RESEARCH PAPER

Increase in leaf temperature opens stomata and decouples net photosynthesis from stomatal conductance in *Pinus taeda* and *Populus deltoides x nigra*

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Abstract

The effect of temperature on stomatal conductance (g_s) and corresponding gas exchange parameters was studied in two tree species with contrasting leaf anatomy and ecophysiology—a broadleaf angiosperm, *Populus deltoides x nigra* (poplar), and a needle-leaf gymnosperm, *Pinus taeda* (loblolly pine). Experiments were conducted in growth chambers across a leaf temperature range of 19–48°C. Manipulations of temperature were done in well-watered and drought soil conditions and under ambient (400 ppm) and elevated (800 ppm) air CO_2 concentrations. Increases in leaf temperature caused stomatal opening at both ambient and elevated [CO_2]. The g_s increased by 42% in poplar and by 40% in loblolly pine when leaf temperature increased from 30°C to 40°C at a vapour pressure difference of 1 kPa. Stomatal limitation to photosynthesis decreased in elevated temperature in loblolly pine but not in poplar. The ratio of net photosynthesis to g_s depended on leaf temperature, especially at high temperatures. Evaporative cooling of transpiring leaves resulted in reductions in leaf temperature up to 9°C in well-watered poplar but only 1°C in drought-stressed poplar and in loblolly pine. As global mean temperatures rise and temperature extremes become more frequent and severe, understanding the effect of temperature on g_s, and modelling that relationship, will become increasingly important.

Key words: Ball–Berry model, elevated temperature, evaporative cooling, global change, heat waves, stomatal conductance.

Introduction

Plant stomata play a key role in water and carbon cycles. On average, plant transpiration accounts for 61% of global evapotranspiration (Schlesinger and Jasechko, 2014). In other words, most water moving from terrestrial ecosystems into the atmosphere passes through plants and the precise amount is regulated by stomata. At the same time, stomatal conductance (g_s) is a key factor determining the rate of net photosynthesis (A) and, therefore, the global carbon cycle and plant

carbon metabolism. As a result, stomatal regulation is one of the main factors which determine local growth and survival of plants and global cycles of mass and energy. Stomatal conductance is so important that it has become central to many models from the leaf level (Ball *et al.*, 1987; Leuning *et al.*, 1995; Jarvis and Davies, 1998; Tuzet *et al.*, 2003), to the tree- and forest-stand level (Mirfenderesgi *et al.*, 2016; Xu *et al.*, 2016), and even up to the global level (Niyogi *et al.*, 2009; Berry, 2012; Verhoef and Egea, 2014). However, the conditions in which plants grow are changing and we still do not know enough about plant stomatal regulation to predict future stomatal responses of plant species and their effects at ecosystem and global scales (Lin *et al.*, 2015).

Temperature is one of the most variable factors in the environment and it affects many plant physiological processes, yet little is known about its effect on g_s, especially at high temperatures (Teskey *et al.*, 2015). Historically, temperatures over 40°C have been recorded in many places in North America. It has been predicted that mean maximum summer temperatures will increase 5°C in the eastern US within this century (Lynn *et al.*, 2007). Here, we studied effects of temperature on the leaf gas exchange of two North American tree species, *Pinus taeda* (loblolly pine) and *Populus deltoides x nigra* (hybrid poplar). Loblolly pine is native to the south-eastern US where the highest temperatures recorded among the 12 states in the region range from 43 to 49°C, with a mean maximum temperature for all 12 states of 45°C (National Climatic Data Center, 2016). Hybrid poplar is widely planted in the Northern Great Plains, which includes the states of Nebraska, Wyoming, Montana, North Dakota and South Dakota. The highest recorded temperatures in those states range from 46 to 49°C with a mean of 48°C. In addition to increases in mean air temperature (T_a), the frequency of extreme temperatures and the severity of heat waves have also increased, and are likely to increase further (Meehl and Tebaldi, 2004; Perkins *et al.*, 2012). Summertime extreme temperatures associated with prolonged heat waves now impact approximately 10% of land surfaces, up from 1% in the 1960s (Hansen *et al.*, 2012). Over recent decades record-breaking monthly temperature extremes have occurred five times more often than during the late 19th through the mid-20th century (Coumou and Robinson, 2013). Heat waves are usually associated with low precipitation and soil drought (Ciais *et al.*, 2005; Stéfanon *et al.*, 2014). However, the frequency of heat waves during wet periods is also increasing. When temperature and precipitation were compared between the periods of 1951–1977 and 1978–2004, it was apparent that both wet/hot and dry/hot conditions were increasing substantially worldwide (Hao *et al.*, 2013). Effects of the increasing frequency and severity of extreme temperature events on g_s are largely unknown.

Results of experiments that examined the direct dependence of g_s on temperature have not been consistent. Previous studies have reported a complete range of responses to increased temperature, including stomatal opening (Schulze *et al.*, 1974; Freeden and Sage, 1999; Lu *et al.*, 2000; Mott and Peak, 2010), no significant response (Teskey *et al.*, 1986; Sage and Sharkey, 1987; Cerasoli *et al.*, 2014; von Caemmerer and Evans, 2015), and stomatal closure (Raven *et al.*, 2005;

Weston and Bauerle, 2007; Lahr *et al.*, 2015). A peaked response with maximum g_s at 20°C (Way *et al.*, 2011) or more complex responses with one peak between 20 and 30°C and an additional increase at extremely high temperatures (Slot *et al.*, 2016) have also been described. One possible explanation for these inconsistent results is that to isolate the direct effect of temperature on g_s requires a well-controlled environment, which is often hard to achieve, particularly with respect to vapour pressure difference (VPD). In addition, differences in sensitivity to heat are likely related to species, whether plants were grown in the laboratory or in the field, and the range of measurement temperature (Slot *et al.*, 2016).

