↔	–	Atkinson et al., 1997[b]
		Quercus robur	abaxial	↑ ~150%	–	
600	↑ 97%	*Pinus palustris*	–	↔	–	Pritchard et al., 1998[c]

Appendix A1 (*continued*)

Experiment length (days)	CO_2 levels (relative to controls[a])	Species used	Side of leaf	SD response	SI response	Source
~730	↑ 100%	*Picea abies*	–	↔	–	Dixon et al., 1995[c]
		Quercus rubra	abaxial	↑ 8%	–	
730	↑ 60%	*Tussilago farfara*	abaxial	–	∗ ↓ 26%	Beerling and Woodward, 1997[b]
750	↑ 97%	*Mangifera indica*	abaxial	∗ ↓ 17%	–	Goodfellow et al., 1997[b]
~840	↑ 99%	*Scirpus olneyi*	–	↔	–	Drake, 1992[c]
3 years	↑ 60%	*Pinus sylvestris*	abaxial	∗ ↓ 16%	–	Beerling, 1997[b]
			adaxial	∗ ↓ 18%	–	
3 years	↑ 60%	*Ginkgo biloba*	abaxial	∗ ↓ 20%	∗ ↓ 7%	Beerling et al., 1998a[b]
1155	↑ 50%	*Pseudotsuga menziesii*	abaxial	↔	–	Apple et al., 2000[b]
~5 years	↑ ~82%	*Citrus aurantium*	abaxial	↔	↔	Estiarte et al., 1994[c]
Meta-analysis		43 species (60% showed SD reductions)		∗ ↓ (9.0 ± 3.3% s.e.)	–	Woodward and Kelly, 1995

∗ response inversely relates ($P < 0.05$) to CO_2 concentration.
↔ no significant change ($P > 0.05$).
– not reported.
[a] Typically between 340 and 360 ppmV.
[b] Plants grown in enclosed greenhouses or chambers.
[c] Plants grown in open-top chambers (OTCs).

Appendix A2

Subfossil stomatal responses

Age of material (years)	CO_2 levels (relative to controls[a])	Species used	Side of leaf	SD response	SI response	Source
#	↓ 5%	*Salix herbacea*	abaxial	↔	–	Beerling et al., 1992
			adaxial	∗ ↑ 83%	–	
#	↓ 13%	*Eucalyptus pauciflora*	combined	∗ ↑ 26%	–	Körner and Cochrane, 1985
#	↓ 6%	*Griselinia littoralis*	combined	↔	–	Körner et al., 1986
	↓ 13%	*Nothofagus menziesii*	abaxial	∗ ↑ 21%	–	
	↓ 8%	*Ranunculus grahamii*	combined	↔	–	
#	↓ 10%	*Vaccinium myrtillus*	abaxial	↓ 20%	–	Woodward, 1986
			adaxial	∗ ↑ 425%	–	
#	↓ 6%	*Nardus stricta*	abaxial	↔	–	Woodward and Bazzaz, 1988
			adaxial	∗ ↑ 19%	–	
@	↑ 194%	*Tussilago farfara*	abaxial	–	∗ ↓ 65%	Beerling and Woodward, 1997
@	↑ 100%	*Scirpus lacustris*	–	∗ ↓ 19%	–	Bettarini et al., 1997
@	↑ 100%	*Allium sphaerocephalon*	abaxial	↔	–	Bettarini et al., 1998
		Buxus sempervirens	abaxial	↔	↔	
		Convolvulus arvensis	abaxial	↔	↑ 26%	
		Convolvulus cantabrica	abaxial	↔	↔	
		Conyza canadensis	abaxial	∗ ↓ 26%	↑ 21%	
		Fraxinus ornus	abaxial	∗ ↓ 35%	↔	
		Geranium molle	abaxial	↔	↔	

Appendix A2 (*continued*)

