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A natural experiment on plant acclimation: Lifetime stomatal frequency response of an individual tree to annual atmospheric CO_2 increase

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ABSTRACT **Carbon dioxide (CO_2) has been increasing in atmospheric concentration since the Industrial Revolution. A decreasing number of stomata on leaves of land plants still provides the only morphological evidence that this man-made increase has already affected the biosphere. The current rate of CO_2 responsiveness in individual long-lived species cannot be accurately determined from field studies or by controlled-environment experiments. However, the required long-term data sets can be obtained from continuous records of buried leaves from living trees in wetland ecosystems. Fine-resolution analysis of the lifetime leaf record of an individual birch (*Betula pendula*) indicates a gradual reduction of stomatal frequency as a phenotypic acclimation to CO_2 increase. During the past four decades, CO_2 increments of 1 part per million by volume resulted in a stomatal density decline of ≈0.6%. It may be hypothesized that this plastic stomatal frequency response of deciduous tree species has evolved in conjunction with the overall Cenozoic reduction of atmospheric CO_2 concentrations.**

An inverse relationship between stomatal frequency in leaves of C_3 plants and man-made increase in atmospheric CO_2 concentration has been repeatedly demonstrated by analyzing herbarium material collected over the past 200 years (1–7) and by growing seedlings under preindustrial CO_2 levels (1, 7, 8). Stomatal density changes in fossil leaves of *Pinus flexilis* and *Salix herbacea* correlate with glacial–interglacial CO_2 dynamics (9, 10). Stomatal indices for fossil *Quercus petraea* reflect fluctuating atmospheric CO_2 concentrations in late Miocene, Pliocene, and early Pleistocene time intervals between ≈10 million and ≈2 million years ago (5–7). In contrast, CO_2-enrichment experiments are not quite equivalent to an ongoing decline in stomatal frequency (1, 7, 8, 11–17). Many experiments suggest only minor responses or no significant change at all. Consequently, it has been assumed that notably in tree species the plateau of stomatal frequency response has already been approached within the past 40 years at CO_2 concentrations between 310 and 350 parts per million by volume (ppmv) (18). It should be realized, however, that the effects of experimental single-step CO_2 enrichment (typically CO_2 doubling) on seedlings of long-lived plants may not be representative of responses to the global increases of 1 to 2 ppmv CO_2 per year or per growing season (19–22). An unambiguous assessment of the current rate of stomatal frequency response among tree species would require their prolonged monitoring, either in the field or experimentally at modest incremental CO_2 increase. Alternatively, among deciduous trees the effect of CO_2 increase may be detected in retrospect from continuous records of leaves shed annually and preserved in favorable sedimentary settings. On the basis of fine-resolution time-series of stomatal parameters derived from both buried and fresh leaves of a solitarily growing birch, we illustrate that individual trees have a capacity to reduce stomatal frequency as a phenotypic acclimation to CO_2 increase during their lifetimes.

Stomatal frequency is conventionally expressed in terms of stomatal density and stomatal index {stomatal index = [stomatal density/(stomatal density + epidermal cell density)] ×100}. We calculated these parameters for buried leaves from a core of a peat deposit, presently formed in a small (10–15 m^2) bog in an abandoned peatery in the Mariapeel National Nature Reserve in the southern Netherlands (Fig. 1). The top 17.5 cm of the peat is largely composed of accumulated birch leaves, in addition to peat moss (*Sphagnum*). The well-preserved leaves can be considered to originate essentially from a solitary birch (*Betula pendula*) that is still growing at the bog-margin with branches overhanging the bog. Tree-ring analysis done in 1995 indicates a maximum age of 47 ± 1 years for the birch tree. Aerial photographs confirm the absence of other trees at the bog-margin during this period. Growth conditions for the tree have remained relatively stable. Its peaty substrate is characterized by a high moisture content during growing seasons; continuous ground water monitoring since 1955 shows no evidence of unidirectional changes in the ground water table. Local weather records for the past decades do not show significant trends in mean seasonal temperature and cloudiness. The canopy of the tree is well-ventilated. Analysis of intraplant variation in leaf form indicates that sun morphotypes dominate the canopy by ≈90%.

The leaf-bearing peat core was sampled at 35 intervals of 0.5 cm each. Leaves from the youngest sample are from 1992. Considering the small amount of foliage of a tree during the first years of its growth, we assume that shedding of the leaf material derived from the basal sample took place not earlier than 1952. Three further age assessments were obtained by comparing palynological and sedimentological data from the leaf-bearing samples with historical information on regional land cultivation (Fig. 2). The pollen record indicates clearance of colonized birch-dominant vegetation in 1980, as well as the shift from rye (*Secale cereale*) to corn (*Zea mays*) monoculture in 1973 (23). Influx of wind-drifted sand into the peat ended in 1961, when arable fields in the surrounding area were converted to grassland. Taking into account observed differences in pollen concentration (24), a time-scale with an estimated accuracy of ± 1 year was constructed by interpolation. In addition to the buried material, we collected fresh leaves during three successive growing seasons (1993–1995).

