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Contrasting responses of stomatal conductance and photosynthetic capacity to warming and elevated CO$_2$ in the tropical tree species *Alchornea glandulosa* under heatwave conditions

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Abstract

Factorial experiments of combined warming and elevated CO$_2$ are rarely performed but essential for our understanding of plant physiological responses to climate change. Studies of tropical species are particularly lacking, hence we grew juvenile trees of *Alchornea glandulosa* under conditions of elevated temperature (+1.5°C, eT) and elevated CO$_2$ (+400ppm, eC) in a factorial open top chamber experiment. We addressed three questions: i) To what extent does stomatal conductance ($g_s$) reduce with eT and eC treatments?; ii) Is there an interactive effect of eT and eC on $g_s$?; iii) Does reduced $g_s$ as a result of eT and/or eC cause an increase in leaf temperature?; iv) Do the photosynthetic temperature optima ($T_{opt}$) and temperature response of
photosynthetic capacities ($V_{\text{cmax}}, J_{\text{max}}$) shift with higher growth temperatures? The experiment was performed during an anomalously hot period, including a heatwave during the acclimation period. Our key findings are that: 1) the eT treatment reduced $g_s$ more than the eC treatment, 2) reduced $g_s$ caused an increase in leaf temperatures, and 3) net photosynthesis and photosynthetic capacities showed very high temperature tolerances with no evidence for acclimation to the eT treatment. Our results suggest that *A. glandulosa* may be able to cope with increases in air temperatures, however reductions in $g_s$ may cause higher leaf temperatures beyond those induced by an air temperature rise over the coming century.

**Keywords:** photosynthesis, climate change, factorial experiment, tropical forest, warming, carbon dioxide, leaf temperature, $v_{\text{cmax}}, j_{\text{max}}$, temperature optima, open top chamber, photosynthetic capacity

**1. Introduction**

Global atmospheric CO$_2$ concentrations are increasing, as are air temperatures, with both patterns expected to continue in the coming decades. Plants are a critical part of global biogeochemical cycles, at the interface of the atmosphere and the land surface, with forests storing 65% of terrestrial aboveground biomass (Liu et al., 2015). Plants respond to environmental stimuli, with long-term adaptation and short-term acclimation to changes in light, temperature and other conditions. Photosynthesis, evapotranspiration, and respiration are the primary functions of leaves. Our understanding of leaf-level physiology is used to drive vegetation and land surface models, and hence to project future climate. Experimental research on the responses
of forests to elevated CO\textsubscript{2} has been heavily focussed on temperate ecosystems (Leakey et al. 2012) despite tropical forests stocking more carbon than temperate and boreal forests combined (Pan et al. 2011). Similarly, there are very few studies of thermal acclimation on tropical species (Dusenge & Way 2017). Although temperature increases in the tropics are predicted to be smaller than in other regions (e.g. boreal zone, Collins et al. 2013), tropical forests experience much lower diurnal and seasonal variation in temperature than temperate or boreal forests, and over geological time have experienced a relatively stable climate, potentially reducing the acclimation potential of tropical tree species (Janzen 1967, Dusenge & Way 2017). Investigating the responses of tropical tree species to temperature and CO\textsubscript{2} is therefore a research priority.

Increasing air temperatures and atmospheric CO\textsubscript{2} concentrations lead to changes in stomatal conductance ($g_s$) over short and long timescales (Way et al., 2015). In the short-term (instantaneous responses), increasing air temperatures typically lead to a reduction in $g_s$ (Way et al., 2015, Slot & Winter 2017a) due to stomatal closure with increasing vapour pressure deficit ($D$), which prevents excessive water loss under high evaporative demand. At very high temperatures, $g_s$ may actually increase in order to avoid reaching dangerously high leaf temperatures (Slot et al., 2016, Slot & Winter 2017b, Urban et al., 2017, Drake et al., 2018). Evidence of acclimation of $g_s$ to higher temperatures in trees over the long-term is varied, however some species show declines (Way et al., 2015). The instantaneous response of $g_s$ to increased CO\textsubscript{2} is to decrease, which reduces water loss while maintaining a high internal leaf CO\textsubscript{2} concentration ($c_i$) (Gaastra, 1959). Similarly, under long-term CO\textsubscript{2} enrichment, $g_s$ reduces. Such declines in $g_s$ may increase leaf temperature ($T_L$) through reduced
evaporative cooling (under increased air temperatures, reduced evaporative cooling would also depend on the extent of increased $D$, Oren et al. 1999). Higher $T_L$ could push leaves beyond their photosynthetic temperature optima ($T_{opt}$) (Doughty & Goulden 2008, Slot & Winter 2017c), and potentially above their physiological temperature tolerances (O’Sullivan et al., 2017) causing permanent leaf damage under extreme heat conditions (Warren et al., 2011). While the response of $g_s$ to combined elevated CO$_2$ (eC) and temperature (eT) has rarely been tested (Way et al., 2015, Becklin et al., 2017), experiments on eucalyptus (Ghannoum et al., 2010), douglas-fir (Lewis et al., 2002) and loblolly pine (Wertin et al. 2010) showed little interactive effect; if the two do interact and lead to even greater decreases in $g_s$, this would increase $T_L$ further.

