



# Plant responses to decadal scale increments in atmospheric CO<sub>2</sub> concentration: comparing two stomatal conductance sampling methods

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## Abstract

**Main conclusion** Our study demonstrated that the species respond non-linearly to increases in CO<sub>2</sub> concentration when exposed to decadal changes in CO<sub>2</sub>, representing the year 1987, 2025, 2051, and 2070, respectively.

**Abstract** There are several lines of evidence suggesting that the vast majority of C3 plants respond to elevated atmospheric CO<sub>2</sub> by decreasing their stomatal conductance ( $g_s$ ). However, in the majority of CO<sub>2</sub> enrichment studies, the response to elevated CO<sub>2</sub> are tested between plants grown under ambient (380–420 ppm) and high (538–680 ppm) CO<sub>2</sub> concentrations and measured usually at single time points in a diurnal cycle. We investigated  $g_s$  responses to simulated decadal increments in CO<sub>2</sub> predicted over the next 4 decades and tested how measurements of  $g_s$  may differ when two alternative sampling methods are employed (infrared gas analyzer [IRGA] vs. leaf porometer). We exposed *Populus tremula*, *Populus tremuloides* and *Sambucus racemosa* to four different CO<sub>2</sub> concentrations over 126 days in experimental growth chambers at 350, 420, 490 and 560 ppm CO<sub>2</sub>; representing the years 1987, 2025, 2051, and 2070, respectively (RCP4.5 scenario). Our study demonstrated that the species respond non-linearly to increases in CO<sub>2</sub> concentration when exposed to decadal changes in CO<sub>2</sub>. Under natural conditions, maximum operational  $g_s$  is often reached in the late morning to early afternoon, with a mid-day depression around noon. However, we showed that the daily maximum  $g_s$  can, in some species, shift later into the day when plants are exposed to only small increases (70 ppm) in CO<sub>2</sub>. A non-linear decreases in  $g_s$  and a shifting diurnal stomatal behavior under elevated CO<sub>2</sub>, could affect the long-term daily water and carbon budget of many plants in the future, and therefore alter soil–plant–atmospheric processes.

**Keywords** Climate change · Water loss · Growth chambers · IRGA · Porometer

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## Introduction

The global land vegetation is a key driver in the hydrological and energy processes on our planet. The transfer of water from the soil, through the plants and into the atmosphere is regulated by stomatal pores on the leaf surface (Brodribb and McAdam 2017) and accounts for up to 80–90% of terrestrial evapotranspiration in some biomes (Jasechko et al. 2013). There are several lines of evidence suggesting that the vast majority of C3 plants respond to elevated CO<sub>2</sub> by decreasing their stomatal conductance ( $g_s$ ) and rates of transpiration and by increasing their assimilation rates ( $A$ ) and overall water use efficiency (WUE) (Ainsworth and Rogers 2007). Under extreme heat and aridity,  $g_s$  can increase in response to elevated CO<sub>2</sub> but a greater magnitude of  $A$  increase still results in improved WUE (Purcell et al. 2018).

Anatomically, elevated CO<sub>2</sub> has been shown to reduce stomatal density (Woodward 1987; Woodward and Kelly 1995; McElwain and Steinthorsdottir 2017) and in some cases alter stomatal pore size (decrease or increase), thereby reducing maximum  $g_s$  to water vapor (Franks and Beerling 2009; Xu et al. 2016; Lammertsma et al. 2011). Stomatal physiological and anatomical responses to elevated CO<sub>2</sub> have been shown to be coordinated (Haworth et al. 2013) and are dependent on the growth environment of the plant (Curtis and Wang 1998) and the plant's underlying degree of plasticity and/or capacity to physiologically acclimate (Stitt and Krapp 1999; Ainsworth and Long 2005). The synergistic/antagonistic effects of other abiotic (e.g., light, vapor pressure deficit [VPD], soil moisture, nutrients etc.) and biotic (e.g., competition, predation etc.) factors can thus substantially alter any predicted direct plant responses to elevated CO<sub>2</sub> (Medlyn et al. 2001; Saxe et al. 1998).

Experiments that try to address the effect of elevated CO<sub>2</sub> on soil–plant–atmosphere water-relations are therefore notoriously difficult to conduct and each study system [e.g., free-air-carbon-enrichment (FACE), greenhouse, laboratory and plant growth chamber] has advantages and disadvantages (Ainsworth et al. 2008; Ainsworth and Long 2005; Porter et al. 2015; Poorter et al. 2016). For example, CO<sub>2</sub> concentrations in FACE systems have been shown to fluctuate substantially (Pepin and Körner 2002) and are usually shut down at night when the air is too still to ensure constant elevated CO<sub>2</sub> treatment, whereas growth chamber environments allow much tighter CO<sub>2</sub> control (Poorter et al. 2016) but poorly represent real field conditions. In most FACE studies, elevated CO<sub>2</sub> conditions are controlled between 538–680 ppm (Purcell et al. 2018), whereas it is only in growth chamber studies where much tighter CO<sub>2</sub> control can be achieved. It has been theoretically demonstrated by Konrad et al. (2008) that small incremental changes in atmospheric CO<sub>2</sub> could result in a non-linear  $g_s$  response to CO<sub>2</sub>, as opposed to a linear response that is currently assumed in Earth System Models. It is therefore important to not just compare plant responses to large, century changes in CO<sub>2</sub> in in situ and ex situ experiments, as currently done in many FACE (Purcell et al. 2018) and chamber experiments, but also to consider investigating  $g_s$  responses to smaller, decadal changes in CO<sub>2</sub> concentration predicted for the next 5–30 years (IPCC 2014).

Measuring the effect of elevated CO<sub>2</sub> on plant physiological traits such as  $g_s$  is of global significance in understanding current, past and future plant responses to a changing climate (Betts et al. 2007; Huntington 2008; McElwain and Steinthorsdottir 2017; Chung et al. 2007; Gornish and Tylianakis 2013). However, collecting data on  $g_s$  across a wide variety of taxonomic groups, biomes and treatments is often hampered by costs and time constraints. Commercially available systems such as hand-held porometers and

portable infrared gas analyzers (IRGA) are amongst the most commonly used devices to measure  $g_s$ . However, the different approaches to gas exchange measurement make them suitable for different purposes. Leaf porometers measure  $g_s$  by placing the conductance of a leaf in series with two known conductance elements, and comparing the humidity measurements between them to estimate water vapor flux. Usually a leaf measurement takes 30 s during which an algorithm can predict the final  $g_s$  reading that would be achieved if unlimited time were allowed for true steady state conditions to occur (Decagon-Devices 2005). In the case of gas analyzers, a reference air mixture is continuously passed through the leaf chamber. Measurements of  $g_s$  are based on differences in H<sub>2</sub>O in the air streams that flow into and out of the leaf cuvette. In other words, the rate of water loss is used to calculate the rate of  $g_s$  (PP-Systems 2007). Since both systems are frequently used in physiological sample protocols in natural, semi-controlled and controlled environments (Long et al. 1996; Lüttge et al. 1986; Yiotis et al. 2017; Bakker 1991; Murray et al. 2019), it is imperative that any deviations in the results between these methods are considered; particularly when interpreted in the light of global change biology (Midgley et al. 1997). Surprisingly, little published data are available comparing porometer and gas analyzer systems (Murray et al. 2019) and, to our knowledge, no published comparison exists that has investigated the difference in derived results under controlled growth chamber environments.