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Abbreviation: ppmv, parts per million by volume.

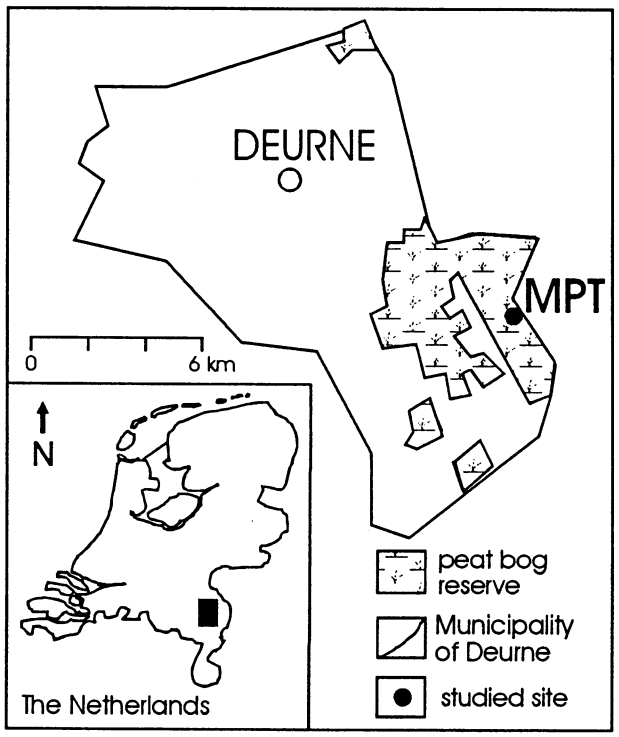

FIG. 1. Location map of site MPT (grid ref. 704025 E/5699525 N) in the Mariapeel National Nature Reserve, southern Netherlands.

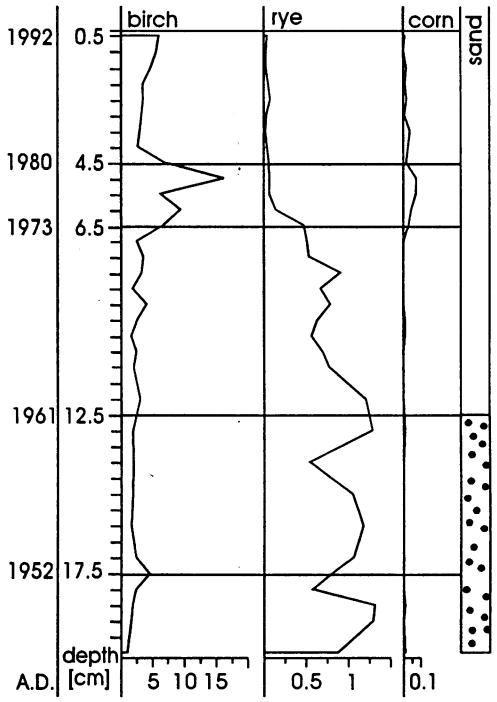

FIG. 2. Frequencies of selected pollen types (birch, rye, and corn; expressed relative to pine pollen numbers) and sand influx in the MPT peat core. For inferred dates see text.

Because the stomatal parameters examined represent the lifetime record of an individual tree, it should be emphasized that variation of stomatal frequency is not directly related to the age of trees. Although seedlings may have relatively low stomatal densities, it has long been known that in deciduous trees there is no significant difference between mean stomatal frequency of saplings and mature trees (7, 25). Also, in the Mariapeel Reserve this stability is confirmed by ongoing research in living birch and oak populations.

The 43-year time series of mean stomatal densities and indices display significant declining trends corresponding to a global atmospheric CO_2-increase of ≈50 ppmv (Fig. 3 *a* and *b*). Calculated relative to the mean CO_2 concentration of 315 ppmv for 1958, the beginning of Mauna Loa monitoring (26), the rate of linear stomatal response corresponds to a relative density reduction of ≈0.6% per ppmv CO_2 increase. Mean stomatal indices show a decline from values of ≈10 for 1952 to ≈7 for 1995. The similarity between the declining trends in stomatal density and index confirms that the action of CO_2 during leaf development is on stomatal formation rather than epidermal cell expansion.

