Within-Canopy Experimental Leaf Warming Induces Photosynthetic Decline Instead of Acclimation in Two Northern Hardwood Species

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Northern hardwood forests are experiencing higher temperatures and more extreme heat waves, potentially altering plant physiological processes. We implemented in-situ leaf-level warming along a vertical gradient within a mature forest canopy to investigate photosynthetic acclimation potential of two northern hardwood species, *Acer saccharum* and *Tilia americana*. After 7 days of +3°C warming, photosynthetic acclimation was assessed by measuring differences between heated and control photosynthetic rates ($A_{\text{opt}}$) at leaf optimum temperatures ($T_{\text{opt}}$). We also measured the effects of warming and height on maximum rates of Rubisco carboxylation, stomatal conductance, transpiration, and leaf traits: leaf area, leaf mass per area, leaf nitrogen, and leaf water content. We found no evidence of photosynthetic acclimation for either species, but rather $A_{\text{opt}}$ declined with warming overall. We found slight shifts in LMA and $N_{\text{area}}$, leaf traits associated with photosynthetic capacity, after 1 week of experimental warming. *T. americana* LMA and $N_{\text{area}}$ was lower in the upper canopy heated leaves than in the control leaves, contributing a shift in $N_{\text{area}}$ height distribution in the heated leaves. *T. americana* showed evidence of greater resiliency to warming, with greater thermoregulation, physiological plasticity, and evaportranspiration. As expected, $A_{\text{opt}}$ of *A. saccharum* increased with height, but $A_{\text{opt}}$ of *T. americana* was highest in the sub canopy, possibly due to constraints on leaf water balance and photosynthetic capacity in the upper canopy. Thus, models relying on canopy height or light environment may incorrectly estimate vertical variation of photosynthetic capacity. If these species are not able to acclimate to warmer temperatures, we could see alteration of plant carbon balance of these two key northern hardwood species.

Keywords: *Acer saccharum*, canopy, experimental warming, leaf traits, photosynthesis, *Tilia americana*, thermal acclimation

INTRODUCTION

Heat waves are expected to become more frequent in the northern hemisphere compared to the southern hemisphere with continued climate change (Meehl and Tebaldi, 2004; Gershunov et al., 2009). Northern hardwood forests are already experiencing more extreme temperature events, potentially altering both plant photosynthetic and respiratory capacities and reducing ecosystem level primary productivity (Ciais et al., 2005; Bastos et al., 2014; Filewod and Thomas, 2014).
In addition, there is evidence that upper canopy leaves in mid-latitude ecosystems are currently operating near their thermal thresholds (O’Sullivan et al., 2017; Mau et al., 2018), and photosynthetic decline could be exacerbated in temperate ecosystems due to their characteristically hot, dry summers. Both the overall elevated temperatures and increased number of heat waves could also contribute to shifts or reductions of species ranges (Thomas et al., 2004; Jump et al., 2006). Specifically, climate change is expected to impact the abundance and distribution of northern hardwood key species, such as *Acer saccharum*, *Fagus grandifolia*, and *Tilia americana*, with some evidence predicting a decline or shift to higher latitudes (Iverson et al., 2008; Tang and Beckage, 2010; Treyger and Nowak, 2011), while other models predict the expansion of species, such as *A. saccharum* (Walker et al., 2002). Understanding how warmer temperatures will affect northern hardwood species’ physiology will give us a better understanding of how these forests will respond to climate change.