Another fundamental issue that plant biologists face when taking measurements of  $g_s$  is that  $g_s$  fluctuates diurnally. Under natural conditions, maximum operational  $g_s$  is often reached in the late morning to early afternoon, with a mid-day depression around noon (Roessler and Monson 1985; Pathre et al. 1998). Konrad et al. (2008) theoretically demonstrated that maximum daily  $g_s$  can shift by approximately 1 h for every 180 ppm increase in CO<sub>2</sub>. The practical implications of this can be quite significant, particularly in studies that aim to identify maximum operational  $g_s$  responses to different experimental treatments such as elevated CO<sub>2</sub>. Although diurnal measurements have the disadvantage of being more time consuming and are restricted in the sense that they require the use of a gas analyzer, they do provide a better account of whole-physiological diurnal plant responses than porometers.

The aims of this study were to investigate  $g_s$  responses to simulated decadal increments in CO<sub>2</sub> predicted over the next 4 decades (IPCC 2014) and to test how measurements of  $g_s$  may differ when two alternative sampling methods are employed (infrared gas analyzer [IRGA] vs. leaf porometer). We focused on both the differences between the recorded values using the two methods and the time-shifts of the maximum daily  $g_s$  value under fluctuating CO<sub>2</sub>. In addition, to compare and build the relationship between gas

analyzer-collected and porometer-collected data, a data set of  $g_s$  from 47 species measured under natural field conditions was used from Murray et al. (2019) and compared to our chamber measured plants. In all cases stomatal conductance was measured with both sampling devices.

## Materials and methods

### Controlled-environment experiment

A total of 54 individuals of bare-rooted *Sambucus racemosa* L. (red elderberry), 54 saplings of *Populus tremula* L. (common aspen) and 18 saplings of *Populus tremuloides* Michx. (quaking aspen) were purchased and grown in controlled experiments. It was not possible to source more individuals of *P. tremuloides* due to very strict importation regulations. These species were chosen as they are each known to occur in more than one global biomes (boreal forest, temperate deciduous forest, temperate grassland/chaparral and temperate rainforest) (Murray et al. 2019) and have also been used in previous CO<sub>2</sub> enrichment studies (Bernacchi et al. 2003). All plants were re-potted into 5 L pots using a growing medium comprising 90% Shamrock® Multi-Purpose Compost (Scotts Horticulture Ltd., Co. Kildare, Ireland) and a 10% combination of Perlite Standard 2–5 mm (Sinclair Pro, Cheshire, UK) and 3 g/l Osmocote® Exact Standard 12–14 M slow release fertilizer (15-9-11 + 2MgO + TE; Scotts International BV, Netherlands). The plants were kept for 2 weeks in the open air at Rosemount Environmental Research Station, University College Dublin (UCD), Ireland, before being treated for pests with an emulsifiable concentrate containing 5% pyrethrin (Pyrethrum 5EC at 20 ml/5 L—Agropharm Ltd., UK). The plants were then moved into CONVIRON (Winnipeg, Manitoba, Canada) BDR-16 and BDW-40 plant growth chambers within the Programme for Experimental Atmospheres and Climate (PÉAC) facility at UCD. The chambers allow monitoring and control of atmospheric conditions including air temperature ( $T$ )

(°C), relative humidity (RH) (%), light (PAR) ( $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) and atmospheric [O<sub>2</sub>] (%) and [CO<sub>2</sub>] (ppm). For the experiment, chambers were programmed to run a 16.5 h/7.5 h day/night cycle. Maximum day time  $T$  and RH were set to 22 °C and 70%, respectively. Maximum night time  $T$  and RH were set to 15 °C and 60%, respectively. Light intensity was set to reach a maximum of 600  $\mu\text{mol m}^{-2} \text{s}^{-1}$  at noon (Table 1) and O<sub>2</sub> concentration was set to ambient concentration of 20.9% in all chambers. A ramping program was used to ensure a uniform diurnal increase in  $T$ , RH and light conditions. CO<sub>2</sub> concentrations were set to 350, 420, 490 and 560 ppm in two chambers per CO<sub>2</sub> treatment (8 chambers in total) and were monitored in each chamber with a PP-Systems WMA-4 CO<sub>2</sub> gas analyzer. The CO<sub>2</sub> concentrations represented the year 1987, 2025, 2051 and 2070, respectively, according to the low-medium stabilization RCP4.5 scenario (IPCC 2014). Supplementary CO<sub>2</sub> for all the chambers was provided by a compressed gas tank containing liquid CO<sub>2</sub>. Since two different chamber types were used in the experiment, an additional control chamber (BDW-40) was added to the 420 ppm treatment to identify any potential confounding effects that might have occurred due to differences in chamber type (Porter et al. 2015). To attain sub-ambient (350 ppm) CO<sub>2</sub> in the chambers, an inline fan with a variable damper regulated the amount of air that was passed from the chambers through an external soda lime unit (2–5 mm Sofnolime™—Molecular Products Group Ltd., Essex, UK). The CO<sub>2</sub> free air was then passed back into the chambers and CO<sub>2</sub> was injected to reach the target sub-ambient set point conditions. All measured chamber conditions are reported in Table 1.

