

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

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- **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.
- **Methods** Measurements of leaf and whole-plant gas exchange, and leaf-stable carbon isotope composition were made on one evergreen (*Sequoia sempervirens*) and two deciduous (*Metasequoia glyptostroboides* and *Taxodium distichum*) ‘living fossil’ coniferous species after 3 years’ growth in controlled-environment simulated Cretaceous Arctic (69°N) conditions at either ambient (400 µmol mol⁻¹) or elevated (800 µmol mol⁻¹) [CO₂].
- **Key Results** Stimulation of whole-plant WUE (WUE_p) 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-scale gas exchange measurements ($iWUE_L$) and $\delta^{13}C$ of foliage ($tWUE_L$). This response was driven largely by increased rates of carbon uptake, because there was no overall CO₂ effect on daily whole-plant transpiration or whole-plant water loss integrated over the study period. Seasonal patterns of $tWUE_L$ differed from those measured for $iWUE_L$. The results suggest caution against over simplistic interpretations of WUE_p based on leaf isotopic 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 (A_L) and lower stomatal conductance to water vapour (g_s) and transpiration rate (E_L ; 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 ($iWUE_L$) 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 A_L and g_s 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 A_L in three ‘living

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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 g_s and E_L 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 ($\delta^{13}\text{C}$; 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 $\delta^{13}\text{C}$ of source CO₂ from the effects of metabolic processes, the $\delta^{13}\text{C}$ signatures of organic material are translated to photosynthetic ^{13}C discrimination ($\Delta^{13}\text{C}$; Farquhar *et al.*, 1982; Farquhar and Richards, 1984). The model of Farquhar *et al.* (1982) relates $\Delta^{13}\text{C}$ linearly to c_i/c_a , the ratio of intercellular (c_i) to atmospheric (c_a) 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 (Wullschlegel and Norby, 2001; Wullschlegel *et al.*, 2002). Explanations for this observation include: energy balance considerations, whereby increased canopy temperatures compensate for lower g_s at elevated [CO₂]; CO₂-induced reductions in g_s 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; Wullschlegel 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 g_s would be translated into reductions in whole-plant transpiration (E_p) over the growing season.

Here, measurements are reported of leaf- and whole-plant gas exchange, and leaf $\delta^{13}\text{C}$, 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 $\mu\text{mol mol}^{-1}$) or elevated (800 $\mu\text{mol mol}^{-1}$) [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, g_s 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 g_s , 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 $\delta^{13}\text{C}$ 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 $\mu\text{mol mol}^{-1}$), and four at elevated [CO₂] (800 $\mu\text{mol mol}^{-1}$), 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 $\mu\text{mol m}^{-2} \text{s}^{-1}$ 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 CO₂ and H₂O 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 μmol m⁻² s⁻¹, which saturates photosynthesis in these species (Osborne and Beerling, 2003). Cuvette [CO₂] 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 CO₂ 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 CO₂ 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 Δ¹³C. Leaf dark respiration rates (μmol m⁻² s⁻¹) were also measured in the same leaves after 10 min dark adaptation. Leaf photorespiration rates (μmol m⁻² s⁻¹) were, then, calculated after Farquhar *et al.* (1980).

Whole-plant transpiration and water-use efficiency

Whole-plant CO₂ and H₂O 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 the use of circulating water jackets. The incoming air was humidified by bubbling through water, and [CO₂] controlled via the injection of pure CO₂ into a mixing volume. An airflow rate of 4 L min⁻¹ resulted in a mean residence time of 3 min for the air in each chamber, which was stirred continuously with two fans.

One plant per species was sampled from each of three replicate growth rooms per CO₂ treatment. Each plant was sealed inside one of the chambers for 24 h, and hourly measurements were then made over the subsequent 24-h period. The hourly fluxes were integrated to obtain daily whole-plant net carbon uptake (A_P) and transpiration (E_P; Royer *et al.*, 2003, 2005), with whole-plant water-use efficiency (WUE_P) being, then, calculated as the quotient between A_P and E_P. Plant water

use over the growing season was approximated by integrating E_P over the study period.