Long-term increasing air temperatures and CO$_2$ concentrations are also predicted to induce changes in net photosynthesis, both directly by impacting biochemical processes and indirectly through changes in $g_s$. Increases in $T_L$ either directly from increased air temperatures or indirectly from a long-term reduction in $g_s$ could shift the leaf beyond $T_{opt}$, leading to reductions in photosynthesis. Some experimental studies have shown partial photosynthetic acclimation to increasing temperatures through increases in $T_{opt}$ (Yamori et al., 2014, Slot & Winter 2017b), which could occur due to alterations in membrane fluidity, expression of heat shock proteins, and production of greater quantities of Rubisco activase or a heat-stable Rubisco activase (Yamori et al. 2014). These changes would lead to altered temperature responses of the photosynthetic capacities $V_{cmax}$ (maximum rate of carboxylation) and $J_{max}$ (maximum rate of electron transport). A recent study of four tropical tree species showed that $g_s$ rather than $V_{cmax}$ or $J_{max}$ limited net photosynthesis beyond $T_{opt}$ (Slot &
Winter 2017a), and hence a change to the temperature (or $D$) response of $g_s$ could also be important for shifts in $T_{opt}$. Photosynthetic capacities are also influenced by growth CO$_2$ concentrations. Under high CO$_2$, Rubisco concentrations typically reduce and hence $V_{cmax}$ declines (Way et al. 2015). Decreases in $g_s$ (as a consequence of increased air temperature or CO$_2$) lead to reduced $c_i$ which can reduce assimilation. Under high CO$_2$ concentrations, this effect could be limited if $c_i$ remains above the Rubisco limited portion of the $A$-$c_i$ curve, however the downregulation of $V_{cmax}$ commonly observed results in plants still being Rubisco limited even at high CO$_2$ (Ainsworth & Rogers 2007) and hence reduced $g_s$ could still reduce assimilation (Way et al., 2015).

The effect of decreased conductance on $T_L$ is well understood biophysically (Jones 1992) and is expected to influence $T_L$ under elevated CO$_2$ (Drake et al. 1997), as has been shown in a small number of experiments (e.g. Siebke et al. 2002, Sigut et al. 2015). However, this effect has not been investigated in any tropical species. Furthermore, because $T_L$ and, to a lesser extent, $g_s$ show high temporal variation with changing microclimate (e.g. Fauset et al., 2018), to fully investigate the effect of altered $g_s$ as a response to elevated temperature and CO$_2$ it is necessary to measure $T_L$ and microclimate with a high temporal resolution.

In this study, we address the following questions using a factorial eT x eC open top chamber experiment with juveniles of tropical tree species *Alchornea glandulosa* (Poepp. & Endl) (Euphorbiaceae): i) To what extent does $g_s$ reduce with elevated temperature (eT) and elevated CO$_2$ (eC) treatments?; ii) Is there an interactive effect of eT and eC on $g_s$?; iii) Does reduced $g_s$ as a result of eT and/or eC cause an increase in $T_L$?; iv) Do the photosynthetic temperature optima ($T_{opt}$) and temperature response
of photosynthetic capacities ($V_{cmax}$, $J_{max}$) shift with higher leaf temperatures? 

$A. glandulosa$ is a pioneer species often found, but not restricted to, riverine environments (Pascotto 2006), distributed in the Atlantic forest, western Amazon/Andes and central America (GBIF Secretariat 2017), with over 100,000,000 individual trees estimated to occur in the Amazon (ter Steege et al., 2013). It is utilized as a timber species, produces medicinal compounds and is used for reforestation in the Atlantic forest region. The fruits of this tree are an important food source for birds (Pascotto 2006). This species was also selected because leaf temperature and stomatal conductance field data for congeneric species $Alchornea triplinervia$ were available from the Atlantic forest (Fauset et al., 2018).

2. Methods

2.1 Experimental setup

The study was carried out at the University of São Paulo from February to March 2017 (23.56° S, 46.73° W, elevation 760 m). $Alchornea glandulosa$ seedlings were sourced from a local plant nursery where they were germinated in shade houses before growing for 12 months outside.

The seedlings were moved to the glasshouse in September 2016 and in November transferred into containers (4l PVC pots with one plant per plot). Hoagland fertilizer solution was added every 2 weeks. The experiment was conducted using four polycarbonate open top chambers (OTCs) with modifications (Aidar et al., 2002) located within the glass house. The four treatments were: i) control (aTaC), ii)
elevated CO\(_2\) (ambient temperature, 800 ppm CO\(_2\), aTeC), iii) elevated temperature (temperature 1.5°C above ambient, ambient CO\(_2\), eTaC), and iv) elevated CO\(_2\) and elevated temperature (temperature 1.5°C above ambient, 800 ppm CO\(_2\), eTeC). Each chamber had an air inlet at the base with a fan, and a spiral heater and/or CO\(_2\) gas inlet was present depending on the treatment (Figure S1). Temperature within the chamber was thermostatically controlled using RICS software (Remote Integrated Control System) with the heater switched on or off to maintain a higher temperature than the unheated chambers. No attempt was made to control for differences in \(D\) due to temperature treatments as increases in temperature would be associated with increases in \(D\) under future conditions assuming no change in relative humidity. CO\(_2\) was passively added to the eC treatments through the use of pressurized CO\(_2\) cylinders. The CO\(_2\) concentrations of the eC chambers was monitored daily and the flow into the chambers altered at a valve if the concentration decreased. Further details of the experimental design can be found in Aidar et al., 2002 and de Souza et al. (2008). Ten seedlings were placed into each chamber on 1 February 2017 and allowed to acclimate for one month before measurements began. Vertical height of each seedling was recorded prior to placement in the OTCs, and placement of seedlings into OTCs was stratified to ensure an even spread of vertical heights.

2.2 Microclimate measurements

Within each OTC air temperature (\(T_a\)), relative humidity (\(h\)) and CO\(_2\) concentration were measured at 5 min intervals (Testo 535, Testo Inc., Flanders, NJ, USA). An additional \(T_a\) sensor (107 thermistor, Campbell Scientific) recorded air temperature every 10 s inside each chamber.
2.3 Physiological Measurements

2.3.1 Leaf temperature and leaf surface PAR

The eight healthiest of the ten seedlings in each chamber were selected for measurement of leaf temperature. On each selected seedling, one fully expanded healthy leaf was chosen (typically the fourth or fifth newest leaf). These leaves were formed inside the glass house but prior to movement of the seedling into the OTCs. Prior to selection, we verified that the leaves were photosynthetically active. A two-junction thermocouple (copper-constantan, type T) that measured leaf-to-air temperature difference ($\Delta T_L$) was attached to the abaxial surface of each sample leaf using a piece of breathable tape (Transpore, 3M, St. Paul MN) following the protocol of Fauset et al. (2018). One thermocouple was used per leaf. Absolute leaf temperatures ($T_L$) were calculated from $\Delta T_L$ and $T_A$ in each chamber measured by the thermistors. A photosynthetically active radiation (PAR) sensor built to the specification of Fielder & Comeau (2000) was positioned adjacent to each sample leaf at the same angle and orientation. PAR sensors were calibrated against a quantum sensor (LightScout, Spectrum Technologies, Aurora, Illinois). $\Delta T_L$ and leaf surface PAR were monitored continuously at 10 s measuring frequency between 24 February – 15 March 2017 using two CR800 data loggers and two AM16/32 multiplexers (Campbell Scientific). Measurements of some leaves were terminated between 10 and 15 March. See Fauset et al. (2017, 2018) for further details of these sensors.