Photosynthetic thermal acclimation could help alleviate some of the negative impacts of supraoptimal temperatures. Photosynthesis rates generally increase with increasing measurement temperatures up to an optimum rate (\( A_{\text{opt}} \)), after which rates will decline (Berry and Bjorkman, 1980). Declines in net photosynthesis above this optimum temperature (\( T_{\text{opt}} \)) occur as a result of several processes, including increased thylakoid membrane permeability (Bukhov et al., 1999; Zhang et al., 2009), Rubisco activase dysfunction (Wang and Portis, 1992; Salvucci et al., 2001; Zhang et al., 2002), higher rates of photorespiration (Ku and Edwards, 1978), stomatal closure (Farquhar and Sharkey, 1982), and higher rates of daytime respiration [reviewed in (Sage and Kubien, 2007)]. Photosynthetic acclimation occurs either through a shift in \( T_{\text{opt}} \) to a higher temperature, or through a greater capacity to photosynthesize at optimum temperatures (i.e., higher rates of \( A_{\text{opt}} \)) (Way and Yamori, 2014). Mechanisms involved in photosynthetic thermal acclimation include increased membrane stability through physical changes in the thylakoid membrane structure (Huner, 1988; Havaux et al., 1996) and production of more stable isoforms of Rubisco activase (Salvucci et al., 2001; Portis, 2003). In addition to physiological acclimation through positive shifts in \( T_{\text{opt}} \) and/or \( A_{\text{opt}} \), some plants can regulate their leaf temperatures through convective cooling. Leaf traits, such as leaf area and shape can influence leaf thermal regulation (Vogel, 1970). Leaves with smaller areas and high complexity, for example, can have lower boundary layer thickness, which can lead to increased evaporative cooling (Gurevitch and Shuepp, 1990; Nicotra et al., 2008; Leigh et al., 2017).

**Abbreviations:** \( A_{\text{max}}, \) light saturated net photosynthesis (\( \mu \text{mol} \text{ m}^{-2} \text{ s}^{-1} \)); \( A_{\text{opt}}, \) photosynthetic rate at the temperature optima (\( \mu \text{mol} \text{ m}^{-2} \text{ s}^{-1} \)); \( E_{\text{sat}}, \) leaf evapotranspiration; \( g_{s}, \) stomatal conductance (\( \mu \text{mol} \text{ m}^{-2} \text{ s}^{-1} \)); \( \text{LMA}, \) leaf mass per area (\( \text{g cm}^{-2} \)); \( \text{N}_\text{max}, \) nitrogen per leaf area (\( \text{g m}^{-2} \)); \( \text{N}_\text{leaf}, \) maximum nitrogen per leaf mass (\( \text{mg} \text{ g}^{-1} \)); \( T_{\text{air}}, \) air temperature (\( ^{\circ} \text{C} \)); \( T_{\text{opt}} \), leaf temperature (\( ^{\circ} \text{C} \)); \( T_{\text{leaf, maximum}} \), maximum temperature (\( ^{\circ} \text{C} \)); \( T_{\text{opt}}, \) optimum temperature (\( ^{\circ} \text{C} \)); \( \text{WUE}, \) instantaneous water use efficiency (\( \text{A}_{\text{max}}/E_{\text{sat}} \)); \( \text{WUE}_{\text{int}}, \) intrinsic water use efficiency (\( \text{A}_{\text{max}}/g_{s} \)); \( V_{\text{p,max}}, \) maximum rate of Rubisco carboxylation; \( \text{VPD}, \) vapor pressure deficit (kPa); \( \Delta T, \) temperature difference between heated and control leaf (\( ^{\circ} \text{C} \)).
Fewer studies have looked at the effects of warming on temperate forest canopies beyond the seedling developmental stage. Studies have implemented warming on immature trees using warming chambers (Gunderson et al., 2010), by pumping heated air through tubes and into an immature tree canopy (Bauerle et al., 2009), or through passive heating (Yamaguchi et al., 2016). Small scale within-canopy warming, either through branch or leaf warming, is a practical method to investigate the plant physiological effects of warming in forest canopies (Cavaleri et al., 2015). Heated cables have been used to warm mature temperate tree branches (Nakamura et al., 2010), and large, infrared heaters have been implemented within canopies to warm branches and leaves (Nakamura et al., 2016). As far as we are aware, there have only been two examples of leaf-level warming in mature forests canopies, both in tropical ecosystems (Doughty, 2011; Slot et al., 2014). These studies used resistance wires covered in aluminum foil (Doughty, 2011) or heat rope and infrared reflective frames (Slot et al., 2014) to heat upper canopy leaves. Leaf-level warming studies can give us important information on the physiological responses of forest ecosystem upper canopies that are currently unattainable through in-situ ecosystem-level warming studies.

