

Stomatal Frequency Change Over Altitudinal Gradients: Prospects for Paleoaltimetry

Lenny L.R. Kouwenberg

*Department of Geology
Field Museum of Natural History
Chicago, Illinois 60605, U.S.A.
lkouwenberg@fieldmuseum.org*

Wolfram M. Kürschner

*Laboratory of Palaeobotany & Palynology,
Utrecht University
3584 CD Utrecht, The Netherlands
w.m.kurschner@bio.uu.nl*

Jennifer C. McElwain

*UCD School of Biology and Environmental Science
University College Dublin
Belfield, Dublin 4, Ireland
Jennifer.McElwain@ucd.ie*

ABSTRACT

Recently, a novel paleoaltimetry method was presented using leaf stomatal frequency response to the decline in CO₂ partial pressure with altitude, and tested on California black oak (*Quercus kelloggii*) (McElwain 2004). Here, we present new data detailing the influence of other climatic variables on leaf stomatal frequency change with altitude in the context of more fully characterizing how stomatal frequencies can be used to infer paleoelevations. A clear increase in stomatal density and stomatal index is observed with increasing elevation for *Q. kelloggii* (black oak) leaves, and *Nothofagus solandri* var. *cliffortioides* (mountain beech) growing over an altitudinal transect on the slope of Mt. Ruapehu (New Zealand). Modern leaves growing in full direct sunlight versus shaded diffuse light for both species show substantial differences in stomatal density and index, however, growth chamber experiments that vary light intensity have revealed that the magnitude of natural increase in radiation with altitude is likely insufficient to explain the overall increase in stomatal frequency (density and index) with elevation. Furthermore, temperature does not have a significant influence on black oak stomatal frequency in growth chamber experiments. Rather changes in stomatal density and index with altitude appear to reflect an adaptation to counteract the limited photosynthetic potential due to the CO₂ partial pressure decrease, further limited by shorter growing seasons and/or increased UV radiation. Our review of the uncertainties associated with the stomatal frequency paleoaltimeter from the literature, together with results from the new plant growth experiments indicate that if sea-level paleoatmospheric CO₂ concentration can be well-constrained, the stomatal frequency method has the potential for very low error margins.

INTRODUCTION

The development of quantitative paleoaltimetry techniques over the last decade offers exciting opportunities to determine the timing and magnitude of topographic development. Quantitative elevation estimates allow testing of models of geodynamic processes, atmospheric circulation patterns and climate, and geochemical cycling (topography may increase weathering rates that affect the carbon cycle). Current paleoaltimetry proxy techniques involve hydrogen and oxygen isotopes (Mulch and Chamberlain 2007), preserved air bubbles in basalts (Sahagian and Proussevitch 2007) and reconstruction of temperature or enthalpy lapse rates with altitude using paleobotanical methods (Wolfe et al. 1997; Forest et al. 1999). Although great strides have been made in the improvement of these methods, problems remain due to a lack of knowledge about regional climate/precipitation patterns, difficulty in finding suitable material, and relatively large uncertainties. Recently, a novel paleobotanical method has been proposed, based on reconstructing the predictable decrease in CO₂ partial pressure with altitude using stomatal density (SD) analysis (McElwain 2004). This method has the potential for high accuracy, as the altitude of modern black oak trees in California from a range of 700-2100 m were predicted with an average error of prediction of ~300 m. Moreover, the stomatal density method can be used in concert with existing paleobotanical methods on a single flora to obtain a suite of independent elevation estimates for a single locality, thereby greatly increasing the confidence in the obtained estimates.

The positive relation between stomatal density and altitude in California black oak, the species used as a paleoaltimeter, has been reported for many other species (Körner and Cochrane 1985; Körner et al. 1986; Woodward 1986; Hovenden and Brodribb 2000; Woodward et al. 2002). However, this relationship is not present in all species and/or localities, which may limit the global applicability of the method (Körner et al. 1986; Hultine and Marshall 2000; Greenwood et al. 2003; Qiang et al. 2003). Furthermore, questions have been raised about the physiological basis of the response of stomatal density to a decrease in CO₂ partial pressure with altitude, considering that other environmental changes with altitude may alleviate the physiological carbon limitation imposed by lower CO₂ partial pressure (Johnson et al. 2004). By assessing the potential influence of different environmental factors changing with altitude on the stomata-altitude relationship, we develop a framework to help explain and predict the conditions under which the stomatal proxy-method can be confidently applied.

To unravel the effect of local climate conditions on the selective pressure driving the stomatal adjustment, we first focus on leaf morphological and stomatal frequency data of two species with contrasting leaf morphology and geographical distribution over altitudinal transects: California black oak (*Quercus kelloggii* Newberry), the species on which this method was originally based (McElwain 2004), and the mountain beech (*N. solandri* var. *cliffortioides* (Hook. f.) Poole) from New Zealand.

Second, we describe the influence of CO₂, irradiation and temperature, the most consistently changing climatic factors with altitude, on stomatal frequency in *N. solandri* var. *cliffortioides* (*N. solandri* var. *cliffortioides* will be referred to as *N. solandri* in the remainder of the text for brevity) and *Q. kelloggii* using material from (1) recent and historical field collections and (2) experiments where *Q. kelloggii* was grown in rigorously controlled environmental growth chambers under different light and temperature settings. These new data are used to consider (1) how environmental factors may change with altitude, (2) whether these factors are known to directly influence stomatal frequency, (3) how climatic factors singularly, and in combination, may affect leaf-air gas-exchange and drive stomatal adjustments, and (4) whether we can infer which leaf types and/or geographical regions may show these adjustments and thus show potential for paleoaltimetry.

Finally we will focus on aspects of the application of both stomatal density (SD: number of stomata per square millimeter) and stomatal index (SI: ratio of number of stomata to

total number of epidermal cells plus stomata expressed as a percentage) for paleoelevation reconstruction. We discuss the key advantages, limitations and calculated uncertainties associated with stomatal-based paleoelevation proxies and make recommendations on the selection of fossil plant taxa, fossil localities and time periods, which will minimize paleoelevation errors using the stomatal-proxy approach.

In the following section, we present new data from California and New Zealand (Figs. 1 and 2) that help resolve contributions of several factors to SD and SI, including elevation, light levels, and temperature. We have chosen to present new results in this review for two reasons. First, we believe they more fully characterize complications that can arise in applying the method. Second, they illustrate the kinds of procedures and data needed to infer elevation. Note that materials and methods are presented in Appendix 1.

NEW RESULTS

Leaf morphology data

Leaf area shows a poorly fitted decreasing trend with altitude for *Quercus kelloggii* ($R^2 = 0.272$), and no significant trend in *N. solandri* (Fig. 3A; 4A). Epidermal cell density increases for both species (Fig. 3B; 4B), although the linear relations are not very consistent. *N. solandri* shows a significant and strong linear increase in stomatal density with altitude (Fig. 3C). Stomatal density in *Quercus kelloggii* also increases with altitude (Fig. 4C). SD in *N. solandri* leaves collected on an elevational transect on Mt. Ruapehu increases linearly from 460 to 600 stomata per mm between 600 and 1470 m (3.5% per 100 m altitude gain; Fig. 3C). In *Q. kelloggii* leaves, SD increases from 250 to 470 stomata per mm² between 1000 and 2400 m (6.3% per 100 m altitude gain; Fig. 4C). Stomatal index in both species also increases with elevation, but the response is more pronounced at higher elevations (Fig. 3D, 4D). In *N. solandri* the SI below 900 m does not change much, and below ~1500 m in *Q. kelloggii*. Non-linear regressions were fitted, as this type of non-linear curve with response limits has been observed before in stomatal index adjustment to increasing CO₂ levels (Kürschner et al. 1997), and the fit improved for both taxa. The response curves for stomatal frequency in *Q. kelloggii* in this data set from 2003 are highly similar to the altitudinal response of *Q. kelloggii* leaves collected in 1934/1935 from the same populations, with a linear response of SD above 1000 m ($R^2 = 0.74$; $p < 0.001$), and a strong SI response starting at ~1500 m ($R^2 = 0.59$; $p < 0.001$) (McElwain 2004). *Q. kelloggii* and *N. solandri* are distributed in different hemispheres and have different morphologies (*Q. kelloggii* has much larger leaves than *N. solandri*), yet still show remarkable similarities in direction and rate of their stomatal response to altitude.

Stomatal density and epidermal cell density, but not stomatal index, of *N. solandri* is found to be significantly higher in sun leaves than in shade leaves when data from three localities on the South Island are combined ($p < 0.05$; Table 1; Fig. 5). For the individual localities, however, stomatal density and index of sun and shade leaves are significantly different. For *Q. kelloggii*, sun leaves collected from a range of modern oak trees in California also show significantly higher stomatal density and index on sun leaves than shade leaves ($p < 0.01$; Table 2; Fig. 6). Under experimental settings, however, the leaves grown under higher light have a slightly increased stomatal density and index, but no significant differences are present in a nested mixed-model ANOVA (Table 3; Fig. 7). Linear regressions of stomatal density and index changes averaged per tree to light intensity are significant, but poorly fitted ($R^2 < 0.2$).

Q. kelloggii tree seedlings grown under experimentally controlled daytime temperatures of 22 °C (low temperature treatment) or 27 °C (high temperature treatment) do not show a significant difference in either stomatal density or stomatal index (Table 4; Fig. 8).