2.3.2 Stomatal Conductance
Stomatal conductance ($g_s$) of each leaf temperature sample leaf was measured under growth conditions inside the chambers on 19 occasions over six days (including four days where $g_s$ of each leaf was measured at least four times, 28 February – 7 March 2017) using an SC-1 porometer (Decagon). For each time point, two measurements of $g_s$ were recorded, one from either side of the midrib, and the mean value was used for analysis.

2.3.3 Photosynthetic measurements

The temperature response of photosynthesis was measured using a LI-COR 6400XT portable photosynthesis measurement system (LI-COR, Nebraska). Data were collected from 10 – 18 March 2018. Light response curves on 3 leaves showed saturating photosynthesis at 800 μmol m$^{-2}$ s$^{-1}$ PAR (Figure S2), hence all measurements were taken at 800 μmol m$^{-2}$ s$^{-1}$ PAR using the standard red-blue LED light source. Note that the glasshouse roof was made of a diffusing plastic which reduced the incoming PAR by c. 60 % compared with the outside, and leaf level PAR reached c. 800 μmol m$^{-2}$ s$^{-1}$, varying with leaf angle and orientation. Three seedlings from each OTC were selected for photosynthesis measurements and the leaf measurements were performed on the same leaf as leaf temperature monitoring. Two sets of measurements were made, net photosynthesis-temperature curves ($A$-$T_L$ curves where net photosynthesis at saturating light intensity is measured at different temperatures), and $A$-$c_i$ curves (where net photosynthesis at saturating light intensity is measured at different CO$_2$ concentrations) at three different temperatures. $A$-$T$ curves were run with the CO$_2$ concentration of the relevant OTC (either 400 or 800 ppm CO$_2$) and assimilation was measured at leaf chamber temperatures of 20, 25, 27, 29, 31, 33, 35 and 40 °C, with 5 measurements recorded at each temperature after the
photosynthetic rate and $g_s$ had stabilized. Measurements at 20, 30 and 35 °C were supplemented using the relevant measurements from the $A-c_i$ curves. $A-c_i$ curves used the following sequence of CO$_2$ concentrations (ppm); 400, 200, 100, 50, 400, 600, 800, 1200, 1500, 2000. $A-c_i$ curves were performed at three temperatures, 20, 30, and 35 °C, and each curve was performed twice for each leaf on either side of the midrib. For all measurements, $h$ was maintained as close as possible to 50 % using a combination of desiccant and adjusting the air flow rate; it was difficult to maintain this $h$ at leaf temperatures above 37 °C (on average 46 %, minimum values were 40 %). The temperature of the chamber was mostly controlled using the inbuilt temperature control system. In addition, for most of the measurements the sensor head was placed inside a specially designed temperature control chamber to enable better control of the chamber temperature (Yepes Mayorga 2010). The temperature control box was switched off during measurements but was used to aid the change of chamber temperature between measurements. Measurements were made at an atmospheric pressure in the greenhouse of 92.6 kPa.

2.3.4 Plant growth

Vertical height (from soil surface) and number of leaves of each seedling was measured three times (1 and 21 February, and 16 March). On the latter two measurement days, the length of the seedling from the soil surface to the end of the longest branch was also recorded, and on 16 March the total plant length including all branches was recorded.

2.4 Data analysis
Differences in microclimate between OTCs (air temperature, CO$_2$ concentration, $h$ and $D$) were tested using ANOVA and Tukey post-hoc test.

The effects of the warming and the elevated CO$_2$ treatments on $g_s$ (porometer measurements pooled from all times of day) were tested using two-way ANOVA with a mixed effects model with leaf as a random factor to account for multiple measurements of the same leaves (function ‘lme’ of the R package nlme, Pinheiro et al. 2017). To investigate the response of $g_s$ to microclimate variables and under different treatments, all possible models of PAR and leaf-to-air vapour pressure deficit $D_L$ (where leaf temperature was taken from thermocouple data), with interactions with CO$_2$ treatment and warming treatment were compared using AIC to select the best model with the function ‘dredge’ in R package MuMIn (Bartoń 2017).

Again, a linear mixed effect model with leaf as a random factor was used to account for multiple measurements of the same leaf/seedling. A quadratic effect of time was also included in the model to account for diurnal changes in $g_s$ not directly linked to PAR, temperature or $D_L$. $R^2$ for mixed-effects models are given using as the marginal pseudo $R^2$ that accounts for fixed factors only rather than the conditional pseudo $R^2$ which also accounts for random effects (Nakagawa & Schielzeth 2013) unless otherwise stated; $R^2$ values for mixed effects models were calculated using the function provided in the R package MuMIn. We also estimated the $g_1$ parameter of the optimal stomatal conductance model (Medlyn et al. 2011, Lin et al. 2015) from the A-T$_L$ curve data collected with the LI-COR 6400.