It has been well established that plants regulate rates of transpiration and photosynthesis in parallel, maintaining a balance between g_s and A (Lawson *et al.*, 2011). Therefore, the effect of temperature on stomata is often considered to be indirect, through VPD, transpiration, leaf water potential, or the effect of temperature on photosynthesis or intercellular [CO_2] (C_i). This parallel regulation results in the conservation C_i at a given atmospheric [CO_2] (C_a) and a close correspondence between g_s and A (Wong *et al.*, 1979; Hetherington and Woodward, 2003). The latter relationship has been central to several models of stomatal control of photosynthesis (Farquhar and Wong, 1984; Ball *et al.*, 1987; Leuning, 1995; Buckley *et al.*, 2003), which assume that the ratio of g_s correlates with A over a wide range of environmental conditions. However, some studies indicated that this relationship was decoupled under extreme temperature during heat waves, such that A decreased, but g_s did not. For example, during an imposed heat wave in which daily maximum T_a ranged from 47 to 53°C and VPD ranged from 6 to 8 kPa, *Pinus taeda* and *Quercus rubra* seedlings exhibited progressively lower A on each day of the heat wave but almost no change in g_s (Ameye *et al.*, 2012). Similarly, g_s of *Acer rubrum* changed very little across a temperature range of 35 to 48°C (Weston and Bauerle, 2007). In a study of five species, g_s either increased or did not decline as T_a increased from 20 to 40°C, even though A initially increased from 20 to 30°C and then decreased (von Caemmerer and Evans, 2015). Collectively these studies suggest that the mechanism modulating stomatal aperture may be independent of A at higher temperatures. However, because VPD varied with temperature in all of these studies, it could not be determined to what degree the observed changes in g_s were due to a change in VPD or in the A, or were a direct response to temperature.

In this study, we addressed the following questions: (i) What is the direct effect of moderate to high temperature on g_s? (ii) Is the effect of moderate to high temperature on g_s altered by water stress or C_a? (iii) How does the response of g_s to temperature link to other related factors such as A, C_i, and water status (transpiration, water potential), and how does the correlation between g_s and A, which is crucial to many models, change with temperature? (iv) What is the magnitude of evaporative cooling under extreme temperatures? To answer these questions we performed leaf gas exchange measurements on two contrasting tree species: poplar (*Populus deltoides x nigra*) and loblolly pine (*Pinus taeda*) across a range of temperature and humidity and under well-watered and drought-stress conditions.

Material and methods

Growth chambers and tree material

Trees were grown, and measurements conducted, in two walk-in growth chambers (EGC 36, Environmental Growth Chambers, Chagrin Falls, OH, USA) at the University of Georgia campus in Athens, GA, USA. Prior to the start of experimental treatments, the trees were grown in the chambers for 30 days at 26°C/ 23°C (day/night) T_a, 1700/560 Pa (day/night) air VPD, and a daily light period of 13 hours. Photosynthetically active radiation (PAR) in the chambers was 520 µmol m^{-2} s^{-1}. Air speed in each chamber was maintained at 1 m s^{-1}. During the growth period the C_a was maintained above 400 µmol mol^{-1} as follows: a CO_2 sensor (GMM 220, Vaisala, Helsinki, Finland) monitored [CO_2] in each chamber and controlled a solenoid valve that released CO_2 from a compressed gas cylinder into the chamber whenever the [CO_2] fell below the 400 µmol mol^{-1} set point. Although this procedure prevented the [CO_2] from decreasing below 400 µmol mol^{-1} during periods of active photosynthesis, it did not prevent increases above 400 µmol mol^{-1}. To mitigate the build-up of CO_2 in the chambers, the exterior room windows were fully opened and a large exhaust fan was placed in one window. We estimate that daytime ambient [CO_2] in the chambers was typically between 400 and 475 µmol mol^{-1}.

Measurements were made on clones of two tree species: a poplar (*Populus deltoides x nigra*) clone obtained as cuttings (OP-367, hybridpoplars.com, Glenmoore, PA, USA) and a loblolly pine (*Pinus taeda*) clone from the South Carolina Coastal Plain (Arborgen, Ridgeville, SC, USA). Two-year-old loblolly pine saplings, originally grown in 4-L pots in a greenhouse under natural temperature fluctuations with temperatures commonly reaching ~ 40°C, and poplar cuttings were planted in March 2014 into 15-L pots in a potting medium (Cofer's Nursery Mix, Cofer's, Athens, GA, USA). Each pot was fertilized with 40 g of 15-9-12 extended release fertilizer (Osmocote Plus #903286, Scotts-Sierra Horticultural Products, Marysville, OH, USA) and 0.2 g of chelated iron (Sprint 138, Becker Underwood, Ames, IA, USA). Trees were watered daily to full soil water capacity. At the beginning of the experiment, in April 2014, the mean stem height of the poplars was 1.05 m, and the diameter 10 cm above soil was 9.2 mm. The mean height and diameter of the loblolly pines were 1.1 m and 13.9 mm, respectively.

Gas exchange measurements

Measurements of light-saturated net photosynthesis (A_{sat}), g_s, rate of transpiration (E), and C_i were made with a portable photosynthesis system equipped with a CO_2 mixer (LI-6400–20, LiCor Biosciences, Lincoln, NE, USA). Leaf cuvette conditions were set as follows: block temperature was set at ambient (growth chamber) temperature; [CO_2] was set at either 400 µmol mol^{-1} or 800 µmol mol^{-1}, equal to the concentration in the growth chamber; relative humidity was maintained equal to that in the growth chamber; and PAR was set at 1200 µmol m^{-2} s^{-1}, resulting in light-saturated photosynthesis and no decline as a result of photorespiration (see figure 2 in Ingwers *et al.* (2016) for the photosynthetic light response curve of loblolly pine trees of the same clone measured in the same growth chambers). Measurements of loblolly pine foliage were made on two fully developed fascicles (six needles total) of the second flush attached to the main stem. The needles were arranged in the cuvette on a flat plane with equal spacing between needles to maximize light interception. After the gas exchange measurement, the widths of each of three sides of the needles were measured with a scale loupe and used to calculate the foliage area in the cuvette. For poplar, measurements were made on approximately the 30th leaf from the top of the plant. Gas exchange measurements were performed on six trees of each species (n=6). Gas exchange results were calculated on a total surface area basis for loblolly pine and a one-sided surface area basis for poplar.