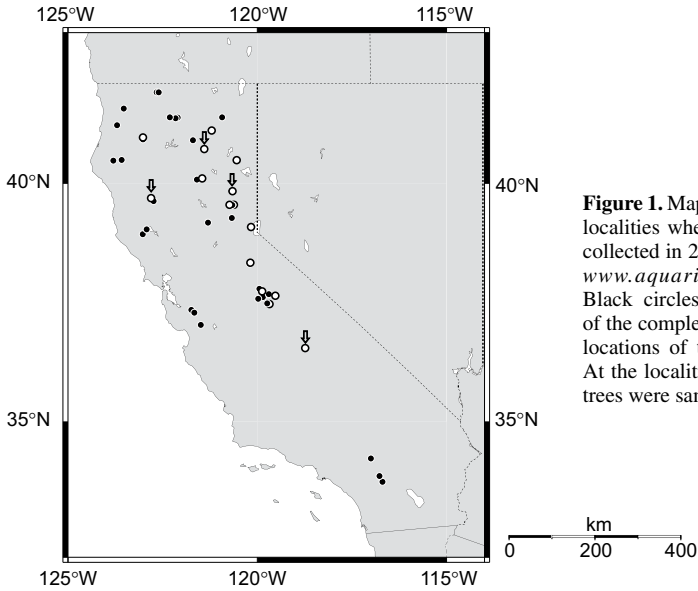


Figure 1. Map of California indicating the localities where *Q. kelloggii* leaves were collected in 2003. (map created on <http://www.aquarius.ifm-geomar.de/omc/>). Black circles indicate sample locations of the complete data set, white circles the locations of the trees used in Figure 4. At the localities indicated by arrows two trees were sampled, instead of one.

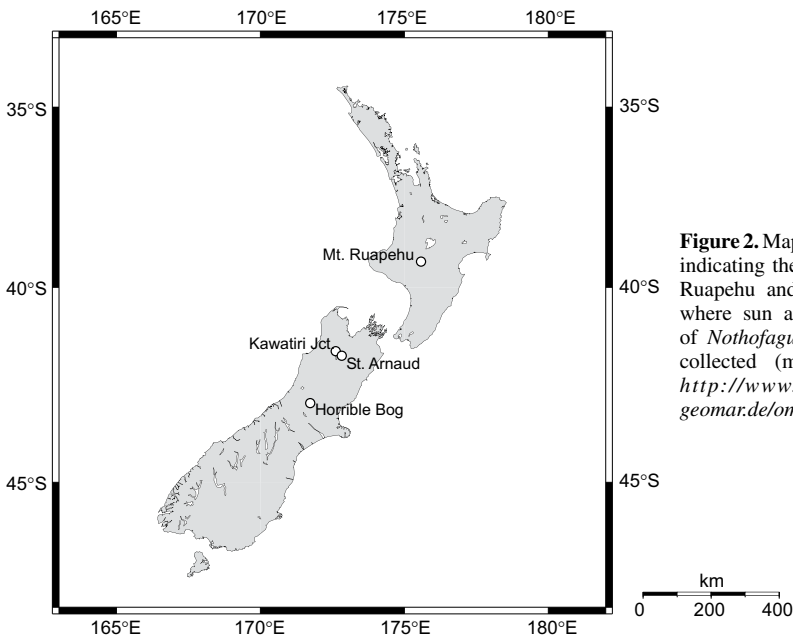


Figure 2. Map of New Zealand indicating the location of Mt. Ruapehu and the three sites where sun and shade leaves of *Nothofagus solandri* were collected (map created on <http://www.aquarius.ifm-geomar.de/omc/>).

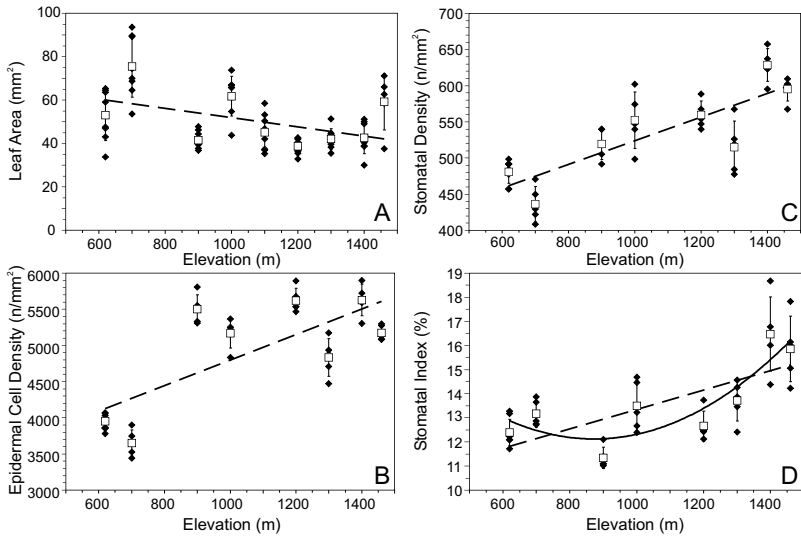


Figure 3. Relationship between leaf area (A), epidermal cell density (B), stomatal density (C) and stomatal index (D) versus altitude for *Nothofagus solandri* leaves growing on the slope of Mt. Ruapehu, New Zealand (collected in 1999). Black diamonds indicate the mean of ten counting fields on each leaf, white squares are the averages of five to eight leaves per elevation, with error bars of ± 1 S.E.M. Nested mixed-model ANOVA with a general linear model indicates significant differences for all factors ($p = 0.000$). Averages per elevation were used for regression analysis: **A.** $y = -0.0212x + 73.1$; $R^2 = 0.276$; $p = 0.147$. **B.** $y = 1.70x + 3122$; $R^2 = 0.505$; $p = 0.048$. **C.** $y = 0.164x + 360$; $R^2 = 0.709$; $p = 0.009$. **D.** linear (dashed): $y = 0.004x + 9.33$; $R^2 = 0.540$; $p = 0.038$; non-linear (solid): $y = 0.00001x^2 - 0.0206x + 21.132$; $R^2 = 0.770$.

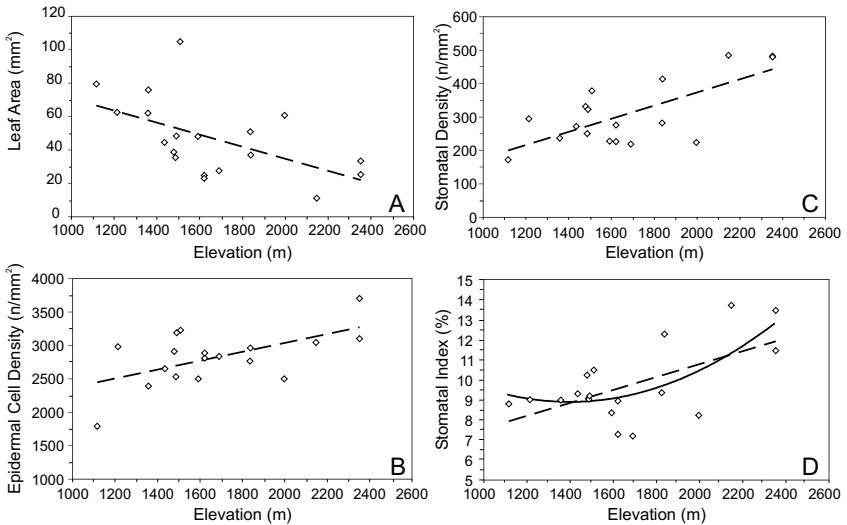


Figure 4. Relationship between leaf area (A), epidermal cell density (B), stomatal density (C) and stomatal index (D) versus altitude for *Quercus kelloggii* shade leaves (collected in 2003). Each point represents the mean of five random counts per leaf. Regressions: **A.** $y = -0.033x + 101.22$; $R^2 = 0.272$; $p = 0.027$. **B.** $y = 0.6608x + 1715$; $R^2 = 0.328$; $p = 0.013$. **C.** linear: $y = 0.1971x + 20.17$; $R^2 = 0.499$; $p = 0.001$. **D.** linear (dashed): $y = 0.0032x + 4.3124$; $R^2 = 0.369$; non-linear (solid): $y = 0.000004x^2 - 0.0123x + 17.55$; $R^2 = 0.466$.

Table 1. Stomatal density (SD), epidermal cell density (ED) and stomatal index (SI) of sun and shade leaves of *Nothofagus solandri* var. *cliffortioides*. Sun and shade leaves were collected at three localities (Fig. 2): Horrible Bog (HOR), Kawatiri Junction (KJ) and St. Arnaud (SA). Values are means of five leaves per light level (seven counts per leaf). The complete data set (total) was analyzed with a nested mixed-model ANOVA based on a general linear model, for comparisons within the individual localities a fully nested ANOVA was used.

locality	light	SD (n/mm ²)	p-value	ED (n/mm ²)	p-value	SI (%)	p-value
HOR	shade	376 ± 36	0.008	4727 ± 325	0.210	7.83 ± 0.61	0.029
HOR	sun	441 ± 21		4994 ± 293		9.22 ± 0.45	
KJ	shade	309 ± 28	0.000	4763 ± 244	0.034	6.95 ± 0.42	0.012
KJ	sun	409 ± 25		5305 ± 406		8.12 ± 0.75	
SA	shade	338 ± 13	0.000	4668 ± 199	0.002	8.07 ± 0.47	0.001
SA	sun	425 ± 16		5119 ± 112		8.61 ± 0.31	
total			0.017		0.011		0.108

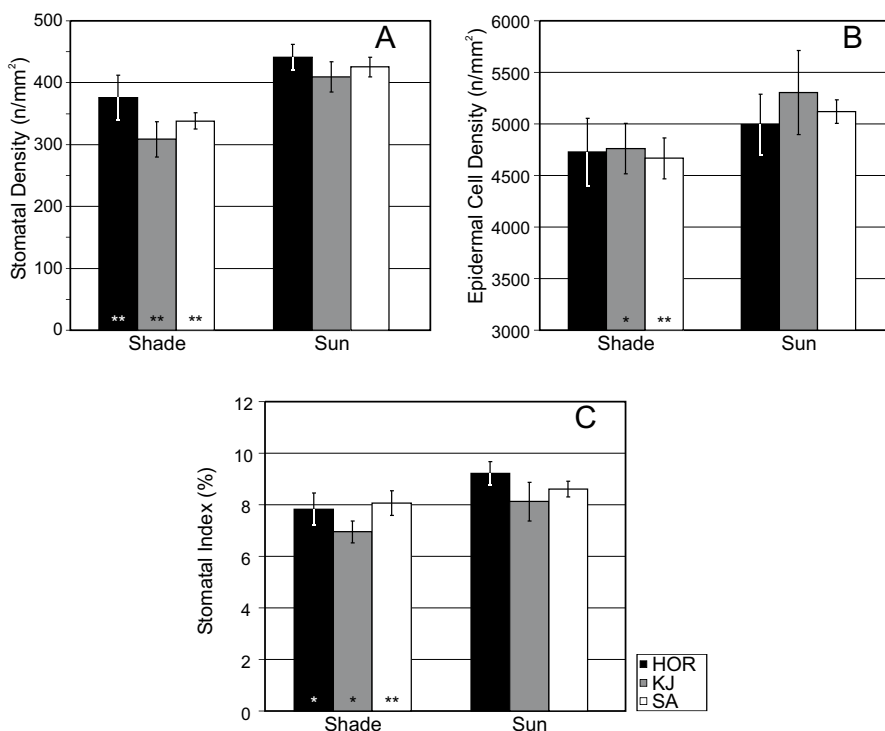


Figure 5. Stomatal density (SD; A), epidermal cell density (ED; B) and stomatal index (SI; C) of sun and shade leaves from modern *Nothofagus solandri* trees at three localities on the South Island of New Zealand (Fig. 2: Horrible Bog (HOR), Kawatiri Junction (KJ) and St. Arnaud (SA)). Nested mixed-model ANOVA of the entire data set indicate significant differences between sun and shade leaves for SD ($p = 0.017$) and ED ($p = 0.011$) but not SI ($p = 0.108$). Asterisks indicate significant (*; $p < 0.05$) and highly significant (**; $p < 0.01$) differences from nested mixed-model ANOVA in that character between the means of sun and shade leaves per location (Table 1). Five sun and five shade leaves per tree were measured (seven random counts per leaf). Error bars represent ± 1 S.E.M.