$$g_s = 1.6 \left(1 + \frac{g_1}{\sqrt{D}} \right) \frac{A}{C_a}$$

where $C_a$ is the atmospheric CO$_2$ concentration in the leaf chamber. The model was fit for each leaf, and the $g_1$ parameter was compared between chambers using ANOVA.
Because leaf temperatures are strongly influenced by microclimate (Jones 1993, Fauset et al. 2018), to assess the influence of treatment on $T_L$ it is necessary to compare $T_L$ within microclimatic envelopes. We subsetted the data into envelopes based on leaf-level PAR, chamber air temperature and $D$. The data was split into low (100 – 200 μmol m$^{-2}$ s$^{-1}$), medium (400 – 500 μmol m$^{-2}$ s$^{-1}$) and high (700 – 800 μmol m$^{-2}$ s$^{-1}$) PAR, and low (28 – 30 °C, 1 - 2 kPa), medium (33 – 35 °C, 2 - 3 kPa), and high (38 – 40 °C, 3 - 4 kPa) air temperature and $D$. An unanticipated effect of the switching on and off of the heater in the warmed chambers was a cycle in leaf temperature. This was particularly clear at night, but also occurred during the day. When the heater was switched on, the $\Delta T_L$ became more negative as the air heated faster than the leaf (Figure S3). The $\Delta T_L$ then rose to reach an equilibrium temperature. Because of this cycle in the $\Delta T_L$ data, it was not possible to compare leaf temperatures directly between the ambient and heated chambers, and hence direct comparisons on $\Delta T_L$ were only made between CO$_2$ treatments within temperature treatments.

The temperature response of photosynthesis is typically modelled as a parabolic curve which provides a $T_{opt}$ parameter (e.g. Robakowski et al. 2012). However, as no evidence of a decline of $A$ with increasing $T_L$ was found (see section 3.4), we could not use the parabolic curve to find $T_{opt}$ (Fig. S4) which was beyond the range of our measurements. Hence, a linear mixed effect model with leaf as a random factor was used to test the relationship between $A$ and $T_L$. As for stomatal conductance we selected the best model based on AIC from all possible models, here including $T_L$ as a continuous fixed effect and interactions with CO$_2$ treatment and warming treatment.
$V_{cmax}$ and $J_{max}$ were estimated for each leaf and each temperature from the $A-c_i$ curve using the Farquhar-von Caemmerer-Berry model using the R package plantecophys (Duursma 2015). For some curves (six for $J_{max}$ and one for $V_{cmax}$, all at 20 °C), the parameters could not be adequately estimated and estimates were not used. Of the remaining fits, the root mean square error ranged 0.18 – 1.57 μmol m$^{-2}$ s$^{-1}$. The temperature responses of $V_{cmax}$ and $J_{max}$ were modelled using the Arrhenius function (Medlyn et al. 2002)

$$f(T_k) = k_{25} \cdot \exp \left( \frac{E_a(T_k - 298)}{(298RT_k)} \right)$$

where $k_{25}$ is the value of $V_{cmax}$ or $J_{max}$ at 25 °C, $E_a$ is the activation energy (kJ mol$^{-1}$), $T_k$ is the leaf temperature (°K) and $R$ is the universal gas constant (8.314 J mol$^{-1}$ K$^{-1}$).

The parameters were fit using non-linear least squares (R function nls). This function was fit separately for each chamber, and significant differences in parameter estimates were tested by comparing the 95 % confidence intervals (following Varhammar et al. 2015). A peaked Arrhenius function was not used as the data did not show a decline in $V_{cmax}$ or $J_{max}$ at high temperatures.

3. Results

3.1 Microclimate over the study period

The experimental period coincided with an anomalously hot summer in São Paulo city including a 4 day heatwave (Figure 1). Using the definition of a heatwave from
Russo et al. (2015) as ≥3 consecutive days where the maximum temperature exceeds the 90th percentile of maximum temperatures from a monthly window for the period 1981-2010, and climate data for the Mirante de Santana weather station (INMET), a four day heatwave period occurred (maximum temperatures above 32.3 °C) in mid-February (Figure 1, Figure S5). The heatwave occurred during the acclimation period but before the initiation of data collection. During this time the maximum daily air temperatures within the OTCs exceeded 45 °C (Figure 1).

Mean daily temperatures within the OTCs over the acclimation and measurement periods were significantly different between chambers ($F = 5.4, P = 0.001$, ANOVA, Figure 2a). Temperatures were significantly lower in the aTeC treatment (28.8 ± 2.3 °C mean ± SD) than the eTeC treatment (30.4 ± 2.3 °C), however the difference between aTaC (29.0 ± 2.2 °C) and eTaC (30.3 ± 2.4 °C) was marginally insignificant ($P = 0.07$, Tukey post-hoc test, Figure 1a). Mean daily CO$_2$ concentration was significantly higher in the aTeC and eTeC treatments (829.9 ± 71.6 ppm and 836.7 ± 70.6 ppm, respectively, Figure 1b) than the ambient CO$_2$ treatments, however the concentration in the eTaC chamber (399.0 ± 8.9 ppm) was significantly lower than the aTaC chamber (459.2 ± 12.2 ppm). Relative humidity also varied by treatment with lower values in the elevated CO$_2$ treatments (Figure 2c), and $D$ was higher in elevated temperature treatments, significantly so for eTeC (Figure 2d).

### 3.2 Stomatal Conductance

Analysing $g_s$ data with measurements at all times of day pooled, $g_s$ was significantly lower under the elevated temperature treatments ($P = 0.0001$, mixed effects model
with leaf as a random factor), with no significant effect of CO$_2$ treatment (Figure 3). Conductance was highest in the control treatment and similarly low in both elevated temperature treatments, with an intermediate $g_s$ in the aTeC treatment (Figure 3).

The best mixed effects model of $g_s$ accounting for microclimate and diurnal changes included time of day, PAR, $D_L$, and interactions between $D_L$, warming treatment and CO$_2$ treatment (Figure 4, Table 1). The overall pseudo marginal $R^2$ of the model was 0.38. If the random effect of leaf is also accounted for, the pseudo conditional $R^2$ increases to 0.67 showing that there is high leaf-to-leaf variation in $g_s$ (Supplementary Figure 6). Interaction plots (Figure 4) of the model show that the relationship between $g_s$ and $D_L$ was weak (with no significant effect of $D_L$ alone, Table 1) and varied between treatments (interactions between heat treatment and $D_L$, and heat treatment, CO$_2$ treatment and $D_L$ were significant, Table 1). Under the aTeC and eTeC treatments $g_s$ was fairly invariant with $D_L$, whilst under the eTaC treatment $g_s$ declined with $D_L$ and under the control aTaC treatment $g_s$ increased with $D_L$.