Although the data confirm a significant correlation of stomatal density decline with CO_2 increase, they also indicate considerable annual and interannual scatter. When studied in lower resolution or in shorter time series, it is obvious that a CO_2-related trend could be masked by variability invoked by changes other than CO_2 increase. In our material, variation is associated with differences in the size of the epidermal cells, both within and between sun morphotypes. In contrast to stomatal density, stomatal index expresses frequency change independently of variation in epidermal cell size, and thus remains a more sensitive parameter for detecting stomatal frequency response to CO_2 increase when limited sets of data are available.

Reduction of stomatal frequency results in declining maximum stomatal conductance. Since this complex parameter (or its reciprocal, stomatal resistance) is determined not only by stomatal frequency but also by stomatal geometry (27), one might expect that a decreasing stomatal density would be accompanied by decreasing dimensions of stomatal pores. In addition to stomatal frequency, we therefore measured mean pore length as an indicator of pore size. Intriguingly, the time series shows a trend of increasing size rather than the expected decline (Fig. 3*c*), implying that the physiological effects of stomatal frequency reduction are partially offset by a concomitant increase in pore size. Although in crop species there is some experimental evidence that stomatal density tends to be negatively correlated with pore size (28), this relationship has so far not been recognized in historical tree-leaf data. Noting the modest increase rate observed in this study, it is likely that medium to long-term shifts remain obscured when few data sets are available.

One of the fundamental questions with respect to the interpretation of the long-term relationship between stomatal frequency and atmospheric CO_2 levels concerns the problem of whether this response reflects phenotypic acclimation of trees or whether natural selection is involved leading to genotypic adaptation (29). Our time series now convincingly confirm that individual deciduous trees are equipped with a plastic phenotype capable of sustained adjustment of stomatal frequency to increasing CO_2 concentration. Herbarium records of stomatal frequency change support the hypothesis that this acclimation is transgenerational, and the experimental results of plant growth under preindustrial CO_2 levels suggest that responsiveness is reversible.

An important consideration in predicting scenarios of future plant performance concerns the question of whether stomatal frequency will decline further as atmospheric CO_2 levels continue to rise. Because stomatal density can be neither zero nor infinite, the rate of stomatal frequency change is likely to be a sigmoid function of changing atmospheric CO_2 concentrations (7). The near-linearity of the realized range of stomatal frequency response in the individual birch indicates that in *B. pendula* the lower plateau of the response has not yet been reached. This observation does not necessarily imply a similar situation in other deciduous tree species. In *Q. petraea*, for example, herbarium