We had two primary study objectives: (1) to develop and test a novel leaf warming device, and (2) to assess whether two northern hardwood species, T. americana and A. saccharum, could acclimate to 7 days of leaf-level +3°C warming. While short-term warming treatments may result in more conservative acclimation responses than warming for longer time periods, studies have found that temperate tree T<sub>opt</sub> can adjust to seasonal temperature variations within 1–5 days (Gunderson et al., 2010; Sendall et al., 2015b). In addition, Smith and Dukes (2017) recently found photosynthetic biochemical acclimation to experimental warming after 7 days. We hypothesized that (i) both species would be able to photosynthetically acclimate to warmer temperatures through shifts in both T<sub>opt</sub> and A<sub>opt</sub>, (ii) T. americana would have a higher resiliency, through higher thermoregulation and higher trait plasticity, to warming in the upper canopy leaves due to characteristic higher stomatal conductance and lower leaf area (Thomas, 2010), and (iii) leaves in the upper canopy for both species would have a higher capacity to acclimate than leaves at lower heights.

**MATERIALS AND METHODS**

**Study Site**

This study was conducted in a mature secondary growth northern hardwood stand dominated by white ash (Fraxinus americana L), sugar maple (Acer saccharum Marshall), basswood (Tilia americana L), and northern red oak (Quercus rubra L), located at the USDA Forest Service Northern Research Station, Forestry Sciences Laboratory in Houghton, MI, USA (N47° 6’ 52.884”, W 88°32’ 52.332’). In 2013, the basal area was 27 m<sup>2</sup> ha<sup>−1</sup> and the stand density was 2,960 trees ha<sup>−1</sup>. Mean stand height was 14 m, with a stand age from 50 to 60 years. A. saccharum comprised 10% of the stand basal area, while T. americana comprised 20% stand basal area. Site elevation is 243 m. The previous 30 years average monthly air temperature ranged from −8.65 to 15.57°C (NOAA National Centers for Environmental Information, 2018). Mean annual rainfall was 86.6 cm and mean annual snowfall is 564 cm. The soil is classified as Michigamme coarse loam (NRCS Soil Survey Staff, 2018). Site description can be found in Potvin and Lilleskov (2017).

**Design**

Experimental warming was conducted on two species, T. americana and A. saccharum, at three canopy positions: understory, sub canopy, and upper canopy (0–2, 6–8, and 12–14 m). Canopy scaffolding (Contur Modular Scaffold, BilJax, Archbold, OH, USA) enabled access to one A. saccharum tree [12.5 m height, 15.6 cm diameter at breast height (DBH)] and two T. americana trees (7.5 and 14 m height; 7.0 and 16.9 cm DBH, respectively). T. americana sub and upper canopy warming was conducted on separate individuals, while A. saccharum sub and upper canopy warming was on the same individual tree. Understory measurements were conducted on three individual saplings per species, ranging from 0.3 to 2.1 m height, located adjacent to the canopy scaffolding. The upper canopy leaves sampled were partially shaded late in the day by an adjacent 16 m tall emergent tree.

Three fully-developed leaves per species per height (18 total) were heated day and night +3.0°C above a nearby control leaf for 7 days. Understory and sub canopy warming was conducted from July 14–21, 2016, and upper canopy warming was conducted August 23–30, 2016. As a result of heater malfunction, one T. americana upper canopy leaf experienced a total of 19 fewer hours of warming than the other heated leaves.