Experimental setup

Responses to changes in temperature and VPD under various [CO_2] and soil moisture

To determine stomatal responses to temperature and VPD, T_a in the growth chamber was controlled at 20, 30, 40, or 49°C and relative humidity was changed from approximately 30 to 80% at each temperature. The sequence of the temperature changes was chosen randomly and individual trees were excluded from further measurements after they had been subjected to 49°C. Six trees were allowed to acclimate for at least 45 minutes after each change in environmental conditions. At every measurement, g_s, A, E, and C_i were recorded. To ensure high water availability, during the measurement period the base of each pot was placed in a 5-cm-tall container that was kept full of water. Pre-light water potential (Ψ_P) and water potential at varying T_a and VPD in the light were measured on foliage using a pressure chamber (model 700, PMS Instrument, Albany, OR, USA). Mean Ψ_P was −0.28 ± 0.02 and −0.13 ± 0.02 MPa (mean ± standard error) for loblolly pine and poplar, respectively. Measurements were conducted under ambient [CO_2] (400 µmol mol^{-1}) and elevated [CO_2] (800 µmol mol^{-1}). For measurements under elevated [CO_2], the [CO_2] was increased in the growth chamber to 800 µmol mol^{-1} as described above by reprogramming the set point of the CO_2 sensor. The plants were allowed to equilibrate to elevated [CO_2] for 24 hours prior to measurements.

In a subsequent experiment the effect of soil water deficit on the stomatal response to temperature was investigated. After withholding water for 5 days, the mean Ψ_P of the poplar plants was −0.81 ± 0.10 MPa. After withholding water for 12 days, the mean Ψ_P of the loblolly pine plants was −0.97 ± 0.06 MPa. On those days, measurements were made using the same combinations of temperature and humidity as in the first experiment. The effect of water deficit was studied only at ambient [CO_2]. The first experiment and this experiment were conducted on different trees (n=6 for each experiment).

Effect of C_i on A_{sat} at various temperatures

Under well-watered conditions, A/C_i curves were measured in the growth chamber on six trees of each species. The VPD was held constant at 1.2 kPa at a leaf temperature (T_l) of 20°C and 3.5 kPa at a T_l of 30°C and 40°C both in the growth chamber and the cuvette. PAR in the cuvette was set at 1200 µmol m^{-2} s^{-1}. The [CO_2] in the cuvette was manipulated from 50 to 100 µmol mol^{-1} and then in 100 µmol mol^{-1} steps to 1800 µmol mol^{-1}. The A/C_i Curve Fitting Utility, version 1.1 (Long and Bernacchi, 2003) was used to determine the maximum rate of Rubisco carboxylation (Vc_{max}, µmol m^{-2} s^{-1}), maximum rate of photosynthetic electron transport (J_{max}, µmol m^{-2} s^{-1}), maximum rate of triose-phosphate utilization (V_{TPU}, µmol m^{-2} s^{-1}), and day respiration in the absence of mitochondrial respiration (R_d^*, µmol m^{-2} s^{-1}).

Stomatal limitation to photosynthesis (L_s) was estimated at [CO_2] 400 µmol mol^{-1} from fitted curves using the equation:

$$L_s = \frac{A_0 - A_{sat}}{A_0} \tag{1}$$

where A_0 is the light-saturated net photosynthesis rate that would occur at infinite g_s (Farquhar and Sharkey, 1982).

Cooling effect

Under lighted conditions, the cooling effect of transpiration was estimated as the difference between the temperature of normal transpiring foliage and foliage greased with petroleum jelly to prevent transpiration (Jones *et al.*, 2002) at the same position on the plant. Leaves and needles were chosen for this comparison at a position on the plant close to the point where gas exchange was measured. T_l was measured with an infrared thermometer (Model 561, Fluke, Everett, WA, USA) with emissivity set to 0.97.

Prior to the analyses, the normality of data was determined using the Shapiro–Wilk test. We used linear and non-linear multiregression analysis to describe the dependence of g_s on external factors (i.e. T_l, VPD). A least-squares regression was used to fit the 3D models to the data. Models used to fit data are listed in Supplementary Table S1 (available at *JXB* online). An F-test was used to test significance of model parameters. Analysis of the generalized linear model was used to test for differences among independent variables and a dependent variable when VPD was a continuous predictor. Tests were performed at α=0.05. Most statistical analyses were performed using SigmaPlot 12.5 software (Systat, San Jose, CA, USA) with the exception of the generalized linear model analysis, which was done in Statistica 12 (StatSoft, Tulsa, OK, USA).

Results

Responses of g_s, E, *and* A_{sat} *to* T_l *and VPD*

The g_s increased with increasing T_l and T_a in both species in all tested environmental conditions (Fig. 1 and Supplementary Fig. S1, available at *JXB* online). Under unlimited soil water availability and a VPD of 1 kPa and [CO_2] of 400 µmol mol^{-1}, an increase in T_l from 30 to 40°C led to an increase in g_s of 42% in poplar and 40% in loblolly pine (Fig. 1a, d; Supplementary Table S1; *P* < 0.001). The rate of increase in g_s with temperature was linear in poplar, but g_s increased more at high than at low T_l in loblolly pine. Increasing the [CO_2] from 400 to 800 µmol mol^{-1} caused partial stomatal closure, which was more pronounced in poplar (mean decrease of 21% at VPD 3.5 kPa, *P* < 0.001) than in loblolly pine (mean decrease of 12% at the same VPD, *P* = 0.030). However, similar to results in ambient [CO_2], g_s increased with increasing T_l in both species under elevated [CO_2] (Fig. 1b, e; *P* < 0.001). Soil water deficit significantly reduced g_s in both species, but more so in poplar than pine (Fig. 1c, f; *P* < 0.001). Even though g_s was reduced in drought conditions, g_s of both species still increased with increasing T_l (*P* = 0.040 for poplar and *P* < 0.001 for loblolly pine).

Transpiration (*E*) increased significantly with increasing T_l (and T_a) or VPD in both species under unlimited soil water availability and ambient [CO_2] (Fig. 2a, b and Supplementary Fig. S2a, b, available at *JXB* online). However, the relationships between *E* and environmental variables differed substantially between poplar and loblolly pine. Transpiration of poplar increased with VPD (*P* < 0.001) but not with T_l (*P* = 0.06). Conversely, in loblolly pine, *E* increased only with T_l (*P* < 0.001) but not with VPD (*P* = 0.15).

