Water-use responses of ‘living fossil’ conifers to CO₂ enrichment in a simulated Cretaceous polar environment

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Methods

Background and Aims During the Mesozoic, the polar regions supported coniferous forests that experienced warm climates, a CO₂-rich atmosphere and extreme seasonal variations in daylight. How the interaction between the last two factors might have influenced water use of these conifers was investigated. An experimental approach was used to test the following hypotheses: (1) the expected beneficial effects of elevated [CO₂] on water-use efficiency (WUE) are reduced or lost during the 24-h light of the high-latitude summer; and (2) elevated [CO₂] reduces plant water use over the growing season.

Key Results Stimulation of whole-plant WUE (WUEp) by CO₂ enrichment was maintained over the growing season for the three studied species but this pattern was not reflected in patterns of WUE inferred from leaf-stable carbon isotope composition.

Conclusions The data suggest that the efficiency of whole-tree water use may be improved by CO₂ enrichment in a simulated high-latitude environment, but that transpiration is relatively insensitive to atmospheric CO₂ in the living fossil species investigated.

Key words: Water-use efficiency, elevated CO₂, living fossil plants, conifers, paleoecology, ancient polar forests, stable carbon isotopes, stomatal conductance, canopy transpiration.

INTRODUCTION

The plant fossil record provides evidence for coniferous forests extending into the polar regions during the Mesozoic (251–65 Myr ago; Axelrod, 1984; Creber and Chaloner, 1985; Spicer and Chapman, 1990), a time when the global atmospheric CO₂ concentration ([CO₂]) was at least double that of the present-day, and the Earth was in a ‘greenhouse’ climate mode (Royer, 2006). These extinct high-latitude forests therefore experienced an environment unlike any on Earth today, characterized by a CO₂-rich atmosphere, extreme seasonal variations in daylight, and winter temperatures above freezing (Tarduno et al., 1998). Although many studies have identified the direct and indirect action of CO₂ enrichment on tree physiology (Medlyn et al., 1999, 2001; Ainsworth and Long, 2005; Ainsworth and Rogers, 2007), few have addressed the interaction between high [CO₂] and a high-latitude light regime representative of these forests.

In the continuous light regime experienced by high-latitude paleoforests during summer, optimum growth would have required continuous gas exchange without excessive risk of catastrophic xylem embolism (Sperry et al., 1993). Extant conifers in present temperate and boreal biomes support part of the high transpirational demand during the day with water stored in stem sapwood, with losses being replenished during night when transpiration is low (Pallardy et al., 1995). However, in the continuous light of an ancient high-latitude summer, the value of a water-relations strategy employing stem capacitance and overnight recharging would be lost, placing a greater adaptive value on higher water-use efficiency (WUE), i.e. the ratio of CO₂-fixation to water-loss (Jagels and Day, 2004).

Because increases in atmospheric CO₂ concentration generally stimulate leaf photosynthesis (Aₗ) and lower stomatal conductance to water vapour (gₛ) and transpiration rate (Eₛ; Ainsworth and Rogers, 2007), we hypothesize that a CO₂-rich atmosphere during the Mesozoic helped coniferous species in the polar regions to maintain a high instantaneous leaf WUE (WUEₗ) throughout the growing period. Stimulation of WUE in these ancient CO₂-rich atmosphere might also have offered the potential for greater tree height (Osborne and Beerling, 2002). However, experimental evidence indicates that the responses of Aₗ and gₛ may be moderated by the extreme variations in daylight characteristics of high-latitude environments. Osborne and Beerling (2003) reported that a strong acclimation in photosynthetic capacity completely offset the CO₂-stimulation of Aₗ in three ‘living
fossil’ coniferous species after several weeks of continuous illumination during a simulated ancient high-latitude summer (69°N). Moreover, conifers generally show weaker effects of elevated [CO2] on gs and E5 than deciduous and evergreen broadleaf species (Saxe et al., 1998; Medlyn et al., 2001; Ainsworth and Long, 2005). Consequently, the expected beneficial effect of elevated [CO2] on WUE might have been reduced or lost during the summer in coniferous species growing in ancient high-latitude environments, but this proposition remains to be tested.