**Leaf Warming**

Individual leaves were heated using 100 watt, 120VAC silicon heating pads (24100, Kat’s, Five Star Manufacturing Group Inc., Springfield, TN). Leaf temperature was monitored using 30 AWG copper-constantan thermocouple wire [TT-T-30 SLE(RH5)], OMEGA Engineering, Inc., Norwalk, CT, USA) wired to a solid-state thermocouple multiplexer (AM25T, Campbell Scientific Inc., Logan, UT, USA) connected to a data logger (CR1000, Campbell Scientific Inc.). Thermocouples were adhered on the abaxial side of the heated and control leaves using breathable medical tape (Slot et al., 2016). Thermocouples were extended using 24 AWG copper-constantan thermocouple wire or 20 AWG for thermocouples that extended more than 15 meters, to ensure that current resistance did not exceed 100 Ω (TT-T-20 and TT-T-24 OMEGA Engineering Inc.) using thermocouple connectors (SMPW-CC-T-MF, OMEGA Engineering Inc.). Heating pad temperature was controlled using a 24-380VAC SSR-25 DA solid state relay module (SSR-25 DA, Fotek Controls Co., Taiwan) wired into a digital output module (SDM-CD 16D, Campbell Scientific Inc.). The heating pad turned off when the heated leaf temperature reached more than 3°C above the control leaf temperature. A datalogger heating program monitored leaf temperature every 15 s, and instantaneous leaf temperatures were recorded every 2 min. The heating pads were attached to a metal frame 7–12 cm below the leaf and secured to the metal frame using metal mesh, which also prevented direct exposure of thermocouple to the heating pad (Figure 1). Control and heated leaves were selected to ensure that the heated leaf was
exposed to a similar ambient environment to the control leaf, including height and shade.

Environmental Monitoring
Air temperature and relative humidity were monitored using HOBO sensors (U23 Pro V2, Onset Corp, Bourne, MA, USA) placed on the canopy access scaffolding at heights of 0.5, 6.25, and 12.5 m. In July, the air temperature sensors were placed on the southeast side of the scaffolding and were moved to the northwest side of the tower in August. Air temperature and relative humidity were also measured in an adjacent open field (Vaisala temperature and relative humidity probe HMP50-L, Campbell Scientific Inc.).

Gas Exchange and Leaf Traits
After 1 week of experimental warming, gas exchange (net photosynthesis, stomatal conductance, and leaf evapotranspiration) response to temperature was measured on each individual heated and control leaf. Gas exchange measurements were conducted using an open-system LI6400 infrared gas analyzer fitted with a 6400-88 expanded temperature kit (Li-COR Inc., Lincoln, NE, USA). Photosynthetic response to temperature was measured at nine temperatures (17, 20, 23, 25, 30, 33, 35, 37°C); although we were unable to reach 37°C for some measurements. Due to difficulties reaching a low enough temperature to extract the parameter $T_{opt}$, we included an additional 15°C temperature measurement to A. saccharum understory and sub canopy temperature curves. Based on photosynthetic light response curves measured prior to leaf warming (data not shown), photosynthetic photon flux density was controlled at 800 µmol m$^{-2}$ s$^{-1}$ for the understory and 1,200 µmol m$^{-2}$ s$^{-1}$ for the upper canopy leaves. CO$_2$ concentration was controlled at 400 ppm. Flow was controlled between 200 and 500 µmol m$^{-2}$ s$^{-1}$ to keep the vapor pressure deficit (VPD) between 1 and 2 kPa; although, at temperatures above 33°C, VPD often reached above 2 kPa. At temperatures below 20°C, VPD was often slightly below 1 kPa.

To gain insight into mechanistic drivers of temperature response and acclimation, we measured a variety of leaf traits on all experimental leaves, including leaf mass per area (LMA), leaf water content, leaf nitrogen on both area and mass bases ($N_{area}$ and $N_{mass}$, respectively), % leaf carbon (%C), maximum rate of Rubisco carboxylation ($V_{max}$), stomatal conductance ($g_s$), and leaf evapotranspiration ($E_{leaf}$). Stomatal conductance and evapotranspiration were measured concurrently with photosynthesis using the LI6400 (Li-COR Inc.). After completing gas exchange measurements, sampled leaves were placed in a sealed plastic bag in an ice cooler for no longer than 2 h, weighed for fresh mass, and immediately placed in a −20°C freezer. At the conclusion of the experiment, frozen leaves were thawed and measured for leaf area using a desktop scanner (HP Deskjet 4480) and ImageJ v1.50 image analysis software (Schneider et al., 2012). Leaves were placed in a 60°C drying oven for at least 72 h for dry mass (g). LMA was obtained by dividing the dry mass (g) by total leaf area (cm$^2$). Leaf water content was calculated by subtracting dry mass (g) from fresh mass (g), dividing by fresh mass (g), and multiplying by 100 to calculate percent water content (%). Dried leaves were ground to a fine powder in a ball bearing grinder (8000 M Mixer/Mill, Spex Sample Prep, Metuchen, NJ, USA) and analyzed for % carbon (C) and % nitrogen (N) using a combustion analyzer (ESC 4010, Costech Analytical Technologies Inc., Valencia, CA, USA).

