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The effect of subambient to elevated atmospheric CO_2 concentration on vascular function in *Helianthus annuus*: implications for plant response to climate change

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Summary

- Plant gas exchange is regulated by stomata, which coordinate leaf-level water loss with xylem transport. Stomatal opening responds to internal concentrations of CO_2 in the leaf, but changing CO_2 can also lead to changes in stomatal density that influence transpiration. Given that stomatal conductance increases under subambient concentrations of CO_2 and, conversely, that plants lose less water at elevated concentrations, can downstream effects of atmospheric CO_2 be observed in xylem tissue?
- We approached this problem by evaluating leaf stomatal density, xylem transport, xylem anatomy and resistance to cavitation in *Helianthus annuus* plants grown under three CO_2 regimes ranging from pre-industrial to elevated concentrations.
- Xylem transport, conduit size and stomatal density all increased at 290 ppm relative to ambient and elevated CO_2 concentrations. The shoots of the 290-ppm-grown plants were most vulnerable to cavitation, whereas xylem cavitation resistance did not differ in 390- and 480-ppm-grown plants.
- Our data indicate that, even as an indirect driver of water loss, CO_2 can affect xylem structure and water transport by coupling stomatal and xylem hydraulic functions during plant development. This plastic response has implications for plant water use under variable concentrations of CO_2, as well as the evolution of efficient xylem transport.

Introduction

Photosynthesis and plant water transport are regulated by stomatal function. For carbon fixation to occur, CO_2 must enter the leaf through open stomata but, unavoidably, this comes at the cost of water loss. This water must be replenished through the xylem stream, otherwise the stomata will close and restrict CO_2 uptake. Stomatal behaviour is complex, and responses vary depending upon light intensities (Guyot *et al.*, 2012), transpiration rates (Mott & Parkhurst, 1991), leaf water potential (Damour *et al.*, 2010; Guyot *et al.*, 2012), xylem transport (Sperry, 2000; Hubbard *et al.*, 2001; Brodribb *et al.*, 2007; Brodribb, 2009) and internal CO_2 concentrations in the leaf (c_i; Farquhar & Sharkey, 1982; Sage, 1994; Ainsworth & Rogers, 2007). Of these variables, the CO_2 response is the least understood mechanistically (Long *et al.*, 2004), but there is evidence that photosynthesis rates (Farquhar & Sharkey, 1982; Sage, 1994; Hetherington & Woodward, 2003; Ainsworth & Rogers, 2007) and a CO_2-based gene-mediated signal cascade (Gray *et al.*, 2000; Lake *et al.*, 2001, 2002) affect stomatal conductance and stomatal development, respectively. Herbarium, fossil and experimental data provide evidence for CO_2-mediated variations in leaf stomatal numbers across both short and geological timescales (Woodward, 1987; Franks & Beerling, 2009), but the question remains: could downstream effects of ambient CO_2 impact the structure and function of plant vascular tissue?

Atmospheric CO_2 concentration ($[CO_2]_{atm}$) has varied dramatically since the evolution of the earliest Devonian tracheophytes, when it peaked at 3300 ppm (Willis & McElwain, 2002; Berner, 2006; Taylor *et al.*, 2009), and has progressively dropped over the past 400 million yr to concentrations below 200 ppm during the Pleistocene glacial maxima (Barnola *et al.*, 1987; Zachos *et al.*, 2008). Although $[CO_2]_{atm}$ is expected to reach *c.* 500–1000 ppm by 2100 (http://www.ipcc-data.org/ddc_co2.html), 96% of the past 400 000 yr experienced $[CO_2]_{atm}$ below 280 ppm (Sage & Coleman, 2001; Zachos *et al.*, 2008). It is conceivable, then, that today's flora – especially long-lived plants – may be more adapted to pre-industrial $[CO_2]_{atm}$ rather than to the elevated $[CO_2]_{atm}$ of the Anthropocene, an idea supported in part by: the absence of prolonged stimulation of photosynthesis; photosynthetic down-regulation; and low growth rates in plants growing under continuously elevated $[CO_2]_{atm}$ (Sage & Coleman, 2001; Ellesworth *et al.*, 2004; Long *et al.*, 2004; Bader *et al.*, 2010). Consistent with this, reduced stomatal conductance

(g) and lower transpiration rates are commonly reported in CO_2 enrichment studies (Dugas *et al.*, 2001; Long *et al.*, 2004; Ainsworth & Rogers, 2007). By contrast, the few studies that have examined the gas-exchange response of plants grown under pre-industrial $[CO_2]_{atm}$ indicate that plants compensate for the CO_2 deficit by increasing g and transpiration per unit of leaf area (Fig. 1 and references therein). All else being equal, photosynthetic processes, including rates of RuBisCo carboxylation, electron transport, and internal phosphorus (P_i) regeneration, regulate c_i, which in turn acts as a signal for stomata to open or close. Consequently, the ratio of internal to external CO_2 concentrations (c_i/c_a) is conserved such that leaf intrinsic water use efficiency increases by a similar fraction to $[CO_2]_{atm}$ (Sage & Reid, 1992; Sage, 1994; Polley *et al.*, 2002; Sperry, 2003; Messinger *et al.*, 2006).

Leaf transpiration is controlled in part by the size of the stomatal aperture and by the number of stomata on the leaf surface, that is, stomatal density, (SD; number mm^{-2}), and both traits have been shown to vary in response to changes in $[CO_2]_{atm}$. Adjustments in stomatal conductance are evident in a matter of minutes, while shifts in stomatal size and stomatal density occur during the development of new leaves (Lake *et al.*, 2001, 2002). Interestingly, stomatal density can vary over time-scales ranging from years to millennia, a response most commonly attributed to changing $[CO_2]_{atm}$. For example, in the leaves of a single *Betula pendula* tree, SD decreased by *c.* 20% from 1952 to 1995, a period during which $[CO_2]_{atm}$ rose by *c.* 50 ppm (Wagner *et al.*, 1996). Similarly, Woodward (1987) used herbarium specimens dating from 1750 to 1981 to document an average 40% decrease in SD in several north temperate tree species, presumably in response to a post-industrial rise in $[CO_2]_{atm}$ from *c.* 280 to 340 ppm. Similar trends have been observed in fossil cuticles from the Mesozoic (McElwain *et al.*, 1999), leading to the development of 'deep time' stomatal function models that predict a *c.* 75% reduction in g under a $[CO_2]_{atm}$ shift from 475 to 2500 ppm (Franks & Beerling, 2009). Because leaf transpiration inexorably links stomatal conductance to overall plant water transport, it is not unreasonable to consider the role of $[CO_2]_{atm}$ as a driver in the evolution of plant vascular function.