Under well-watered conditions, C_i increased with increasing temperature in both species (Fig. 3a, c, *P* < 0.001, and Supplementary Fig. S2c, d). A decrease in C_i with increasing VPD was observed in poplar (*P* < 0.001) but not in loblolly pine (*P* = 0.15). In addition, the range of C_i was smaller in poplar than in loblolly pine. T_l (and T_a) had an effect on *A* in both species (Fig. 3b, d, *P* < 0.001, and Supplementary Fig. S3, available at *JXB* online). In both species, at a given T_l there was a specific relationship between A_{sat} and g_s. However, this relationship between A_{sat} and g_s changed with T_l (Fig. 3b, d, *P* < 0.001).

A/C$_i$ curves and L$_s$ *to* A$_{sat}$ *at various* T$_l$

Temperature had a large effect on the parameters of A/C_i curves in both poplar and loblolly pine (Table 1). Stomata of poplar imposed a smaller limitation on the diffusion of CO_2 than stomata of loblolly pine. The relative L_s in poplar did not exceed 20% while in loblolly pine they were between 23 and 78%. L_s was directly comparable between 30 and 40°C because it was measured at the same VPD. While L_s in poplar did not change (*P* = 0.21) with a T_l increase from 30 to 40°C, L_s in loblolly pine declined under the same temperature increase (*P* < 0.001). The values of parameters related to biochemical processes of photosynthesis (Vc_{max}, J_{max}, V_{TPU}, and R_d^*) consistently increased with T_l in both species, with the exception of V_{TPU} in poplar.

Effect of E *on* T$_l$

The temperature of transpiring leaves was lower than the temperature of foliage that did not transpire (Fig. 4). The magnitude of the temperature difference in poplars in wet soil reached up to 9.0°C and scaled with VPD (*P* < 0.001) but not with T_a (Fig. 4a). Transpiring leaves of poplar in dry soil were an average of 1.1°C cooler than non-transpiring leaves (*P* = 0.02) and the magnitude of the cooling effect depended on neither temperature nor VPD (Fig. 4b). In loblolly pine, transpiring needles were an average of 0.9°C cooler than those that did not transpire (*P* = 0.002). There was no effect of soil water availability and the magnitude of the cooling did not depend on temperature or VPD (Fig. 4c).

*Leaf water potential*

Leaf water potential decreased with increasing T_l and VPD in both species when the soil was wet (Fig. 5a, c). When soil was dry, leaf water potential scaled with both T_l and VPD in poplar, but in loblolly pine only VPD had an effect on water potential (Fig. 5b, d). At the same T_l and VPD, poplar maintained higher water potential than loblolly pine.

Discussion

Stomatal conductance, stomatal limitations, and photosynthesis

Stomata play a key role in regulating fluxes of water and carbon dioxide between plant and atmosphere. They regulate both plant growth and cycles of mass and energy. Therefore, much attention has been focused on principles of stomatal regulation and several regulatory mechanisms have been identified. Most research has centred on the stomatal responses to various indices of water status and carbon balance (Farquhar and Sharkey, 1982; Jones, 1998; Buckley *et al.*, 2003). Surprisingly little attention has been paid to the responses of g_s to temperature, even though it is one of the most variable environmental factors. A few previous studies suggested a dependence of g_s on temperature. However, these studies have often provided conflicting results. While some evidence
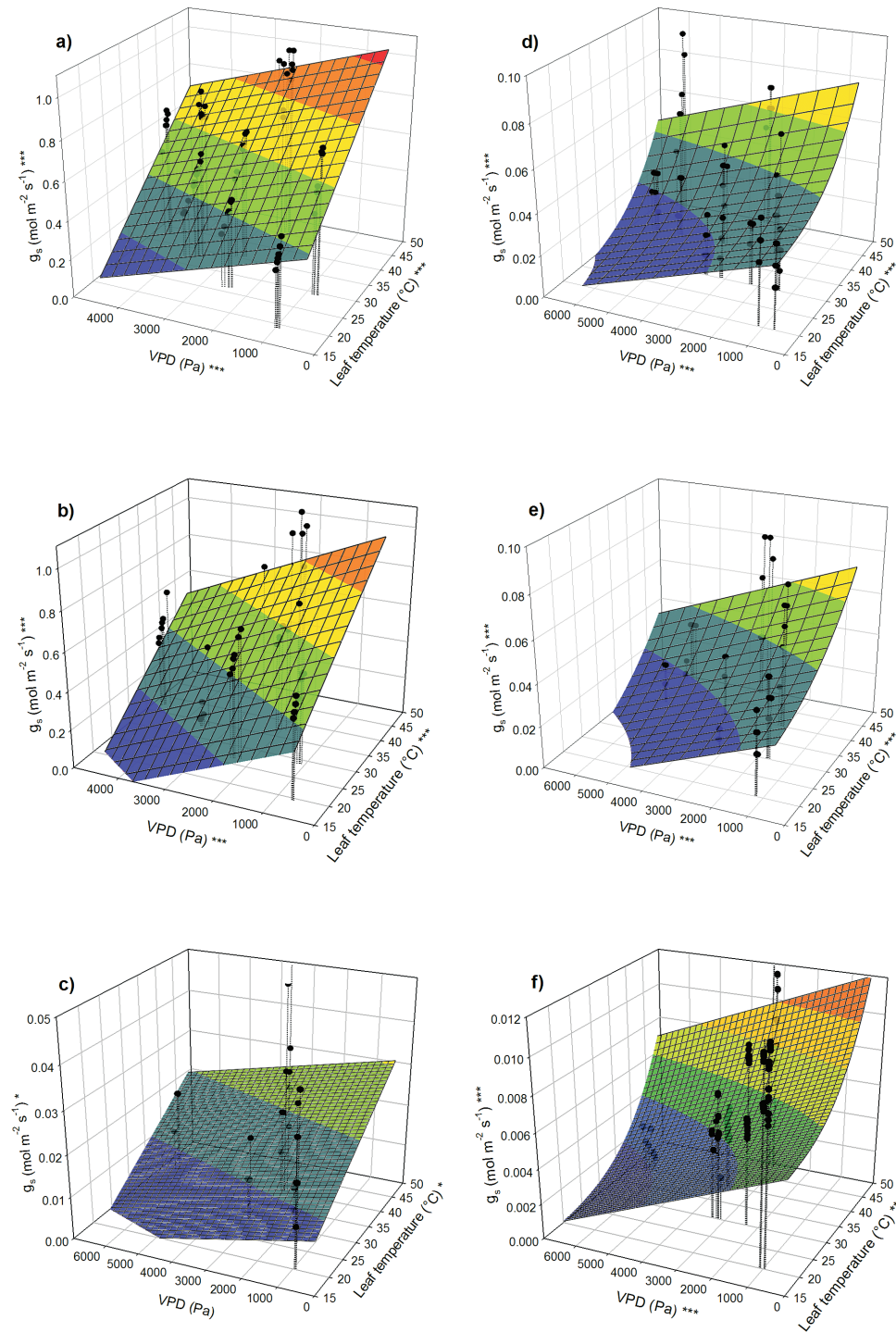
Fig. 1. Stomatal conductance (g_s) of poplar (left panels) and loblolly pine (right panels) and its dependence on T_l and VPD. Plants were measured in high soil moisture conditions and (**a**, **d**) ambient [CO_2] or (**b**, **e**) elevated [CO_2]. (**c**, **f**) Measurements made on drought-stressed trees at ambient [CO_2]. Linear regression was used to fit the data for poplar and non-linear regression was used for loblolly pine. Asterisks at the z-axis label indicate overall significance of the model; asterisks at the x-and y-axes indicate significance of the respective parameters (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