Age of material (years)	CO_2 levels (relative to controls[a])	Species used	Side of leaf	SD response	SI response	Source
		Globularia punctata	abaxial	↔	↔	
			adaxial	↔	↔	
		Hypericum perforatum	abaxial	↔	↔	
		Plantago lanceolata	abaxial	↔	↔	
			adaxial	↔	↔	
		Potentilla reptans	abaxial	↔	↔	
		Pulicaria sicula	abaxial	↔	↔	
		Ruscus aculeatus	abaxial	↔	–	
		Scabiosa columbaria	abaxial	↔	↔	
		Silene vulgaris	abaxial	↔	↔	
		Stachys recta	abaxial	* ↓ 11%	↔	
		Trifolium pratense	abaxial	↔	↔	
@	↑ ~130%	*Bauhinia multinervia*	abaxial	↑ 62%	↑ 41%	Fernández et al., 1998
			adaxial	* ↓ 71%	* ↓ 73%	
		Spathiphylum cannifolium	abaxial	↔	↔	
			adaxial	* ↓ 72%	* ↓ 85%	
@	↑ 40%	*Quercus pubescens*	abaxial	↔	↔	Miglietta and Rasci, 1993
@	↑ 515%	*Arbutus unedo*	abaxial	* ↓ 29%	* ↓ 20%	Jones et al., 1995
@	↑ 114%	*Quercus ilex*	abaxial	* ↓ 26%	–	Paoletti et al., 1998
@	↑ 50%	*Boehmeria cylinderica*	abaxial	↔	–	Woodward and Beerling, 1997
@	↑ ~71%	*Phragmites australis*	abaxial	↔	–	van Gardingen et al., 1997
			adaxial	* ↓ 45%	–	
37	↑ 15%[b]	*Metasequoia glyptostroboides*	abaxial	↔	* ↓ 17%	D.L. Royer, unpublished data
43	↑ 15%[b]	*Betula pendula*	abaxial	* ↓ 30%	* ↓ 32%	Wagner et al., 1996
70	↑ 18%[c]	*Acer campestre*	abaxial	↔	–	Beerling and Kelly, 1997
		Acer pseudoplatanus	abaxial	↔	–	
		Alliaria petiolata	abaxial	↑ 22%	–	
		Allium ursinum	abaxial	↔	–	
		Alnus glutinosa	abaxial	↑ 132%	–	
		Anemone nemorosa	abaxial	↔	–	
		Arum maculatum	abaxial	* ↓ 61%	–	
			adaxial	* ↓ 80%	–	
		Betula pendula	abaxial	* ↓ 39%	–	
		Betula pendula	abaxial	* ↓ 43%	–	
		Betula pubescens	abaxial	* ↓ 56%	–	
		Carpinus betulus	abaxial	↑ 13%	–	
		Castanea sativa	abaxial	* ↓ 24%	–	
		Chamaenerion angustifolium	abaxial	↔	–	
		Circaea lutetiana	abaxial	* ↓ 25%	–	
		Cirsium palustre	abaxial	* ↓ 22%	–	
		Cornus sanguinea	abaxial	* ↓ 16%	–	
		Corylus avellana	abaxial	* ↓ 50%	–	
		Crataegus monogyna	abaxial	* ↓ 36%	–	
		Dipsacus fullonum	abaxial	↑ 54%	–	
			adaxial	↑ 550%	–	
		Epilobium montanum	abaxial	* ↓ 28%	–	
			adaxial	↔	–	
		Fagus sylvatica	abaxial	↑ 33%	–	
		Fagus sylvatica	abaxial	↔	–	
		Fraxinus excelsior	abaxial	↑ 39%	–	
		Geranium dissectum	abaxial	↔	–	
		Geranium robertianum	abaxial	* ↓ 58%	–	
			adaxial	↑	–	
		Geum rubanum	abaxial	* ↓ 21%	–	
			adaxial	↔	–	
		Glechoma hederacea	abaxial	* ↓ 23%	–	
		Hedera helix	abaxial	↑ 101%	–	

Appendix A2 (*continued*)