Stable carbon isotope analyses

Foliar δ¹³C values were determined on the same leaves that were used for gas exchange. Oven-dried leaves were ground to a homogenized fine powder and δ¹³C determinations made on a 20-mg sub-sample using an ANCA GSL preparation module coupled to a 20–20 stable isotope analyser (PDZ Europa, Cheshire, UK). Measurements on the same sample had a reproducibility of 0.5‰. The isotopic composition of the sample (δ¹³C_s) was calculated as the sample ¹³C/¹²C ratio relative to the PDB standard (‰), and converted to discrimination values (Δ¹³C) using equation of Farquhar *et al.* (1989):

$$\Delta^{13}\text{C} = (\delta^{13}\text{C}_a - \delta^{13}\text{C}_s) / [1 + (\delta^{13}\text{C}_s / 1000)] \quad (1)$$

where δ¹³C_a is the isotopic composition of the atmosphere, estimated using flask samples taken from each of the eight growth rooms over two 6-h time periods, together with atmospheric and tank CO₂ end-members. Concurrent measurements of [CO₂] were obtained using the gas-exchange system. The δ¹³C_a of flask samples was measured with the same mass spectrometer, and mean values used to construct a Keeling plot (Keeling, 1958; Yakir and Sternberg, 2000). This yielded the following regression, which was then used to estimate δ¹³C_a from growth room records of [CO₂] on a monthly basis equivalent to the sampling of leaf tissue:

$$\delta^{13}\text{C}_a = -32.8 + 9011.2 / [\text{CO}_2] \quad (r^2 = 0.97) \quad (2)$$

Farquhar *et al.* (1982) formalized the relationship between Δ¹³C and leaf gas exchange with the well-validated model:

$$\Delta^{13}\text{C} = a - d + (b - a)(c_i/c_a) \quad (3)$$

where a and b are constants representing isotope fractionation by diffusion through the stomatal pores (4.4‰) and by the enzyme ribulose-1,5-biphosphate carboxylase-oxygenase (Rubisco; 29‰), and d summarizes the effects of discrimination by CO₂ dissolution, 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, Δ¹³C is estimated as:

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

tWUE_L was calculated following Farquhar and Richards (1984):

$$\text{tWUE}_L = \{[\text{CO}_2](1 - c_i/c_a)\} / (1 \cdot 6D) \quad (5)$$

where [CO₂] is the CO₂ concentration in each growth room, the factor 1.6 is the ratio of gaseous diffusivities of CO₂ and water vapour in the air, c_i/c_a is obtained from Δ¹³C 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_i/c_a , derived from the leaf gas exchange measurements, showed a significant CO₂ effect on the balance between A_L and g_s , with higher overall c_i/c_a values in elevated than ambient [CO₂] (Table 1 and Fig. 1D–F). Values of c_i/c_a 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_i/c_a decreased as a linear function of photosynthesis (A_L) for plants grown at either ambient or elevated [CO₂] (Fig. 2B).

Elevated [CO₂] significantly increased $iWUE_L$ under standardized conditions (Table 1). However, this effect was restricted to spring and autumn in *S. sempervirens* 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_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

	[CO ₂]		Species		Month		[CO ₂] × species		[CO ₂] × month		Species × month	
	F	P	F	P	F	P	F	P	F	P	F	P
	g_s (mmol m ⁻² s ⁻¹)	5.5	0.031	53.1	< 0.001	4.3	0.008	0.9	0.411	1.0	0.401	3.2
Instantaneous c_i/c_a	13.1	0.002	26.2	< 0.001	24.2	< 0.001	2.2	0.135	2.6	0.058	8.8	< 0.001
$iWUE_L$ (mmol CO ₂ mol ⁻¹ H ₂ O)	51.2	< 0.001	14.7	< 0.001	23.6	< 0.001	0.9	0.403	5.8	0.002	5.7	< 0.001
E_P (mol plant ⁻¹ day ⁻¹)	3.4	0.091	9.2	0.004	33.5	< 0.001	0.3	0.77	1.5	0.23	1.8	0.135
WUE_P (mmol CO ₂ mol ⁻¹ H ₂ O)	12.4	0.004	1.2	0.33	7.9	< 0.001	1.4	0.289	2.3	0.088	8.1	< 0.001
$\delta^{13}C$ (‰)	19.8	0.001	6.1	0.013	7.5	< 0.001	0.03	0.966	1.5	0.226	1.2	0.340
$tWUE_L$ (mmol CO ₂ mol ⁻¹ H ₂ O)	1.9	0.190	8.3	0.004	11.0	< 0.001	1.2	0.331	4.7	0.006	1.1	0.397
Photorespiration (mmol plant ⁻¹ d ⁻¹)	57.5	< 0.001	18.9	< 0.001	17.4	< 0.001	3.9	0.038	5.2	0.003	5.8	< 0.001

Three-way interactions are not shown.
Results are highlighted when significant ($P \leq 0.05$).