However, there is large scatter in the data (Figure 4, Figure S6).

The parameter $g_1$ (inversely proportional with the carbon cost of transpiration and hence low when a plant is conservative in its water use) estimated from the A-T$_L$ curves did not show any significant differences between chambers, despite a lower mean for the eTaC chamber (Figure 5).

### 3.3 Observed Leaf Temperatures
Diurnal patterns of average $\Delta T_L$, $T_L$, PAR and $D$ are shown for all chambers in Figure 6 based on the period 24 February – 15 March 2017. There are differences in the patterns of average $\Delta T_L$ for each chamber (Figure 6c,d), and these patterns are linked to the patterns of average PAR (Figure 6e,f). In order to properly compare the leaf temperatures between different leaves and chambers, the varying microclimate needs to be accounted for.

Mean $\Delta T_L$ values were not significantly different between elevated and ambient CO$_2$ within the warming treatment under any specified microclimate (Figure 7, eTeC versus eTaC). In contrast, under the majority of microclimates tested $\Delta T_L$ values were significantly higher in the elevated CO$_2$ treatment compared to the ambient CO$_2$ treatment when under ambient temperatures (Figure 7, aTeC versus aTaC). The microclimate conditions under which no significant differences were found were both in the high PAR category where there were much fewer data points, and the pattern in the data was similar to other microclimates. The extent of the difference in $\Delta T_L$ between aTaC and aTeC increased under increasing air temperature and increasing PAR, with a difference of 2.8 °C under high PAR and high air temperature. Analysing the data for $T_L$ rather than $\Delta T_L$ produced the same results (data not shown).

### 3.4 Photosynthetic Temperature Response Curves

Despite measuring photosynthesis at leaf temperatures up to 40 °C, there was no evidence of reaching $T_{opt}$ as $A$ continued to increase with $T_L$ for the majority of leaves (Figure 8). Consequently, estimation of $T_{opt}$ was not attempted and linear models were used to analyse the $A$-$T_L$ curves. There was no significant effect of temperature
treatment, however $T_L$, CO$_2$ treatment and their interaction were included in the best model. $A$ (measured at the growth CO$_2$ concentration) was higher and the slope of the $A$-$T$ relationship was steeper under the elevated CO$_2$ treatments (Figure 8). The marginal pseudo-R$^2$ of the model was 0.53, and all model terms ($T_L$, CO$_2$ treatment and their interaction) were significant (Table 2).

3.5 Temperature Responses of $V_{cmax}$ and $J_{max}$

As for $A$, both $V_{cmax}$ and $J_{max}$ increased with measurement temperature (Figure 9) and no optimum temperature was found within the measurement range (20 – 35 °C). $V_{cmax}$ varied ranged 6.1 – 51.8 μmol m$^{-2}$ s$^{-1}$ and $J_{max}$ ranged 16.3 – 46.3 μmol m$^{-2}$ s$^{-1}$ with standard errors ranging 0.097 – 6.88 μmol m$^{-2}$ s$^{-1}$ for $V_{cmax}$ and 0.29 - 2.76 μmol m$^{-2}$ s$^{-1}$ for $J_{max}$. The higher SE values correspond with higher parameter values. Temperature treatment had no significant effect on either of the two variables, however $V_{cmax}$ was lower and the temperature response of $V_{cmax}$ was weaker (lower activation energy) under elevated CO$_2$, with significant differences between eTaC and eTeC treatments (Figure 9, Table 3). The ratio of $J_{max}/V_{cmax}$ decreased with increasing temperature (30 - 35 °C, not sufficient $J_{max}$ data at 20°C), and was significantly higher in the elevated CO$_2$ treatment (Figure S7).

3.6 Seedling growth

There were no significant effects of treatment on seedling size at any time point during the experiment (vertical height, total branch length, number of leaves, Figure S8).
In this study we present a factorial elevated temperature and elevated CO\(_2\) experiment with juveniles of a tropical pioneer species. The study was performed under high temperature conditions including a heatwave during the acclimation period (Figure 1). Our key findings are i) that the elevated temperature treatment had a stronger influence on \(g_s\) than elevated CO\(_2\) (Figure 3, Table 1), ii) that reduced \(g_s\) caused a change in leaf temperatures (Figure 7), iii) that net photosynthesis and photosynthetic capacities show very high temperature tolerances with no evidence for acclimation to the elevated temperature treatment (Figure 8), and iv) that there was no interactive effect of temperature and CO\(_2\) treatment on \(g_s\) (Figure 3, Table 1).

4.1 Temperature and CO\(_2\) impacts on stomatal conductance

As expected, \(g_s\) declined in the eC treatments compared with the control, as has been shown in many other studies. Here we find a 21.2 \% reduction (95\% CI 10.6 – 30.2 \% based on bootstrapping) in our aTeC treatment compared with the control (Figure 2). In forest free air CO\(_2\) enrichment (FACE) experiments with CO\(_2\) elevated by 200 ppm \(g_s\) declines on average by c. 20 \% (Ainsworth & Rogers 2007), with stronger declines in angiosperm than gymnosperm species (Brodribb et al. 2009). Past chamber experiments performed on angiosperm trees with a doubling of CO\(_2\) show an average \(g_s\) reduction of c. 18 \% (from data in Saxe et al. 1998). Our data therefore shows consistency with species from other biomes, but with few tropical species included in existing studies. The literature on tropical species shows wide variation (Berryman et
from eight publications covering 22 tropical angiosperm species with CO₂ enrichment
in the range 300-400 ppm showed an average change in gs of 28.6 ± 18.4 % SD
reduction. One species (Chrysophyllum cainito) showed a very small increase
(Dalling et al. 2016), and the largest reduction of 61 % was shown by Inga punctata
(Cernusak et al. 2011). Hence, the reduction we observed was below average but well
within the range of observations of other tropical species in experiments.