Age of material (years)	CO_2 levels (relative to controls[a])	Species used	Side of leaf	SD response	SI response	Source
		Heracleum sphondylium	abaxial	∗ ↓ 14%	–	
			adaxial	↔	–	
		Hyacinthoides non-scripta	abaxial	↑ 56%	–	
			adaxial	↔	–	
		Hypericum hirsutum	abaxial	∗ ↓ 11%	–	
		Hypericum perforatum	abaxial	∗ ↓ 56%	–	
		Ilex aquifolium	abaxial	↑ 31%	–	
		Lamiastrum galeobdolon	abaxial	↔	–	
		Lathyrus pratensis	abaxial	↔	–	
			adaxial	∗ ↓ 38%	–	
		Ligustrum vulgare	abaxial	∗ ↓ 67%	–	
		Lonicera periclymenum	abaxial	∗ ↓ 27%	–	
		Luzula sylvatica	abaxial	∗ ↓ 44%	–	
		Lysimachia nummularia	abaxial	∗ ↓ 56%	–	
			adaxial	∗ ↓ 67%	–	
		Mercurialis perennis	abaxial	∗ ↓ 17%	–	
		Oxalis acetosella	abaxial	↔	–	
		Populus nigra	abaxial	↑ 46%	–	
		Primula vulgaris	abaxial	∗ ↓ 14%	–	
		Prunella vulgaris	abaxial	∗ ↓ 47%	–	
			adaxial	∗ ↓ 55%	–	
		Prunus avium	abaxial	∗ ↓ 20%	–	
		Pteridium aquilinum	abaxial	↔	–	
		Quercus petraea	abaxial	∗ ↓ 14%	–	
		Quercus robur	abaxial	↔	–	
		Ranunculus ficaria	abaxial	∗ ↓ 21%	–	
			adaxial	↔	–	
		Rosa canina	abaxial	∗ ↓ 28%	–	
		Sambucus nigra(sun)	abaxial	↔	–	
		sambucus nigra(shade)	abaxial	↔	–	
		Scrophularia nodosa	abaxial	∗ ↓ 18%	–	
		Silene dioica	abaxial	↑ 49%	–	
			adaxial	∗ ↓	–	
		Sorbus aucuparia	abaxial	↔	–	
		Stellaria holostea	abaxial	∗ ↓ 28%	–	
		Taxus baccata	abaxial	↔	–	
		Tilia cordata	abaxial	∗ ↓ 34%	–	
		Ulmus glabra	abaxial	↔	–	
		Vaccinium myrtillus	abaxial	↔	–	
			adaxial	↔	–	
		Vicia cracca	abaxial	∗ ↓ 57%	–	
			adaxial	∗ ↓ 20%	–	
		Vicia sepium	abaxial	∗ ↓ 43%	–	
		Viola odorata	abaxial	↔	–	
91	↑ 20%[c]	*Betula nana*	abaxial	∗ ↓ 29%	–	Beerling, 1993
98	↑ 24%[c]	*Salix herbacea*	combined	–	∗ ↓ 21%	Rundgren and Beerling, 1999
110	↑ 25%[c]	*Betula pubescens*	abaxial	∗ ↓ 45%	∗ ↓ 35%	Kürschner, 1996
118	↑ 24%[c]	*Quercus petraea*	abaxial	–	∗ ↓ 34%	van der Burgh et al., 1993
126	↑ 14%[c]	*Salix herbacea*	combined	∗ ↓ 22%	–	Beerling et al., 1993
~127	↑ 24%[c]	*Salix cinerea*	abaxial	∗ ↓ 22%	∗ ↓ 17%	McElwain et al., 1995
144	↑ 23%[c]	*Salsola kali*(C_4)	abaxial	–	↔	Raven and Ramsden, 1988
144	↑ 27%[c]	*Ginkgo biloba*	abaxial	↔	∗ ↓ 44%	D.L. Royer, unpublished data
150	↑ 14%[c]	*Salix herbacea*	combined	∗ ↓ 26%	–	Beerling et al., 1995
151	↑ 27%[c]	*Quercus robur*	abaxial	∗ ↓ 23%	–	Beerling and Chaloner, 1993b
173	↑ 25%[c]	*Olea europaea*	abaxial	∗ ↓ 24%	–	Beerling and Chaloner, 1993c
181	↑ 26%[c]	*Fagus sylvatica*	abaxial	∗ ↓ 43%	–	Paoletti and Gellini, 1993
		Quercus ilex	abaxial	∗ ↓ 28%	–	

Appendix A2 (*continued*)

Age of material (years)	CO_2 levels (relative to controls[a])	Species used	Side of leaf	SD response	SI response	Source
190	↑ 27%[c]	*Quercus petraea*	abaxial	∗ ↓ 40%	∗ ↓ 31%	Kürschner et al., 1996
200	↑ 24%[c]	*Acer pseudoplatanus, Carpinus betulus, Fagus sylvatica, Populus nigra, Quercus petraea, Q. robur, Rhamnus catharticus, Tilia cordata*	abaxial	∗ ↓ 40% (mean)	–	Woodward, 1987
240	↑ 25%[c]	*Alnus glutinosa, Amaranthus caudatus, Betula pendula, Buxus sempervirens, Ceratonia siliqua, Cynodon dactylon, Gentiana alpina, Helleborus foetidus, Juniperus communis, Papaver alpinum, Pinus pinea, P. uncinata, Pistacia lentiscus, Rhododendron ferrugineum*	combined	∗ ↓ 17% (mean)	↔ (mean)	Peñuelas and Matamala, 1990
3318	↑ 22%[d]	*Olea europaea*	abaxial	∗ ↓ 33%	–	Beerling and Chaloner, 1993c