Table 2. Stomatal density (SD), Epidermal cell density (ED) and stomatal index (SI) of modern *Quercus kelloggii* leaves, assigned to light regime during growth by degree of undulation, and p-values from a pairwise comparison using a nested mixed-model ANOVA based on a general linear model.

Light regime	No. of leaves	SD (n/mm ²)	ED (n/mm ²)	SI (%)
sun	16	414 ± 58	3059 ± 490	12.0 ± 1.4
neutral	23	352 ± 73	2963 ± 487	10.6 ± 1.0
shade	21	301 ± 94	2773 ± 426	9.7 ± 1.6
sun vs. shade (p-value)		0.001	0.099	0.000
sun vs. neutral (p-value)		0.023	0.592	0.005
neutral vs. shade (p-value)		0.063	0.222	0.042

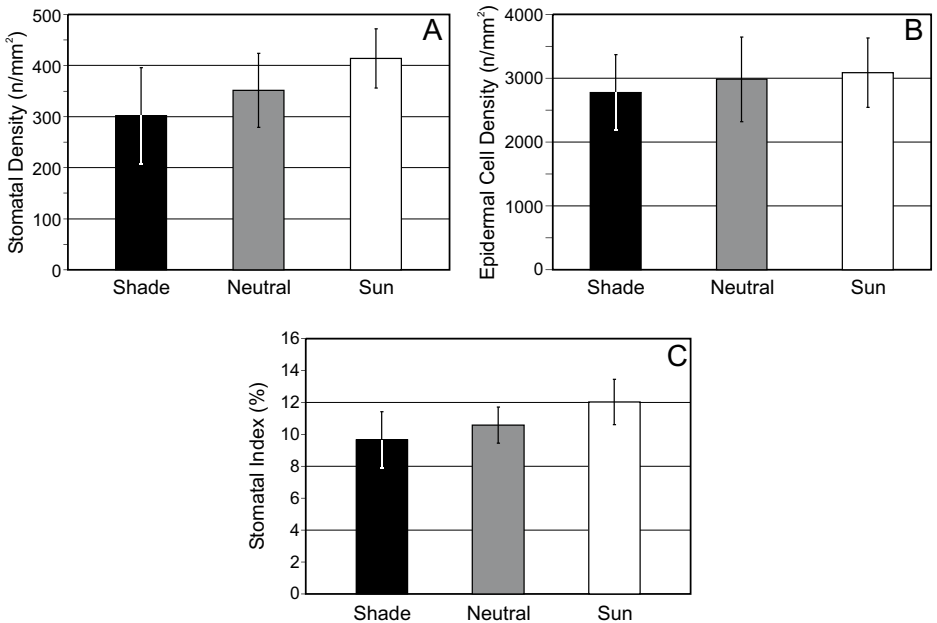


Figure 6. Stomatal density (SD; A), epidermal cell density (ED; B) and stomatal index (SI; C) of 61 modern *Quercus kelloggii* leaves collected in California in 2003 (Fig. 1). Leaves were assigned to sun, shade or neutral type by degree of undulation of the epidermal cell walls (e.g., Kürschner 1997). Nested mixed-model ANOVA showed significant differences for SD ($p = 0.001$) and SI ($p = 0.000$), but not ED ($p = 0.217$). Paired comparison of the means of stomatal density and index in a nested mixed-model ANOVA showed significant differences for SD and SI between sun and shade leaves ($p < 0.01$; Table 2). Error bars represent ± 1 S.E.M.

Table 3. Average stomatal density (SD), epidermal cell density (ED) and stomatal index (SI) of *Quercus kelloggii* leaves grown experimentally under low and high light conditions. The data set was analyzed with a nested mixed-model ANOVA based on a general linear model.

Light intensity ($\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$)	No. of leaves	SD (n/mm ²)	ED (n/mm ²)	SI (%)
49	8	178 ± 36	1706 ± 168	9.4 ± 1.2
85	8	226 ± 33	1997 ± 88	10.2 ± 1.3
156	11	206 ± 34	1733 ± 207	10.6 ± 1.6
204	10	237 ± 19	1949 ± 162	10.8 ± 0.6
p-value		0.202	0.032	0.654

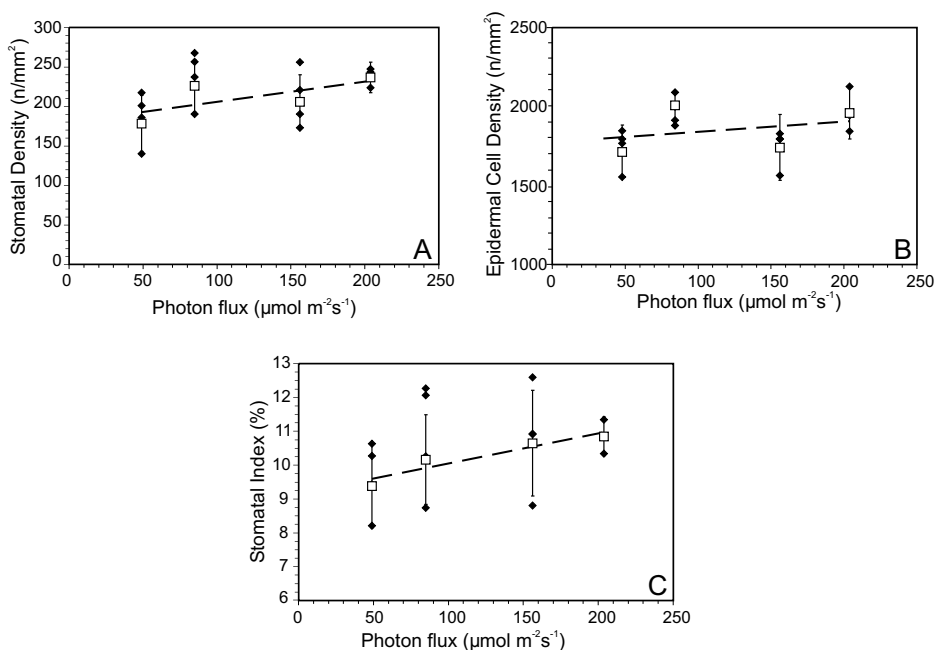


Figure 7. Stomatal density (SD; A), epidermal cell density (ED; B) and stomatal index (SI; C) of *Quercus kelloggii* leaves in relation to light intensity (photon flux) in four growth chambers. Black diamonds represent averages per tree (based on seven random counts per leaf on two to four leaves per tree), white squares averages per chamber. Nested mixed-model ANOVA based on a general linear model indicates no significant differences in SD ($p = 0.202$) or SI ($p = 0.654$), but in ED ($p = 0.032$). Linear regression of the means per tree is significant for SD ($p = 0.011$) and SI ($p = 0.015$), but R^2 values are very low (SD: $R^2 = 0.173$; SI: $R^2 = 0.156$). Linear regressions for the means per chamber: **A.** $y = 0.2574x + 179.98$, $R^2 = 0.489$; $p = 0.301$; **B.** $y = 0.6316x + 1768$, $R^2 = 0.088$; $p = 0.703$; **C.** $y = 0.0089x + 9.1652$, $R^2 = 0.896$; $p = 0.054$).

Table 4. Average stomatal density (SD), epidermal cell density (ED) and stomatal index (SI) of *Quercus kelloggii* leaves grown experimentally under low and high temperature regimes. The data set was analyzed with a nested mixed-model ANOVA based on a general linear model.