Leaf Scorching
Due to an artifact of this leaf warming method, the temperature difference between paired heated and control leaves ($ΔT$) was often >3°C before the digital output module turned the heater off. Leaf scorching, defined as visible leaf necrosis, was assessed on all heated leaves to account for possible damage to photosynthetic apparatus caused by spikes in leaf temperature. Percent leaf area scorched was calculated on scanned leaf images using ImageJ software.

Data Analysis
Warming device efficacy was determined by examining the average $ΔT$ across species, canopy position, sample month, and time of day (daytime or nighttime). To assess the effect of species and height on temperature spiking, heated leaf maximum temperature ($T_{LeafMax}$) and the frequency of time points where heated leaves were 10°C higher than control leaves ($ΔT >10°C$) for each leaf pair were compared between species and canopy positions using two-way ANOVAs and post-hoc contrasts.

Photosynthetic acclimation is denoted by a positive shift in $T_{opt}$ or an increase in the photosynthetic rate at $T_{opt}$ ($A_{opt}$). In order for a $T_{opt}$ shift to result in enhanced photosynthetic performance, an upregulation of photosynthesis at the new growth temperature must also occur. For example, a positive shift in $T_{opt}$ could occur at the same time as decreased $A_{opt}$, which is considered a detractive adjustment to photosynthesis, as opposed to constructive adjustments that would occur with positive shifts in $T_{opt}$ and $A_{opt}$ (Way and Yamori, 2014). $T_{opt}$ and $A_{opt}$ were determined by fitting individual temperature response curves to
the second order polynomial regression model (Cavieres et al., 2000) (Figures 2A,B):

\[ A_{max} = aT_{leaf}^2 + bT_{leaf} + c \]  

(1)

where \( A_{max} \) is light saturated CO\(_2\) assimilation (\( \mu \)mol m\(^{-2}\) s\(^{-1}\)) at leaf measurement temperature (\( T_{leaf} \)) (°C). \( T_{opt} \) is calculated from the first derivative of the polynomial equation:

\[ T_{opt} = \frac{-b}{2a} \]  

(2)

\( A_{opt} \) is extracted by setting \( T_{leaf} = T_{opt} \) in Equation (1) and solving for \( A_{max} \). The polynomial regression model was not able to capture \( T_{opt} \) of 9 of the 18 \( A. \) saccharum curves. For these curves, we took the temperature at the maximum \( A_{max} \) value from each individual curve and treated this value as \( T_{opt} \). The inability to fit positive polynomial curves to these data is likely due to the very low response to temperature in \( A. \) saccharum understory and sub canopy leaves (Figure 2A); which, can likely, in part, be due to the very low stomatal conductance recorded in these leaves (Figure S1A).

In order to explain any possible differences in photosynthetic rates between heated and control leaves, stomatal conductance at the photosynthetic optimum temperature (\( g_s \) at \( T_{opt} \)), maximum rate of Rubisco carboxylation (\( V_{cmax} \)) at the photosynthetic optimum temperature, and equation parameters of evapotranspiration (\( E_{leaf} \)) response to temperature was compared between treatment, species, and canopy position. \( g_s \) was modeled using Equation (1) and substituting \( g_s \) for \( A_{max} \) (Figures S1A,B). In the cases where \( T_{opt} \) was extracted at the maximum \( A_{max} \) value, \( g_s \) at \( T_{opt} \) was also taken as this leaf temperature.