∗ response inversely relates ($P < 0.05$) to CO_2 concentration.
↔ no significant change ($P > 0.05$).
– not reported.
data from an altitudinal study; thus, the 'age' is however long the population has existed at the sampled altitudes.
@ data from a natural CO_2 spring area; thus, the 'age' is however long the population has existed at the location, assuming constant CO_2 emissions.
[a] Typically between 340 and 360 ppmV; for herbarium studies, control corresponds with oldest material.
[b] From direct measurements from Mauna Loa Observatory, Hawaii and South Pole (Keeling et al., 1995).
[c] From Siple Station ice core (Neftel et al., 1985; Friedli et al., 1986).
[d] From Taylor Dome ice core (Indermühle et al., 1999).

Appendix A3

Fossil stomatal responses

Age of material (years)	CO_2 levels (relative to controls[a])	Species used	Side of leaf	SD response	SI response	Source
9000	↓ 25%[b,c]	*Salix herbacea*	combined	–	∗ ↑ 55%	Rundgren and Beerling, 1999
9190	↓ 27%[b]	*Salix cinerea*	abaxial	∗ ↑ 57%	∗ ↑ 32%	McElwain et al., 1995
9800	↑ 20%[d,j]	*Betula pubescens, B. pendula*	abaxial	–	∗ ↓ 32% (mean)	Wagner et al., 1999
10750 (Allerød/Y. Dryas)	↓ 25%[c,k]	*Salix herbacea*	combined	∗ ↑ 27%	–	Beerling et al., 1995
11500	↓ 24%[b]	*Salix herbacea*	combined	↓ 46%	↔	Beerling et al., 1992
13000	↓ 29%[b]	*Betula nana*	abaxial	∗ ↑ 60%	–	Beerling, 1993
16500	↓ 47%[b]	*Salix herbacea*	combined	∗ ↑ 54%	∗ ↑ 25%	Beerling et al., 1993
28000	↓ 46%[b]	*Pinus flexilis*	–	∗ ↑ 31%	–	van de Water et al., 1994
140,000	↓ 47%[b]	*Salix herbacea*	combined	∗ ↑ 73%	∗ ↑ 39%	Beerling et al., 1993
2.5 m.y.	↑ 4%[e]	*Quercus petraea*	abaxial	–	∗ ↓ 10%	van der Burgh et al., 1993; Kürschner et al., 1996

Appendix A3 (*continued*)