Temperature	No. of leaves	SD (n/mm ²)	ED (n/mm ²)	SI (%)
Low	11	345 ± 48	2395 ± 248	12.6 ± 1.0
High	9	290 ± 76	2224 ± 322	11.4 ± 1.7
p-value		0.323	0.442	0.313

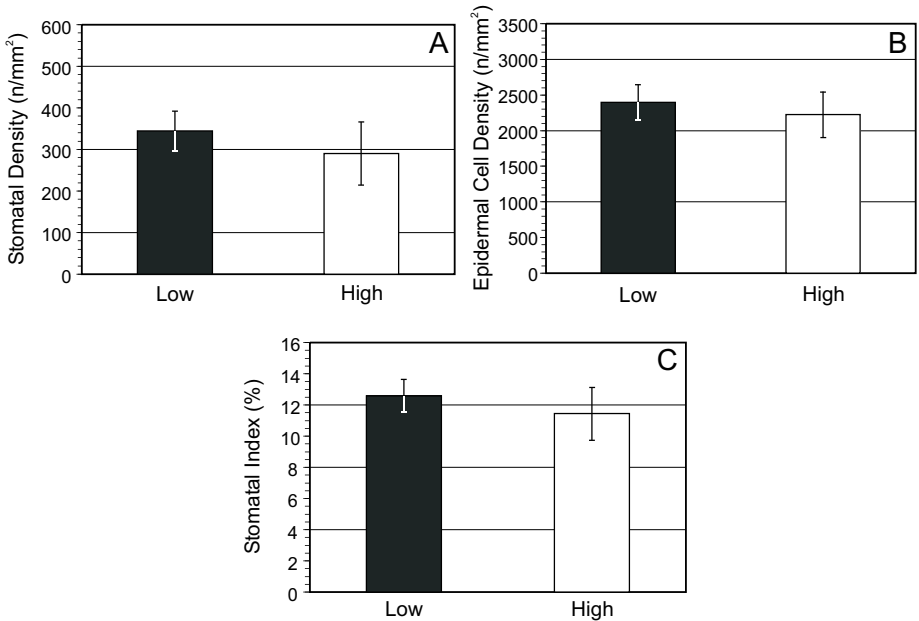


Figure 8. Stomatal density (SD; A), epidermal cell density (ED; B) and stomatal index (SI; C) of *Quercus kelloggii* leaves grown in growth chambers under low temperature (day: 20°C, night: 15°C) and high temperature regimes (day: 27°C, night: 22°C). Measurements for low temperature are based on eleven leaves (seven random counts each) from six trees and for the high temperature on nine leaves from five trees. Nested mixed-model ANOVA indicates no significant differences between low and high temperature treatments for SD (0.323), ED (0.442) or SI (0.313). Error bars represent ± 1 S.E.M.

CLIMATIC PARAMETERS, GAS EXCHANGE AND STOMATAL RESPONSE

Many abiotic factors change with elevation, such as temperature, humidity, irradiance (long-wave and UV-B) and wind speed, but atmospheric pressure decreases independent of micro-climatic conditions and can thus be the most accurately predicted without any substantial regional differences. The potential of each of these factors for explaining the observed changes in stomatal density and index will be discussed by comparing our experimental results and data from field-collected leaves with documented effects of climatic factors on stomatal frequency from the literature. Since stomatal frequency is closely linked to gas-exchange and water-use efficiency through its influence on stomatal conductance, the potential selective pressure of the combination of abiotic changes with altitude will then be discussed in terms of photosynthetic uptake and evaporative demand.

Changes in abiotic factors with altitude and direct effects on stomatal density and initiation

CO₂ partial pressure. Atmospheric pressure (P_{air}) decreases with altitude in a predictable manner (Jones 1992):

$$P_{air,z} = 101325 \left[\frac{-MW_{air} \cdot g \cdot z}{RT} \right] \quad (1)$$

where MW_{air} is the molecular weight of air (28.964×10^{-3} kg/mol), z is altitude in m, g is acceleration due to gravity in m/s, R is the gas constant (8.3144 J/mol) and T is mean July temperature in Kelvin.

The CO₂ partial pressure depends on the altitude as follows (McElwain 2004):

$$P_{CO_2,z} = \left[\frac{P_{air,z}}{101325} \right] P_{CO_2,sea-level} \quad (2)$$

where $P_{CO_2,sea-level}$ is the CO₂ partial pressure at sea-level and $P_{CO_2,z}$ is CO₂ partial pressure at unknown altitude z .

Many studies have shown genetically controlled adjustments in stomatal density and/or index for different plant taxa to changing CO₂ mixing ratios, although the degree and sign of response varies greatly between species (Peñuelas and Matamala 1990; Malone et al. 1993; Greenwood et al. 2003; Kouwenberg et al. 2003; Reid et al. 2003; Marchi et al. 2004; Wagner et al. 2005). The stomatal response to CO₂ concentration (at constant atmospheric pressure) and the developmental pathway involved has been reviewed extensively in recent literature (Gray et al. 2000; Brownlee 2001; Royer 2001; Lake et al. 2002; Woodward et al. 2002; Roth-Nebelsick 2005; Coupe et al. 2006) and a detailed discussion will thus not be included here. Stomatal density and index of fossil leaves have been used as a proxy to reconstruct paleoatmospheric CO₂ levels on timescales ranging from the Paleozoic to the last millennium (Van der Burgh et al. 1993; McElwain and Chaloner 1995; Rundgren and Beerling 1999; Wagner et al. 1999, 2004; Royer et al. 2001; McElwain et al. 2002, 2005; Roth-Nebelsick et al. 2004; Haworth et al. 2005; Kouwenberg et al. 2005; Rundgren et al. 2005; Van Hoof et al. 2006). Fewer studies have been carried out on SD and SI responses to CO₂ partial pressure decreases with elevation (i.e., *decreasing* atmospheric pressure), which is the focus of this paper because of the obvious application as a paleoaltimeter.

In a highly innovative experiment, Woodward and Bazzaz (1988) demonstrated that plants that were grown under lower air pressure (CO₂ partial pressure was changed, but CO₂ mixing ratios remained constant), showed significantly increased stomatal densities to retain comparable photosynthetic rates, under lower CO₂ availability. Since changes in CO₂ partial pressure have been shown experimentally to adjust stomatal frequencies, the decrease in CO₂ partial pressure can reasonably be invoked to have influenced the observed stomatal frequency increase with elevation for *N. solandri* and *Q. kelloggii*. The lack of stomatal index reduction at lower elevations in both species corresponds well with the documented response limits around 330–340 ppmV for other *Quercus* species (Kürschner et al. 1997). Considering the experimental evidence that CO₂ partial pressure under reduced air pressure can strongly affect stomatal density, and the simultaneous response of stomatal density and index for both species described here, decreasing CO₂ partial pressure is very likely to play a major role in the stomatal frequency increase with altitude.

Temperature. Temperature consistently decreases with altitude, although the specific temperature lapse rates are highly dependent on local geographical and climatic conditions. Humidity has a very prominent influence; theoretically, lapse rates can vary from ~10 °C/km in extremely dry conditions to 0 °C/km in extremely wet conditions, or can even be negative

in special cases (Meyer 1992; Leuschner 2000). More realistic lapse rates are about 7.5 °C/km for dry and 5 °C/km for wetter mountain ranges (Meyer 1992; Leuschner 2000; Meyer 2007). In Tongariro National Park (New Zealand), where Mt. Ruapehu is located, the temperature lapse rate is close to the global mean of 6 °C/km (Druitt et al. 1990), and in northern California including the Sierra Nevada, where most *Q. kelloggii* grows, the temperature lapse rate is 5.9-6.0 °C/km (Meyer 1992).

Quercus kelloggii grown in a controlled environment under daytime temperatures of 20 °C and 27 °C did not show significant changes in either stomatal density or index (Table 4; Fig. 8). In temperature experiments on other species varying effects have been observed: no change in stomatal density is found (Apple et al. 2000; Luomala et al. 2005), or a negative correlation for certain age cohorts (Luomala et al. 2005), or a positive relationship for stomatal density (Ferris et al. 1996; Reddy et al. 1998) and stomatal index (Wagner 1998). The Tasmanian southern beech, *Nothofagus cunninghamii* shows an increase in stomatal density with altitude (Hovenden and Brodribb 2000), but no change in specific leaf area, stomatal density or stomatal index under an experimental controlled daytime temperature increase of 5 °C (Hovenden 2001). Since there is no experimental evidence for a consistent negative relationship between stomatal frequency and temperature, temperature decrease with altitude can not explain stomatal frequency increases.

Moreover, in natural habitats, any influence of temperature on stomatal initiation may be of little consequence, since most plants compensate for fluctuating temperatures by adjusting the timing of leaf development (Wagner 1998). At higher altitudes bud swelling and leaf expansion start later in the year (4.4 to 7 days/100 m for New Zealand beeches (Wardle 1984)), when temperatures are closer to temperatures at the start of the growing season at lower altitudes (Barrera et al. 2000 and references therein). Any potential direct effect of temperature on stomatal frequency is therefore likely negated by these phenological adjustments.

Precipitation. Water availability strongly affects stomatal density, but not stomatal index, because of its influence on epidermal cell expansion (Royer 2001). If lower precipitation and water availability with altitude strongly influenced stomatal density and epidermal cell density, then SD and cell density should be tightly correlated, and epidermal cell density and leaf size should show a large adjustment to altitude. Epidermal cell densities are indeed higher at higher altitude in both *N. solandri* and *Q. kelloggii*, and *Q. kelloggii* leaves are smaller, but the relations are weak or not consistently linear, and therefore do not reflect a predictable precipitation change with altitude. Moreover the increase in stomatal index indicates that stomatal initiation rates increased with altitude as well which can not be explained by water availability as only light and CO₂ are known to control stomatal initiation (Lake et al. 2002).

In general, cloud cover and rainfall do not show a general, consistent decrease with elevation. In many temperate mountain regions, as in California and New Zealand, cloud cover and precipitation actually increase with elevation (which should decrease stomatal densities), and in tropical systems rainfall increases up to 1000-1500 m and then decreases (Leuschner 2000). In the region of Mt. Ruapehu, rainfall increases strongly between elevations of ~600 and ~1120 m (Druitt et al. 1990). Precipitation data from 26 climate stations in Northern California (closest to the sample localities in Fig. 1) do not show a significant relation between precipitation and elevation in any direction ($R^2 = 0.07$; climate data from: <http://www.wrcc.dri.edu>). Thus, the increase in stomatal frequency in both *Q. kelloggii* and *N. solandri* to altitude is unlikely to be a direct result of changes in precipitation. If water availability were a primary factor affecting stomatal densities and caused larger variability due to microclimatic differences at sites, stomatal density and leaf size would be strongly correlated. The extremely weak direct correlation between stomatal density and leaf size for both species ($R^2 = 0.119$ for *Nothofagus* and 0.09 for *Quercus*) argues against water availability as an important influence on stomatal densities by inducing smaller epidermal cell size (and consequently smaller leaves) during drought (Fig. 9).