suggested that g_s increased with increasing temperature (Schulze *et al.*, 1974; Lu *et al.*, 2000; Mott and Peak, 2010), other studies found that temperature had no effect on stomata (Teskey *et al.*, 1986; Sage and Sharkey, 1987; Cerasoli *et al.*, 2014; von Caemmerer and Evans, 2015), or that increased temperature triggered stomatal closure (Weston and Bauerle, 2007; Lahr *et al.*, 2015). One explanation for the conflicting results across these studies might be that the experiments were

often conducted in uncontrolled environmental conditions in the field. The design of our experiment, in which the response of g_s to T_l was separated from the effect of VPD and all measurements were made under constant illumination, allowed us to separate the effect of temperature from the effects of other factors.

Our results conclusively demonstrated that there is a strong direct positive response of g_s to increasing T_l in two
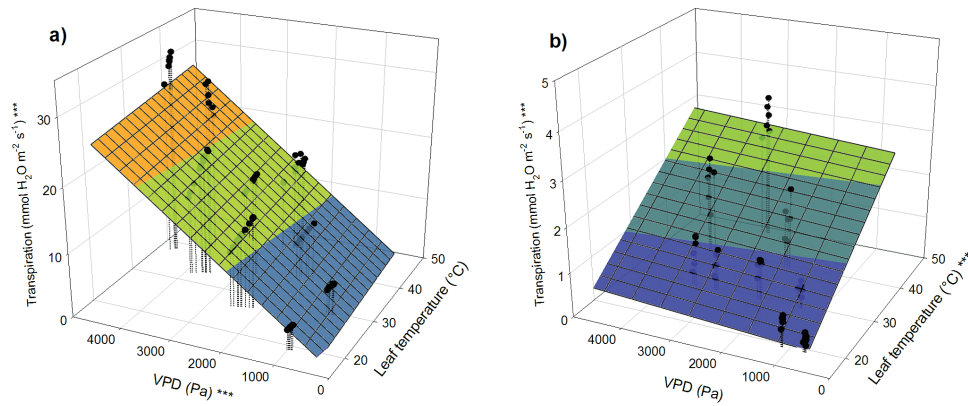
Fig. 2. Response of *E* to VPD in (**a**) poplar and (**b**) loblolly pine at varying T_l and VPD. Asterisks at the *z*-axis label indicate overall significance of the model; asterisks at the *x*- and *y*-axes indicate significance of the respective parameters (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

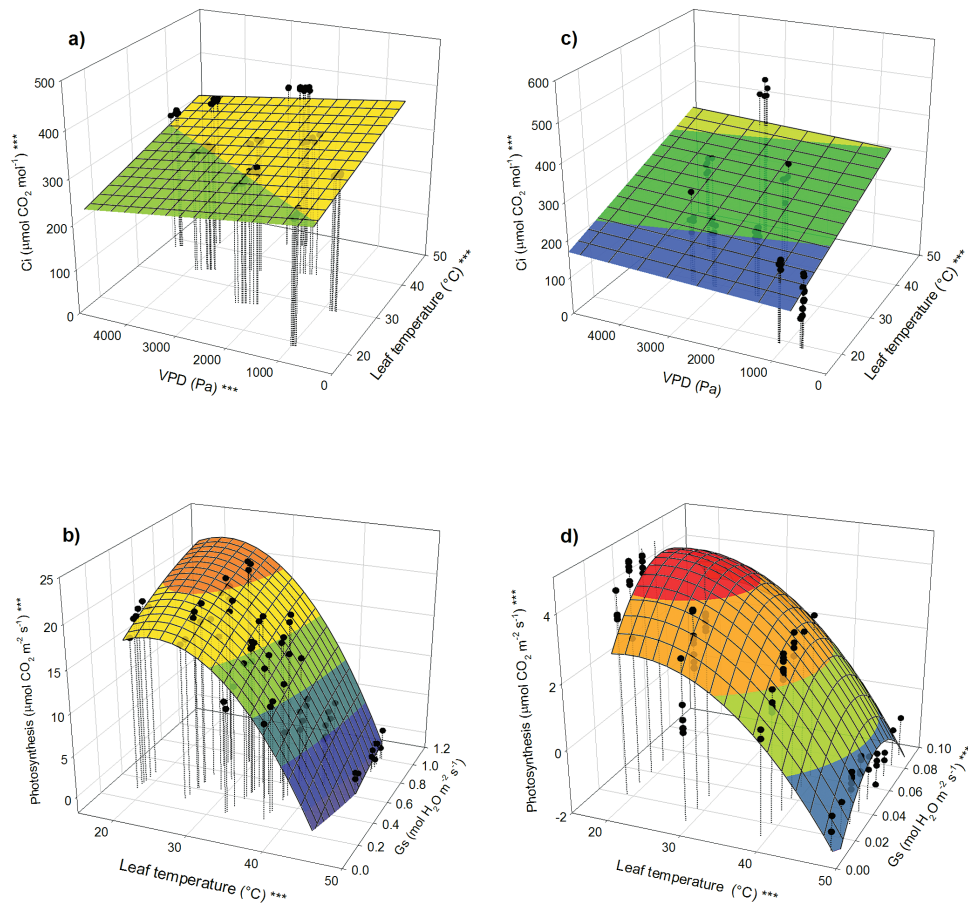**Fig. 3.** (**a**, **c**) Relationship between C_i, T_l, and VPD for poplar (left panels) and loblolly pine (right panels). (**b**, **d**) Relationship between A_{sat}, T_l, and g_s. Asterisks at the *z*-axis label indicate overall significance of the model; asterisks at the *x*- and *y*-axes indicate significance of the respective parameters (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