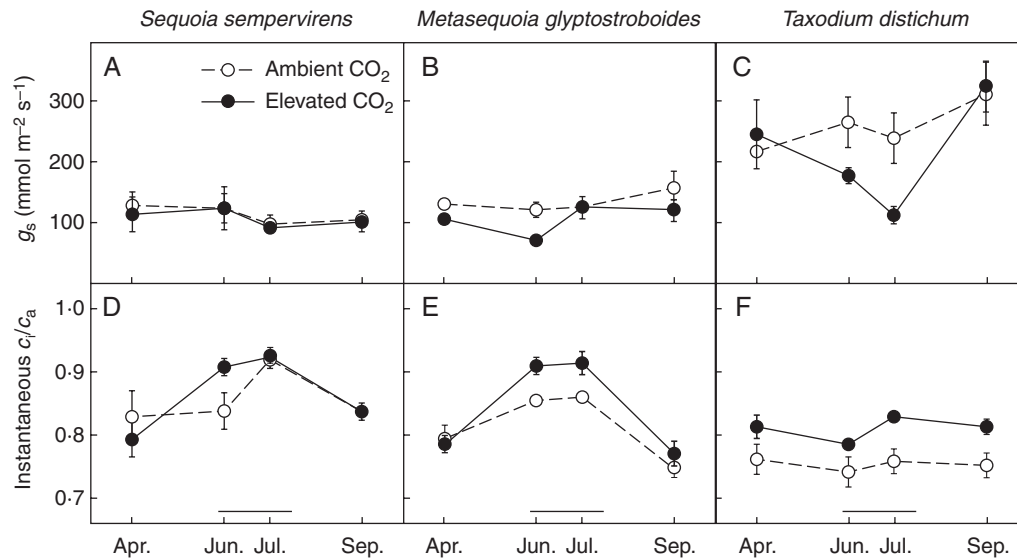


FIG. 1. Seasonal changes in (A–C) leaf stomatal conductance (g_s), and (D–F) the ratio c_i/c_a derived from leaf gas exchange measurements for *S. sempervirens*, *M. glyptostroboides* and *T. distichum* growing at ambient or elevated [CO₂] in a simulated Cretaceous warm-Arctic environment. Values are means \pm s.e.m. for four replicated growth rooms. The line above the months depicts the period of 24 h continuous light.

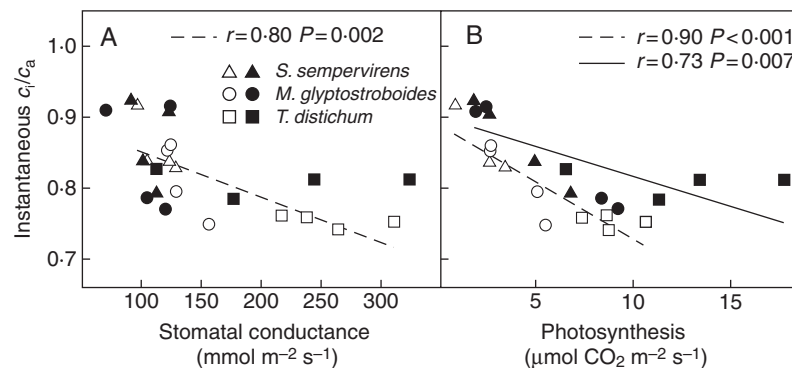


FIG. 2. Instantaneous measurements of c_i/c_a plotted against leaf (A) stomatal conductance and (B) photosynthesis for *S. sempervirens* (triangles), *M. glyptostroboides* (circles) and *T. distichum* (squares) at ambient (open symbols) and elevated (closed symbols) [CO₂]. Each symbol represents the mean \pm s.e.m. for each species and sampling date. The dashed and continuous lines are linear regressions fitted to ambient and elevated [CO₂] data points, respectively.