Leaf WUE can be assessed through instantaneous leaf gas-exchange measurements, and from leaf carbon isotope measurements (δ13C; Farquhar et al., 1982) which integrates the balance between carbon dioxide and water fluxes over long periods (for a review, see Dawson et al., 2002). To distinguish variations in the δ13C of source CO2 from the effects of metabolic processes, the δ13C signatures of organic material are translated to photosynthetic 13C discrimination (Δ13C; Farquhar et al., 1982; Farquhar and Richards, 1984). The model of Farquhar et al. (1982) relates Δ13C linearly to cic/cia, the ratio of intercellular (cia) to atmospheric (cia) CO2 mole fractions, which reflect leaf WUE integrated over the lifetime of the leaf. In the present study, both gas exchange and isotopic measurements are used to estimate leaf-scale WUE, and they are compared with WUE values derived from whole-plant gas exchange measurements to investigate the relationship between leaf-scale WUE and overall plant water use.

Previous studies indicated that large reductions in leaf-scale stomatal conductance and/or WUE at elevated [CO2] do not necessarily translate to reductions in rates of whole-tree transpiration (Wullschleger and Norby, 2001; Wullschleger et al., 2002). Explanations for this observation include: energy balance considerations, whereby increased canopy temperatures compensate for lower gs at elevated [CO2]; CO2-induced reductions in gs being limited to upper canopy leaves; and increases in leaf area for plants grown at elevated compared with ambient [CO2] that offset the reductions in water use due to partial stomatal closure (Field et al., 1995; Wilson et al., 1999; Wullschleger and Norby, 2001). However, we would not expect this last effect would be important in ancient paleoforests, since Royer et al. (2005) reported that, under simulated high-latitude conditions (69°N), elevated [CO2] decreased canopy leaf area in two out of five ‘living fossil’ tree species, while there were no statistically distinguishable changes in the others. Therefore, we might hypothesize that, in a warm high-latitude environment, decreases in gs would be translated into reductions in whole-plant transpiration (E5) over the growing season.

Here, measurements are reported of leaf- and whole-plant gas exchange, and leaf δ13C, made at bimonthly intervals throughout the growing season after 3 years’ exposure of saplings of three ‘living fossil’ coniferous species to either current ambient (400 μmol mol⁻¹) or elevated (800 μmol mol⁻¹) [CO2] in controlled-environment simulated Cretaceous high-latitude conditions (69°N). The three studied species (Sequoia sempervirens, Metasequoia glyptostroboides and Taxodium distichum) belong to three genera known to occur in Cretaceous and Paleogene Arctic forests (Miller, 1977; Schweitzer, 1980; Aulenback and LePage, 1998; Yang and Jin, 2000). The use of ‘living fossil’ species, or extant taxa belonging to lineages characterized by little or no phenotypic change since the Mesozoic, as modern analogues for their congeneric ancestors provides a system to explore the ecophysiology of high-latitude paleoforests (Beerling and Osborne, 2002; Osborne and Beerling, 2003; Royer et al., 2003, 2005; Jagels and Day, 2004; Vann et al., 2004; Equiza et al., 2005; Jagels and Equiza, 2005; Llorens et al., 2009).

In the present study, the following hypotheses were tested: (1) atmospheric CO2 enrichment improves WUE of coniferous species growing in a simulated ancient high-latitude environment only in spring and autumn because, during summer, gs and transpiration rate are insufficiently reduced by elevated [CO2] to compensate for the acclimation of photosynthesis; and (2) despite the relatively low CO2 sensitivity of gs, a doubling of atmospheric CO2 reduces whole-plant water loss over the growing season. These two hypotheses also provide a framework for evaluating: (a) inferences about leaf or plant water use made using leaf δ13C measurements, and (b) the validity of instantaneous leaf-scale measurements to assess overall plant water use.