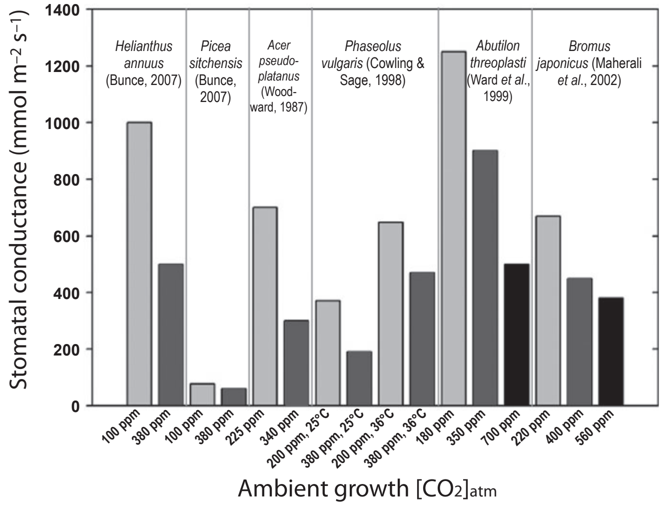

Fig. 1 Previously reported stomatal conductance values in woody and herbaceous plants grown at subambient, ambient and/or elevated CO_2 concentrations. Vapour pressure deficits (VPDs) varied from 0.9 to 1.2 kPa. See also Bunce (2006) for stomatal response data under variation in VPD and atmospheric CO_2 concentration ($[CO_2]_{atm}$).

Leaf evapotranspiration is tightly coupled to the water potential gradient driving water transport from the soil to the leaf, but under hydrated conditions, it is the hydraulic conductance of the xylem tissue that defines the upper limits of g (Sperry *et al.*, 1998; Hubbard *et al.*, 2001; Brodribb, 2009). Indeed, the coordination between leaf-level water loss and the xylem-mediated water supply has been demonstrated across both conifer and angiosperm taxa from tropical to temperate environments (Meinzer & Grantz, 1990; Brodribb & Feild, 2000; Santiago *et al.*, 2004; Brodribb *et al.*, 2007; Pittermann *et al.*, 2012). One might expect similar hydraulic coordination from plants growing under a range of $[CO_2]_{atm}$ regimes, where under equivalent intensities of light, nutrients and hydration, it is $[CO_2]_{atm}$ that drives the hydraulic response through a top-down effect on leaf water loss via stomatal function (Pittermann, 2010). Numerous studies have documented a 10–100% increase in g in plants grown under subambient $[CO_2]_{atm}$ (Fig. 1), so the basic tenets of leaf-xylem hydraulic coordination can lead to some testable predictions. In plants grown under subambient $[CO_2]_{atm}$ ($<$ 390 ppm) where higher SD and consequently higher g lead to a greater requirement for water, the hydraulic demand may be satisfied by: a greater proportion of large-diameter conduits; a higher number of conduits for a given xylem area; or a greater ratio of xylem area to stem cross-sectional area. The opposite may be true for plants grown under elevated $[CO_2]_{atm}$, where lower g lessens the demand for efficient hydraulic transport (Fig. 1).

Here, we tested these ideas by evaluating the xylem structure–function relationships in *Helianthus annuus* plants grown in an outdoor facility where $[CO_2]_{atm}$ is controlled along a continuous gradient ranging from *c.* 250 to 500 ppm (Fay *et al.*, 2009). Anatomical and hydraulic traits including leaf stomatal density, xylem anatomy, xylem transport efficiency, and xylem cavitation resistance were examined in plants grown under 290, 390 and 480 ppm. Growth $[CO_2]_{atm}$ may affect the development of the xylem tissue, and its vulnerability to hydraulic failure caused by the entry of air into vessels through inter-vessel pit membranes (i.e. cavitation; Sperry, 2000). To test this effect, we used the centrifuge method to characterize the cavitation response of the sunflowers grown in each of the three $[CO_2]_{atm}$ treatments. Our results are discussed broadly in the context of plant response to climate change, as well as the developmental implications of $[CO_2]_{atm}$ for the evolution of plant structure and function.

Materials and Methods

Plants and experimental facility

Sunflower (*Helianthus annuus* L.) plants were grown in two structurally identical elongated field chambers designed to expose plants to a $[CO_2]_{atm}$ gradient that spans pre-industrial to elevated concentrations (Fay *et al.*, 2009). Detailed photographs, schematics and other information about the facility can be found in Fay *et al.* (2009) and Johnson *et al.* (2000). Located in central Texas (Temple; 31°05′N, 97°20′W), each chamber is *c.* 1.2 m wide by 1.6 m tall by 50 m long, and transmits > 90% of incident irradiance to the enclosed plants with minimal effects on spectral quality (Polley *et al.*, 2008; Fay *et al.*, 2009). The system relies upon photosynthesis such that the CO_2 concentration of air introduced into chambers (enriched in CO_2 in one chamber, and ambient CO_2 in the other) is progressively depleted during the day by assimilating plants as it is carried towards the system outlet. Desired CO_2 concentrations are maintained by regulating the rate of air flow (*c.* 0.2–0.5 $m\ s^{-1}$) through each of the two chambers. Air temperature and vapour pressure deficit are kept near ambient values by cooling and dehumidifying air at 5-m intervals along the length of each chamber. Plants are housed in ground-level compartments that span the length of the chambers.