tree species. In well-watered trees, temperature and VPD had major effects on g_s, as suggested by Freeden and Sage (1999). Elevated C_a caused a decline in g_s but did not fully mitigate increased stomatal opening in response to increased temperature. The increase in g_s with increased T_l was found in both species despite large differences in leaf morphology, xylem structure, and physiology. However, because of these differences, the magnitude of stomatal opening in response to T_l and closing in response to elevated [CO_2], along with the effects on associated physiological processes (such as *E* and

A), differed between the two species. The interplay between elevated T_l, which increased g_s, and elevated [CO_2], which decreased g_s, differed between the two species, suggesting that it could contribute to differences in behaviour among species in the predicted future climate.

The two experimental species stand at opposite ends of the range of mechanisms for stomatal adjustment of water loss. *E* in poplar continuously increased with increasing VPD, while *E* of loblolly pine remained the same over a large range of VPD within a given T_l and increased with increases in T_l

Table 1. *Parameters related to biochemical processes of photosynthesis in poplar and loblolly pine plants measured at three leaf temperatures*

Species	T_l (°C)	Vc_{max}	J_{max}	V_{TPU}	$R_d{}^*$	L_s
Poplar	20	66	132	10.05	2.10	0.19
	30	165	151	11.11	1.9	0.16
	40	301	165	11.46	3.25	0.2
P-value		**<0.001**	**<0.001**	0.07	**<0.001**	0.21
Loblolly pine	20	21	45	3.62	1.55	0.41
	30	67	71	4.57	2.73	0.78
	40	163	75	4.99	6.52	0.23
P-value		**<0.000**	**<0.001**	**<0.001**	**0.011**	**<0.001**

Significant differences between measurements at different temperatures indicated in bold.

Fig. 4. Evaporative cooling effect (temperature difference) of transpiration on (**a**) well-watered poplar, (**b**) drought-stressed poplar, and (**c**) loblolly pine at varying T_a and VPD. Asterisks at the *z*-axis label indicate overall significance of the model; asterisks at the *x*- and *y*-axes indicate significance of the respective parameters (*P < 0.05; **P < 0.01; ***P < 0.001).

(Fig. 2, Supplementary Fig. S2). These results suggest that g_s is regulated by more complex mechanisms than simply E (Mott and Parkhust, 1991), and that temperature changes affect the relationship between E and g_s.