**MATERIALS AND METHODS**

**Experimental design**

A Cretaceous Arctic environment was simulated in eight replicated growth rooms located in Sheffield, UK (53°N). Four were maintained at the current ambient [CO2] (400 μmol mol⁻¹), and four at elevated [CO2] (800 μmol mol⁻¹), similar to estimates for the Cretaceous based on geochemical proxies (Royer et al., 2001). In all the growth rooms, a daytime photosynthetic photon flux density (PPFD) of 300–400 μmol m⁻² s⁻¹ was provided using water-cooled sodium lamps (Sunbeam Hydrostar; Avon Gro-Lite Systems, Bristol, UK), and the photoperiod was changed weekly to simulate 69°N. Temperature within the growth rooms was raised by 5°C compared with the outside air, and maintained above a minimum of +5°C in the winter, to match proxy-based estimates of palaeotemperature at high latitudes for the Cretaceous (Beerling and Osborne, 2002; Royer et al., 2003). Relative humidity within the growth rooms was maintained above 75% by using an automated misting system. Plants were grown in a silica sand–vermiculite–peat medium (13:5:2), watered twice daily via an automated drip irrigator. Fertilizer was applied as Rorison’s nutrient solution, with a gradual increase in strength from 10% to 50% during the experiment as the plants grew larger, with the aim of providing a non-limiting nutrient supply. Full experimental details are provided elsewhere (Beerling and Osborne, 2002; Osborne and Beerling, 2003; Royer et al., 2003, 2005).

All saplings were grown from seed at the Llangwm Arboretum (Usk, UK) and acclimated to Sheffield climate for 2 months before transfer to the growth rooms (Beerling and Osborne, 2002). Saplings were 1 year old at the start of the experiment and were grown for further 3 years first in 2-L pots and later in 6-L pots. The coastal redwood (Sequoia sempervirens [D. Don] Endl) is evergreen, whereas the others, the dawn redwood (Metasequoia glyptostroboides Hu and Cheng) and the swamp cypress (Taxodium distichum
Rich), are deciduous. Every year, leaf growth started in April and continued until September in all species. The three species are all members of the family Cupressaceae and have long fossil records at the generic level (>65 Myr), with ancestors that formed Cretaceous and Paleogene Arctic forests (Miller, 1977; Schweitzer, 1980; Aulenback and LePage, 1998; Yang and Jin, 2000).

**Leaf gas exchange**

Instantaneous measurements of leaf CO$_2$ and H$_2$O exchange rates were made using an open gas-exchange system (CIRAS-1; PP Systems, Hitchin, Herts, UK) on recently expanded upper canopy leaves during the third growing season. The leaves were illuminated with a quartz halide source providing a PPFD of 600 $\mu$mol m$^{-2}$ s$^{-1}$, which saturates photosynthesis in these species (Osborne and Beerling, 2003). Cuvette [CO$_2$] and leaf-to-air vapour pressure difference were set to match growth conditions. Leaf temperature was maintained at 25 °C with a feedback control system. Values of $A_L$, $E_L$ and the internal-to-ambient CO$_2$ concentration ($c_{i,c_a}$) were calculated following von Caemmerer and Farquhar (1981), with $iWUE_L$ defined as the ratio of $A_L$ to $E_L$.

Measurements were taken in April (14 h light d$^{-1}$), June (24 h light d$^{-1}$), July (24 h light d$^{-1}$) and September (12 h light d$^{-1}$), on a single leaf from two plants per species in each of four replicated growth rooms per CO$_2$ treatment. Values of the in vivo carboxylation capacity of Rubisco ($V_{c,max}$) were calculated using the gas exchange data for each date, as reported previously by Osborne and Beerling (2003). Here $V_{c,max}$ values were used to explore their relationship with $\Delta^{13}$C. Leaf dark respiration rates ($\mu$mol m$^{-2}$ s$^{-1}$) were also measured in the same leaves after 10 min dark adaptation. Leaf photospiration rates ($\mu$mol m$^{-2}$ s$^{-1}$) were, then, calculated after Farquhar et al. (1980).