Individuals of *H. annuus* var. 'Lemon Queen' were grown from seed in 0.5 m × 1 m spaces in chamber compartments with daytime $[CO_2]_{atm}$ at 293 ± 15.9 (mean ± SD), 392.6 ± 9.1 and 479.7 ± 7.7 ppm $[CO_2]_{atm}$ (pooled data for 2010 and 2011; Supporting Information Fig. S1). Nighttime $[CO_2]_{atm}$ values were 414 ± 23.4, 474.8 ± 9.2 and 605 ± 35.2 ppm, respectively. Seeds were planted in 15-cm-diameter pots that were 45 cm deep, containing a silty clay Mollisol from the Austin soil series. Plants were watered daily to saturation by a drip-line irrigation system for the 7–11-wk duration of experiments. Sunflower was selected because the species exhibits a strong main axis that can tolerate the mechanical stress imposed by centrifugation experiments needed to determine xylem cavitation resistance (Rosenthal *et al.*, 2010). The 'Lemon Queen' variety of *H. annuus* is tolerant of the high temperatures characteristic of summers in central Texas. The chamber designated for the ambient to subambient CO_2 gradient housed sunflowers grown in the 290-ppm treatment, while the second chamber (ambient to elevated CO_2 gradient) housed plants grown at 390 and 480 ppm. Six individuals per $[CO_2]_{atm}$ treatment could be accommodated in chambers at any one time, so two batches of plants were grown in May–August 2010, and a third group was grown in June–July 2011. Data for healthy plants from all three trials were pooled, and additional sampling details are provided in the figure legends. Gas-exchange measurements were attempted but technical setbacks prevented us from collecting reliable data.

Biomass measurements

Plants were harvested at maturity. We measured total leaf area, and leaf, stem and root dry weights (Fig. S2). Select, fully expanded leaves were tagged and preserved in a plant press for stomatal size and density measurements.

Stomatal density measurements

Stomatal density was measured on a leaf area basis according to standard protocols described by Woodward (1987), whereby 1 cm by 1 cm samples were removed from the central portion of fully expanded leaves (one per plant), and cleared in a solution of 1 M KOH for 1–2 wk as per Brodribb *et al.* (2007). The samples were subsequently rinsed, stained lightly with toluidine blue, rinsed again and mounted on a glass slide. The abaxial side of the samples was photographed at 100× using a digital microscope (BA210; Motic, Richmond, BC, Canada) and the stomatal number counted over an average total leaf area of 1–2 mm^2 using IMAGEJ (Rasband, 1997–2012). The majority of guard cells in our samples were closed, so stomatal length was measured from one end of the aperture to the other.

In place of gas-exchange-derived measurements of stomatal conductance, the maximum stomatal conductance to water vapour (g_{wmax}) was calculated on the basis of stomatal size and density per the approach of Franks & Beerling (2009):

$$g_{wmax} = (d/v) \cdot D \cdot a_{max} / (l + (\pi/2) \cdot \sqrt{(a_{max}/\pi)}) \qquad \text{Eqn 1}$$

(*d*, the diffusion coefficient of water vapour in air ($2.42 \times 10^{-5}\ m^2\ s^{-1}$ at 20°C; Nobel, 1999); *v*, the volume of one mole of air ($0.0224\ m^3\ mol^{-1}$); *D*, stomatal density (m^{-2}); a_{max}, the maximum area of the open stomatal pore (m^2); *l*, the depth of the stomatal chamber (m; equivalent to the width of a single guard cell).) The a_{max} was estimated as $\pi\ (p/2)^2$, where *p* is the length of the stomatal pore. Modelling g_{wmax} is reasonable as Franks *et al.* (2009) found strong agreement between g_{wmax} and time-integrated stomatal conductance in *Eucalyptus* sp., while reductions in stomatal density were consistent with a drop in stomatal conductance in conifers (Haworth *et al.*, 2011; but see also Tricker *et al.*, 2005) .

Hydraulic measurements

The main axes of all vigorous sunflowers were subject to hydraulic measurements. Internode segments ranging from 7 to 12 mm in diameter and up to 20 cm in length were sampled from the main axis of the plant at 10–15 cm above the soil, wrapped immediately in wet paper towels, bundled in plastic sacks and refrigerated until they were shipped overnight in a chilled styrofoam cooler to U.C. Santa Cruz later that same day. The segments remained at 4–7°C until the hydraulic analysis was completed.

Upon arrival, sunflower segments were immersed in distilled and filtered water (0.22 μm; E-Pure filtration system; Barnstead International, Dubuque, IA, USA) and degassed under vacuum overnight to remove any air (embolisms) trapped in the xylem. These segments were subsequently re-cut to a length of 14.2 cm, and the distal ends were shaved smooth with a razor blade.

Hydraulic conductivity (*K*) was measured according to Sperry (1993) and computed as the flow rate per unit of pressure, adjusted for segment length. The segments were mounted on a tubing apparatus where flow was measured gravimetrically using 20 mM KCl solution. The flow rate was determined without a pressure head before and following each gravimetric flow measurement to obtain a 'background' flow. Background flows were then averaged and subtracted from the gravimetric flow.

Xylem- and leaf-area-specific conductivity (K_s and K_{leaf}, respectively) was computed by dividing K by the total measured xylem or leaf area.

The centrifuge method was used to determine the xylem cavitation resistance in response to progressively more negative xylem pressures (P_x; Alder *et al.*, 1997; Rosenthal *et al.*, 2010). Segments were secured in a custom rotor and spun for 3 min at speeds that induce a known P_x. The percentage loss of conductivity (PLC) caused by cavitation was calculated from the K following each spin, and relativized to the maximum conductivity (K_{max}) at $P_x = 0$ MPa following degassing treatment, such that

$$\mathrm{PLC} = 100 \times (1 - K/K_{max}) \qquad \text{Eqn 2}$$

The segments were spun to progressively greater P_x until PLC exceeded 90% or until we reached P_x of −5 to −7 MPa. Stems showed a tendency to disintegrate during the centrifugal spin at P_x below −5 MPa, particularly segments collected from 290-ppm-grown plants. We minimized breakage by slowly increasing the rotor speed during centrifugation, and by spinning to P_x no lower than −5 to −7 MPa. One response curve was obtained from each stem, and the P_x at which a segment exhibited a 50% loss of conductivity (P_{50}) was determined by applying the curve-fitting routine of Pammenter & Vander Willigen (1998).