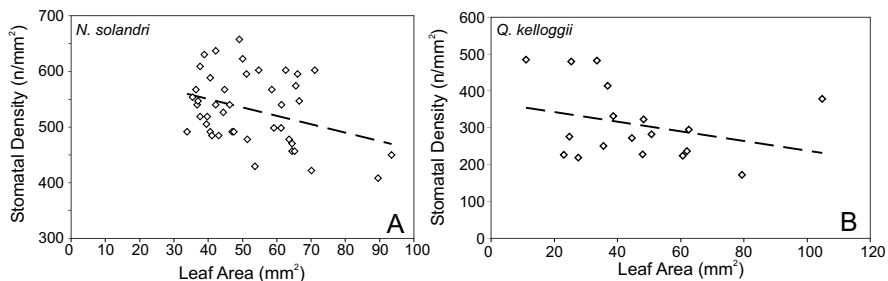


Figure 9. Relationship between stomatal density and leaf area for *Nothofagus solandri* leaves (A), grown at an altitudinal transect on the slope of Mt. Ruapehu, New Zealand, and *Quercus kelloggii* leaves (B) from California. **A.** Linear regression: $y = -1.5148x + 611.22$; $R^2 = 0.119$; $p = 0.023$ **B.** Linear regression: $y = -1.3121x + 369.36$; $R^2 = 0.090$; $p = 0.225$.

Light intensity. Regarding the influence of possible irradiation changes on stomatal frequency, a clearly higher SD and SI are observed in the field-collected sun leaves compared with shade leaves, for both *Q. kelloggii* (SD +38%; SI +24%) and *N. solandri* (SD +25%; SI +14%; averaged over the three sites). The positive effect of high light intensity on stomatal density and index of sun and shade leaves has been a long-standing observation in many other species (e.g., Kürschner 1997; Royer 2001; Lake et al. 2002). The light treatments in the *Q. kelloggii* light experiment covered a 300% increase in irradiation intensity from the equivalent of shade conditions to sunlight on an overcast day (because of their understorey habitat, black oak seedlings do not grow well under higher light intensities). These relatively low light levels are also reflected in the undulating epidermal cell walls observed in both treatments, lacking a clear “sun” type epidermal morphology. Under these light intensity increases, SD in *Q. kelloggii* increased by +16% and SI by +10%, but due to the large variability between trees in the chambers, this effect was not statistically significant (Table 3; Fig. 7). We can therefore conclude from these experiments that although irradiation clearly affects both stomatal density and index, the magnitude of change in light intensity required to elicit a significant stomatal frequency response in leaves far exceeds the typical irradiation changes observed along elevational gradients in either California or New Zealand.

In theory, solar radiation intensity gradually increases with altitude, but this trend can locally be very different due to changes in cloudiness with elevation. Long term instrumental measurements indicate that yearly global horizontal irradiation increases 10% with altitude between 200 and 1700 m (Delacasiniere et al. 1993), and measurements in the Swiss Alps show an altitude gradient over 370–3580 m for mean annual global radiation of $1.3 \text{ Wm}^{-2}/100 \text{ m}$ (Marty et al. 2002). However, in summer, this gradient is slightly negative above 2000 m due to increased cloudiness. Regional differences tend to override any expected irradiation increases with altitude, however. For example, the net radiation in the Austrian Alps stays constant with altitude (Leuschner 2000), because cloud cover gradually increases at higher elevations. In Hawaii, on the other hand, cloud cover increases up to 1000–1500 m, so radiation decreases over this range, and only at higher elevations does radiation begin to increase again. These regional trends illustrate that in mountain ranges proximal to oceans, such as in California and New Zealand, irradiance does not necessarily increase linearly with elevation, due to increased cloudiness and therefore can not explain the linear increase in SD and SI with altitude.

The increase in stomatal density and index with altitude could be a result of opening up of the higher elevation landscape, which increases the amount of intercepted radiation, as has been suggested for *Nothofagus cunninghamii* forests in Tasmania (Hovenden and Vander Schoor 2006). Both *N. solandri* and *Q. kelloggii* show substantial differences in stomatal frequency

between sun and shade leaves, and if the collected leaves become predominated by the sun morphotype, SD and SI increase with altitude (although the irradiation-related differences in stomatal density decrease for *Nothofagus cunninghamii* trees at higher elevations (Hovenden and Vander Schoor 2006). However, the sampling strategy for *N. solandri* and *Q. kelloggii* minimized local irradiation difference by sampling leaves from a similar position in the crown, and trees were selected that grew along an open road. In *Q. kelloggii* sun and shade leaves can be recognized and separated according to the degree of cell wall undulation, and the leaves analyzed in this study and depicted in Figure 4 are all shade leaves. Moreover, there is no relation between elevation and the degree of undulation of the oak leaves (One-way ANOVA on elevation vs. undulation class: $p = 0.217$), which unambiguously indicates that in this data set from California incepted light does not vary in a predictable manner with elevation.

Even though light intensity can influence stomatal density and stomata index, the weak experimental response of SD and SI to light intensity demonstrated for *Q. kelloggii*, together with the oceanic geographical settings of the study areas suggest that direct effects of irradiation on the overall altitudinal increase in stomatal frequency for both *Q. kelloggii* and *N. solandri* were probably not very pronounced. Microclimatic differences in incepted radiation per sampling site, however, may explain much of the variation found between sites.

UV-B. UV-B radiation increases with elevation by about 7-9% per km (Blumthaler et al. 1997; Alexandris et al. 1999; Zaratti et al. 2003). High UV-B levels are suggested to reduce the activity of photosynthetic enzymes and down-regulate photosynthetic genes (DeLucia et al. 1992; Jansen et al. 1998), but the effects vary for different species (Searles et al. 2001). Plants at high altitudes may suffer increased vulnerability to the enhanced UV-B levels as the short and cool growing seasons can delay the development of protective epidermis, cuticles and epicuticular wax (DeLucia et al. 1992; Turunen and Latola 2005). As a response to enhanced UV-B in experimental settings, most plant species exhibit a decrease in stomatal density (Dai et al. 1995; Visser et al. 1997; Keiller and Holmes 2001; Poulson et al. 2002; Gitz et al. 2005), but increases have also been observed (Stewart and Hoddinott 1993; Kostina et al. 2001). According to Nogues et al. (1998, 1999) the increased UV-B radiation at higher altitudes limits stomatal opening, and hence reduces stomatal conductance. An increase in SD with altitude could counterbalance this decreased stomatal conductance in order to retain optimized photosynthetic rates.

Effects of climatic changes with altitude on air-leaf gas exchange

CO₂ assimilation. The amount of CO₂ available for photosynthesis decreases with decreasing CO₂ partial pressure at higher elevations, but this effect is offset by the increase in diffusion speed at lower air pressure (Gale 1972, 1973). The lower temperature at higher altitudes, however, decreases diffusion speed, and therefore the temperature lapse rate of the particular mountain determines whether CO₂ availability decreases (dry-moist lapse rate) or stays relatively constant (very wet lapse rate) (Smith and Donahue 1991). The lower air pressure at altitude does not just decrease CO₂ partial pressure but also O₂ partial pressure, which results in lower photorespiration rates and more efficient photosynthesis. When all these effects are modeled, photosynthetic rates generally decrease with altitude, unless the temperature lapse rate is very low (which could occur in extremely wet mountain ranges), but the photosynthetic limitation is much less than expected based on just the partial pressure decrease (Terashima et al. 1995; Smith and Johnson 2007).

Growth experiments on three plant species under decreased air pressure, where CO₂ partial pressure was equivalent to high altitudes, but mixing ratio was unchanged, showed a significant increase in stomatal density, even though temperature was kept constant, creating a "wet" lapse rate situation, and CO₂ uptake was limited to a lesser degree than at high altitude under realistic lapse rates (Woodward and Bazzaz 1988). The increase in stomatal density in these species under low air pressure was as large as the experimental response to an equivalent

CO₂ decrease by adjusting the CO₂ mixing ratio (Woodward and Bazzaz 1988). These results strongly argue in favor of a central role of CO₂ partial pressure decreases with elevation on plant SD and SI. Under the relatively dry temperature lapse rates in California and New Zealand, therefore, the decrease in CO₂ partial pressure is expected to increase stomatal density and index, as observed in *Q. kelloggii* and *N. solandri* (Figs. 3, 4).

Transpiration. Two opposing effects work on transpiration at higher altitudes: the decreasing air pressure increases the diffusion speed of water (D_{wv}), but the lower temperature in turn decreases the diffusion speed. The importance of temperature lapse rates is illustrated by biophysical leaf models showing a significant increase in evaporation under wet lapse rates (3 °C), and a strong decrease in evaporation under dry lapse rates (8 °C) (Smith and Johnson 2007). However, an additional parameter stimulating transpiration is the increasing difference between leaf temperature (T_{leaf}) and air temperature (T_{air}). Therefore, factors affecting T_{leaf} , such as the radiation load, change D_{wv} too. The potentially higher radiation load at higher altitude will generally increase T_{leaf} (Smith and Johnson 2007) especially in large leaves, as they receive more irradiation than smaller leaves, to increase transpiration (Smith and Geller 1979). Perhaps the decrease in leaf size that is often seen at higher altitudes functions to keep the leaf cooler and cause less excess evaporation. Thus, transpiration effects of altitude depend greatly on local conditions, such as the temperature lapse rate (high/dry lapse rates cause lower evaporation) and radiation change (higher irradiation leads to increased evaporation), and on plant anatomical features such as leaf size (determining leaf temperature) and epidermal structure (affects the boundary layer thickness). For example, potential evapotranspiration for a 4 cm² leaf is modeled to be higher in equatorial mountains than in temperate areas, due to higher radiation loads and a larger leaf to air water vapor concentration gradient (Leuschner 2000). If radiation does not increase because of increased cloudiness, evaporation can easily decrease with altitude. High evaporation rates at high altitudes occur mainly in (1) dry tropical mountain chains because of high irradiation and low cloudiness and rainfall and (2) small oceanic mountains with strong upward convection of low air and thus low temperature lapse rates (Leuschner 2000).

Even if actual evaporation rates for the sites in this study are difficult to predict because of the dependence on the specific microclimatic conditions, we can infer general trends for the altitudinal transects in New Zealand and California. Both are located in temperate areas and have relatively dry temperature lapse rates (6 °C), and in California, cloudiness increases with altitude. Comparing these conditions to the modeled environments discussed above would suggest that evaporation is likely to decrease with altitude, or at least not increase significantly. The larger leaf size of the oak leaves may increase their evaporation rates relative to the smaller mountain beech leaves, but this remains speculative as no irradiation data available were available for either site.