To acclimatize plants to chamber conditions, six plants of *P. tremula* and *S. racemosa* and two plants of *P. tremuloides* were transferred into each chamber (Table 2) and grown under ambient local conditions (420 ppm CO<sub>2</sub> and 21% O<sub>2</sub>) for 14 days before treatment conditions were initiated. The plants were then grown for 126 days under treatment. Plants were watered and fertilized (N:P:K; 22:4:22) every 2 days and 2 weeks, respectively. Soil moisture content was monitored using a

**Table 1** Plant growth chamber parameter settings

| Type                         | Set point     | CO <sub>2</sub> set point<br>350–560 ppm | CO <sub>2</sub> measured | Light<br>600 $\mu\text{mol}$ | Temp. day<br>22 °C | RH day<br>70% | Temp. night<br>15 °C | RH night<br>60% |       |
|------------------------------|---------------|--|--------------------------|------------------------------|--------------------|---------------|----------------------|-----------------|-------|
| Chamber 1–2<br>( $n=2$ )     | BDW 40        | Mean                                     | 350                      | 345.59                       | 600.08             | 20.46         | 68.12                | 16.28           | 65.86 |
|                              |               | SD                                       |                          | 34.27                        | 30.72              | 0.27          | 3.36                 | 0.27            | 3.42  |
| Chamber 3–5<br>( $n=1/n=2$ ) | BDW 40/BDR 16 | Mean                                     | 420                      | 434.06                       | 551.60             | 20.41         | 67.44                | 16.24           | 66.01 |
|                              |               | SD                                       |                          | 19.09                        | 16.83              | 0.54          | 5.56                 | 0.73            | 6.22  |
| Chamber 6–7<br>( $n=2$ )     | BDR 16        | Mean                                     | 490                      | 481.76                       | 568.87             | 20.58         | 68.75                | 16.66           | 64.14 |
|                              |               | SD                                       |                          | 23.26                        | 19.70              | 1.04          | 6.86                 | 2.43            | 9.82  |
| Chamber 8–9<br>( $n=2$ )     | BDR 16        | Mean                                     | 560                      | 544.34                       | 560.09             | 20.45         | 69.95                | 16.27           | 66.10 |
|                              |               | SD                                       |                          | 30.96                        | 25.74              | 0.38          | 6.74                 | 0.53            | 7.74  |

**Table 2** Number of individuals grown under different CO<sub>2</sub> conditions and chamber types

|             | CO <sub>2</sub> (ppm) | Type   | <i>S. racemosa</i> | <i>P. tremula</i> | <i>P. tremuloides</i> |
|-------------|-----------------------|--------|--------------------|-------------------|-----------------------|
| Chamber 1–2 | 350                   | BDW 40 | 12                 | 12                | 4                     |
| Chamber 3   | 420                   | BDW 40 | 6                  | 6                 | 4                     |
| Chamber 4–5 | 420                   | BDR 16 | 12                 | 12                | 2                     |
| Chamber 6–7 | 490                   | BDR 16 | 12                 | 12                | 4                     |
| Chamber 8–9 | 560                   | BDR 16 | 12                 | 12                | 4                     |
| Total       |                       |        | 54                 | 54                | 18                    |

Delta-T Devices HH2 Moisture Meter (Delta-T Devices Ltd., Cambridge, UK) ( $P. tremula = 0.25 \pm 0.12 \text{ m}^3 \cdot \text{m}^{-3}$ ;  $P. tremuloides = 0.29 \pm 0.11 \text{ m}^3 \cdot \text{m}^{-3}$ ;  $S. racemosa = 0.25 \pm 0.13 \text{ m}^3 \cdot \text{m}^{-3}$ ). Plants were rotated randomly twice within each chamber to avoid spatial acclimation (Hammer and Hopper 1997) or within-chamber variability (Porter et al. 2015).

Stomatal conductance was measured using a PP-Systems Ciras-2 infrared gas analyzer attached to a PLC6(U) Automatic Universal Leaf Cuvette and a hand-held Decagon Devices SC-1 Leaf Porometer. Measurements were taken inside the chambers on a minimum of two, fully expanded leaves per individual plant that had developed fully under treatment conditions. Each leaf was labelled and measured repeatedly throughout the experiment. The IRGA was used to measure  $g_s$  over 24 h for each species in all treatments ( $n = 10$ ), except the 490 ppm treatment as we were restricted by the availability of equipment. The light emitting diode (LED) unit was removed from the leaf cuvette to attain ambient light conditions in the cuvette (see supplementary figure). Leaves were allowed to equilibrate within the cuvette for a minimum of 20 min. until  $g_s$  had remained stable for approximately 15 min. at a VPD of 1 kPa. CO<sub>2</sub> concentrations in the leaf cuvette were fixed to the ambient treatment condition of each of the growth chambers (Table 1). Leaf temperature was determined using the energy balance setting. To avoid temporal differences between measurements, plants were measured in rotation across treatments. In combination with the 24 h measurements, a leaf porometer was used to make additional spot measurements at 11 am in the morning on two leaves per individual plant over several days. To ensure plants were measured at the same time of the day, the time setting of the plant growth chambers was staggered by 1 hour.

## Field data

See Murray et al. (2019) for a detailed account of the field data collection protocol. Briefly,  $g_s$  measurements were carried out in North and Central America in the summer of 2014. A total of 47 C3 woody angiosperm tree and shrub species were sampled in two boreal forest sites (Bird Creek [60°58' N, 149°28' W] and Kenai [60°33.3' N, 151°12.8' W],

Alaska, USA), one temperate deciduous forest site (Smithsonian Environmental Research Centre [38°53' N, 76°32' W], Maryland, USA), two tropical seasonal forest (wet) sites (Cambalache [18°27' N, 66°35' W] and Guajataca [18°24' N, 66°58' W], Puerto Rico) and one tropical seasonal forest (dry) site (Guanica [17°93' N, 66°92' W], Puerto Rico). Stomatal conductance was measured with a CIRAS-2 gas analyzer (PP-Systems, Amesbury, MA, USA) attached to a PLC6(U) cuvette fitted with a 1.7 cm<sup>2</sup> measurement window and a red/white light LED unit. Stomatal conductance was measured at ambient atmospheric CO<sub>2</sub> of 400 ppm on an average of four individuals per species between 9:00 am and 13:00 pm on a sun exposed leaf following standard sample protocols (Berveiller et al. 2007; Domingues et al. 2010; Koch et al. 2004; Rowland et al. 2015; Dang et al. 1997). Cuvette conditions were set at 200 cm<sup>3</sup> min<sup>-1</sup> air flow, 1000 μmol m<sup>-2</sup> s<sup>-1</sup> light intensity and 80–90% incoming mole fraction of water vapor. To standardize our measurement protocol for each site, regardless of the temperature changes during the daily measurement time window, they calculated the average site-specific leaf temperature at 9:00 am by recording the leaf temperature of at least ten leaves belonging to ten different species grown at each site. For the final  $g_s$  measurements each leaf was left to equilibrate for at least 15 min before values were recorded.

In addition to the IRGA  $g_s$  measurements, a Decagon Devices SC-1 steady state Leaf Porometer was used to measure  $g_s$  on the same species and site, on fully exposed leaves (Murray et al. 2019). One leaf on three individuals per species was measured consecutively over 4 days. As with the IRGA, measurements were not taken on wet days or on wet leaves. Where moisture was a factor, excess moisture was blotted off and the leaf was left to dry before it was measured (Murray et al. 2019).