Anatomical measurements

The functional xylem area was measured on hand-cut, transverse sections made from the centre of stem segments (stems had little to no taper) that were stained in phloroglucinol to highlight the xylem tissue. We observed mostly primary xylem, although some secondary xylem fibres were evident in plants grown under 480 ppm. Digital photographs were obtained at 20× and the xylem area measured using IMAGEJ software. Phloem was easily visible above the xylem so phloem area was measured in the same manner. The lumen areas of all the vessels present in all of the primary xylem bundles in three radial stem sectors were measured using digital photographs taken at 200× from centrally located transverse sections. These lumen areas were converted to equivalent circle diameters and the hydraulic mean diameter (D_h) was computed from vessel diameter distributions whereby $D_h = \sum D^5 / \sum D^4$ (Kolb & Sperry, 1999).

Previous work has shown a strong relationship between species' cavitation resistance (P_{50}) and the vessel double-wall-to-lumen-span ratios $(t/D_h)^2$ in woody plants, suggesting that this ratio indicates an appropriate degree of reinforcement against conduit implosion when the water column is under tension as a result of negative P_x. The $(t/D_h)^2$ ratio was measured according to Hacke *et al.* (2001), whereby wall thickness (t) was obtained from the double wall between two adjacent conduits, at least one of which was within 10% of D_h.

Statistical analysis

Statistical analyses were performed in the R environment (R Core Team, 2012). Standard tests for one-way analysis of variance (ANOVA) were followed by Fisher's least significant difference test to identify significant differences between treatments (Zar, 1999).

Results

A statistically significant 52% increase in stomatal density was observed in the leaves of *H. annuus* growing at subambient

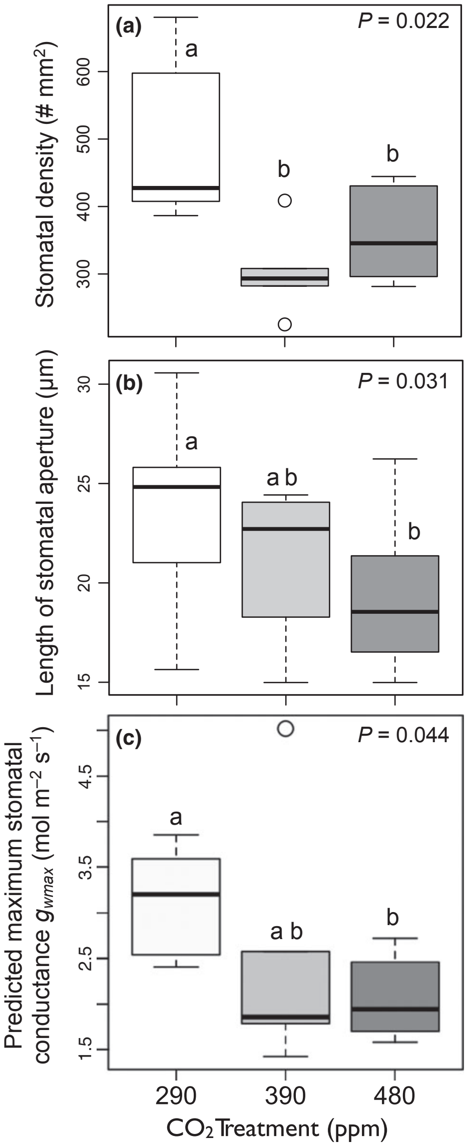

Fig. 2 Boxplots of stomatal density (a), the length of the stomatal aperture (b) and predicted maximum stomatal conductance (c) in *Helianthus annuus* plants grown under three atmospheric CO_2 concentration ($[CO_2]_{atm}$) treatments. The dark bar indicates the median, the top and bottom of the box indicate the upper and lower quartiles, respectively, and the whiskers indicate the minimum and maximum data values. Single data points indicate outliers. The mean stomatal density values ± SE for the three treatments are as follows: 290 ppm, 500.7 ± 45.7, $n = 7$; 390 ppm, 301.8 ± 24.4, $n = 6$; 480 ppm, 357.3 ± 28, $n = 6$. The mean lengths of the stomatal apertures ± SE are as follows: 290 ppm, 23.7 ± 1.57, $n = 8$; 390 ppm, 21.2 ± 1.27, $n = 8$; 480, ppm, 19.26 ± 1.3, $n = 8$.

$[CO_2]_{atm}$ relative to plants from 390 and 480 ppm, but elevated $[CO_2]_{atm}$ imposed no further effect on this trait relative to plants grown at ambient $[CO_2]_{atm}$ (Fig. 2a). Similar trends were observed in the size of the stomatal pore, whereby plants grown at 290 ppm had a tendency to exhibit apertures that were on average 2.5 and 4.3 μm longer than in plants from the 360- and 480-ppm treatments, respectively (Fig. 2b). Lastly, the predicted values of g_{wmax} were highest at 290 ppm, averaging 3.12 ± 0.63 mol m^{-2} s^{-1} (mean ± SD) relative to 2.42 ± 1.33 and 2.06 ± 0.43 mol m^{-2} s^{-1} at 390 and 480 ppm, respectively (Fig. 2c). These data fall within the range of modelled g_{wmax} values predicted by Beerling and Franks (2009).

The xylem-specific hydraulic conductivity was 35% higher in *H. annuus* grown at subambient $[CO_2]_{atm}$ than in plants grown at ambient and elevated $[CO_2]_{atm}$ (Fig. 3a). Again, no depression in K_s was observed in plants grown at 480 relative to 390 ppm, indicating a saturating response at ambient $[CO_2]_{atm}$ in this species. Consequently, we observed no correlation between K_s and $[CO_2]_{atm}$ in *H. annuus* as a result of this CO_2 'ceiling' effect. Surprisingly, we observed no significant effect of $[CO_2]_{atm}$ on leaf-specific conductivity, although there was a slight tendency for K_{leaf} to increase with higher $[CO_2]_{atm}$ (Fig. 3b). This is because the total leaf area was highest in 290-ppm-grown plants (Fig. 3c). *Helianthus annuus* preferentially allocated resources from the main stem to the leaves at 290 ppm such that plants grown at the lowest $[CO_2]_{atm}$ had the highest total leaf biomass (Fig. S2F). The greater leaf area presumably served to improve carbon gain under reduced $[CO_2]_{atm}$ availability. Interestingly, this also means that the ratio of total phloem area (per stem cross-section) to leaf area was lowest in the 290-ppm-grown plants, Fig. 3d). It is important to note, however, that, consistent with expectations, the overall shoot biomass was lowest in the 290-ppm-grown plants on account of much reduced carbon allocation to the main stem (Fig. S2C,F).