**Whole-plant transpiration and water-use efficiency**

Whole-plant CO$_2$ and H$_2$O fluxes were quantified using custom-built enclosures attached to a differential infrared gas analyser (CIRAS-1; PP Systems), using the same time intervals and a subset of the plants used for leaf measurements (for full details, see Royer et al., 2003, 2005). The gas exchange chambers were set-up within one of the growth rooms, and subjected to the same light and temperature regime. Radiant heating from the lamps was offset through radiant heating from the lamps was offset through radiant heating from the lamps was offset through liquid phase diffusion, and possible discriminations during respiration and photorespiration (O’Leary, 1993). Since the value of $d$ has been typically considered small, it is usually excluded from (3) and, thus, $\Delta^{13}$C is estimated as:

$$\Delta^{13}C = a - d + (b - a)(c_i/c_a)$$  (4)

$tWUE_L$ was calculated following Farquhar and Richards (1984):

$$tWUE_L = (([CO_2](1 - c_i/c_a))/(1 \cdot 6D)$$  (5)

where $[CO_2]$ is the CO$_2$ concentration in each growth room, the factor 1-6 is the ratio of gaseous diffusivities of CO$_2$ and water vapour in the air, $c_i/c_a$ is obtained from $\Delta^{13}$C by re-arranging (4), and $D$ is the mean leaf-to-air water vapour pressure difference for each growth chamber, estimated from temperature and relative humidity measurements.
following Buck (1981), by assuming that leaf temperature equals air temperature.

**Statistical analyses**

Effects of [CO₂] and species were assessed throughout the study period (except for total plant water loss) with two-way repeated-measures analysis of variance (ANOVAR) using means for each sampling date and growth chamber. The effects of [CO₂] and species on whole-plant transpiration integrated over the study period were tested with two-way analysis of variance (ANOVA). Significant differences between means, as well as significant interactions between factors, were identified by taking \( P \leq 0.05 \) as the level of significance. Relationships between variables were also analysed by means of least-square linear regressions using means for each species, sampling date and CO₂ treatment.

**RESULTS**

**Leaf gas exchange**

Overall, \( g_s \) measured under standardized conditions was lower in trees grown at elevated [CO₂] than those from the ambient treatment (Table 1 and Fig. 1A–C). The effect of elevated [CO₂] was largest in *T. distichum* in June and July, when \( g_s \) fell by nearly 50% from 244 mmol m\(^{-2}\) s\(^{-1}\) to 113 mmol m\(^{-2}\) s\(^{-1}\) (Fig. 1C). Instantaneous values of \( c_a/c_s \), derived from the leaf gas exchange measurements, showed a significant CO₂ effect on the balance between \( i/\varphi \) and \( WUE_{\lambda} \), with higher overall \( c_a/c_s \) values in elevated than ambient [CO₂] (Table 1 and Fig. 1D–F). Values of \( c_a/c_s \) decreased as a linear function of \( g_s \) for plants grown at ambient [CO₂], but this relationship was not significant in the case of plants grown at elevated [CO₂] (Fig. 2A). However, instantaneous \( c_a/c_s \) decreased as a linear function of photosynthesis (\( A_s \)) for plants grown at either ambient or elevated [CO₂] (Fig. 2B).

Elevated [CO₂] significantly increased \( iWUE_{\lambda} \) under standardized conditions (Table 1). However, this effect was restricted to spring and autumn in *S. sempervires* and *M. glyptostroboides* (Fig. 3A and B), while it persisted throughout the year in *T. distichum* (Fig. 3C).

Estimated leaf photorespiration rates were significantly lower in elevated than ambient [CO₂], although the magnitude of this effect was more or less pronounced depending on the species and the sampling month (Table 1).

**Whole-plant gas exchange**

Direct measurements of whole-plant gas exchange under growth conditions showed that CO₂ enrichment did not significantly affect daily transpiration (\( E_p; \) Table 1 and Fig. 4A–C). After integration of these measurements over the study period, no significant CO₂ effect was found on overall plant water use during this period (Fig. 5).