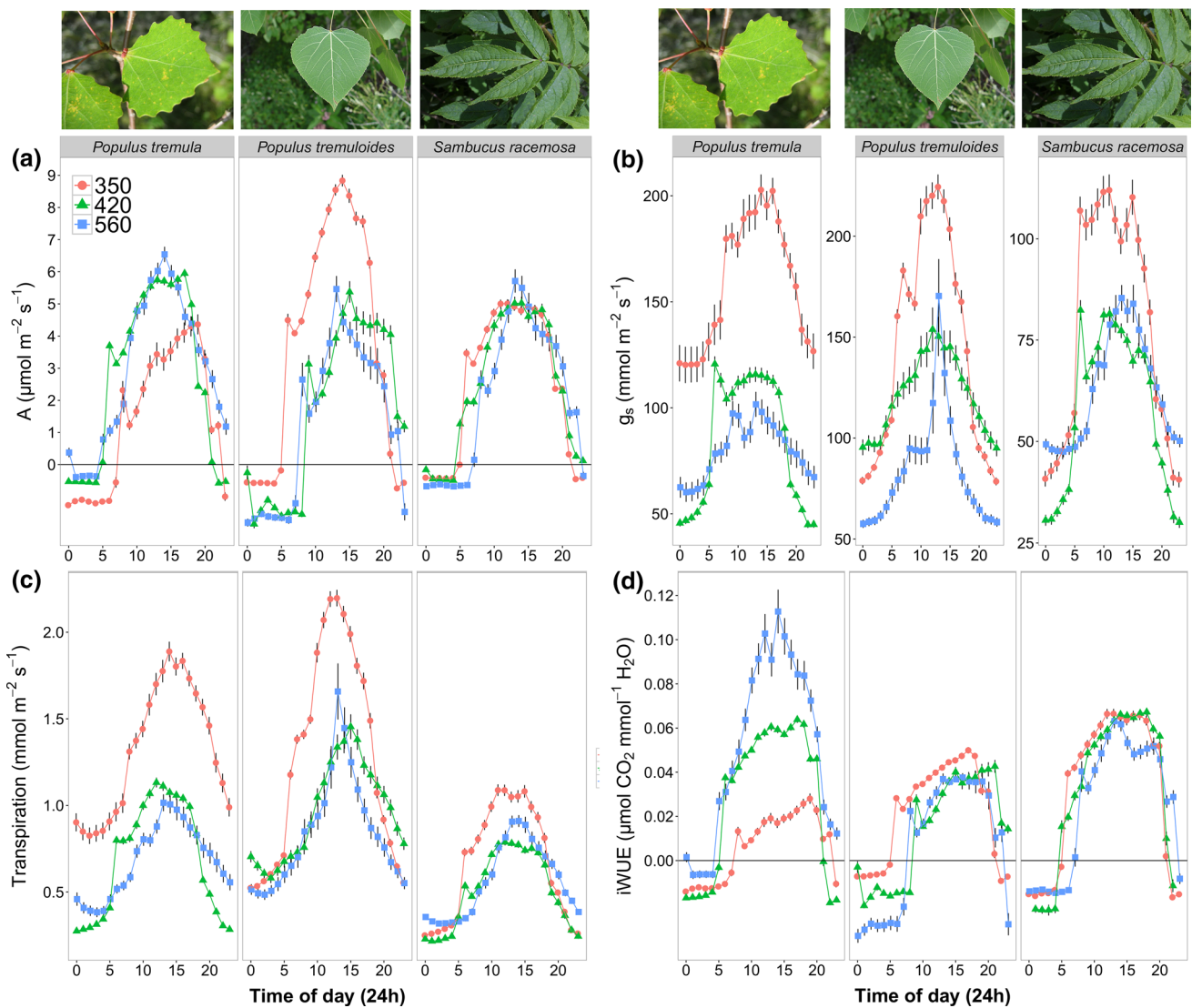
## Analysis

Data were tested for normality and equal variance. Difference in  $g_s$  between treatments was tested separately for each species using ANOVA comparison. The ANOVA was weighted by the soil moisture content of each plant to account for variability in soil moisture between  $g_s$  measurements. ANOVA comparisons that were significant

were further analyzed using pairwise tests with Bonferroni corrections. To identify how maximum diurnal  $g_s$  shifted between treatments, a polynomial surface was fitted using non-parametric locally weighted regression. Maximum  $g_s$  was then calculated for each fit (species and treatment) separately. To test for chamber effects as a result of using different chamber types, mixed effect models were used. All analysis was performed using the statistical package ‘R’ version 3.4 (R Developing Core Team 2017).

### Results

In the chambers, diurnal physiological responses of photosynthetic assimilation ( $A$ ) ( $\mu\text{mol m}^{-2} \text{s}^{-1}$ ),  $g_s$  ( $\text{mmol m}^{-2} \text{s}^{-1}$ ), transpiration ( $\text{mmol m}^{-2} \text{s}^{-1}$ ) and  $i\text{WUE}$  ( $=A/g_s$ ) ( $\mu\text{mol m}^{-2} \text{s}^{-1}/(\text{mmol m}^{-2} \text{s}^{-1})$ ) contrasted between and within species grown at different  $\text{CO}_2$  concentrations (Fig. 1). Assimilation was lowest at the 350 ppm treatment for *P. tremula* and higher at the 420 and 560 ppm treatments (Fig. 1a);  $i\text{WUE}$  was therefore greatest for individuals grown under 560 ppm conditions (Fig. 1d). Assimilation and  $i\text{WUE}$  for *P. tremuloides* showed the opposite response with higher  $A$  and  $i\text{WUE}$  under 350 ppm  $\text{CO}_2$  conditions. Although  $A$



**Fig. 1** Diurnal physiological responses of plants grown under 350 (circle and red), 420 (triangle and green) and 560 ppm (square and blue)  $\text{CO}_2$ . No IRGA data for the 490 ppm treatment was collected due to restricted access to equipment. **a** assimilation ( $A$ ), **b** stomatal

conductance ( $g_s$ ), **c** transpiration and **d** intrinsic water use efficiency ( $i\text{WUE}$ ). Each value is the mean of approximately ten measurements per treatment ( $n=10$ ). Vertical bars represent the 95% confidence interval