Helianthus annuus plants grown at 480 ppm exhibited the highest cavitation resistance, with an average P_{50} of −4.27 MPa, whereas plants grown at 290 ppm were the most vulnerable to air-seeding (Fig. 4). These data are consistent with anatomical measurements indicating that conduit implosion resistance, that is, the conduit double wall thickness to diameter ratio $(t/D_h)^2$ per Hacke *et al.* (2001), is significantly lower in 290-ppm-grown plants than those subject to the 390- and 480-ppm treatments (Fig. 5b). Two traits explain this trend. First, the 290-ppm-grown plants allocated fewer carbon resources to conduit reinforcement than their 390- and 480-ppm counterparts, as evidenced by their thinner walls (Fig. 5c). Secondly, the 290-ppm-grown plants exhibited larger vessels (Figs 5a, S4).

Consistent with the hypothesis that $[CO_2]_{atm}$-driven demand for water will be supplied by increased xylem capacity, our data show that plants grown at 290 ppm had an average vessel D_h of 53.7 ± 7.97 μm (± SD), whereas those grown at ambient and elevated $[CO_2]_{atm}$ exhibited vessel D_h of 47.8 ± 6.94 and 44.5 ± 2.13 μm, respectively (Fig. 5a). These anatomical data are in line with the increased xylem-specific conductivity exhibited by the plants grown at subambient $[CO_2]_{atm}$ (Fig. 3a). Similar anatomical trends were observed in pilot experiments with two herbs, *Solidago canadensis* L. and *Desmanthus illinoensis* (Michx.) MaMill. ex B.L. Rob. & Fernald (Fig. S3), where average vessel diameters in both species were largest at subambient $[CO_2]_{atm}$. In *H. annuus*, the ratio of xylem area to cross-sectional stem area

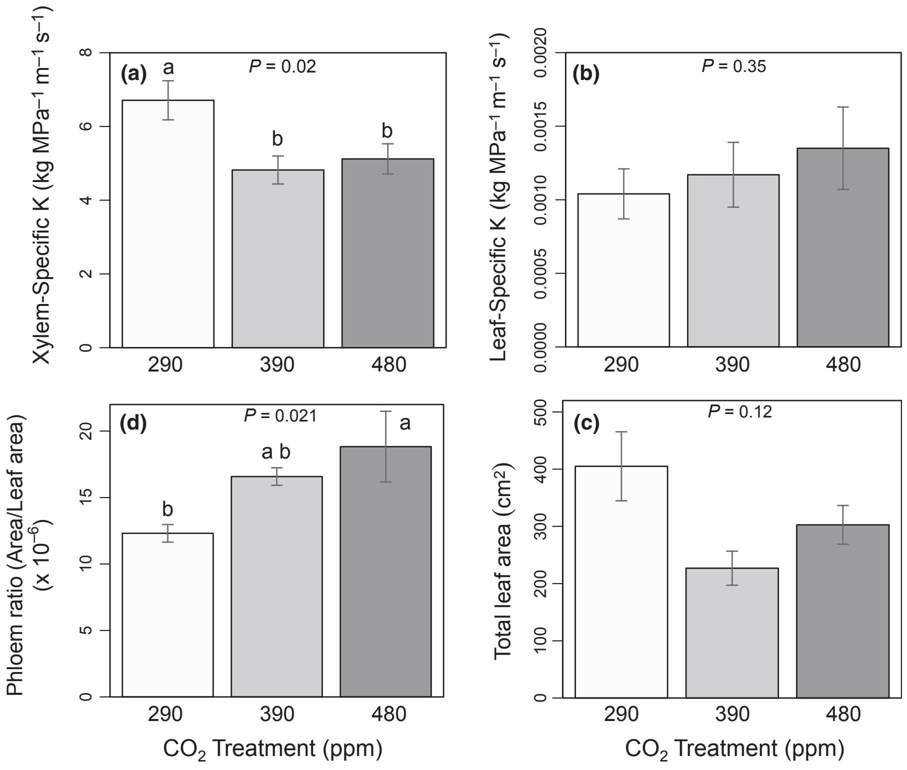

Fig. 3 The vascular response of xylem and phloem in *Helianthus annuus* plants with respect to total leaf area presented as mean ± SE. Xylem-specific conductivity of plants subjected to the three atmospheric CO_2 concentration ($[CO_2]_{atm}$) treatments is presented in (a) (290 ppm, *n* = 14; 390 ppm, *n* = 16; 480 ppm, *n* = 12), the leaf-specific conductivity in (b) (290, ppm, *n* = 11; 390 ppm, *n* = 13; 480 ppm, *n* = 9), the total leaf area in (c) (same sample size as b) and the phloem:leaf area ratio in (d) (290 ppm, *n* = 4; 390 ppm, *n* = 5; 480 ppm, *n* = 5).