Calculation of \( WUE_{E_p} \) from whole-plant measurements of \( A_p \) and \( E_p \) under growth conditions (Fig. 3D–F) gave lower values to those estimated from leaf gas exchange measurements (Fig. 3A–C) and showed a statistically significant, but attenuated, stimulation by elevated [CO₂] across all species

### Table 1. Results of two-way repeated-measures analysis of variance (ANOVAR)

<table>
<thead>
<tr>
<th>Species</th>
<th>[CO₂] × Species</th>
<th>[CO₂] × Species</th>
<th>Month</th>
<th>[CO₂] × Species</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>F</td>
<td>P</td>
<td></td>
<td>F</td>
</tr>
<tr>
<td>M. glyptostroboides</td>
<td>5.5</td>
<td>0.031</td>
<td>4.3</td>
<td>0.001</td>
</tr>
<tr>
<td>S. sempervires</td>
<td>13.1</td>
<td>0.002</td>
<td>24.2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>T. distichum</td>
<td>3.4</td>
<td>0.044</td>
<td>9.2</td>
<td>0.004</td>
</tr>
<tr>
<td></td>
<td>12.4</td>
<td>0.004</td>
<td>8.2</td>
<td>0.008</td>
</tr>
<tr>
<td></td>
<td>10.8</td>
<td>0.002</td>
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</tr>
<tr>
<td></td>
<td>9.6</td>
<td>0.033</td>
<td>4.3</td>
<td>0.031</td>
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<tr>
<td></td>
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<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>5.5</td>
<td>0.002</td>
<td>17.4</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>5.1</td>
<td>0.001</td>
<td>18.9</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Results are highlighted when significant (\( P \leq 0.05 \)).
The non-significant CO$_2$ × sampling month interaction indicated that CO$_2$ effects on WUE$_P$ did not change significantly over the growing season, in contrast to what was found for iWUE$_L$.

**Stable carbon isotope discrimination**

Values of Δ$^{13}$C were significantly higher in plants growing under elevated [CO$_2$] than those in the ambient treatment, in all species, and throughout the growing season (Table 1 and Fig. 6A–C). There was a significant negative relationship between Δ$^{13}$C and $V_{c,max}$ at both ambient and elevated [CO$_2$] (Fig. 7A). Variation in Δ$^{13}$C was also significantly correlated with variation in iWUE$_L$ in both CO$_2$ treatments (Fig. 7B), but not with variation in WUE$_P$ ($P = 0.624$ and $P = 0.358$ for plants grown at ambient and elevated [CO$_2$], respectively). However, trends in tWUE$_L$ (Fig. 3G–I) differed substantially from those found for iWUE$_L$ (Fig. 3A–C). Values of tWUE$_L$ were greater in the elevated than ambient CO$_2$ treatment in the two deciduous species in the spring and at the beginning of the summer, but this CO$_2$ effect was lost as the growing season progressed (Fig. 3H, I).

As predicted by eqn (3), Δ$^{13}$C values were significantly correlated with instantaneous measurements of $c_i/c_a$ (Fig. 8). The regression equations explained 48% and 36% of variation in Δ$^{13}$C for ambient and elevated [CO$_2$] plants, respectively. However, trends in tWUE$_L$ (Fig. 3G–I) differed from those found for iWUE$_L$ (Fig. 3A–C). Values of tWUE$_L$ were greater in the elevated than ambient CO$_2$ treatment in the two deciduous species in the spring and at the beginning of the summer, but this CO$_2$ effect was lost as the growing season progressed (Fig. 3H, I).

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Leaf Δ$^{13}$C values at ambient [CO$_2$] were poorly predicted from instantaneous $c_i/c_a$ when using $d = 0$% (either assuming $b = 29$% or $b = 27$%) in eqn (3) (Fig. 8). By contrast,
\[ d = 4\%_{\text{o}} \text{ (assuming } b = 29\%_{\text{o}}) \] provided a much better fit between predicted $\Delta^{13}$C values from instantaneous $c_i/c_a$ and observed $\Delta^{13}$C values at ambient [CO$_2$].