Apart from air pressure, temperature and radiation, as discussed above, potential higher wind speed in the more open alpine environments can also influence evaporation. The boundary layer thickness of leaves is determined by the leaf dimensions and wind speed. Generally, wind speed increases with elevation (Leuschner 2000) and the greater wind speed will initially decrease the thickness of the boundary layer and enhance evapotranspiration (Gates 1976). However, the relation between transpiration rate and wind speed depends on the radiation absorption. In case of a high radiation absorption (and high leaf temperatures typical of larger leaves) or high leaf resistance, evaporation decreases with wind speed because of the convective cooling of the leaves (Baig and Tranquillini 1980). Under a high radiation load, transpiration can increase with wind speed in dry air, while decreasing in humid air (Gates 1968). Only when the low amounts of radiation are absorbed, for example by small leaves, resulting in relatively low leaf temperatures, does evaporation increase with wind speed due to the boundary layer thinning (Gates 1976). Leuschner (2000) modeled for a standard 4 cm² leaf that elevational increases in wind speed reduces potential transpiration by 11% in equatorial

and 25% in middle-latitude mountain ranges. Thus, the increase in wind speed with altitude can offset the higher transpiration rates due to the larger leaf-air temperature difference created by the increase in radiation with elevation.

A decreased stomatal conductance, and thus transpiration under higher wind speeds has been reported in several experimental studies (Retuerto et al. 1996; Campbell-Clause 1998; Hoad et al. 1998), although no effect was detected in vegetation from high elevation in Puerto Rico (Cordero 1999), and grasses and shrubs showed enhanced transpiration despite decreased leaf temperatures (Yu et al. 1998). The varying effects of wind speed on transpiration in these studies could depend on the different leaf sizes studied. In summary therefore if wind speeds indeed increase with altitude in California and New Zealand, this could increase evaporation for the small *N. solandri* leaves, and decrease evaporation for the *Q. kelloggii* leaves, thus compensating for the higher radiation inception and leaf temperature of the larger leaves.

Gas exchange and stomatal density. Overall, the geographical setting of the studied areas and the subsequent relatively dry temperature lapse rates indicate that altitude affects gas-exchange in *Q. kelloggii* and *N. solandri* in similar fashion. The lower CO₂ partial pressure combined with the lower temperatures limit photosynthetic uptake at higher altitudes, although not as much as might be expected since the uptake is enhanced by the increased diffusion speed at higher altitude. Evaporation probably does not significantly increase, and may even be reduced, especially with increased cloudiness. Considering that experiments have shown that plants are as sensitive to CO₂ partial pressure as CO₂ mole fraction changes, the relatively small photosynthetic limitation due to reduced CO₂ availability at altitude is expected to increase stomatal densities and index in the two species studied, that are not hampered by excessive evaporation rates. The selective pressure for maintaining adequate carbon gain at high elevation due to decreased CO₂ partial pressure would obviously be higher in times of the geological past when sea-level CO₂ partial pressure was lower than current ambient levels.

However, even if photosynthetic capacity at higher altitudes might not be as severely hampered as the decrease in CO₂ partial pressure would imply, high altitude does restrict photosynthetic capacity by shortening the growing season due to low winter temperatures. Near treeline, in more open alpine landscapes, the thin snowpack is less effective at isolating the soil, and the resulting lower soil temperatures cause frost-drought to occur for a longer time (frost drought results in stomatal closure on trees living around treeline from November to April (Tranquilini 1976)). In extreme cases, such as the high elevation forests in the Basin and Range, Nevada, maximum photosynthetic rates are only attained for one month (Smith and Knapp 1990). Leaf gas exchange models indicate that stomatal aperture controls transpiration rates more effectively than photosynthetic rates (Pachepsky 1995), and therefore plants adapted to high elevation may increase stomatal density (and maximum stomatal conductance) to maximize photosynthetic carbon gain during the short period available (e.g., Woodward et al. 2002).

Higher stomatal densities allow the plants to maximize photosynthetic activity during the time when photosynthesis is not hampered by climatic conditions. Several aspects of higher altitude environments limit the time of photosynthetic activity, and the combination of these may explain the necessary compensation through increasing the maximum photosynthetic potential. Factors that change with increasing elevation affecting gas exchange are: (1) lower CO₂ partial pressure, especially under relatively high temperature lapse rates, (2) higher UV levels decreasing stomatal conductance, (3) higher irradiation that limits daytime photosynthetic hours because of extremely high transpiration rates in the middle of the day, and (4) the shorter growing seasons due to lower spring temperatures and later snowmelt than at lower elevations. The capability for higher maximum photosynthetic rates at altitude has been noted before, and is associated with higher nitrogen levels in leaves (Körner et al. 1986).

The two taxa studied here show a clear increase in stomatal density with altitude, as do many other species (Körner et al. 1986; Woodward 1986; Hovenden and Brodribb 2000), but

this relation is not ubiquitous (Körner et al. 1986; Hultine and Marshall 2000; Greenwood et al. 2003; Qiang et al. 2003). It is obvious that the photosynthetic and gas exchange limitations vary for local conditions, such as temperature lapse rates, altitudinal irradiation profiles and leaf anatomy. In areas with low temperature lapse rates, for example, where the CO₂ exchange rate does not decrease with altitude and/or evaporation rates increase strongly, stomatal frequency changes with altitude may be much less pronounced, absent or even reversed in direction. These differences in selective pressure may explain the widely varying observed trends in stable carbon isotopes (reflecting water use efficiency), nitrogen content and stomatal parameters reported for different taxa over altitudinal ranges (Körner et al. 1986, 1991; Marshall and Zhang 1994; Sparks and Ehleringer 1997; Hultine and Marshall 2000; Kogami et al. 2001; Greenwood et al. 2003; Qiang et al. 2003).

APPLICATION OF STOMATAL FREQUENCY AS A PALEO-ALTIMETER: RECOMMENDATIONS AND LIMITATIONS

Stomatal frequency techniques

Stomatal density. The linear relation between stomatal density of *Q. kelloggii* and elevation above 1000 m (Fig. 4) suggests great potential for its use to reconstruct paleoelevations. As discussed above, however, it is unlikely that the stomatal density increase is the sole result of the decrease in CO₂ partial pressure. The stomatal increase may be an adjustment in gas exchange capability to counter the lower availability of CO₂ under shorter growing seasons, higher longwave radiation and higher UV radiation that would all decrease the maximum photosynthetic capacity. In this way, the observed stomatal density increase is definitely linked to the CO₂ partial pressure decrease, but can not be converted directly into a CO₂ partial pressure value using the relation in Figure 10 to calibrate leaves from different regions or sea-level CO₂ regimes. Indeed, when stomatal density data from Californian black oak leaves, collected between 1891 and recent are plotted against CO₂ partial pressure, an offset in the y intercepts is apparent between stomatal density–CO₂ partial pressure curves for each time period (Fig. 10). If CO₂ was the only factor directly determining the stomatal density decrease, the combined data from different sea-level CO₂ regimes and elevations would be expected to show a single linear response curve. Instead, all time periods, which only differ in sea-level CO₂ pressure, show the same SD–CO₂ slope, but with significant y offsets.

Therefore, to calibrate stomatal density to paleoelevation using the training set in Figure 4C, a correction factor is required. The elevation of eleven recent oak trees, geographically widely spread, could be predicted with an average error of estimation of ~300 m once $P_{\text{CO}_2,z}$ in Equation (2) was adjusted by adding the sea-level CO₂ concentration at the time of fossil growth minus the sea-level CO₂ concentration of the training set [$P_{\text{CO}_2,\text{sea-level (fossil)}} - P_{\text{CO}_2,\text{sea-level (calibration)}}$] (McElwain 2004).

Stomatal index. The stomatal index represents the rate of stomatal initiation, and being independent of cell expansion, is less influenced by environmental factors that relate to gas exchange than stomatal density. In experiments, stomatal index is usually not influenced by factors other than CO₂ levels and, to a lesser extent, light intensity (Royer 2001; Lake et al. 2002). Thus, since stomatal index is directly related to CO₂ levels, prediction of paleoelevation using stomatal index should be feasible without extra corrections. The only other factor known to influence stomatal index apart from CO₂ is light intensity (Royer 2001). The additional error introduced by the potential influence of light intensity can be minimized when (1) a region is selected where light intensity does not increase much over altitude due to increasing cloud cover and/or fogginess, and (2) by distinguishing sun and shade leaf morphotypes which have a distinctive epidermal cell morphology, where anticlinal epidermal cell walls show more undulation (become less straight and more “jigsaw piece”-like), and

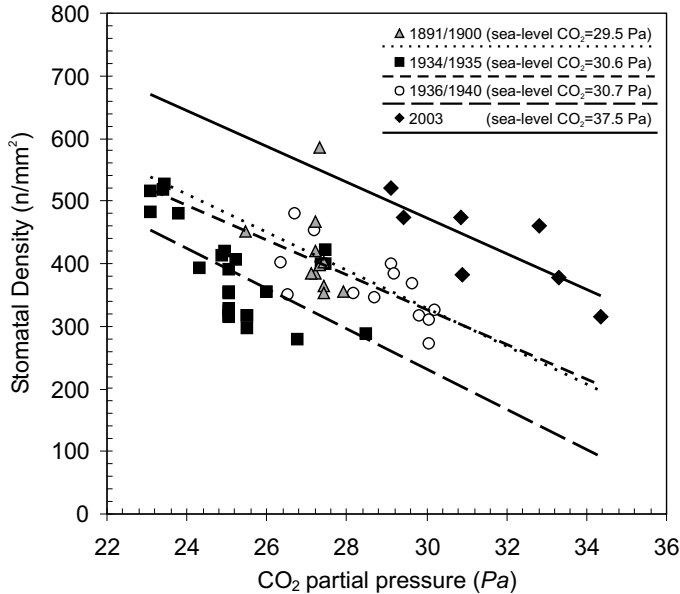


Figure 10. Historical and modern stomatal density (SD) data sets of *Quercus kelloggii* vs. CO_2 partial pressure from herbarium leaves (sun + shade) collected in (1) 1891-1900, (2) 1934-1935, (3) 1936-1940, and 2003, demonstrate similar SD response rates, but different intercepts. The 2003 data in this figure are not included in the present study [Used by permission of GSA, from McElwain (2004), *Geology*, Vol. 32, Fig. 3, p. 1019].

can thus be separated for the calibration. The application of stomatal index instead of density has not been tested using *Q. kelloggii* leaves, because stomatal index in this species shows no response at altitudes under ~ 1500 m a.s.l., leaving a very short elevation range and data set for informative tests.