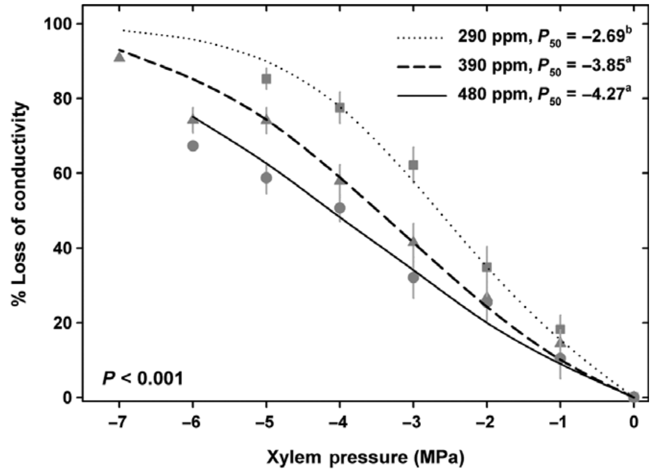

Fig. 4 The cavitation response curves for *Helianthus annuus* stems grown under three atmospheric CO_2 concentration ($[CO_2]_{atm}$) regimes, where the data at each xylem pressure are presented as mean ± SE (290 ppm, $n = 15$; 390 ppm, $n = 12$; 480 ppm, $n = 8$).

was statistically equivalent across the three treatments (Fig. S2H), whereas conduit density (vessels/xylem area) was lowest in plants grown at 290 ppm, a trend consistent with the larger conduits observed in this treatment (Figs S2I, S4).

Discussion

Plant water relations under elevated $[CO_2]_{atm}$ have received much attention, with many studies showing a 30–50% reduction in transpiration and/or stomatal conductance rates in both herbaceous C3 and woody plants (Jackson *et al.*, 1994; Field *et al.*, 1995; Dugas *et al.*, 2001; Maherali *et al.*, 2002; Long *et al.*, 2004; Leuzinger & Korner, 2007). By contrast, relatively few studies have examined the water relations and/or the gas-exchange response of plants grown across $[CO_2]_{atm}$ gradients that include subambient $[CO_2]_{atm}$ (Bunce & Ziska, 1998; Cowling & Sage, 1998; Maherali *et al.*, 2002; Polley *et al.*, 2002; Bunce, 2006, 2007; Gerhart & Ward, 2010; Phillips *et al.*, 2011). However, evaluating plant responses along a continuum of $[CO_2]_{atm}$ can reveal the degree to which plasticity can accommodate the predicted future rise in $[CO_2]_{atm}$, broaden the interpretative power of fossil wood and growth rings, inform predictions of plant biogeography under elevated $[CO_2]_{atm}$ and add further insight to studies of xylem evolution (Sperry, 2003; Pittermann, 2010; Fonti & Jansen, 2012; Voelker *et al.*, 2012). Hence, the goal of this study was to contribute an organismal perspective on the hydraulic effects of $[CO_2]_{atm}$, with the hypothesis that $[CO_2]_{atm}$ forcing will shift xylem function in a manner consistent with changes in leaf-level demand for water.

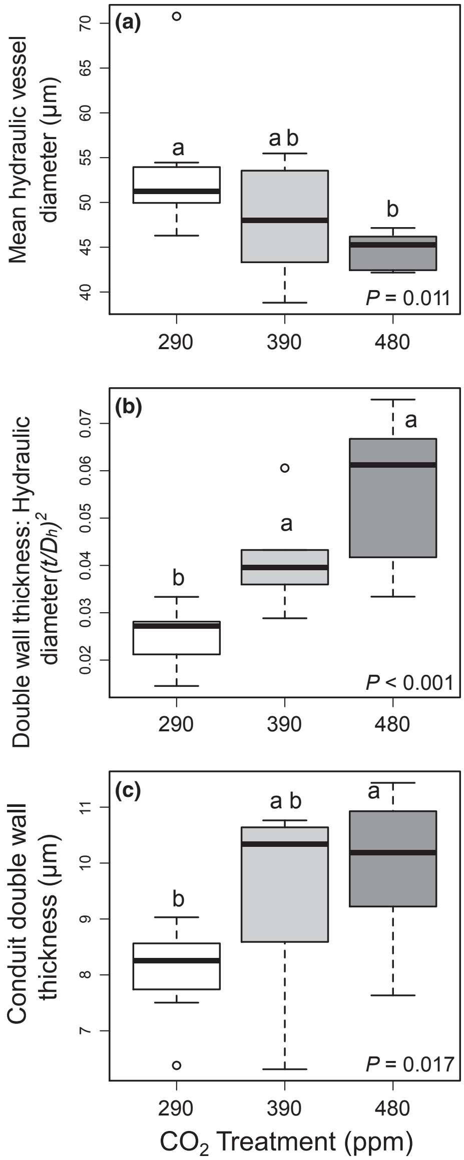

Fig. 5 Boxplots showing the variation in vessel anatomy in response to growth under variable atmospheric CO_2 concentration ($[CO_2]_{atm}$) in *Helianthus annuus.* The mean hydraulic vessel diameters are presented in (a), the vessel double-wall-to-lumen-span ratio $(t/D_h)^2$ in (b), and the thickness of the conduit double wall in (c). The mean hydraulic conduit diameter values ± SE for each of the three treatments are as follows: 290 ppm, 53.7 ± 7.97; 390 ppm, 47.8 ± 6.9; 480 ppm, 44.54 ± 2.12. The mean $(t/D_h)^2$ ratios ± SE are as follows: 290 ppm, 0.0248 ± 0.0067; 390 ppm, 0.0416 ± 0.0118; 480 ppm, 0.0552 ± 0.0161. The mean wall thickness values ± SE are: 290 ppm, 8.04 ± 0.89; 390 ppm, 9.32 ± 1.9; 480 ppm, 9.94 ± 1.336. Samples sizes for each of the treatments are $n = 7$, 5 and 7, respectively.

Our results indicate that subambient $[CO_2]_{atm}$ invokes a greater demand for water in *H. annuus* plants, as evidenced by greater leaf area, a tendency towards larger stomata and higher stomatal density. This demand is supplied by higher xylem-specific conductivity, the result of a modest increase in vessel diameter, and a decrease in conduit mechanical reinforcement. A potentially significant cost of anatomical adjustments at subambient $[CO_2]_{atm}$ is greater vulnerability to cavitation relative to plants grown at ambient and elevated $[CO_2]_{atm}$. Our findings imply that plants inhabiting low $[CO_2]_{atm}$ climates were capable of transporting more water, though perhaps at the cost of increased sensitivity to drought. By contrast, elevated $[CO_2]_{atm}$ is likely to reduce demand for water as well as fortify xylem conduits such that plants may be more resilient to drought stress.