\( V_{cmax} \) was calculated for each \( A_{max} \) value using the one point method (De Kauwe et al., 2016a,b). The one-point method works under the assumption that light saturated photosynthesis (\( A_{max} \)) is limited by Rubisco carboxylation instead of RuBP regeneration, or photosynthetic electron transport. The constants for the apparent \( V_{cmax} \) (\( \hat{V}_{cmax} \)) were estimated based on Bernacchi et al. (2001) estimation of Michalis constants for CO\(_2\) and O\(_2\) temperature dependencies. The CO\(_2\) compensation point was estimated from Crous et al. (2013). Because the one-point method uses internal CO\(_2\) concentration to calculate \( \hat{V}_{cmax} \), we removed all data points that had CO\(_2\) concentration <50 and >500 ppm, which resulted in the removal of 12 out of 192 data points (4.1% of the data). \( \hat{V}_{cmax} \) was fitted to \( T_{leaf} \) using an Arrhenius equation (Medlyn et al., 2002) (Figures S1 C,D):

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

(3)

Where \( T_k \) is the temperature in Kelvin, \( k_{25} \) is the rate of \( \hat{V}_{cmax} \) at 25 °C, and \( E_a \) is the activation energy, or exponential rise, of the \( \hat{V}_{cmax} \) to increasing temperature. \( \hat{V}_{cmax} \) at \( T_{opt} \) was calculated by substituting \( k_{25} \) and \( E_a \), estimated from individual temperature response curves, and substituting \( T_{opt} \) (K) into Equation (3). Evapotranspiration (\( E_{leaf} \)) parameters were estimated for each individual \( E_{leaf} \)-\( T_{leaf} \) response curve by fitting a regression equation where the leaf temperature was exponentially transformed to each response curve (Figures 2C,D):

\[ E_{leaf} = \beta_0 + \beta_1 T_{leaf} \]  

(4)

where \( \beta_0 \) is the intercept and \( \beta_1 \) describes the exponential rise in \( E_{leaf} \) with increasing temperature. Differences between \( A_{opt} \), \( T_{opt} \), \( \hat{V}_{cmax} \) at \( T_{opt} \), \( g_s \) at \( T_{opt} \), \( E_{leaf} \) (intercept, \( \beta_0 \)), and \( E_{leaf} \) (exponential rise, \( \beta_1 \)) values between treatment, species, and canopy position were compared using mixed effects models that accounted for individual tree as the random effect and species, treatment, and canopy position as the fixed effects. Mean separation was compared using post-hoc contrasts.

To examine if the response of water use efficiency (WUE) to temperature varied between treatments, species, and canopy position, we calculated instantaneous water use efficiency (WUE), calculated as \( A_{max}/E_{leaf} \), and intrinsic water use efficiency (WUE\(_{int} \)), calculated as \( A_{max}/g_s \). WUE and WUE\(_{int} \) response to temperature was modeled using a mixed effects model where \( T_{leaf} \), species, treatment, and canopy position were the fixed effects and individual tree was the random effect. WUE slopes and intercepts were extracted and compared with post-hoc contrasts using the FSA package (Ogle, 2018) in R Statistical software (R Core Team, 2015).

To identify environmental differences between species and across canopy vertical gradients, we investigated maximum and mean leaf temperatures between species and maximum and mean leaf and air temperatures along the canopy vertical gradient. Average and maximum leaf temperatures of control leaves were compared for each canopy position and species using two-way ANOVAs and post-hoc contrasts. Average and maximum air temperatures were compared at each canopy position using a one-way ANOVA and Tukey's post-hoc mean separation. The canopy air temperature and relative humidity sensors were moved between the July and August sampling. To account for differences in canopy temperatures between sample months, we used a Welch’s t-test to measure the difference between in July and August using air temperature measurements collected in an adjacent open field.