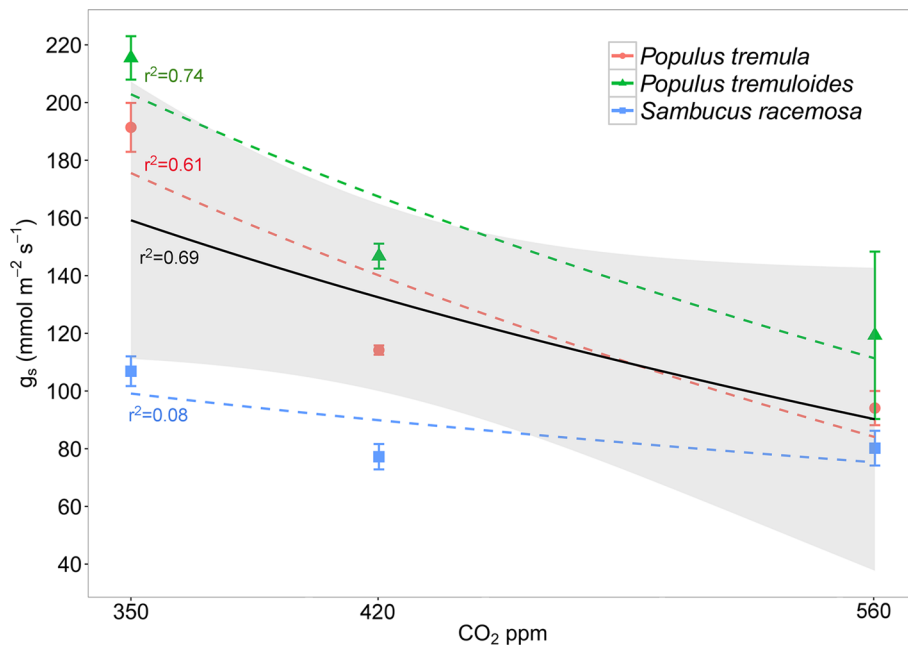
and iWUE were also higher for *S. racemosa* in the 350 ppm CO<sub>2</sub> treatment, the differences between treatments were much smaller (Fig. 1a, d).

Generally, *g<sub>s</sub>* and transpiration decreased under elevated CO<sub>2</sub> (Fig. 1b, c) for all three species, with the greatest difference observed between the 350 ppm and 560 ppm CO<sub>2</sub> treatments. The decrease in *g<sub>s</sub>* under elevated CO<sub>2</sub> was best explained by a log regression (overall  $F=6.825$ ,  $r^2=0.689$ ,  $p=0.03$ ), showing that the mean *g<sub>s</sub>* response to CO<sub>2</sub> when measured at 11 am was non-linear for all three species in the IRGA measurements (Fig. 2). This non-linear decrease was

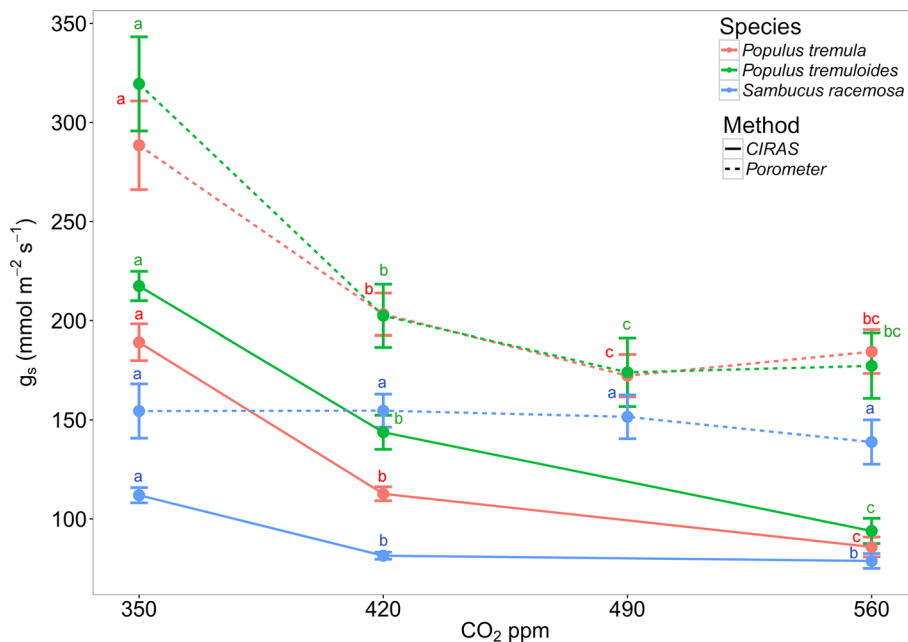
also reflected for two species (*P. tremula* and *P. tremuloides*) in the porometer measurements (Fig. 3).