The hydraulic coordination of leaf gas exchange and xylem conductivity and anatomy has been well documented in angiosperms and gymnosperms (Meinzer & Grantz, 1990; Brodribb & Feild, 2000; Santiago *et al.*, 2004; Brodribb *et al.*, 2007; Pittermann *et al.*, 2012), but our study is the first to show that CO_2 forcing can elicit a similar, plastic response that synchronizes hydraulic supply with demand. In *H. annuus,* conduit diameter varied concurrently with both hydraulic capacity and leaf-level demand for water, whereby plants grown at 290 ppm exhibited a developmental trend towards larger diameter vessels (Fig. 5) and a tendency to develop conduits in excess of 100 µm (Fig. S4). It appears that increasing water transport capacity with wider conduits is preferable to investing resources into additional xylem and/or increasing conduit density (Fig. S2H,I). Indeed, varying conduit size may be the most efficient way of maximizing hydraulic efficiency because as Fig. 6 shows, all else being equal, even minor adjustments in conduit diameter can result in improved leaf hydration, as evidenced by a four-fold increase in leaf transpiration rates. Conduit hydraulic conductance is largely a factor of conduit diameter raised to the fourth power, so, developmentally, a slight increase in diameter will result in a much quicker hydraulic pay-off than the construction of additional conduits or more xylem (Tyree *et al.*, 1994).

Despite shifts in conduit diameter and xylem-specific conductivity, we found no clear effect of $[CO_2]_{atm}$ on K_{leaf} (Fig. 3), a trait that directly reflects leaf-level demand for water. We expected higher K_{leaf} in plants grown at 290 ppm than at 390 and 480 ppm but, instead, we observed a slight shift in the opposite direction, whereby greater resource allocation to leaves reduced K_{leaf} in 290-ppm-grown *H. annuus* plants. Although the subambient $[CO_2]_{atm}$ plants had greater leaf area, their leaves were thinner and, when combined with the stem tissue, their overall shoot biomass was lower (Supporting information Fig. S2C,F; see also Dippery *et al.*, 1995). It may be that the hydraulic response varies with varied resource allocation strategies across different species. For example, Phillips *et al.* (2011) showed that K_{leaf} increased in *Eucalyptus sideroxylon* seedlings grown at elevated CO_2, whereas no treatment response was observed in seedlings of *Eucalyptus saligna*. Unfortunately, it was not possible to tease out changes in leaf area and stomatal density because they co-varied at 290 ppm. Whether or not K_{leaf} responds to changes in $[CO_2]_{atm}$ may depend in part on species-specific variation in xylem function, stomatal response and leaf area, so evaluating the $[CO_2]_{atm}$ response across a broad sampling of woody and herbaceous species is needed.

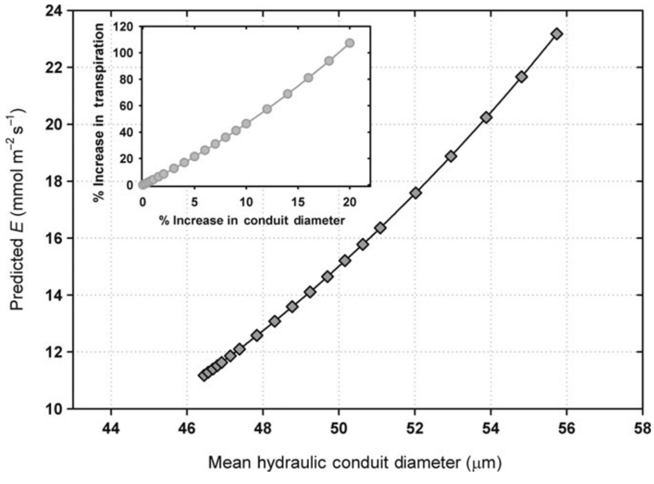

Fig. 6 The predicted transpiration rates (E) as a function of increasing mean hydraulic conduit diameter. The inset plot indicates the relative per cent gain in both conduit diameter and the predicted transpiration rates. Assuming no changes in leaf area or stomatal conductance, very minor shifts in hydraulic capacity can have profound effects on leaf water loss. E was calculated using D'Arcy's law, $E = K_L \times d\Psi/dx$, where K_L represents leaf-specific conductivity and $d\Psi/dx$ represents a gradient of 1 MPa/ 1 m (Sperry *et al.*, 1998). K_L was modelled by applying the Hagen–Poiseuille equation to a population of vessels from a *Helianthus annuus* plant grown at 390 ppm and dividing by total leaf area. This value was then multiplied by 0.56 to factor in the effect of pit membrane resistance (Sperry *et al.*, 2006). Predicted E was computed by incrementally increasing the diameters of all the vessels in the sample population.

A secondary goal of this study was to examine the cavitation response of sunflower, as cavitation resistance is related to interconduit pit membrane traits and conduit biomechanics (Hacke *et al.*, 2001; Choat *et al.*, 2008; Lens *et al.*, 2011). To our knowledge, this is the first study to examine the cavitation resistance of xylem in plants grown at a range of $[CO_2]_{atm}$ including subambient $[CO_2]_{atm}$. Predictably, plants grown at 290 ppm exhibited the highest vulnerability to cavitation. This is probably because insufficient carbon availability weakened the conduit pit membranes and/or increased their porosity, making the entry of air more likely (Choat *et al.*, 2008; Lens *et al.*, 2011). Larger conduits are also more likely to have higher pit area fractions, thereby increasing the probability of large membrane pores (Wheeler *et al.*, 2005; Christman *et al.*, 2009). By contrast, greater carbon availability and reduced demand for water explain the thicker conduit walls, narrower conduit diameters, and greater likelihood of the presence of robust pit membranes in sunflowers grown at 390 and 480 ppm.