(Table 1). The non-significant CO₂ \times sampling month interaction indicated that CO₂ 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 $\Delta^{13}C$ were significantly higher in plants growing under elevated [CO₂] 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 $\Delta^{13}C$ and $V_{c,max}$ at both ambient and elevated [CO₂] (Fig. 7A). Variation in $\Delta^{13}C$ was also significantly correlated with variation in $iWUE_L$ in both CO₂ treatments (Fig. 7B), but not with variation in WUE_P ($P = 0.624$ and $P = 0.358$ for plants grown at ambient and elevated [CO₂],

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 CO₂ treatment in the two deciduous species in the spring and at the beginning of the summer, but this CO₂ effect was lost as the growing season progressed (Fig. 3H, I).

As predicted by eqn (3), $\Delta^{13}C$ values were significantly correlated with instantaneous measurements of c_i/c_a (Fig. 8). The regression equations explained 48% and 36% of variation in $\Delta^{13}C$ for ambient and elevated [CO₂] plants, respectively. Overall, $\Delta^{13}C$ at a given c_i/c_a value was greater in the elevated than ambient [CO₂] treatment, which is consistent with an influence of [CO₂] on the y-intercept (offset) of eqn (3). Leaf $\Delta^{13}C$ values at ambient [CO₂] were poorly predicted from instantaneous c_i/c_a when using $d = 0\text{‰}$ (either assuming $b = 29\text{‰}$ or $b = 27\text{‰}$) in eqn (3) (Fig. 8). By contrast,