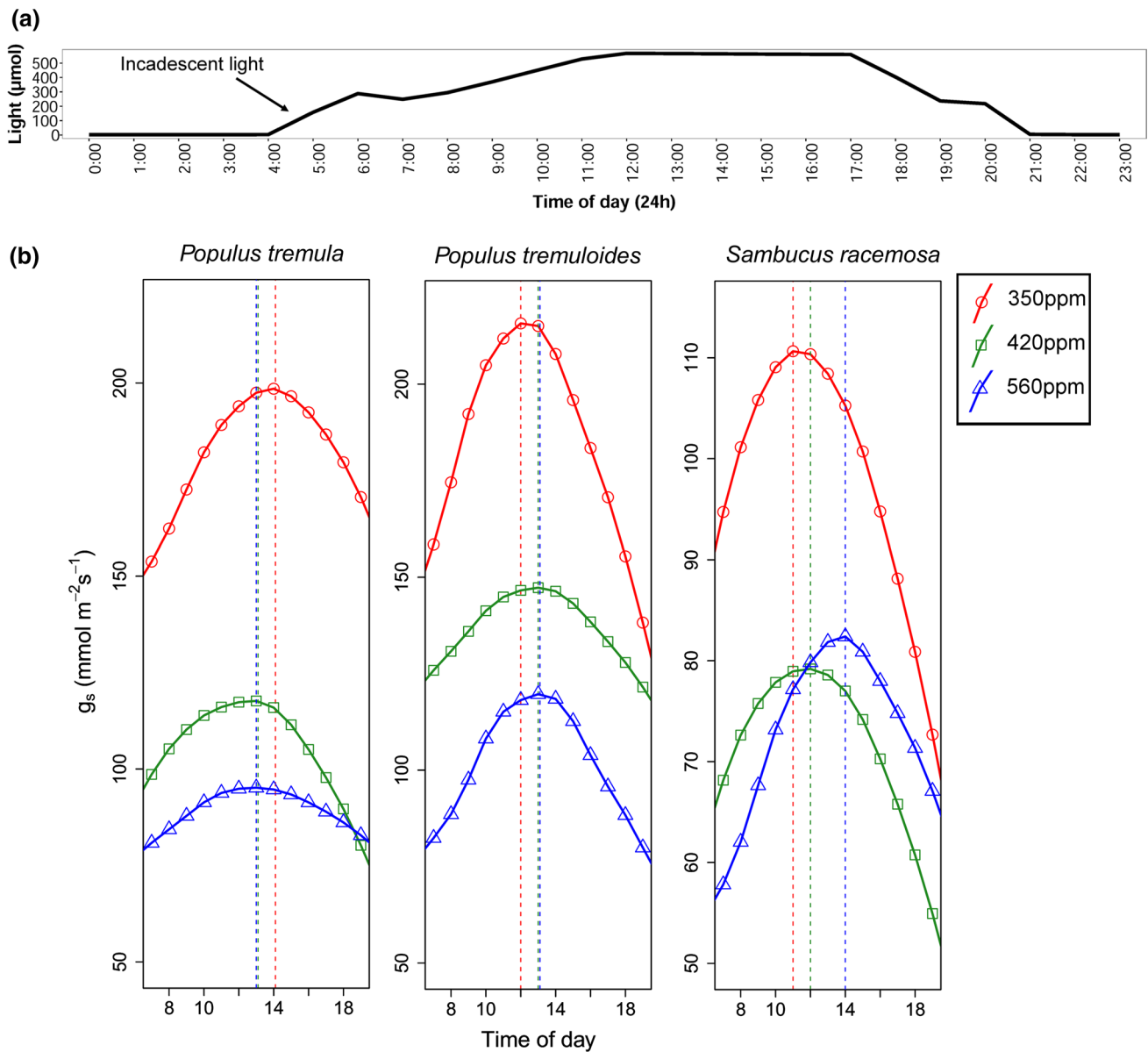
The time of the maximum operational *g<sub>s</sub>* also differed between treatments and was species-specific (Fig. 4b). For *S. racemosa*, the maximum operational *g<sub>s</sub>* shifted across the day by 1–2 h for each 70 ppm increase in CO<sub>2</sub>. For *P. tremuloides*, maximum operational *g<sub>s</sub>* shifted across the day by 1–2 h from 350 to 420 ppm CO<sub>2</sub> but no shift was observed between 420 and 560 ppm CO<sub>2</sub>. There was a shift in maximum *g<sub>s</sub>* to earlier hours for *P. tremula* in response to changes in CO<sub>2</sub> concentrations (Fig. 4b).

**Fig. 2** Fitted species and mean (black solid line) log regression ( $F=6.825$ ,  $r^2=0.69$ ,  $p=0.03$ ) of *g<sub>s</sub>* across CO<sub>2</sub> treatments. Grey-shaded area represents the standard error for the mean fit of all three species. Individual measurements represent the mean and 95% confidence intervals of daytime *g<sub>s</sub>* measured using a PP Systems CIRAS-2 ( $n=10$ )



**Fig. 3** Stomatal conductance measured with a porometer (dashed lines) and an IRGA (solid lines) for each species (different colors) and within each CO<sub>2</sub> (ppm) treatment. The means and 95% confidence intervals are shown. Different letters indicate significant differences between treatments (Bonferroni post-hoc test at  $p < 0.05$ ). The spot measurements were taken between 11 am and noon every second day. Each value is the mean of approximately 160 (porometer) and 10 (IRGA) repeated measurements per treatment



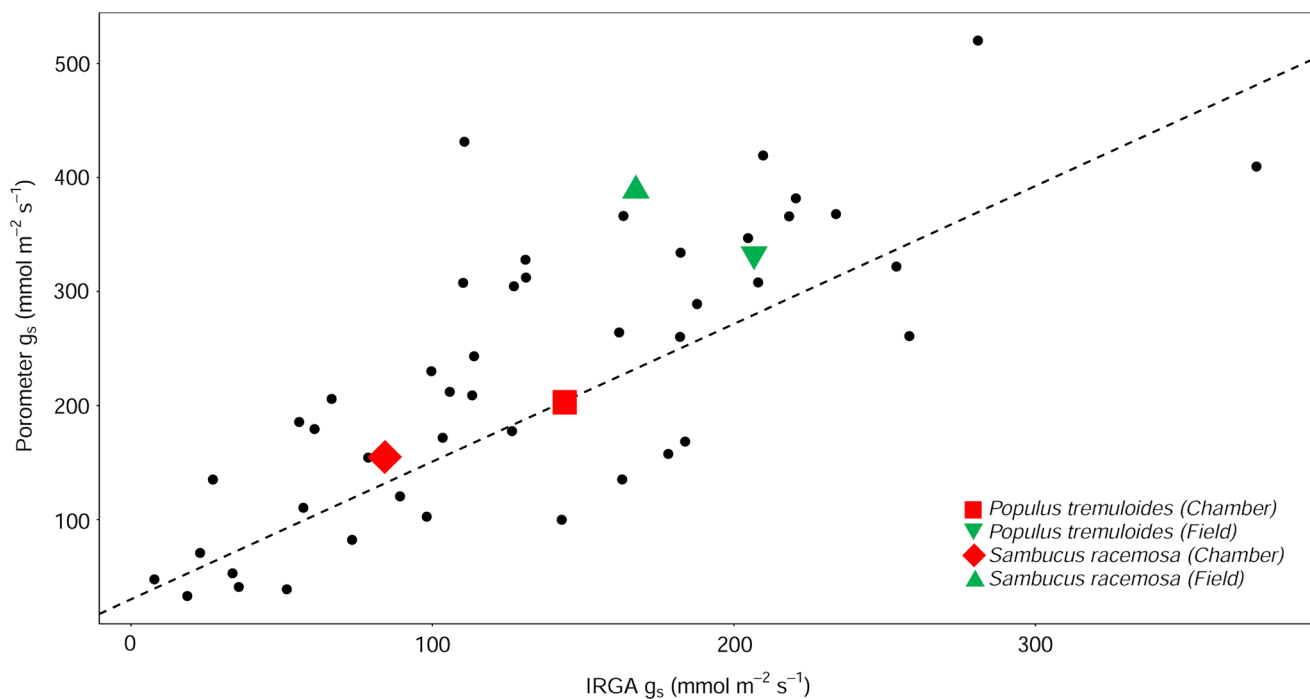


**Fig. 4** **a** Diurnal chamber light pattern. **b** Diurnal non-parametric locally weighted polynomial regression curves of  $g_s$  calculated for plants grown under 350 (circle and red), 420 (square and green) and

560 ppm (triangle and blue) CO<sub>2</sub>. The dashed lines represent the maximum  $g_s$  at time  $t$

The comparison of measured  $g_s$  between the porometer and IRGA in the growth chambers showed that, in general, the measured  $g_s$  responses under elevated CO<sub>2</sub> were proportionally very similar. Both methods detected a decrease in  $g_s$  with elevated CO<sub>2</sub>. However, the magnitude of measured  $g_s$  responses varied between the porometer and CIRAS, with an average of ~25% higher values in  $g_s$  observed when the porometer was used (Fig. 3). Although no statistically significant difference ( $p > 0.05$ ) was observed between the three treatments, the relative difference in measured  $g_s$  between the porometer and the IRGA increased with elevated CO<sub>2</sub> (~20–30%).