Leaf trait differences between treatments, species, and canopy positions were analyzed to investigate possible drivers of photosynthetic rates across canopy positions and photosynthetic acclimation. Leaf traits (\( N_{mass} \), \( N_{area} \), leaf area, LMA, %C, and leaf water content) were compared for differences across treatment, species, and canopy position using a mixed effects model where treatment, species, and canopy position were the fixed effects and individual tree was the random effect and post-hoc contrasts. In order to elucidate drivers of photosynthetic rates and leaf water content, \( A_{opt} \) responses to leaf traits (LMA, \( N_{area} \), and \( N_{mass} \)) and leaf water content correlation with \( E_{leaf} \) (Intercept) were assessed for differences in species and treatment using mixed effects models with tree as the random effect. All statistical analyses were performed using R Statistical Software (R Core Team, 2015). Mixed effects models were analyzed using the “nlme” package in R (Pinheiro et al., 2018).
RESULTS

Air Temperature, Leaf Temperature, and Warming Device Performance

Maximum and mean daily leaf and air temperatures were consistent across all canopy heights for both of our study species. Neither daily maximum nor daily mean air temperatures differed across canopy positions (Figures S2A,C; Table S1), and we found no difference in mean daily $T_{air}$ between canopy months when measured in an adjacent open field [20.15 ± 1.31°C for July, 19.56 ± 0.80°C for August (mean ± SEM)] (Table S1). Mean daily $T_{leaf}$ of unheated foliage did not differ across canopy position or between species (Figure S2B; Table S1). Control leaf maximum daily temperatures ($T_{LeafMax}$) showed an almost significant species × canopy position interaction; however, post-hoc mean separation found no significant differences (Figure S2D; Table S1).

The leaf-level warming device effectively increased temperatures of treated leaves +3°C compared to paired control leaves over 24 h for both species at all canopy positions. Mean differences between heated and control leaves ($\Delta T$) across species, canopy position, sample month (July and August), and daytime vs. nighttime ranged from 2.91 to 3.14°C (Table S2). Temperatures of the warmed leaves were more variable than controls (Figure 3A); however, average $\Delta T$ values were close to 3°C at all canopy positions (Table S2; Figure 3B).

Moderate temperature spiking did occur in all heated leaves, and we found some evidence of leaf scorch, primarily in $A.$ saccharum. The occurrences of temperature spiking differed across canopy positions and between species. While $A.$ saccharum heated leaf $T_{LeafMax}$ was higher in the upper canopy than the sub canopy and understory, $T.$ americana $T_{LeafMax}$ did not vary with canopy height (Figure 4A). There was no difference in $A.$ saccharum and $T.$ americana heated $T_{LeafMax}$ in the understory or sub canopy, but $A.$ saccharum heated $T_{LeafMax}$ was higher than $T.$ americana in the upper canopy by ~9°C. The mean % frequency $\Delta T$ > 10°C was <1.2% for all canopy positions for both species (Figure 4B), and there were no effects of species, canopy position, or their interaction (Table 1). Leaf scorching was found on 5 of the 18 total heated leaves: four $A.$ saccharum and one $T.$
americana. A. saccharum showed some degree of scorching on one leaf in the upper canopy (9% of leaf area scorched), two leaves in the sub canopy (10% and 2%), and one leaf in the understory (17%). Only one T. americana leaf, located in the understory, exhibited scorching on 1% of its leaf area.

Leaf Level Acclimation and Within-Canopy Differences in Gas Exchange Parameters

There was no evidence of photosynthetic acclimation for either A. saccharum or T. americana after 1 week of experimental warming, but there was evidence of overall reduced rates of photosynthetic capacity in the warmed leaves, indicating detractive adjustment with experimental warming. We found no warming treatment effects on optimum temperature for photosynthesis (T_{opt}), stomatal conductance (g_s) at T_{opt}, maximum rate of Rubisco carboxylation (V_{cmax}) at T_{opt}, or leaf evapotranspiration (E_{leaf}) parameters for either study species (Table 2; Figure 5). We did, however, find a significant overall treatment effect for rates of photosynthesis at optimum temperatures, where warmed leaves showed slightly lower A_{opt} than control leaves (p = 0.020; Table 2; Figure 5B).