A limitation of our experiment and its comparability with other studies is the short
duration of exposure to the treatments. We measured the physiological responses on
leaves formed before initiation of the experiment, which had been exposed to the
treatments for c. 5 weeks. As stomatal properties (e.g. density) often differ on leaves
formed in high CO₂ environments (Saxe et al. 1998), there could potentially be
greater changes than we observed, had new leaves formed. Whilst this is quite
possible, the long-term response of gs to CO₂ is typically similar to the short term
response (Way et al. 2015), and hence while the mechanism of reduced gs may be
different in short and long-term studies, the gs may be similar. However, a caveat to
our results is that to truly observe the acclimation of leaves to the treatments, longer
acclimation periods and production of new leaves is necessary.

The observed responses of gs to elevated temperature vary considerably in the few
studies available (Way et al. 2015). Here we find strong reductions in gs in the
temperature treatments with a 49.6 % (95% CI 42.2 – 56.5 %) reduction under the
eTaC treatment and 53.0 % (95% CI 52.9 – 58.3 %) reduction in the combined eTeC
treatment, although we did not find any significant difference between treatments for
the $g_t$ parameter value. This may be because the Medlyn et al. (2011) model
incorporates the ambient CO$_2$ concentration, and if the short-term and long term $g_s$
response to CO$_2$ is the same there would not be a difference. The declines in $g_s$ are not
driven purely by higher $D_L$ in the eT chambers as there are significant differences
even when $D_L$ is controlled for (Table 1, Figure 4) or when $g_s$ is analysed within a
narrow $D_L$ range (data not shown). This shows acclimation of $g_s$ due to higher air
temperature and/or $D_L$ (both quantities strongly co-varied) which will reduce water
loss from the plants. There were no significant differences in $g_s$ between the eTaC and
eTeC treatments, hence the response to the temperature treatment (with significant
differences) was stronger than the response to the CO$_2$ treatment. The result is
surprising given the very mixed results in the limited literature on elevated
temperature impacts on $g_s$, and even more so given that in this study the temperature
treatment was fairly modest (+1.5 °C) compared to the CO$_2$ treatment (+ 400 ppm),
although the effect of eC on $g_s$ may have been limited by the lack of new leaf
development (as stated above). This finding could also be because the ambient
temperatures were very hot inside the chambers throughout the experiment and
especially during the acclimation phase (Figure 1), which meant that a small increase
in air temperature had a large impact, with stomata closing to reduce water loss. An
experimental study of gas exchange of Solanum lycopersicum (cherry tomato)
measured during and following a +14 °C heatwave showed reduced $g_s$ during the
heatwave, which remained low when measured 5 days after the heatwave (Duan et al.,
2016). Similarly, Duarte et al. (2016) found reduced $g_s$ of Pseudotsuga menziesii
(Douglas fir) during +12 °C heatwaves which remained when measured one month
later. This is somewhat in contrast with recent research suggesting stomata remain
open under very high air temperatures for increased evaporative cooling (Slot et al., 2016, Slot & Winter 2017b, Urban et al., 2017, Drake et al, 2018). Responses are likely to be species specific, with an example of a late successional species reducing $g_s$ under heatwave conditions while a pioneer species showed increased $g_s$ (Vargas & Cordero 2013). However these studies are assessing the instantaneous response of $g_s$ to short-term warming rather than the long-term response. A field study reporting the impact of four months of experimentally elevated temperature on $g_s$ of existing leaves showed a c. 25% reduction with 2 °C temperature increase averaged across six tropical species (Doughty 2011), lower than we observed. However, in contrast to our results for Alchornea glandulosa, Yepes Mayorga (2010) found that $g_s$ of Hymenea courbaril was more strongly controlled by elevated CO$_2$ than elevated temperature in a similar study, as did Ameye et al. (2012) in a study of temperate species Quercus rubra and Pinus taeda in treatments of elevated by 320 ppm and $T_A$ elevated by 3 °C or with heat waves. Two studies of subtropical/temperate Eucalyptus spp. found no difference in $g_s$ of under treatments of CO$_2$ elevated by 240 ppm and $T_A$ elevated by 3 °C or 4 °C after 15 and 7 months of acclimation respectively (Quentin et al. 2013, Duan et al. 2018). While more studies are needed to see if there is a general pattern for tropical broadleaf species, the results of this study suggest that there could be larger implications of rising temperature than rising CO$_2$ for water use of at least some species of tropical tree, and even implications of modest temperature rises such as the ambitious aims of the Paris Agreement (UNFCCC 2015).

The $g_s$ dataset also showed a weak relationship with respect to $D_L$, which varied with treatment (Table 1, Figure 3). Other studies with a congeneric species show that $g_s$ of *A. triplinervia* is more weakly linked to $D_L$ than other measured species (Garcia-
Núñez et al. 1995, Fauset et al. 2018). A weaker relationship between \(g_s\) and \(D_L\) is expected for low wood density pioneer species compared to species with higher wood density (Lin et al. 2015). In addition, as the species is commonly found in riparian areas (and therefore with access to a good water supply), its lack of stomatal control is not surprising. Our results show that despite a weak instantaneous response of \(g_s\) to microclimate, \(A. glandulosa\) still showed acclimation and reduction in \(g_s\) in response to long-term microclimate change. Hence, the short-term response of \(g_s\) does not provide information on the long-term response.