Following the work of Murray et al. (2019), there was a strong positive correlation between the IRGA and the porometer measured  $g_s$  of our 47 measured species in the field (Fig. 5;  $F = 64.05$ ,  $r^2 = 0.58$ ,  $p < 0.01$ ). Figure 5 also suggests that low conducting species display greater proportional difference between IRGA and porometer observations due to the fact that the relationship crosses the y axis at a value of 30 mmol m<sup>-2</sup> s<sup>-1</sup> ( $F = 7.29$ ,  $r^2 = 0.12$ ,  $p < 0.01$ ). The mean differences in measured  $g_s$  between the IRGA and the porometer for *P. tremuloides* and *S. racemosa* measured in the field in woody C3 angiosperm taxa was 24.3% and 40.6%, respectively. This was of a similar magnitude



**Fig. 5** Comparison of mean porometer and IRGA measured  $g_s$  in field and chamber conditions. The black dots show the porometer and IRGA comparison of 45 species in the field by Murray et al. (2019). The green symbols show the same comparison in the field for *P. tremuloides* (upside down triangle) and *S. racemosa* (upright triangle). The red symbols show the porometer and IRGA comparison in the chambers for *P. tremuloides* (square) and *S. racemosa* (diamond). Note that the field measurements were done under 400 ppm,

whereas the chamber measurements for this comparison was done with individuals in the 420 ppm treatment. The y-intercept was fixed to  $30 \text{ mmol m}^{-2} \text{ s}^{-1}$ . This was done because preliminary measurements using dry pieces of paper and plastic revealed that porometer measurements were on average  $30 \text{ mmol m}^{-2} \text{ s}^{-1}$  higher from zero compared to IRGA measurements ( $g_s=0$ ) [see Murray et al. (2019) for more detail]

compared to the difference of  $g_s$  between the IRGA and the porometer for *P. tremuloides* (17%) and *S. racemosa* (31%) measured in the growth chambers at 420 ppm (Fig. 5).

## Discussion

The non-linearity of  $g_s$  response to increasing  $\text{CO}_2$  has been predicted in empirical explorations and modelling studies (Konrad et al. 2008; Maherali et al. 2002; Gill et al. 2002; Medlyn et al. 2011; de Boer et al. 2011). However, in many published studies plants are often exposed to large step increases in  $\text{CO}_2$ , mostly comparing ambient to high ( $\sim 600$  ppm)  $\text{CO}_2$  manipulations; representing centurial increase in  $\text{CO}_2$  (Ainsworth et al. 2008; Ainsworth and Long 2005). Since atmospheric  $\text{CO}_2$  is expected to increase gradually in the future, large step increases from  $\text{CO}_2$  studies are not easily extrapolated to intermediate  $\text{CO}_2$  concentration increases; hence why in some studies a non-linear response has often gone undetected (Long et al. 2004). The non-linear decrease in  $g_s$  can only be detected when plants are exposed to decadal rather than centurial magnitude  $\text{CO}_2$  change. Growth chamber experiments compared to other

experimental systems (e.g., FACE) have a technical advantage when it comes to subtle (e.g., decadal) manipulations of the  $\text{CO}_2$  environment, as they can control  $\text{CO}_2$  more tightly (increments of +50 to +70 ppm are possible, see Table 1). We found clear evidence that at least two of the species measured in this experiment are responding to elevated  $\text{CO}_2$  with a non-linear decrease in  $g_s$ . Understanding and demonstrating this non-linear decrease through experimental manipulation can help to refine predictions to ecosystem responses (Bonan et al. 2014).

The decrease in  $g_s$  under elevated  $\text{CO}_2$  was in some cases accompanied by an increase in  $A$  and  $i\text{WUE}$  (Fig. 1). The adjustment of  $g_s$  to  $\text{CO}_2$ , via feedback regulation of stomatal aperture and or stomatal density and pore size, is part of the mechanism for optimizing  $\text{CO}_2$  uptake with respect to water loss (Haworth et al. 2013). The decrease of  $A$  in *P. tremuloides* under elevated  $\text{CO}_2$  can possibly be attributed to a strong down-regulation of photosynthesis (Ainsworth and Long 2005) or increased stomatal limitation (e.g., speed or stomatal anatomy) compared to the two other species. The latter is less likely to be the case, as  $g_s$  in *P. tremuloides* at 420 and 560 ppm are equal or higher compared to the  $g_s$  values of the other two species. In *S. racemosa*, where  $A$



did not change and  $g_s$  was generally lower compared to the other species, the possible increased stomatal limitation at elevated  $\text{CO}_2$  had potentially a greater impact on  $A$  values. When plotting  $A$  against  $g_s$  for each species, the species that had the strongest control/limitation of  $g_s$  on  $A$  was *P. tremuloides*, followed by *P. tremula* and *S. racemosa* (not shown). Furthermore, it has been demonstrated that long-term exposure to  $\text{CO}_2$  in C3 plants can, in some instances, result in a reduction or even complete suppressing of  $A$  (Makino and Mae 1999; Faria et al. 1996). Such responses have been attributed to secondary responses related to decreased nitrogen content or excess carbohydrate accumulation in the leaves, and has often been documented in drought conditions (Cregger et al. 2014; Griffin et al. 2004). Drought is less likely to be the cause of the responses observed here, as the soil moisture was monitored when  $g_s$  measurements were taken throughout the experiment and did not change between treatments ( $p > 0.05$ ) or throughout the duration of the experiment. Also, sink limitations as a result of pot experiments are a likely cause of the observed down regulation of  $A$  in *S. racemosa* under elevated  $\text{CO}_2$  (Ruiz-Vera et al. 2017; Schaz et al. 2014). All plants were provided with liquid fertilizer and were potted in soil that contained slow release fertilizer. However, no soil characteristics (e.g., nitrogen, pH) were measured to exclude this possibility. *S. racemosa* grew much faster compared to the two other species (pers. obs.), which could have resulted in less optimal growing conditions, as the fertilizer input was not changed throughout the duration of the experiment.