Leaf water potential declined with both increased temperature and increased VPD in both species (Fig. 5). Typically, g_s declines with a decline in water potential across a wide range of both iso- and anisohydric species (Klein, 2014). But in our study, despite a decline in water potential, g_s increased with temperature. Stomata may have opened with increasing temperature owing to, in part, changes in hydraulic conductivity. When temperature increases, the viscosity of water declines and mesophyll conductance (g_m) increases, which may improve the supply of water to sites of evaporation and thus increase stomatal aperture (Cochard *et al.*, 2000; von Caemmerer and Evans, 2015). However, this increase was not great enough to prevent a decline in leaf water potential. Therefore, it was proposed that resistance to water vapour and heat transfer among sites of evaporation and guard cells, which induce differences in temperature and VPD at these sites, may also regulate stomatal opening in response to transpiration and T_l (Mott and Peak, 2013). The general increase in overall tree hydraulic conductance due to water viscosity may be further modified by temperature-dependent variability in tree xylem hydraulic conductance, which, due to differences in vascular traits, may contribute to differences in the responses of conifers and angiosperm trees (Wolf *et al.*, 2016). Changes in leaf g_m may be further paired with xylem resistance to embolism and the safety margin against cavitation, which is higher in conifers than in angiosperms (Choat *et al.*, 2012). Trees adjust their g_s to maximize CO_2 uptake (resulting in higher E) but still protect xylem against excessive cavitation (Brodribb *et al.*, 2016). Loblolly pine strictly regulated transpiration such that it did not change with variation in VPD, thus protecting xylem against cavitation and maintaining a broad safety margin. However, when temperature increased, loblolly pine was not able to maintain this strict control over water loss, so E increased. This result may suggest that in the pine, overall resistance of the hydraulic pathway (including xylem and mesophyll resistance) significantly contributed to regulation of transpiration and that stomatal regulation was
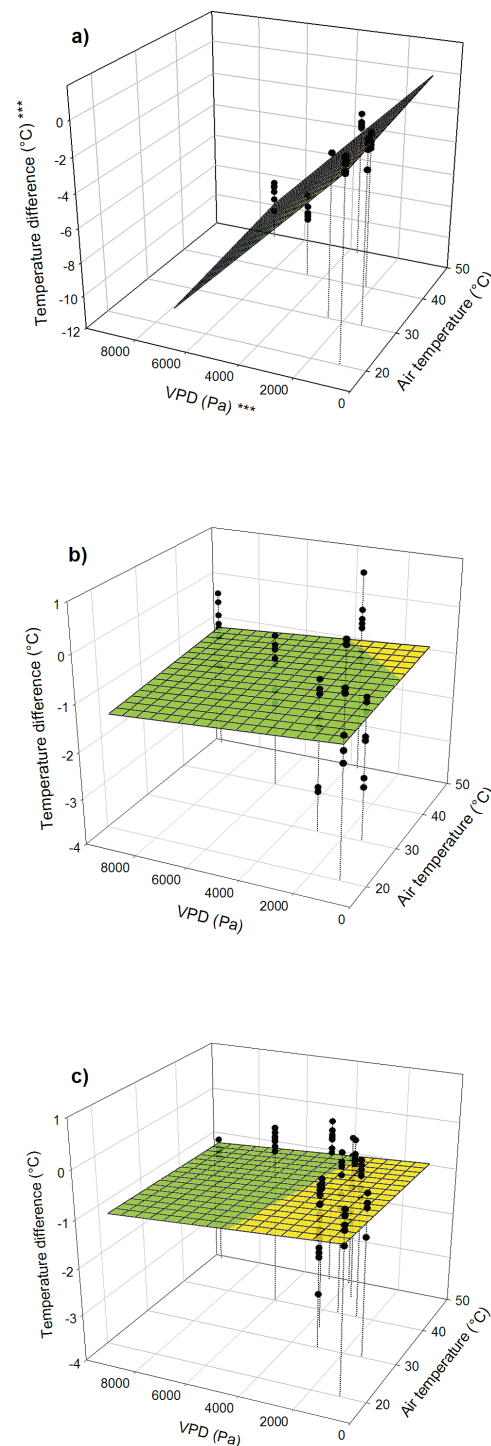
at least partly independent of E. In contrast, the broadleaf poplar exerted the same degree of stomatal control on E at all temperatures. The inability of loblolly pine to regulate E when temperature increases may negatively impact survival with climate change and may contribute to succession by angiosperm tree species (Carnicer *et al.*, 2013).
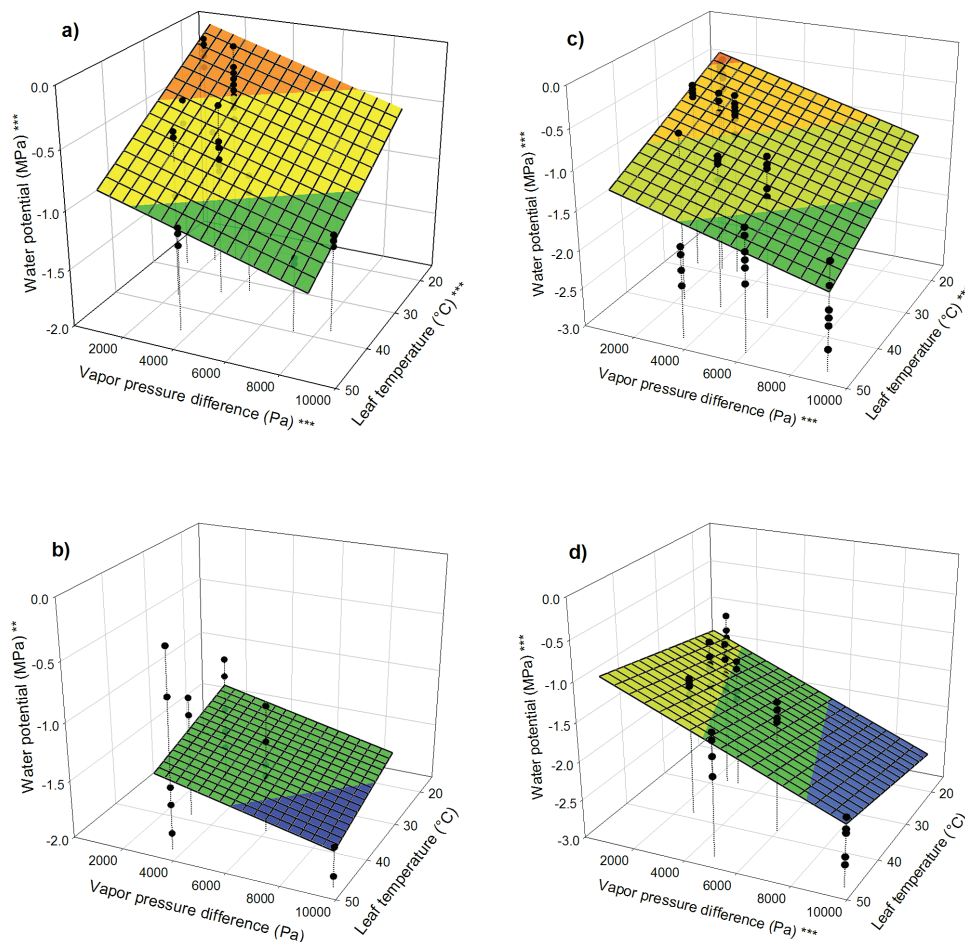
Fig. 5. Leaf water potential of (**a**, **b**) poplar and (**c**, **d**) loblolly pine in wet soil (a, c) and dry soil (b, d). Asterisks at the *z*-axis label indicate overall significance of the model; asterisks at the *x*- and *y*-axes indicate significance of the respective parameters (*P < 0.05; **P < 0.01; ***P < 0.001).

Apart from plant water status, other mechanisms known to regulate g_s are related to photosynthesis, to which stomata often present a large limitation. L_s in loblolly pine is usually lower than 65%. Higher L_s may occur but it is usually attributed to low soil water potential or low temperature (Teskey *et al.*, 1986; Sasek and Richardson, 1989; Ellsworth, 2000). In this study, when VPD was high, L_s of 78% was observed at 30°C (Table 1), indicating strong stomatal control of carbon gain in the range of temperature which is optimal for photosynthesis. With increasing T_l, L_s declined. Therefore, photosynthesis of loblolly pine may partly benefit from the decline in L_s at increased temperature, even though the extremely high temperature will set biochemical limits to A and the resulting A may be the same or lower. In contrast to loblolly pine, L_s in poplar was unaffected by T_l and was generally lower than 20%. Low L_s in poplar in this study corresponded to low L_s in poplar observed previously; for example, L_s averaged 10% in two clones of *Populus* (Noormets and Sober, 2001). The lower L_s in poplar compared with loblolly pine may have been related to the ratio of g_s to g_m. Although we did not measure g_m, it is generally lower in conifers than in angiosperm trees (Flexas *et al.*, 2012), suggesting L_s should also be lower. However, because L_s was not lower, we speculate that the ratio of g_s to g_m also differed between the species. The high A_{sat} in poplar might be related to high g_s/g_m,

which could support increased photosynthesis by increasing C_i and keeping [CO_2] at the chloroplasts high. It could also increase nutrient acquisition through increased E, which would enhance photosynthetic capacity. g_m also increases with temperature in a wide range of species (von Caemmerer and Evans, 2015). However, this mechanism does not explain the increase in g_s at supra-optimal temperatures at which A_{sat} becomes low or negative.