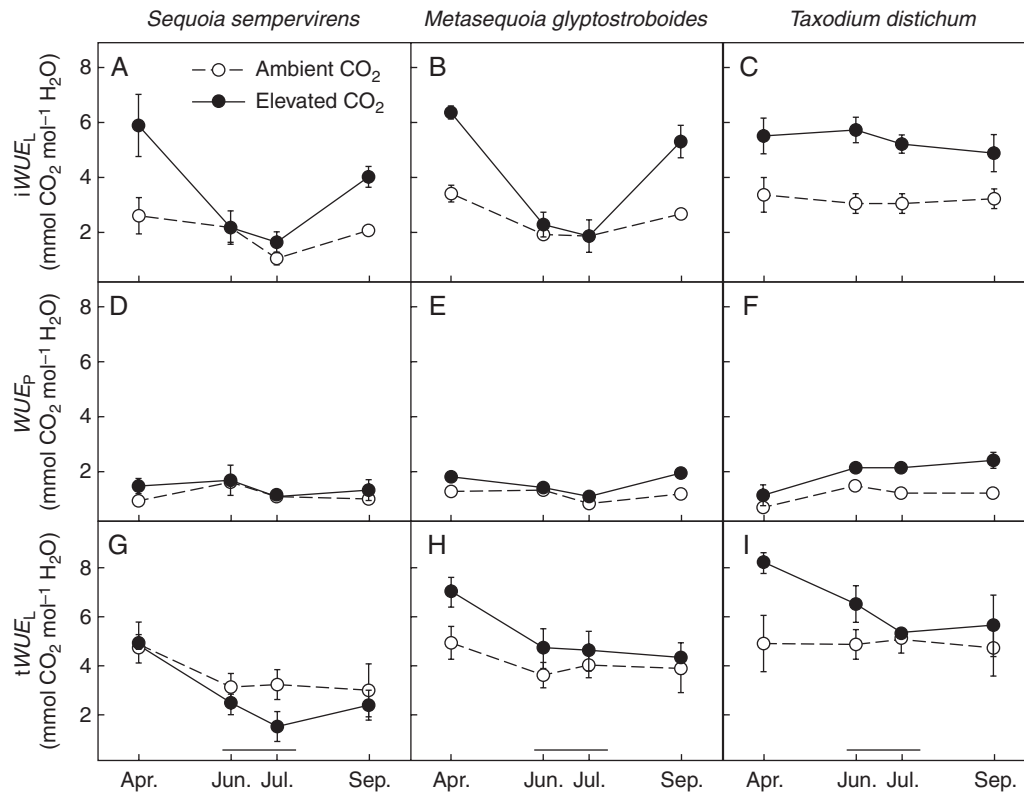


FIG. 3. Seasonal changes in (A–C) instantaneous leaf WUE ($iWUE_L$), (D–F) whole-plant WUE (WUE_P), and (G–I) time-integrated leaf WUE ($tWUE_L$) for *S. sempervirens*, *M. glyptostroboides* and *T. distichum* growing at ambient or elevated [CO_2] in a simulated Cretaceous warm-Arctic environment. $tWUE_L$ values were calculated using $b = 29\%$ in eqn (4). Values are means \pm s.e.m. for three or four replicated growth rooms. The line above the months depicts the period of 24 h continuous light.

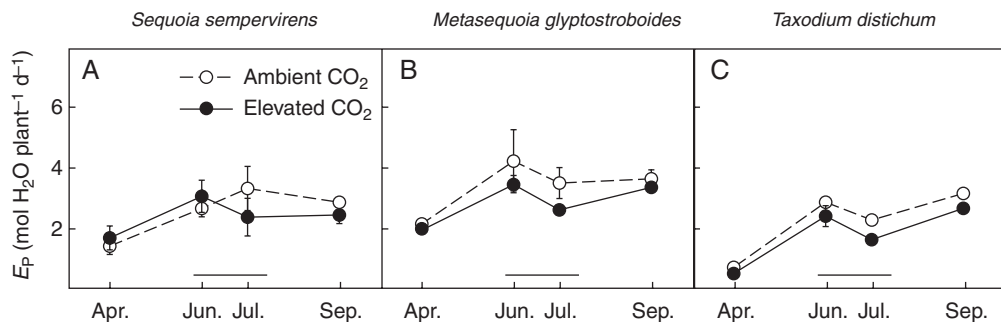


FIG. 4. Seasonal changes in daily whole-plant transpiration (E_P) for (A) *S. sempervirens*, (B) *M. glyptostroboides* and (C) *T. distichum* growing at ambient or elevated [CO_2] in a simulated Cretaceous warm-Arctic environment. Values plotted are means \pm s.e.m. for three replicated growth rooms. The line above the months depicts the period of 24 h continuous light.

$d = 4\%$ (assuming $b = 29\%$) 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 $iWUE_L$ 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 $iWUE_L$ 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 atmospheric [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

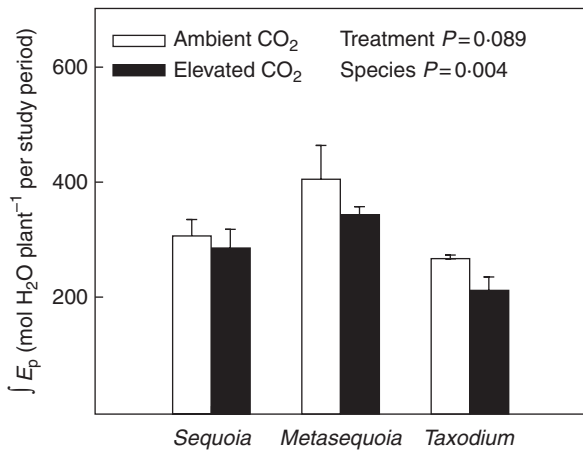


FIG. 5. Whole-plant transpiration integrated over the study period using measurements of E_P (Fig. 4) for *S. sempervirens*, *M. glyptostroboides* and *T. distichum* at ambient or elevated [CO₂] in a simulated Cretaceous warm-Arctic environment. Values plotted are means \pm s.e.m. ($n = 3$).