4.2 \(CO_2\) impacts on leaf temperature

The lower \(g_s\) as a result of elevated \(CO_2\) caused increases in leaf temperatures (Figure 5). The differences in \(\Delta T_L\) increased with increasing PAR at the leaf surface, and to a lesser extent with increasing air temperature and \(D\). This shows that the differences in leaf temperatures due to \(CO_2\)-altered \(g_s\) are more apparent under high thermal stress conditions (high PAR and high air temperature), and therefore that this impact is likely to be stronger under heat waves, which are expected to increase in frequency during the 21st century (Coumou & Robinson 2013). When at high air temperatures, differences in \(\Delta T_L\) due to reduced \(g_s\) could have significant consequences, as seen in observations of premature leaf senescence during a heatwave in a temperate FACE experiment (Warren et al. 2011). While the average differences in \(\Delta T_L\) between aTaC and aTeC reached 2.8 °C under high light and air temperature, the light conditions were limited by the greenhouse environment which reached only 1000 \(\mu mol m^{-2} s^{-1}\). Under field conditions where incoming PAR can reach over 2500 \(\mu mol m^{-2} s^{-1}\) the impact of reduced \(g_s\) on \(\Delta T_L\) could be much higher. Unfortunately due to \(\Delta T_L\)
fluctuations induced by heating the air (Figure S3) it was not possible to assess the impact of the high temperature treatment compared to the control. Within the two high temperature treatments there were no significant differences in $\Delta T_L$ under any microclimate between the elevated and ambient CO$_2$ treatments, which is expected as they did not show any significant differences in $g_s$.

4.3 Temperature and CO$_2$ impacts on photosynthesis

The elevated temperature treatment had no discernible effect on $A$ or photosynthetic capacity and their responses to elevated temperatures. The high temperature tolerance of both $A$ and photosynthetic capacity was marked, with no decline in $A$ found even at 40 °C. Consequently, we were not able to assess shifts in $T_{opt}$ with treatment as $T_{opt}$ was above the maximum temperature under which we performed measurements. It is worth noting that such high leaf temperatures are often considered to be detrimental to photosynthetic functions (e.g. Rubisco activase activity is strongly temperature sensitive with inhibition found above 35 °C [Crafts-Brandner & Salvucci 2000]). Moreover, photosystem II (PSII) activity declines rapidly above temperature thresholds of 41.5 – 50.8 °C (O’Sullivan et al. 2017). However, plants are well adapted to their environment, with temperature thresholds of PSII increasing from arctic to tropical habitats (O’Sullivan et al. 2017), and even increasing thermal tolerance of PSII over very short timescales (days) in response to high temperatures (Drake et al. 2018). Slot et al. (2017c) found that $T_{opt}$ measured in the field in Panama was around the mean maximum daily temperature (30-32 °C) for all 42 species measured, and that, for a smaller sample of four species, it was $g_s$ rather than Rubisco activase, $J_{max}$, $V_{cmax}$ or light respiration that limited the photosynthetic rates at high
temperatures (Slot & Winter 2017a). In another study, $T_{opt}$ was higher than daily maximum air temperature in moist and wet tropical forest sites in Puerto Rico (Mau et al. 2018). In the case of the *A. glandulosa* seedlings measured here, the mean maximum daily temperature over the acclimation and measurement period was 40 – 42 °C (varying by treatment, Figure 1), matching the minimum potential $T_{opt}$ of 40 °C, and showing tolerance to the high temperatures to which they were exposed. Measurement under higher temperatures would be necessary to find the $T_{opt}$ for these plants. Over the measured temperature range, $g_s$, $V_{cmax}$ and $J_{max}$ did not decline. Yet, it should also be noted that the rates of $A$, $V_{cmax}$ and $J_{max}$ were fairly low (c. 3, 40 and 35 μmol m$^{-2}$ s$^{-1}$ respectively, at the highest values and under ambient CO$_2$). For example, these are lower than $A$ of 12 – 16 μmol m$^{-2}$ s$^{-1}$ across 42 Panamanian species (Slot & Winter 2017c) and 5 – 12 μmol m$^{-2}$ s$^{-1}$ for four species in Puerto Rico (Mau et al. 2018), and $V_{cmax}$ of 70 – 300 μmol m$^{-2}$ s$^{-1}$ and $J_{max}$ of 80 – 220 μmol m$^{-2}$ s$^{-1}$ across four Panamanian species (Slot & Winter 2017a), all at their optimum temperatures. The measured rates are also lower than plants in other high temperature environments e.g. five desert species with $A$ ranging 19 – 35 μmol m$^{-2}$ s$^{-1}$ (Mooney et al. 1981), and Mediterranean cork oak with $V_{cmax}$ and $J_{max}$ both over 150 μmol m$^{-2}$ s$^{-1}$ (Ghouil et al. 2003). Thus, high temperature tolerance of photosynthetic machinery in *A. glandulosa* may come at a cost of lower photosynthetic rates. An alternative explanation for the low photosynthetic rates is the low light conditions within the greenhouse, with maximum leaf surface PAR of 800-1000 μmol m$^{-2}$ s$^{-1}$. In the field, maximum PAR is likely to be much higher (> 2000 μmol m$^{-2}$ s$^{-1}$), and leaves may achieve higher photosynthetic rates. The low light conditions in the greenhouse also have implications for the high temperature tolerance observed. Because at high temperatures photosynthetic biochemistry is under greater stress, there is a greater
need for photoprotection from high incoming radiation. Perhaps under the higher light conditions found in the field, very high temperature tolerance of photosynthesis may be more difficult to achieve. Field studies under high temperature conditions are needed to establish whether the high tolerance we find here also occurs under natural conditions.

As for $g_s$, the impacts of increased CO$_2$ followed expectations from previous studies with increased net photosynthesis when measured at growth CO$_2$, a steeper slope of $A$ in response to temperature, and downregulation of photosynthetic capacity (Figure 8, 9). The steeper slope is due to the reduction in oxygenation of Rubisco due to higher $c_i$ under elevated CO$_2$, which otherwise increases with temperature due to the reduced affinity of Rubisco for CO$_2$ with higher temperature (Long 1991). The effect of the downregulation can be seen when the temperature response of $A$ is plotted with added points taken from the $A$-$c_i$ curves at 400 and 800 ppm CO$_2$ for the elevated and ambient CO$_2$ treatments respectively, showing that without the downregulation of photosynthetic capacity $A$ would have been higher in the elevated CO$_2$ treatment (Figure S9).