**DISCUSSION**

*Water-use efficiency and transpiration estimated from gas exchange measurements*

Increases in $i$WUE$_L$ with CO$_2$ enrichment were restricted to the beginning and end of the growing season for *S. sempervirens* and *M. glyptostroboides* (Fig. 3A, B), in agreement with our first hypothesis. As anticipated, these patterns tracked seasonal variations in $A_L$ for both species (Osborne and Beerling, 2003), rather than $g_s$ (Fig. 1A, B). By contrast, CO$_2$ enhancement of $i$WUE$_L$ in *T. distichum* was maintained throughout the study period (Fig. 3C) because continuous reductions in $g_s$ during the polar summer (Fig. 1C) offset the effects of acclimation on $A_L$. The observed loss of $A_L$ stimulation by a doubling of CO$_2$ in these coniferous species during summer (Osborne and Beerling, 2003) differ markedly from the situation in field-grown conifers of temperate environments, where $A_L$ typically shows a significant, season-long stimulation of $>40\%$ in response to a doubling of CO$_2$,
which persists over a number of years (Ellsworth, 1999; Medlyn et al., 1999; Crous and Ellsworth, 2004). However, the low CO2 sensitivity of $g_s$ relative to that of $A_L$ is consistent with previous field studies showing that most conifer species respond to CO2 enrichment with small or non-significant decreases in $g_s$ (Saxe et al., 1998; Ellsworth, 1999; Medlyn et al., 2001), in contrast to the general pattern for angiosperms of significant decreases in this parameter (Ainsworth and Rogers, 2007).

At the whole-plant scale, the present results failed to support hypothesis (1), because the stimulation of $WUE_P$ by CO2 enrichment did not change significantly between sampling dates (Table 1). Because respiration of non-photosynthetic organs throughout the day, and respiration of autotrophic tissues in the dark, affect plant carbon balance, and non-transpirational (cuticular and lenticellular) water losses influence plant water economy, $WUE_P$ is expected to be lower than $iWUE_L$ (Ripullone et al., 2004). Accordingly, lower values of $WUE_P$ compared with $iWUE_L$ were found over the study period, irrespective of species and CO2 treatment, with differences between $WUE_P$ and $iWUE_L$ being greater in spring and autumn, i.e. when plants had some hours of darkness (Fig. 3A–F).

Despite values of $WUE_P$ for the three coniferous species being significantly higher at elevated than ambient [CO2], the relative insensitivity of $E_P$ to the CO2 treatment (Table 1 and Fig. 4) suggest that this response was driven largely by increased canopy assimilation rates (Royer et al., 2005). The non-significant effect of CO2 enrichment on plant water loss over the study period (Fig. 5) is in accordance with previous studies showing that canopies may be substantially decoupled from the atmosphere, with transpiration from the canopy being

![Fig. 5](image-url) Whole-plant transpiration integrated over the study period using measurements of $E_p$ (Fig. 4) for *S. sempervirens*, *M. glyptostroboides* and *T. distichum* at ambient or elevated [CO2] in a simulated Cretaceous warm-Arctic environment. Values plotted are means ± s.e.m. ($n = 3$).

![Fig. 6](image-url) Seasonal changes in leaf carbon isotope discrimination ($\Delta^{13}C$) calculated from $\delta^{13}C$ values for (A) *S. sempervirens*, (B) *M. glyptostroboides* and (C) *T. distichum* growing at ambient or elevated [CO2] in a simulated Cretaceous warm-Arctic environment. Values are means ± s.e.m. for four replicated growth rooms. The line above the months depicts the period of 24 h continuous light.