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

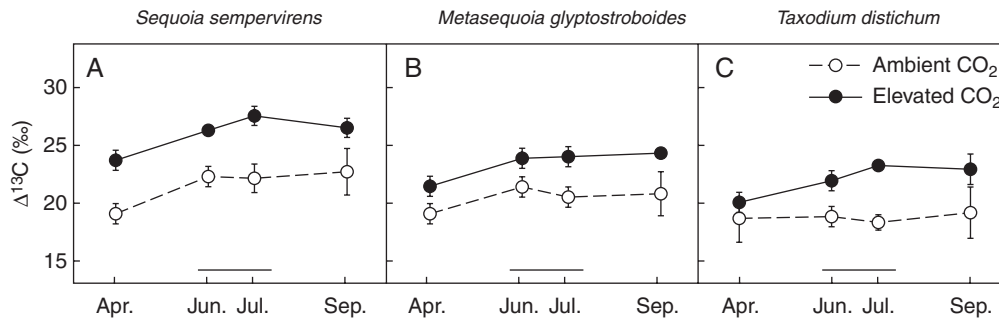


FIG. 6. Seasonal changes in leaf carbon isotope discrimination ($\Delta^{13}C$) calculated from $\delta^{13}C$ values for (A) *S. sempervirens*, (B) *M. glyptostroboides* and (C) *T. distichum* growing at ambient or elevated [CO₂] in a simulated Cretaceous warm-Arctic environment. Values are means \pm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

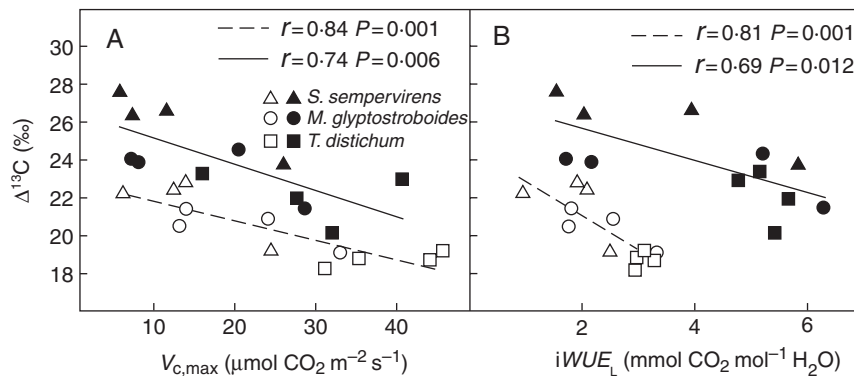


FIG. 7. Leaf carbon isotope discrimination ($\Delta^{13}C$) calculated from $\delta^{13}C$ values plotted against (A) Rubisco carboxylation capacity ($V_{c,max}$) and (B) instantaneous leaf WUE ($iWUE_L$) for *S. sempervirens* (triangles), *M. glyptostroboides* (circles) and *T. distichum* (squares) 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.

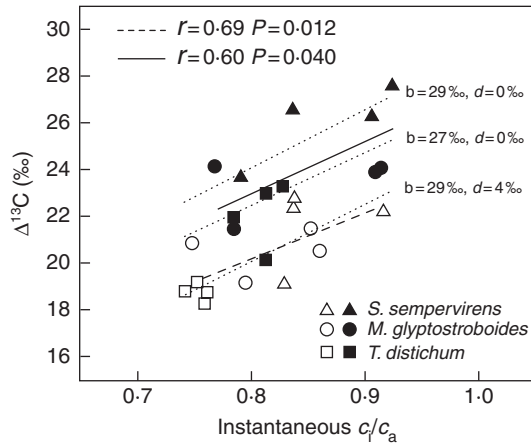


FIG. 8. Leaf carbon isotope discrimination, ($\Delta^{13}\text{C}$, from $\delta^{13}\text{C}$ values) vs. instantaneous c_i/c_a ratio (from leaf gas exchange measurements) for *S. sempervirens* (triangles), *M. glyptostroboides* (circles) and *T. distichum* (squares) at ambient (open symbols) and elevated (closed symbols) $[\text{CO}_2]$. Each symbol represents the mean for each species and sampling date. The dashed and continuous lines are linear regressions fitted to ambient and elevated $[\text{CO}_2]$ data points, respectively. Dotted lines depict predicted $\Delta^{13}\text{C}$ values estimated from instantaneous c_i/c_a measurements using either, $d=0\%$ and $b=29\%$ or $b=27\%$, or $d=4\%$ and $b=29\%$ in eqn (3) (assuming $a=4.4\%$).

relatively insensitive to changes in g_s (Eamus, 1991, Wullschlegel *et al.*, 2002). Therefore hypothesis (2) is rejected as an important component of the physiological ecology of conifer species growing under CO_2 enrichment in a warm, high-latitude light environment.