Constraints on the selection of method and material

Method. Whether to use stomatal density or stomatal index from leaf fossils for paleoelevation reconstruction depends on the availability of modern reference material and the quality of the fossil cuticle. Ideally both should be used on the same material to increase the confidence in the provided estimates. In cases, however, when the quality of the fossil cuticle does not allow epidermal cells to be recognized with the necessary accuracy, and stomatal index can not be determined, stomatal density measurements are the only available option. To use stomatal index, a training set of modern and herbarium material grown under different CO_2 pressure provides the necessary calibration. Because stomatal density is influenced by more factors than just the CO_2 partial pressure, the actual response of stomatal density of the chosen fossil taxon has to be confirmed by analyzing leaves from actual elevation transects. Stomatal density is physiologically more informative than stomatal index as SD is strongly related to maximum stomatal conductance.

Taxa. Although many species show the classic inverse relationship between stomatal frequency and CO_2 , not all species do so. Therefore, the sensitivity of the species of fossil leaves needs to be tested when selecting fossil species to work with. Moreover, since plants have morphological and physiological constraints that do not allow adjustment of stomatal numbers to infinitely high or low CO_2 , plant species have species-specific response limits to CO_2 (Kürschner et al. 1997). For instance, the nonlinearity of the stomatal index-elevation

relation for both *Q. kelloggii* and *N. solandri* indicates that both species have already reached their response limit under modern ambient CO₂ levels. These species are therefore not suitable to reconstruct (1) low elevations under modern or higher CO₂ levels and (2) any elevations in time periods with much higher than present CO₂ levels, such as during the Mesozoic or Paleogene. To reconstruct paleoelevations during “greenhouse” ages, fossil taxa need to be used that are still sensitive at higher CO₂ levels, such as gymnosperms in general (Kouwenberg et al. 2003), or more specifically *Metasequoia glyptostroboides* or *Ginkgo biloba* (Royer et al. 2001). Contrary to CLAMP-based paleobotanical methods, the stomatal frequency method is not restricted to floras dominated by angiosperm taxa. A strong stomatal density response to maintain adequate carbon gain at high elevation due to decreased CO₂ partial pressure would be expected in species that do not suffer from high evaporative demands at higher altitudes, due to leaf anatomical adjustments such as increasing trichome density that can decrease transpiration by increasing boundary layer thickness.

Age range of applicability. Theoretically, this paleoaltimetric method could be used throughout the Late Cretaceous and Cenozoic, as long as the fossil species have extant counterparts for calibration purposes and cuticle is preserved. However, the accuracy and reliability of the method worsen further back in time. When selecting fossil floras for application of the stomatal frequency paleoelevation proxy, a number of potential setbacks need to be considered. First, the method requires calibration of stomatal frequency versus CO₂ pressure and/or altitude based on modern material of the same or a very closely related species. A few gymnosperm taxa, such as *Metasequoia* and *Ginkgo*, likely have evolved little since the Mesozoic. However, these are exceptions to the rule and for most species the Neogene may be the only period with a representative fossil record. Secondly, as mentioned earlier, finding suitable taxa, with modern equivalents for quantitative calibration, that also still respond to the (very) high CO₂ levels in greenhouse periods such as the Mesozoic and Paleogene might prove more of a challenge. Finally, sea-level CO₂ pressure, as the largest potential uncertainty associated with the method (see below), needs to be relatively well-constrained for the time period, requiring (1) good agreement on estimates of sea-level CO₂ pressure derived from other methods, or (2) in the ideal case, low-elevation floras containing the preferred taxon that are contemporaneous with the flora from unknown altitude. These requirements may be harder to fulfill further back in the Phanerozoic, as both stratigraphic control and certainty of paleoatmospheric CO₂ concentration decrease with age.

Sources of error and quantification of uncertainty

Error of prediction. McElwain (2004) used stomatal density counts to predict the known altitude of eleven modern *Q. kelloggii* trees across California from single leaves. This test revealed that, after using the correction for sea-level CO₂ differences between the calibration curve and the modern data, the average error in prediction was ~ 300 m. This prediction error is one of the smallest in any currently available paleoaltimetry method, but some other sources of uncertainty may increase this error.

Sun and shade leaves. Having a mixture of sun and shade leaves in the fossil assemblages could introduce a larger variability in the stomatal frequency data and subsequent elevation reconstruction, because they significantly differ in stomatal density and index (Royer 2001) and in sensitivity to CO₂ partial pressure (McElwain 2004). For *Quercus kelloggii*, the 38% difference in stomatal density between sun and shade leaves observed in this study would result in a 558 m difference in elevation, when converted to elevation estimates using Figure 4C. For *Nothofagus solandri*, converting stomatal frequency of either sun or shade leaves to estimate elevation, using Figure 3C and 3D, would result in a difference of 512 m for stomatal density and 250 m for stomatal index. This error may be smaller when using other species that show less difference in stomatal frequency between sun and shade morphotypes.

This added uncertainty can be greatly decreased, however, when sun and shade leaves

can be distinguished by the undulation of the epidermal cell walls, as is the case in the oaks (Kürschner 1997). Often, sun-morphotypes are preferentially preserved in the fossil record (Kürschner 1997). All oak leaves in the light intensity experiment would be classified as shade leaves, and may provide us with an estimate of the effect of varying light intensity within the morphotypes. The observed change in SD of 16% equals an uncertainty in elevation estimates of 162 m. The exact light intensity related uncertainties vary for different species, but should be taken into account as an addition to the error margin.

Uncertainty in sea-level CO₂ estimates. The calculation of unknown paleo-elevations hinges on the difference in CO₂ partial pressure between sea-level and the site of unknown elevation (Eqn. 1). Therefore, not knowing exact sea-level CO₂ concentrations will introduce a significant additional error into the estimation. This uncertainty could be minimized by estimating sea-level CO₂ using stomatal frequency analysis on the same species from a contemporaneous low-elevation flora. However, if such fossil material is not available, CO₂ estimates based on other plant species or other proxies have to be used for calibration.

To explore the margin of error that uncertainty in sea-level CO₂ proxy data can introduce, we have calculated the offset in elevation estimates for a sea-level CO₂ regime of 365 ppmV with a hypothetical uncertainty of ± 40 ppmV, and a sea-level CO₂ mixing ratio of 750 ppmV, in combination with uncertainties of ± 40 ppmV and ± 80 ppmV (Table 5; Fig. 11). These calculations indicate that under the low CO₂ regime, a 40 ppmV uncertainty results in an error ranging from ± 900 m to ± 1200 m, depending on the reconstructed elevation. The same uncertainty (± 40 ppmV) in the high CO₂ regime results an error range of ± 450 m to ± 700 m, which would indicate that the stomatal method would be more precise for higher CO₂ regimes. However, usually error margins increase with the absolute value of the reconstructed CO₂ levels, and when the uncertainty in the high CO₂ regime is doubled to ± 80 ppmV, the elevation error range increases to ± 1000 m – ± 1400 m.

This exercise clearly suggests that uncertainty in sea-level CO₂ estimates is potentially the largest source of error connected to the stomatal density paleoaltimeter, and when the uncertainty is very high, the utility of the method is severely compromised. Reliable and well-constrained CO₂ estimates or the availability of contemporaneous low-elevation leaf material can reduce or altogether eliminate this error source.

Table 5. Estimated elevation ranges using different uncertainties in sea-level CO₂ partial pressure for modern and high (Eocene) CO₂ levels.

Sea-level CO ₂ pressure (Pa)		1000 m elevation	2000 m elevation	3000 m elevation	4000 m elevation
37.5	CO ₂ decrease (Pa)	3.7	8	11.5	15
37.5	CO ₂ decrease (%)	9.9	22.1	30.7	38.7
37.5	Elevation range (± 40 ppmV) (m)	100-1950	1000-3150	1900-4150	2700-5300
77	CO ₂ decrease (Pa)	8.3	16.5	23	29
77	CO ₂ decrease (%)	10.8	21.4	29.9	37.7
77	Elevation range (± 40 ppmV) (m)	550-1500	1520-2600	2360-3600	3300-4700
77	Elevation range (± 80 ppmV) (m)	0-2000	930-3160	1770-4250	2600-5400

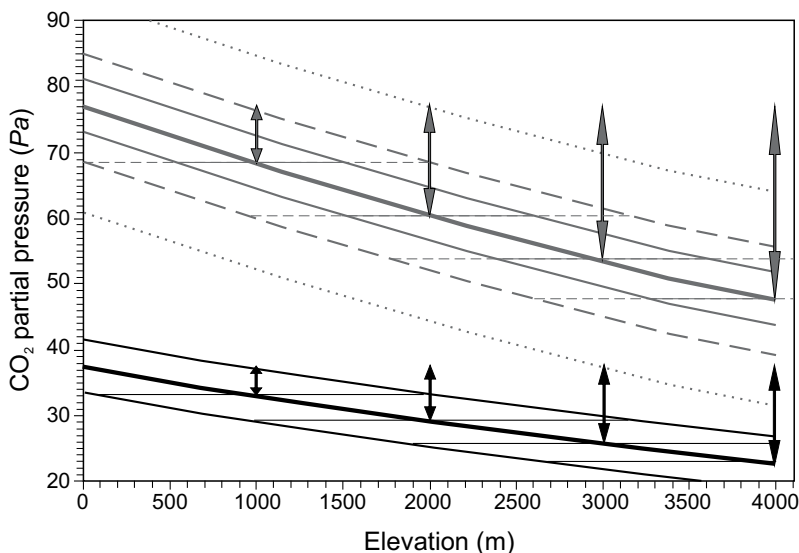


Figure 11. CO₂ partial pressure along an altitudinal range for different sea level CO₂ regimes. Thick black line was calculated using current global atmospheric CO₂ mixing ratios (365 ppmV), thick grey line represents global atmospheric CO₂ ratios comparable to estimated Eocene values (760 ppmV). Solid black and grey lines represent a sea-level CO₂ uncertainty of ± 40 ppmV, grey dashed lines a sea-level uncertainty of ± 80 ppmV. The arrows indicate the difference in CO₂ partial pressure between sea-level and the elevation. Horizontal solid lines indicates range in reconstructed elevation with the 40 ppmV uncertainty, horizontal dashed line the range for the 80 ppmV uncertainty. Dotted line indicates a ± 160 ppmV uncertainty envelope, where the range in elevation estimates exceeds ± 2000 m.