Age of material (years)	CO_2 levels (relative to controls[a])	Species used	Side of leaf	SD response	SI response	Source
6.5 m.y.	↓ 20%[e]	*Quercus petraea*	abaxial	–	∗ ↑ 55%	van der Burgh et al., 1993; Kürschner et al., 1996
	↓ 24%[e,l]	*Fagus attenuata*	abaxial	–	∗ ↑ 41%	
6.5 m.y.	↓ 20%[d]	*Betula subpubescens*	abaxial	∗ ↑ 72%	∗ ↑ 45%	Kürschner, 1996
10 m.y.	↑ 4%[e]	*Quercus petraea*	abaxial	–	∗ ↓ 9%	van der Burgh et al., 1993; Kürschner et al., 1996
10 m.y.	↔ [d]	*Betula subpubescens*	abaxial	∗ ↔	∗ ↔	Kürschner, 1996
15.5 m.y.	↑[f]	*Chamaecyparis linguaefolia, Cunninghamia chaneyi, Metasequoia occidentalis, Pinus harneyana, Pinus* sp., *Taxodium dubium*	combined	∗ ↓ (mean)	–	Huggins, 1985
44–50 m.y. (M. Eocene)	↑ 43%[g]	*Lindera cinnamomifolia, Lindera* sp.[n]	abaxial	∗ ↓ 36% (mean)	∗ ↓ 47% (mean)	McElwain, 1998
		Litsea bournensis, L. edwardsii, L. hirsuta[n]	abaxial	∗ ↓ 27% (mean)	∗ ↓ 38% (mean)	
160–185 m.y. (M. Jurassic)	↑ 149%[g]	*Brachyphyllum crucis*[n]	abaxial	∗ ↓ 54%	∗ ↓ 39%	McElwain and Chaloner, 1996
		B. mamillare[n]	abaxial	∗ ↓ 39%	∗ ↓ 52%	
		Ginkgo huttonii[n]	abaxial	∗ ↓ 32%	–	
160–185 m.y. (M. Jurassic)	↑ 149%[g]	*Baeira furcata*[n]	abaxial	∗ ↓ 44%	–	McElwain, 1998
			adaxial	∗ ↓ 67%	–	
		Ctenis exilis, C. kaneharai, C. sulcicaulis[n]	abaxial	∗ ↓ 46% (mean)	↑ 14% (mean)	
		Pagiophyllum kurrii, P. maculosum, P. ordinatum[n]	abaxial	∗ ↓ 36% (mean)	∗ ↓ 39% (mean)	
~205 m.y. (Latest Triassic)	↑ 69%[h]	*Baeira boeggildiana*[n]	abaxial	–	∗ ↓ 44%	McElwain et al., 1999
		B. minuta[n]	abaxial	–	∗ ↓ 49%	
		B. paucipartiata[n]	abaxial	–	∗ ↓ 25%	
		Baeira sp.[n]	abaxial	–	∗ ↓ 36%	
		Ctenis minuta, C. nilssonii[n]	abaxial	–	∗ ↓ 43% (mean)	
		C. nilssonii[n]	abaxial	–	∗ ↓ 21%	
		Ginkgo acosmica[n]	abaxial	–	∗ ↓ 26%	
		G. obovatus[n]	abaxial	–	∗ ↓ 57%	
~205 m.y. (Earliest Jurassic)	↑ 567%[h]	*Baeira longifolia*[n]	abaxial	–	∗ ↓ 60%	
		B. spectabilis[n]	abaxial	–	∗ ↓ 71%	
		Nilssonia polymorpha[n]	abaxial	–	∗ ↓ 70%	
		Stenopteris dinosaurensis[n]	abaxial	–	∗ ↓ 11%	
285–290 m.y. (E. Permian)	↔ [g]	*Lebachia frondosa*[n]	abaxial	↑ 120%	∗ ↔	McElwain and Chaloner, 1995
290–303 m.y. (L. Penn.)	↑[l]	*Neuropteris ovata*	abaxial	∗ ↓ 40%	∗ ↓ 27%	Cleal et al., 1999
310 m.y. (L. Penn.)	↔ [g]	*Swillingtonia denticulata*[n]	abaxial	↑ 460%	∗ ↔	McElwain and Chaloner, 1995
388–373 m.y. (M. Devonian)	↓ 61%[g,m]	*Drepanophycus spinaeformis*	–	∗ ↑ 42%	∗ ↑ 38%	Edwards et al., 1998

Appendix A3 (*continued*)

Age of material (years)	CO_2 levels (relative to controls[a])	Species used	Side of leaf	SD response	SI response	Source
390–403 m.y. (E. Devonian)	↑ 657%[g]	*Aglaophyton major*[n]	combined	∗ ↓ 99%	∗ ↓ 84%	McElwain and Chaloner, 1995
		Sawdonia ornata[n]	combined	∗ ↓ 98%	∗ ↓ 78%	

∗ response inversely relates ($P < 0.05$) to CO_2 concentration.
↔ no significant change ($P > 0.05$).
– not reported.
[a] Typically between 340 and 360 ppmV.
[b] From Vostok (Barnola et al., 1987) and Taylor Dome (Indermühle et al., 1999) ice cores.
[c] From stomatal response of recent *Salix herbacea*, where CO_2 concentrations are known; values match ice core data (refer table footnote 9).
[d] From stomatal responses of recent *Betula pubescens* and *Betula pendula*, where CO_2 concentrations are known.
[e] From stomatal response of recent *Quercus petraea*, where CO_2 concentrations are known; values correlate with temperature curve.
[f] From Freeman and Hayes, 1992; Cerling et al., 1997 (c.f. Pagani et al., 1999a).
[g] From 'best estimate' of Berner (1994, 1998).
[h] From stomatal ratios (McElwain and Chaloner, 1995, 1996; McElwain, 1998).
[j] The control group is prior to the CO_2 spike (260 ppmV CO_2 (refer table footnote 11)).
[k] The control group is the late Allerød material, prior to CO_2 drop (273 ppmV CO_2 (refer table footnote [10])).
[l] The control group is the 10 Ma material (370 ppmV CO_2 (refer table footnote 12)).
[m] The control group is the 388 Ma material (2600 ppmV CO_2 (refer table footnote 14)).
[n] Stomatal responses compared with corresponding Nearest Living Equivalents (NLEs); method described in text.

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