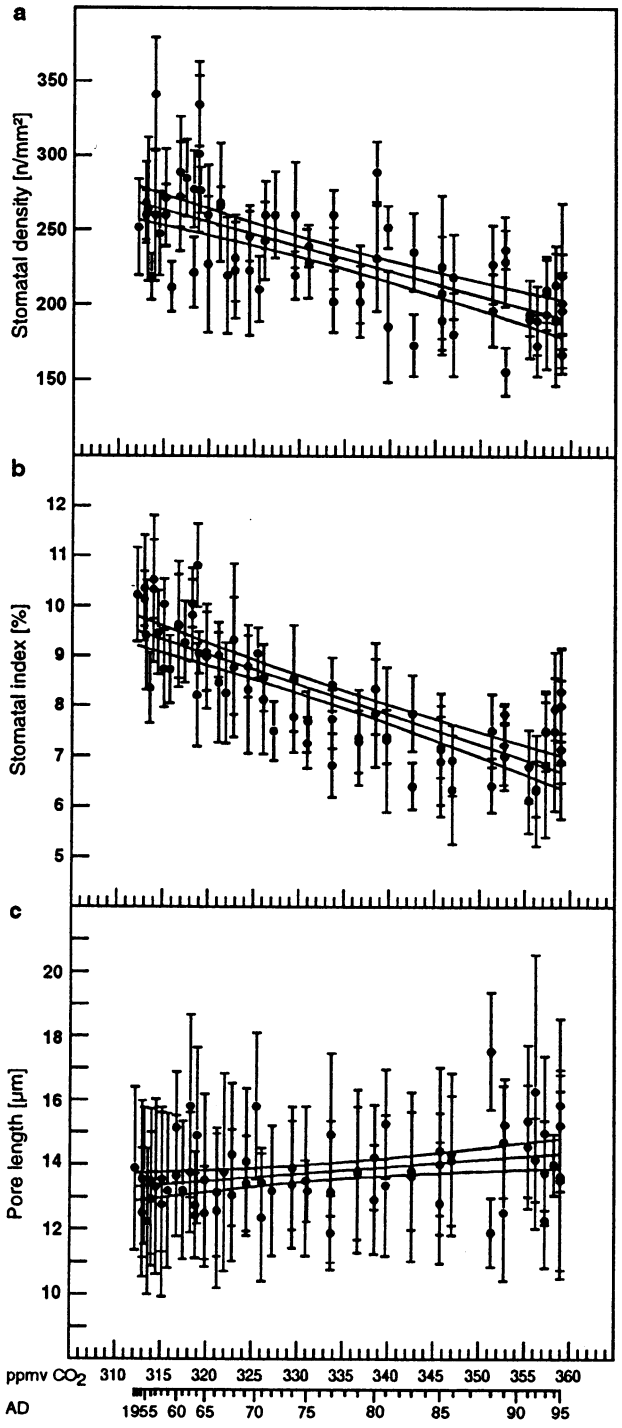

FIG. 3. Response of stomatal parameters for *B. pendula* to global atmospheric CO_2 increase in the period 1952–1995. Computer-aided determination of stomatal parameters was performed on a Leica Quantimet 500C/500+ Image Analysis System. Measured parameters include pore length, stomatal density, and epidermal cell density (including stomatal guard cells). Calculated stomatal index = [stomatal density/(stomatal density + epidermal cell density)] × 100. Stomatal densities and stomatal indices are mean values for seven counted digitized images (field area 0.035 mm^2) per sample (standard deviations are constant after seven counts); counted areas are restricted to stomata bearing areoles. Pore length data are mean values for 50 measured stomata. Statistic analysis with SPSS 6.1. Mean global atmospheric CO_2 concentrations as measured at Mauna Loa, Hawaii, are from ref. 26. The linear regression line for the stomatal density (*a*), with 95% confidence limits, shows a reduction from mean maximum 275 to mean minimum 198 stomata per mm^2 ($n = 69$); slope, -1.645 ± 0.204. The linear regression, with 95% confidence limits, for the stomatal index (*b*) decreases from mean maximum 9.9 to mean minimum 6.7 ($n = 69$); slope, -0.061 ± 0.005. The linear regression line, with 95% confidence limits, for the pore length (*c*) shows an increase from mean minimum 13.2 to mean maximum 14.4 µm ($n = 69$); slope, $+0.022 \pm 0.007$. (*a*) The goodness of the linear model for the stomatal density is: $R^2 = 0.49$, $R^2_{adj} = 0.48$; ANOVA results, $F_{(1, 67)} = 65.09$ ($P < 0.0001$). (*b*) The goodness of the linear model for the stomatal index is: $R^2 = 0.66$, $R^2_{adj} = 0.65$; ANOVA results, $F_{(1, 67)} = 132.32$ ($P < 0.0001$). (*c*) The goodness of the linear model for pore length is: $R^2 = 0.09$, $R^2_{adj} = 0.11$; ANOVA results, $F_{(1, 67)} = 8.28$ ($P < 0.005$).

data suggest that response rates have markedly declined in the last decades (6, 7).

It may be hypothesized that a plastic stomatal frequency response is a conservative property that has evolved in conjunction with the overall Cenozoic reduction of atmospheric CO_2 (30–32). Models of CO_2 fixation in C_3 plants suggest that photosynthetic performance would be decreased rapidly as atmospheric CO_2 concentration declined below 300 ppmv and became a limiting factor to plant growth (33). Possibly only since the late Miocene have such low CO_2 concentrations been of any influence on angiosperms (5–7). For all modern species, minimum concentrations of 180–190 ppmv, which are known for the ultimate and penultimate glacial maxima (34), may have been approaching the threshold level of natural conditions of plant growth. Adaptation to CO_2-depleted atmospheres should impose selective pressure for high stomatal conductance to enable rapid CO_2 uptake. Developmental plasticity capable of producing high stomatal frequencies may therefore be regarded as an adaptive trait for maintaining optimal performance under low CO_2 concentrations. Because high stomatal conductance incurs the risk of rapid and poorly controlled water loss, this trait must develop in association with the refined mechanisms of fast and well-tuned stomatal openings regulated by guard cells that characterize modern land plants (33).

Stomatal conductance (or stomatal resistance) is increasingly emphasized as an important variable in modeling future environmental and biotic consequences of man-made CO_2 increase (35–37). In contrast to herbaceous plants, including most agricultural crop species, trees experience significant atmospheric change during their lifetime. Time series for stomatal frequency and stomatal geometry of ecologically and taxonomically contrasting tree categories could therefore be profitable research tools for model calibration and validation. Bogs and other wetland ecosystems throughout the world may contain continuous and prolonged records of buried leaves that are uninterruptedly linked to living trees, not only to populations but even to individuals. The example presented here illustrates that such records can provide unambiguous near-annual documentation of long-term responses of stomatal characteristics to atmospheric CO_2 increase that is impossible to replicate in conventional short-term controlled-environment experiments. Records of buried leaves that can be linked to living populations of *B. pubescens* and *Q. robur* in the Mariapeel Reserve are currently under investigation.

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