CONCLUSIONS

- The increase in stomatal density (SD) and stomatal index (SI) of *Quercus kelloggii* and *Nothofagus solandri* with elevation is most likely to be an adaptation to counteract the limited photosynthetic potential due to the CO₂ partial pressure decrease, further limited by shorter growing seasons and/or increased UV radiation. Growth experiments on *Q. kelloggii* show that temperature and light intensity differences comparable to gradients along elevational transects do not significantly affect stomatal density and index, and thus can not explain the increase in stomatal frequency with altitude. However, local differences in light inception, as reflected in typical sun and shade leaf morphology, may be an important source of variability in stomatal frequency.
- Leaf gas exchange rates are highly dependent on local climatic factors influencing CO₂ diffusion and evaporation rates, especially temperature lapse rates. The dependency of gas-exchange parameters on local climatic factors and leaf anatomy may account for the wide variability in leaf stomatal responses and stable isotope composition over elevation transects found in different species and different regions.
- For plants at high elevation, where evaporative demand is not excessively high (such as under high temperature lapse rates, increases in cloudiness and for small leaves) a strong SD response would be expected based on current ecophysiological understanding of plant growth along elevation gradients. Under low temperature lapse rates, strong irradiation increases and for large leaves, evaporative demand is high and selection pressure for an SD response to declining CO₂ partial pressure would not be expected.

- Stomatal density and index show great potential for paleoelevation reconstructions with low error margins, if additional error sources such as the presence of sun and shade morphotypes and especially uncertainty in sea-level CO₂ concentrations can be well constrained. Unlike other paleobotanical methods, stomatal frequency analysis is not restricted to angiosperm dominated floras, and has no requirements for a minimum amount of taxa present. The method will be most reliable when applied to fossil taxa that are closely related to extant species, and suitable taxa are most likely to be found for periods when CO₂ concentrations were not much higher than ambient (380 ppmV).

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APPENDIX 1 MATERIAL AND METHODS

Collected material

Leaves from 61 georeferenced *Q. kelloggii* trees were collected from multiple populations over an altitudinal range of 1100–2400 m across California in the summer of 2003 (Fig. 1). The leaf samples were divided into “sun,” “neutral” or “shade” types based on the degree of undulation of the epidermal anticlinal cell walls, which is indicative of light levels during growth (Kürschner 1997).

One branch with fully expanded leaves was collected in September 1999 from nine *N. solandri* var. *cliffortioides* trees growing between 600 and 1460 m (treeline) on Mt. Ruapehu (North Island, New Zealand; 39°18'S 175°35'E; Fig. 2). All samples were taken at ~1.5 m height from the outer north side of the canopy to minimize non-altitude related variation in irradiation levels. Five to eight leaves per tree were processed for SD and SI investigation using standard protocols (McElwain et al. 1995; Poole and Kürschner 1999) and analyzed. *N. solandri* var. *cliffortioides* will be referred to as *N. solandri* in the remainder of the text for brevity.

Branches with sun and shade leaves from three *N. solandri* trees were collected in October 1999 from localities on the South Island, New Zealand: Horrible Bog (S 43°01'12.8" E 171°43'44.6"; 650 m a.s.l.), Kawatiri Junction (S 41°041' E 172°37'; 380 m) and St. Arnaud ((S 41°048' E 172°50'; 700 m) (Fig. 2). Five leaves from each branch were processed for cuticular analysis.

Light experiment

A light experiment was conducted on four-year-old *Q. kelloggii* seedlings in four CONVIRON E8 growth chambers from March to July 2006. Plants were grown in six liter containers filled with a mixture of peat moss, perlite and sand. Nutrients (Miracle Grow) were added twice a year, but not during the experiment. Average light intensity in the four chambers over the five month growth period was 49 (chamber B), 85 (chamber C), 156 (chamber A) and 204 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ (chamber D). Temperature in all four chambers was set to mimic a typical 24-hr cycle in the northern Californian habitat during the growing season (22 °C during the day and 17 °C at night with a 16 hr photoperiod). Humidity levels in all four chambers were maintained between 40 and 80%. Atmospheric CO₂ concentration in the chambers was ~400 ppmV. Plants were watered twice a week (0.25 l per seedling) using an automatic irrigation system. All four chambers contained 15 replicate plants from two different source populations in California.

Temperature experiment

A temperature experiment was conducted from February to May 2004 on two-year-old seedlings of *Quercus kelloggii* (the same plants used in the light experiment, but randomized between experiments). Plants in two Conviron E8 chambers were manually watered twice a week, and light intensity was set at a ~200 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ to mimic the natural understory habitat of *Q. kelloggii* seedlings. In the low temperature treatment, temperature followed a 24-hr cycle, consisting of a 16 hour day of 22 °C and a 6 hour night of 15 °C, with a stepped temperature increment between day and night to simulate dawn and dusk transitions. In the high temperature treatment the same photoperiod settings in the high temperature setting were applied with daytime and nighttime temperature of 27 °C and 20 °C respectively. Growth room relative humidity and seedling nutrient and watering regime were the same for both chambers as in the light experiment.

Processing and analytical methods

Small leaf disks (~1 cm²) were cut out of the central part of the *Q. kelloggii* leaves using a hole punch. Edges were cut off of the *N. solandri* leaves (complete leaves were small enough

to be processed whole), and, if necessary, the dense layer of trichomes on the abaxial surface was “shaved” by gentle scraping with a scalpel. All leaf samples were then treated with either 4% sodium hypochloride (*N. solandri*) or a 50/50 mixture of concentrated glacial acetic acid and 30% hydrogen peroxide (*Q. kelloggii*) at ~50 °C for one to two days to remove mesophyll tissue and separate the cuticle. The abaxial cuticle was stained with saffranin, mounted in glycerin jelly or water on a slide and computer-aided analysis was performed using transmitted light on a Leica DMLB epifluorescence microscope (*Q. kelloggii*) and a Leica Quantimet 500C/500+ Image Analysis system (*N. solandri*).

Stomatal density, epidermal cell density, and stomatal index were measured in five to seven digitally captured counting fields (0.068 mm²) on each *Q. kelloggii* leaf and seven (sun and shade leaves) to ten (transect) counting fields (0.014 mm²) on each *N. solandri* leaf. ImageJ freeware software was used to digitally stack cuticle images in the z plane and to assist in counting epidermal and stomatal cells.

Leaf area of the *N. solandri* leaves from Mt. Ruapehu was measured using a Wild Leitz model area meter. Three measurements were averaged for each leaf, and seven leaves from each elevation were analyzed. Leaf area of *Q. kelloggii* was measured digitally using SigmaScan software from scanned images (800 dpi) of leaves (including petiole).

The stomatal index reported for *N. solandri* leaves in this paper has been adjusted to solely reflect stomatal initiation rates without the influence of trichome density (TD). *N. solandri* leaves have widely varying numbers of trichomes, and since trichome density affects the number and distribution of epidermal pavement cells (Glover and Martin 2000), this also influences stomatal index values. In a data set of 480 counts on 88 *N. solandri* leaves, correlations between epidermal cell density and trichome numbers were significant and positive within groups of leaves with the same stomatal densities. This shows that the trichome-epidermal cell number correlation is not simply an effect of the degree of cell and leaf expansion. Using the regressed trichome-epidermal cell density correlation from the total data set, “trichome-free” epidermal cell numbers were calculated ($ED_{adjusted} = ED - 1.18 \times TD$) to express stomatal initiation rates, and the Stomatal Index for *N. solandri* in this paper is thus a “trichome-free” SI based on corrected epidermal cell numbers.

Results were analyzed by nested mixed-model ANOVA’s using general linear procedures, in the MINITAB 15 statistical program. Nested mixed-model ANOVA was used when multiple leaves per tree and multiple trees per treatment were available. Additional analyses were linear and quadratic regressions (performed in MINITAB 15 and Excel), and when significant differences occurred, means were compared using Student’s t-test or nested mixed-model ANOVA.

Temperature and precipitation data for selected climate stations in northern California was obtained from the Western Regional Climate Center (<http://www.wrcc.dri.edu/>).

APPENDIX 2
APPLICATION MANUAL

1. Identify cuticle-bearing fossil taxa in flora.
2. Select nearest living relative taxon.
3. Collect a modern training set from a reasonably similar geographical area (i.e. expecting comparable temperature lapse rates) to check if the taxon is indeed adjusting stomatal frequency to elevation and establish a calibration curve.
4. Collect modern reference material from different light intensity regimes (sun and shade leaves) and check for the possibility to recognize and separate these (for instance through cell wall undulation index (Kürschner 1997). Often, sun-morphotypes are preferentially preserved in the fossil record. If sun and shade leaves can be separated, develop morphotype-specific calibration curves to minimize potential error.
5. Analyze stomatal density and/or stomatal index on fossil material. (abaxial and/or adaxial). For processing methods, see Kouwenberg et al. 2007.
6. Convert measured SD and/or SI to paleo-CO₂ levels, and calculate paleoelevation using either (preferably) leaf material from the same taxon from a contemporaneous low elevation flora, or sea-level CO₂ reconstructions from other sources. Use CO₂ correction factor for stomatal density if necessary.
7. Calculate the applicable and taxon-specific error margins:
 - a. error of prediction (uncertainty from curve fit in training set)
 - b. additional error from potential mixing up of sun and shade leaves
 - c. error introduced through uncertainty in sea-level CO₂ estimate (in absence of contemporaneous low elevation leaf material)