Our data demonstrated that the porometer generally over-estimates  $g_s$  in growth chamber conditions (Fig. 3), and this is independently confirmed in field measurements of the same taxa and other woody angiosperms (Fig. 5). Similar to our findings, Ramírez et al. (2006) demonstrated that porometer measurements over-estimated  $g_s$  by approximately 32% compared to IRGA measurements in *Stipa tenacissima* L. The over-estimation of  $g_s$  values using the porometer is ~25% on average and is more pronounced in species with low  $g_s$  and under conditions that lead to decreased  $g_s$  (e.g., elevated  $\text{CO}_2$ —Fig. 5). Yet, this difference was not statistically significant. The  $\text{CO}_2$  effect on the relative  $g_s$  difference is likely the result of a stomatal closing response when  $\text{CO}_2$  is increased, thus decreasing  $g_s$  and increasing the difference in measured  $g_s$  between the IRGA and the porometer. In addition, the relative differences in  $g_s$  are the result of the differences in how  $g_s$  is measured between the devices. The IRGA system allows more time for the leaf-chamber conditions to equilibrate before the measurement is taken (usually within 15 min.), whereas it only takes a few seconds for equilibrium to occur in the porometer chamber. Steady-state porometers are used frequently in plant studies (Jones 1999; Grant et al. 2007; Maes et al. 2016; Keel et al. 2006; Nijs et al. 1997), making the findings presented here

relevant to many eco-physiologists, who rely on the accuracy of these devices. Using the proposed relationship adjustment by Murray et al. (2019) to account for this observed difference would help future comparative studies that would like to make use of measurements from both porometer and IRGA studies.

The porometer can be temporally limiting as it only provides an instantaneous measurement of  $g_s$  at a given time, whereas the IRGA can provide multiple  $g_s$  measurements over a longer time scale (up to 28 h depending on settings). When the measured species display large fluctuations in  $g_s$  across the diurnal cycle, this becomes important, as porometer protocols, which usually involve a single spot measurement per day, are less likely to capture this variability. For example, the diurnal shift in maximum  $g_s$  with elevated  $\text{CO}_2$  was previously suggested by Konrad et al. (2008) using an optimization model. He showed that under given environmental conditions, maximum  $g_s$  happens between 7.00 and 10.00 am for  $\text{CO}_2$  values < 700 ppm, but that  $g_s$  shifted to 10.00–13.00 for atmospheric  $\text{CO}_2$  values > 700 ppm. Similarly, we demonstrated that for *P. tremuloides* and *S. racemosa*, maximum  $g_s$  shifted into the afternoon as a result of elevated  $\text{CO}_2$  (Fig. 4). Short-term changes in stomatal aperture are often caused by diurnal variations of temperature, insolation, atmospheric humidity, light and wind speed (Konrad et al. 2008). ‘Long-term’ exposure in atmospheric  $\text{CO}_2$  on the other hand, has shown to affect stomatal anatomy and thus maximum theoretical conductance ( $g_{\text{max}}$ ) (Franks and Beerling 2009). The species-specific difference in maximum  $g_s$  to an increase in  $\text{CO}_2$  observed here, is likely to be a response of anatomical stomatal changes (e.g., density and size). For example, an increase in  $\text{CO}_2$  is often associated with a decrease in stomatal density and an increase in stomatal size (Franks and Beerling 2009). Although we did not measure anatomical traits here, Konrad et al. (2008) showed that the timing of maximum  $g_s$  is strongly dependent on the environmental conditions, stomatal traits and the rate of assimilation. It is likely that our species did adjust their  $g_{\text{max}}$  and thus optimized their timings of physiological responses to elevated  $\text{CO}_2$ .

Changes of the time of the day when water is lost by plants could potentially influence the timing of precipitation in some biomes by altering plant-atmospheric dynamics, particularly in biomes where evaporation makes up a large proportion of the evapotranspiration flux (Schlesinger and Jasechko 2014). In addition, the shift in  $g_s$  with elevated  $\text{CO}_2$  has important consequences for the way  $g_s$  is measured in studies that are interested in the effect of elevated  $\text{CO}_2$  on  $g_s$ . In particular it is critical to know the diurnal  $g_s$  pattern of the species to be studied, if the aim of the research is to measure maximum  $g_s$ . For example, our species do not show a very strong mid-day depression, which has been observed in other taxa (Pathre et al.

1998; Franco and Lüttge 2002; Tucci et al. 2010; Kosugi and Matsuo 2006). As previously mentioned, the absolute  $g_s$  value may be overestimated, but more importantly the optimal time (i.e.  $g_s$  is at its maximum) when measurements are taken between the ambient and high  $\text{CO}_2$  treatments will differ as a result. For example, we found that an increase of 70 ppm  $\text{CO}_2$  in *S. racemosa* shifted the maximum  $g_s$  into the afternoon by approximately 1–2 h. This response can be very species-specific (Fig. 4) and is also likely to depend on the model fitted. An experiment that aims to identify how maximum  $g_s$  differs between  $\text{CO}_2$  treatments, using a sample protocol with fixed time-points across all treatments, is likely not to capture the actual maximum  $g_s$  in a day as a result of such a shift. It would therefore be advisable to combine porometry with 24 h response measurements from an IRGA to ensure that the measuring times between treatments are adjusted. However, it remains to be seen whether our findings are applicable across different environmental conditions and taxonomic groups.

## Conclusion

Our study demonstrated that the species in this study respond non-linearly to increases in  $\text{CO}_2$  concentration when exposed to decadal changes in  $\text{CO}_2$ ; small  $\text{CO}_2$  concentrations increases (70 ppm) often not tested by other studies. In addition, we showed that the daily maximum  $g_s$  can, in some species, shift later into the day when plants are exposed to only 70 ppm increases in  $\text{CO}_2$ . Our findings have potential important implications to the diurnal water and carbon budget of plants, and the feedback of these across the soil–plant–atmospheric continuum; specifically under future changes in atmospheric  $\text{CO}_2$ . Due to the importance of stomata regulating global water fluxes,  $\text{CO}_2$  effects that result in  $g_s$  (a) decreasing non-linearly and (b) possibly shifting diurnally, need to be considered, as shown here and elsewhere, when attempting to refine predictions of plant responses to  $\text{CO}_2$  across ecosystems.

**Author contribution statement** SB and JM conceived and designed the research. SB, CY and CE-K conducted the experiment. SB and CY analyzed the data. SB wrote the manuscript. All authors edited and approved the manuscript.

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