4.4 Conclusions

This study demonstrates that the tropical tree species *Alchornea glandulosa* shows strong responses of stomatal conductance to elevated temperature and of photosynthetic parameters to elevated CO$_2$. While a very high temperature tolerance of photosynthesis was observed in this species, photosynthetic rates were low under the high growth temperatures. These results show that this species will be able to cope
with the predicted atmospheric changes over the coming century. Therefore, it is an
appropriate species for reforestation activities, which are planned and ongoing in the
Atlantic forest (Rodrigues et al. 2009). More studies of other species are required to
determine whether similar results occur in other forest trees.

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Table 1. ANOVA results for stomatal conductance linear mixed effects model. Temperature and CO\(_2\) refer to treatment effects. Asterisks denote P values: *** P < 0.0001, ** P < 0.001, * P < 0.05, • P < 0.1, ns not significant.

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<th>Model Term</th>
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<th>Denominator DF</th>
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<tr>
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Table 2. ANOVA results for \(A-T_L\) linear mixed effects model. \(CO_2\) refers to treatment. Asterisks denote P values: *** P < 0.0001, ** P < 0.001.

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<td>Leaf Temperature:(CO_2)</td>
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<td>11.8**</td>
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Table 3. Parameter estimates of Arrhenius functions of the temperature sensitivity of $V_{c\text{max}}$ and $J_{\text{max}}$. Standard errors are given in brackets. Significance of between treatment effects are shown: * $P < 0.05$, • $P < 0.1$, ns not significant. Letters denote differences between treatments.

<table>
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<tr>
<th>Chamber</th>
<th>$V_{c\text{max}25}$</th>
<th>$E_a (V_{c\text{max}})$</th>
<th>$J_{\text{max}25}$</th>
<th>$E_a (J_{\text{max}})$</th>
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<td>81702 (16778) AB</td>
<td>24.95 (1.14)</td>
<td>32187 (4279)</td>
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<td>eTaC</td>
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<td>80644 (7551) A</td>
<td>24.0 (1.84)</td>
<td>27499 (7183)</td>
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<td>aTeC</td>
<td>11.0 (1.75) AB</td>
<td>71004 (13689) AB</td>
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<td>-</td>
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<tr>
<td>eTeC</td>
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<td>62050 (2171) B</td>
<td>21.8 (3.35)</td>
<td>25840 (14184)</td>
</tr>
<tr>
<td>Among Chambers</td>
<td>*</td>
<td>•</td>
<td>ns</td>
<td>ns</td>
</tr>
</tbody>
</table>
Figure 1. Time series of daily maximum temperatures in São Paulo (Mirante de Santana weather station, data from INMET, http://www.inmet.gov.br/portal/, accessed 22/05/2018) and in each experimental chamber during the experiment. The experiment was initiated on 1 Feb 2017. The period classified as a heatwave, periods of leaf temperature and photosynthesis data collection, and days in which diurnal cycles of stomatal conductance were performed are shown.
Figure 2. Differences in microclimate variables between chambers a) mean air temperature, b) mean CO$_2$ concentration, c) mean relative humidity, d) mean $D$. Box plots show daily averaged values from both the acclimation and measurement periods. Treatments: aTaC – ambient temperature and CO$_2$, eTaC – elevated temperature and ambient CO$_2$, aTeC – ambient temperature and elevated CO$_2$, eTeC – elevated temperature and CO$_2$. 
Figure 3. Effect of treatment on stomatal conductance where measurements from all times of day are pooled. Treatments: aTaC – ambient temperature and CO\textsubscript{2}, eTaC – elevated temperature and ambient CO\textsubscript{2}, aTeC – ambient temperature and elevated CO\textsubscript{2}, eTeC – elevated temperature and CO\textsubscript{2}.
Figure 4. The relationship between $g_s$ and leaf-to-air $D$ for each treatment (accounting for time of day ($t$, hours) and PAR as fixed effects and leaf as a random factor). Grey points show the partial residuals of the model. Full model equations: 

- $-1053.2 + 215.6t - 8.6t^2 + 0.13PAR + 45.9D$ (aTaC);
- $-1029.7 + 215.6t - 8.6t^2 + 0.13PAR - 7.0D$ (aTeC);
- $-1074.1 + 215.6t - 8.6t^2 + 0.13PAR - 17.3D$ (eTaC);
- $-1142.5 + 215.6t - 8.6t^2 + 0.13PAR - 2.6D$ (eTeC).

Figure 5. Comparison of the $g_1$ stomatal conductance parameter (unitless) between chambers. Bars show the mean value and error bars the standard deviation.
Figure 6. Diurnal cycles of leaf-to-air temperature difference (a,b), leaf temperature (c,d), PAR (e,f), $D$ (g,h), for chambers with ambient air temperature (a,c,e,g) and elevated temperatures (b,d,f,h).
Figure 7. Leaf to air temperature differences for each treatment under a range of microclimate conditions. Contrasts are made between aC and eC under ambient the temperature treatment, and between aC and eC under the elevated temperature treatment, using mixed effects models with leaf as a random factor. Data is from $\Delta T_L$ measurements at 10 s temporal resolution subsetted for specific chamber air temperature ($T_A$) and $D$ conditions, and leaf surface PAR conditions. Asterisks denote $P$ values: *** $P < 0.0001$, ** $P < 0.001$, * $P < 0.05$, ns not significant.
Figure 8. Temperature response of net photosynthesis. Under high CO$_2$ net photosynthesis is higher and the temperature response is steeper. For ambient CO$_2$ $A = 1.86 + 0.032 \cdot T_L$; for elevated CO$_2$ $A = 1.75 + 0.078 \cdot T_L$.

Figure 9. Temperature response of $V_{\text{max}}$ and $J_{\text{max}}$ fit with Arrhenius functions. For $J_{\text{max}}$ in the high CO$_2$ treatment no values at 20 °C were obtainable. Equation parameters are given in Table 3.