The maximum rate of plant water transport is constrained by the hydraulic capacity of the xylem, but it is the dynamic behaviour of the stomata that regulates plant water loss in response to changing conditions. This regulation is achieved by varying stomatal size, stomatal density and stomatal conductance, all of which can shift in a manner that reflects species-specific developmental patterns, water availability, light intensities and atmospheric $[CO_2]_{atm}$ (Körner *et al.*, 1986; Poole *et al.*, 1996; Maherali *et al.*, 2002; Franks & Beerling, 2009). Indeed, stomatal density has been found to increase, decrease or remain unchanged across a range of $[CO_2]_{atm}$ (Körner *et al.*, 1986; Maherali *et al.*, 2002; Reid *et al.*, 2003; Tricker *et al.*, 2005), possibly for reasons related to species evolutionary history, leaf longevity, and variable respiratory costs associated with stomatal function as well as intrinsic behaviour, that is, whether the stomatal response is passive or responsive to abscissic acid signalling (Brodribb & McAdam, 2011; Haworth *et al.*, 2011, 2013). Nocturnal $[CO_2]_{atm}$ values in our chambers were 20–30% higher than daytime values, possibly contributing to some of the variation observed in stomatal traits, while other factors such as seed size, seed quality, and possible fluctuations in growth conditions within the chambers may also have affected our results. Given these caveats, our findings are consistent with theory, as well as many studies that report decreasing stomatal density with increasing $[CO_2]_{atm}$ (Woodward, 1987; Wagner *et al.*, 1996; Franks & Beerling, 2009; Franks *et al.*, 2009; Franks *et al.* 2012; Haworth *et al.*, 2011; Lammertsma *et al.*, 2011).

Modelling exercises show that, if changes in stomatal density are not accompanied by appropriate modifications in leaf structure, the leaf may experience a drop in carbon gain that increases the costs associated with higher rates of leaf water loss (Pachepsky & Acock, 1996). Hence it can be argued that shifts in SD will only occur in species developmentally predisposed to capitalize on the increase in carbon gain. In our experiment, where $[CO_2]_{atm}$ was the only experimental variable and all plants experienced equal light intensities, temperatures and vapour pressure deficits, we observed a rise in stomatal size, stomatal density and the predicted maximum rate of conductance to water vapour in plants grown at 290 ppm (Fig. 2). However, given that low $[CO_2]_{atm}$ has been shown to override stomatal closure even under water stress (Bunce, 2007), it is possible that carbon starvation combined with the associated higher rates of water loss and drop in P_x may predispose these plants to hydraulic failure by cavitation, even if no changes occur in pit membrane porosity or the implosion resistance of the xylem conduits.

In *H. annuus*, changes in hydraulic capacity, stomatal traits and leaf area co-occurred in response to variable $[CO_2]_{atm}$, particularly in the 290-ppm-grown plants, so our data provide initial evidence for a potential developmental link between stomata, leaf development and xylem. Our results imply that CO_2-mediated water loss through leaves (or cotyledons) can affect the transport efficiency of the xylem. The mechanism by which this occurs remains unclear, but evidence for a CO_2-sensing gene (*HIC*, high carbon dioxide; Gray *et al.*, 2000) combined with a systemic CO_2 signal that moves from older leaves to meristems (Lake *et al.*, 2002) suggests that stomatal development in younger leaves is guided by a number of mobile cues. We can speculate that some combination of stimuli arising from transpiration rates (Mott & Parkhurst, 1991), cell expansion (Cosgrove, 1999) or auxin gradients (Sieburth & Deyholos, 2006) acts on developing xylem tissue such that transport capacity scales allometrically with foliar demand.

It is highly probable that genotypes of today's plants have diverged from their pre-industrial relatives, but studies such as ours can shed light on plant function in climates in which $[CO_2]_{atm}$ differed significantly from today. $[CO_2]_{atm}$ varied dramatically over the Phanerozoic, dropping precipitously by the early- to mid-Devonian as a result in part of CO_2 sequestration by the colonization of land plants (Berner, 2005, 2006). Hence, phenotypic plasticity in response to $[CO_2]_{atm}$ may have been an adaptive trait that contributed in part to the evolution of xylem tissue, specifically selection for the large conduits evident in late-Devonian tracheophytes, and possibly vessels in extinct and extant plant lineages including the Selaginellales, Sphenopsids, Pteridophytes, Gnetales and Angiosperms (Niklas, 1985; Li *et al.*, 1996; Sperry, 2003). Low values of $[CO_2]_{atm}$ during the Pleistocene are thought to have reduced plant productivity and thus limited widespread agriculture before 12 000 yr ago (Sage, 1995), but the increased demand for water under low $[CO_2]_{atm}$ may have also challenged early attempts at successful, large-scale crop cultivation. By contrast, lower rates of transpiration, higher water-use efficiency and increased resistance to cavitation may be evident in herbaceous vegetation under elevated $[CO_2]_{atm}$. Indeed, elevated $[CO_2]_{atm}$ has been shown to increase plant water potential in C3 crops (Bunce & Ziska, 1998; Long *et al.*, 2004), while plants grown at subambient concentrations exhibit increased drought stress (Bunce, 2007). Interestingly, it is postulated that the evolution of angiosperms and the subsequent rise of angiosperm forests occurred during a time of declining atmospheric $[CO_2]_{atm}$ (McElwain *et al.*, 2005). Based on our findings, we speculate that the $[CO_2]_{atm}$-mediated increase in hydraulic demand may have contributed to the evolution of complex reticulate venation networks observed in angiosperms (Brodribb & Feild, 2010; Feild *et al.*, 2011). It follows, then, that selection would favour the evolution of an efficient vascular system in order to maintain effective levels of plant hydration. Such coordination would have been critical during the Cenozoic as $[CO_2]_{atm}$ continually dropped (Zachos *et al.*, 2008), and the present research indicates that larger conduits were the most efficient means for meeting these hydraulic demands. Our data support the findings of the many studies reporting a stomatal response to variable $[CO_2]_{atm}$, and provide evidence for a functional coupling between leaf transpiration rates, stomatal density, hydraulic efficiency and xylem anatomy.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Nocturnal and diurnal ambient CO_2 concentrations under which *H. annuus* plants were grown.

Fig. S2 Biomass and additional anatomy data for *H. annuus* plants grown under the three $[CO_2]_{atm}$ regimes.

Fig. S3 Conduit diameter data for *Solidago canadensis* and *Desmanthus illinoensis* plants grown under three $[CO_2]_{atm}$ regimes.

Fig. S4 Vessel diameter distributions of *H. annuus* plants subjected to the three $[CO_2]_{atm}$ treatments.