![Fig. 7](image-url) Leaf carbon isotope discrimination ($\Delta^{13}C$) calculated from $\delta^{13}C$ values plotted against (A) Rubisco carboxylation capacity ($V_{c,max}$) and (B) instantaneous leaf WUE ($iWUE_L$) for *S. sempervirens* (triangles), *M. glyptostroboides* (circles) and *T. distichum* (squares) at ambient (open symbols) and elevated (closed symbols) [CO2]. Each symbol represents the mean for each species and sampling date. The dashed and continuous lines are linear regressions fitted to ambient and elevated [CO2] data points, respectively.
et al. on photosynthesis (Koch high-latitude light environment. This result is in accordance with: (et al. Wullschleger Greenwood and Basinger, 1994; Williams that of living temperate deciduous forests, with trees reaching ductivity of high-latitude paleoforests was comparable with Beerling, 2002). Indeed, it has been reported that the pro-

constraint on stomata and therefore a CO2-diffusion limitation determining ancient high-latitude environments, even during the con-
might have benefited the growth of coniferous species inhab-

istics to these regions, it might have helped trees to reach heights comparable to those found in present temperate deciduous forests.

Inference of water use from isotope data

Throughout the study period, but especially during the high-

latitude summer, all three coniferous species showed higher foliage $\Delta^{13}C$ values when grown under high [CO$_2$] (Table 1 and Fig. 6). This result is in accordance with: (a) the observed reduction of carboxylation capacity in plants growing under CO$_2$ enrichment during summer (Osborne and Beerling, 2003), and (b) the marginal effect of elevated [CO$_2$] on the stomatal conductance of these species (Table 1 and Fig. 1A–C).

A decrease in plant carboxylation capacity, in the absence of a significant change in $g_s$, should lead to a rise in $\Delta^{13}C$ (Farquhar et al., 1989) and, accordingly, a significant negative relationship was found between $\Delta^{13}C$ and the maximum rate of RuBP carboxylation ($V_{c,max}$; Fig. 7A). The higher $c_i/c_a$ ratios (Fig. 1D–F) and $\Delta^{13}C$ values observed in plants growing under elevated [CO$_2$] compared with controls are in agreement with some previous studies (Picon et al., 1996; Bryant et al., 1998), although chamber and FACE (free-air CO$_2$ enrichment) studies have usually shown that the co-ordination of stomatal conductance and photosynthesis is not substantially altered by CO$_2$ enrichment, with $c_i/c_a$ being typically insensitive to atmospheric CO$_2$ (Drake et al., 1997; Xu and Hsiao, 2004; Ainsworth and Long, 2005).

As predicted by theory (Farquhar et al., 1989), a significant negative relationship was found between $\Delta^{13}C$ and iWUE$_{L}$ (Fig. 7B). However, plants grown at ambient [CO$_2$] showed lower iWUE$_{L}$, but also lower $\Delta^{13}C$ values when compared with plants grown at elevated [CO$_2$]. Therefore, the present data support previous studies showing that lower $\Delta^{13}C$ may not always indicate higher iWUE$_{L}$, or translate into higher WUE at the whole-plant level (Condon et al., 2004; Seibt et al., 2008).