Low L_s in poplar was linked to high g_s, which results in low water use efficiency of photosynthesis. The advantage of low L_s, which favours fast-growing species under an unlimited soil water supply, may jeopardize their existence during heat waves when high E depletes available soil water, resulting in increased drought stress, especially under initial conditions of low soil moisture. The effect of variable L_s was further demonstrated by the alteration of C_i in loblolly pine. Normally the ratio of $C_i:C_a$ is highly conserved (Liu and Teskey, 1995), as was observed in poplar when C_i consistently remained at ~300 µmol mol^{-1} at all temperatures (Fig. 3a). However, C_i in loblolly pine was highly variable, ranging from ~165 µmol mol^{-1} at 20°C to ~240 µmol mol^{-1} at 40°C (Fig. 3c), which corresponds with prior observations of high variability in C_i with changing environmental conditions in this species (Green and Mitchell, 1992).

Evaporative cooling

Evaporation of water from the leaf surface can significantly lower T_l (Monteith, 1981; Jones, 1999). As long as stomata remain open, evaporative cooling can mitigate the negative effect of supra-optimal T_a on A during heat waves and can positively affect photosynthesis, yield, and plant survival (Lu *et al.*, 1994; Ameye *et al.*, 2012). Maintaining T_l through regulation of E to minimize stress at high T_a was theoretically suggested (Mahan and Upchurch, 1988) and observations in *Arabidopsis thaliana* indicated that plants regulate water loss and even adjust their architecture to achieve the best cooling effect (Crawford *et al.*, 2012). The magnitude of the cooling effect is often several degrees (Jones, 1999; Feller, 2006). In our study the maximum cooling, 9°C, was observed in poplar at high T_a and high VPD (Fig. 4). This rate of cooling lowered T_l from 49 to 40°C and positive A was observed at this extreme T_a. In contrast to poplar, g_s of loblolly pine was roughly 10 times lower and therefore the maximum cooling effect was only 0.9°C. Consequently, at T_a of 49°C, poplar had positive A and loblolly pine did not. The cooling effect due to stomatal opening at high temperature (under well-watered conditions) is likely to be much more beneficial in species with high g_s than those with low g_s.

Evaporative cooling may help plants survive heat waves, especially when the air is dry. However, this mechanism requires sufficient soil water supply, which relies on high soil water capacity and sufficient hydraulic conductivity. With a long-duration heat wave, high E may result in the depletion of soil water storage and plants will no longer be able to utilize this mechanism to minimize heat stress. This effect was observed in our study: only a very small cooling effect (1.1°C) was observed in drought-stressed trees (Fig. 4b). Nevertheless, evaporative cooling proved to have a significant effect on A and may play an important role in the diurnal regulation of T_l during short-duration heat waves. In addition to soil water availability, elevated $[CO_2]$ affects g_s. Stomatal closure resulting from elevated $[CO_2]$ will to some degree counteract the opening effect of elevated temperature. Results of this study, demonstrating that stomata of poplar are more sensitive to $[CO_2]$ than stomata of loblolly pine, were similar to previous findings on broadleaf and conifer species in general (Medlyn *et al.*, 2001). Therefore, if stomata in broadleaf species close in response to future predicted increases in $[CO_2]$, the difference in the rate of evaporative cooling between broadleaf and conifer species may shrink.

Relationships among g_s, C_i, *and* A

In both species we found that the positive relationship between A_{sat} and g_s observed at lower temperatures was not present at extremely high temperatures. The most obvious impairment occurred at a $T_l > 40$°C, when A_{sat} became negative and yet the stomata remained open (Fig. 3). C_i at this temperature increased and approached the ambient $[CO_2]$ of 400 μmol mol^{-1}. Under these conditions a reduction in g_s would be expected (Hashimoto *et al.*, 2006), but instead the stomata opened even more. These results do not imply that stomata do not react to C_i. Rather, it appears that there was

a direct stomatal response to supra-optimal temperature that overrode the response to C_i.

Many models of g_s assume a fixed relationship between A and g_s regardless of temperature (Ball *et al.*, 1987; Leuning, 1995; Buckley *et al.*, 2003). These models have been widely used and, in a comparison with other models of g_s, provided the best results (Way *et al.*, 2011). Our study also provided evidence of a stable relationship between A_{sat} and g_s at low temperatures (Fig. 3). However, that stability did not hold true at high temperature. As an extreme example, when A_{sat} became negative at temperatures over ~40°C, the ratio $A:C_i$ also became negative in both species. In such a case, the Ball–Berry–Leuning model, which uses that ratio to predict g_s, would provide negative values of g_s. Correctly predicting g_s from photosynthesis and *vice versa*, especially at extreme temperatures during heat waves, will require detailed study of the interplay among A, C_i, VPD, T_l, and possibly other factors driving stomatal regulation, which, when applied simultaneously, can have complex effects (Merilo *et al.*, 2014).

Conclusions

We conclude that T_l has a direct effect on stomatal opening in the two tree species we examined. For accurate predictions of g_s and plant water use this temperature dependency should be taken into account, especially at high temperatures. Elevated $[CO_2]$ reduced g_s of both species but general trends of increasing g_s with increasing T_l remained similar regardless of $[CO_2]$. Along with changes in g_s, T_l also affected L_s to photosynthesis, C_i, and corresponding A. A became negative in both species at extremely high T_l. However, the effect of evaporative cooling, which lowered T_l in the rapidly transpiring poplar, significantly increased A. g_s was decoupled from A at high T_l in both species, which is an indication that substantial changes are likely in gas exchange physiology at high temperatures. Further research should focus on verifying results of this laboratory study in the field, as well as discovering the principles of temperature dependency of stomatal regulation and implementing temperature functions into the models of g_s.

Supplementary data

Supplementary data are available at *JXB* online.

Fig. S1. G_s of poplar and loblolly pine and its dependence on T_a and VPD.

Fig. S2. E and C_i of poplar and loblolly pine and their dependence on T_a and VPD.

Fig. S3. A_{sat} of poplar and loblolly pine and its dependence on g_s at T_a 20–49°C.

Table S1. Regression equations and parameters of models used in Figs 1–5.

Acknowledgements

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