Nevertheless, the sustained improvement of WUE_P over the growing season in response to a CO_2 -enriched atmosphere might have benefited the growth of coniferous species inhabiting ancient high-latitude environments, even during the continuous light period. WUE_P is likely to be an important determinant of maximum tree height, because xylem water potential declines markedly with height, imposing a hydraulic constraint on stomata and therefore a CO_2 -diffusion limitation on photosynthesis (Koch *et al.*, 2004). Stimulation of photosynthesis and WUE via enhanced CO_2 offsets this hydraulic mechanism, offering the potential for greater maximum tree height in ancient CO_2 -rich atmospheres (Osborne and Beerling, 2002). Indeed, it has been reported that the productivity of high-latitude paleoforests was comparable with that of living temperate deciduous forests, with trees reaching diameters of 1 m and heights of up to 40 m (Francis, 1991; Greenwood and Basinger, 1994; Williams *et al.*, 2003). Therefore, although a CO_2 -enriched atmosphere might have brought about no significant improvement in water economy of coniferous species growing in Cretaceous high-latitude 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 $\Delta^{13}\text{C}$ values when grown under high $[\text{CO}_2]$ (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 $[\text{CO}_2]$ 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 $\Delta^{13}\text{C}$ (Farquhar *et al.*, 1989) and, accordingly, a significant negative relationship was found between $\Delta^{13}\text{C}$ and the maximum rate of RuBP carboxylation ($V_{c,\text{max}}$; Fig. 7A). The higher c_i/c_a ratios (Fig. 1D–F) and $\Delta^{13}\text{C}$ values observed in plants growing under elevated $[\text{CO}_2]$ 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 $\Delta^{13}\text{C}$ and $iWUE_L$ (Fig. 7B). However, plants grown at ambient $[\text{CO}_2]$ showed lower $iWUE_L$, but also lower $\Delta^{13}\text{C}$ values when compared with plants grown at elevated $[\text{CO}_2]$. Therefore, the present data support previous studies showing that lower $\Delta^{13}\text{C}$ 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 $\Delta^{13}\text{C}$ and the predictions of eqn (4), with eqn (4) overestimating discrimination under ambient $[\text{CO}_2]$ conditions (von Caemmerer and Evans, 1991; Farquhar and Lloyd, 1993; Gillon and Griffiths, 1997; Lauteri *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 $[\text{CO}_2]$: 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/c_a) and observed $\Delta^{13}\text{C}$ values at ambient [CO₂] than using $d = 0\text{‰}$ (either assuming $b = 29$ or 27‰ ; Fig. 8). An effect of [CO₂] on d would be consistent with the suppression of photorespiration at elevated [CO₂] 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 [CO₂] 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 CO₂ enrichment in this system, transpiration is relatively insensitive to atmospheric CO₂.

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APPENDIX

Abbreviations used

A_P	Daily whole-plant net carbon uptake (mmol CO ₂ plant ⁻¹ d ⁻¹)
A_L	Net leaf photosynthetic rate (μmol CO ₂ m ⁻² s ⁻¹)
E_P	Daily whole-plant transpiration (mol H ₂ O plant ⁻¹ d ⁻¹)
E_L	Leaf transpiration rate (mmol H ₂ O m ⁻² s ⁻¹)
g_{sL}	Leaf stomatal conductance (mmol H ₂ O m ⁻² s ⁻¹)
$iWUE_L$	Instantaneous leaf water-use efficiency (mmol CO ₂ mol ⁻¹ H ₂ O)
PPFD	Photosynthetic photon flux density (μmol m ⁻² s ⁻¹)
$tWUE_L$	Time-integrated leaf water-use efficiency (mmol CO ₂ mol ⁻¹ H ₂ O)
WUE_P	Whole-plant water-use efficiency (mmol CO ₂ mol ⁻¹ H ₂ O)
$\Delta^{13}C$	Discrimination against ¹³ C (‰)
$\delta^{13}C$	Stable carbon isotope composition (‰)
$\delta^{13}C_a$	¹³ C of the atmosphere
$\delta^{13}C_s$	¹³ C of the sample
$V_{c,max}$	Rubisco carboxylation capacity (μmol CO ₂ m ⁻² s ⁻¹)