Seasonal patterns of tWUE$_{L}$ also differed from those measured for iWUE$_{L}$ for the three species studied (Fig. 3). All of these discrepancies may be interpreted from different, but not mutually exclusive, perspectives. The first potential mechanism is a ‘smoothing’ effect in the isotopic composition of tree leaves, whereby the carbohydrate used to construct new foliage is a mixture of recently fixed and stored carbon pools (Keel et al., 2007). Secondly, discrepancies between tWUE$_{L}$ and iWUE$_{L}$ may have resulted from differences between $D$ (leaf-to-air water vapour pressure difference) and PPFD averaged over the course of the experiment, as compared with values in the cuvette during instantaneous measurements (Xu and Hsiao, 2004; Cernusak et al., 2007). Thirdly, part of the discrepancy may relate to the fact that $c_i/c_a$ is calculated differently from instantaneous gas exchange measurements than from isotopic measurements (Cernusak et al., 2007). Finally, discrepancies may arise from the fact that eqn (4) excludes the term $d$, which summarizes the fractionation caused by dissolution of CO$_2$ and liquid phase diffusion, photorespiration and dark respiration (Farquhar et al., 1989). Previous studies have reported substantial differences between observed $\Delta^{13}C$ and the predictions of eqn (4), with eqn (4) overestimating discrimination under ambient [CO$_2$] conditions (von Caemmerer and Evans, 1991; Farquhar and Lloyd, 1993; Giller and Griffiths, 1997; Lauter et al., 1997; Seibt et al., 2008). Few direct estimates of $d$ exist in the literature and, as far as we know, all of them are for species growing at current [CO$_2$]: approx. 3 % in wheat (Evans et al., 1986), near-zero for barley (Hubick and Farquhar, 1989), approx. 1 % for peanut (Hubick, 1990), 4 % for Ficus insipida (Cernusak et al., 2007) and 3 % for several tropical species (Cernusak et al., 2008). In the present data, a value of $d = 4 \%$ provided a much better fit (assuming $b = 29 \%$) between predicted
(from instantaneous $c_{i}a_{d}$) and observed $\Delta^{13}C$ values at ambient $[CO_2]$ than using $d = 0 \%$ (either assuming $b = 29$ or $27 \%$; Fig. 8). An effect of $[CO_2]$ on $d$ would be consistent with the suppression of photospiration at elevated $[CO_2]$ inferred from the present data (Table 1), but this aspect of the results requires further investigation.

Conclusions

The data indicate that leaf- and whole-plant-scale WUE in conifers are significantly enhanced by elevated $[CO_2]$ under a simulated warm Cretaceous high-latitude environment. These responses are driven primarily by photosynthesis rather than stomatal conductance, and are highly seasonal and species-specific at the leaf but not the plant scale. Overall, the present data suggest that, while the efficiency of whole-tree water use may be improved by $CO_2$ enrichment in this system, transpiration is relatively insensitive to atmospheric $CO_2$.

ACKNOWLEDGEMENTS

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LITERATURE CITED


APPENDIX

Abbreviations used

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>A_p</td>
<td>Daily whole-plant net carbon uptake (mmol CO2 plant^{-1} d^{-1})</td>
</tr>
<tr>
<td>A_l</td>
<td>Net leaf photosynthetic rate (mmol CO2 m^{-2} s^{-1})</td>
</tr>
<tr>
<td>E_p</td>
<td>Daily whole-plant transpiration (mol H2O plant^{-1} d^{-1})</td>
</tr>
<tr>
<td>E_l</td>
<td>Leaf transpiration rate (mmol H2O m^{-2} s^{-1})</td>
</tr>
<tr>
<td>g_(st)</td>
<td>Leaf stomatal conductance (mmol H2O m^{-2} s^{-1})</td>
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<tr>
<td>iW/Ue</td>
<td>Instantaneous leaf water-use efficiency (mmol CO2 mol^{-1} H2O)</td>
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<tr>
<td>PPF</td>
<td>Photosynthetic photon flux density (µmol m^{-2} s^{-1})</td>
</tr>
<tr>
<td>tW/Ue</td>
<td>Time-integrated leaf water-use efficiency (mmol CO2 mol^{-1} H2O)</td>
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<td>W/UE</td>
<td>Whole-plant water-use efficiency (mmol CO2 mol^{-1} H2O)</td>
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<td>Δ13C</td>
<td>Discrimination against 13C (%o)</td>
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<td>ε13Ca</td>
<td>Stable carbon isotope composition (%o)</td>
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<td>ε13Ca_o</td>
<td>δ13C of the atmosphere</td>
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<tr>
<td>δ13C</td>
<td>Stable carbon isotope composition (%o)</td>
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<tr>
<td>\delta^{13}C</td>
<td>Stable carbon isotope composition (%o)</td>
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<tr>
<td>V_c,max</td>
<td>Rubisco carboxylation capacity (µmol CO2 m^{-2} s^{-1})</td>
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