Optimum temperature was consistent throughout all canopy positions, while patterns with height of both optimum rates of photosynthesis, stomatal conductance, evapotranspiration, and the rate of Rubisco carboxylation differed by species. There were no significant treatment interactions for T_{opt}, A_{opt}, V_{cmax} at T_{opt}, g_s at T_{opt}, or the intercept term of E_{leaf} (Table 2); therefore, treatments were pooled and analyzed for differences between canopy positions and species (Figures 6, 7). Neither the mixed effects model nor post-hoc contrast detected any differences between species or between canopy positions in the values of T_{opt} (Figure 6A; Table 2). A. saccharum upper canopy A_{opt} was twice that of understory and sub canopy levels, while T. americana sub and upper canopy A_{opt} values were more than double understory A_{opt} (Figure 6B). Rates of A_{opt} of T. americana were greater than rates of A. saccharum in the upper and sub canopy (Figure 6B). V_{cmax} at T_{opt} was higher in T. americana than A. saccharum at all canopy positions. T. americana sub canopy V_{cmax} was double the rate of the understory and upper canopy and A. saccharum V_{cmax} at T_{opt} was consistent throughout all canopy positions (Figure 6C). Stomatal conductance at optimum temperatures (g_s at T_{opt}) showed similar patterns with species and canopy position as did A_{opt}; however, there were no differences between species in the understory and upper canopy (Figure 7A). A. saccharum E_{leaf} intercept followed a similar pattern to A. saccharum g_s at T_{opt}, where the upper canopy had higher rates of E_{leaf} compared to the understory and sub canopy. T. americana E_{leaf} intercept was highest in the sub canopy and the upper canopy E_{leaf} intercept was higher than the understory (Figure 7B).

The mixed effects model comparing the effects of T_{leaf}, treatment, species, and canopy position on both instantaneous water use efficiency (WUE, A_{max}/E_{leaf}) and intrinsic water use efficiency (WUE_{int}, A_{max}/g_s) showed interaction effects across most variables (Table S3); however, post-hoc analyses primarily distinguished differences between A. saccharum sub canopy heated and control leaves (Figure S3). Overall, WUE and WUE_{int}
were weakly correlated with \( T_{leaf} \), and significant correlations only occurred for some species, canopy position, and treatment combinations (Table S4). The only significant *A. saccharum* WUE vs. \( T_{leaf} \) correlation occurred in heated leaves located in sub (WUE \( p = 0.016 \) and WUE\(_{int} \) \( p < 0.001 \)) and upper (WUE \( p = 0.011 \)) canopy position, which all had decreasing slopes (Table S4; Figures S3A,C). *T. americana* water use efficiency tended to have a negative response to temperature, where only the sub canopy heated leaf (both WUE and WUE\(_{int} \)) and the upper canopy heated leaf (WUE\(_{int} \) only) did not decrease with increasing temperature (Table S4; Figures S3B–D). The mixed effects model showed some treatment effects; however, post-hoc analysis only distinguished treatment differences between *A. saccharum* sub canopy (WUE slope \( p = 0.030 \), WUE intercept \( p < 0.001 \), WUE\(_{int} \) slope \( p = 0.004 \), and WUE\(_{int} \) intercept \( p < 0.001 \)) and *T. americana* upper canopy WUE intercept (\( p = 0.050 \)) (upper case letters, Table S4). *A. saccharum* sub canopy heated leaf had a more negative slope than the control leaf, while *T. americana* upper canopy heated leaf intercept was higher than the control leaf (Figure S3A). There were very few differences detected between species (Table S3).

### Leaf Traits

Experimental warming had no effect on leaf area or leaf water content; however, both leaf traits were higher for *T. americana* than *A. saccharum*. *A. saccharum* leaf area did not vary with height (Table 3; Figure 7C); however, leaf area in the *T. americana* sub canopy was \( \sim60\% \) higher than in the understory or upper canopy and was greater than that of *A. saccharum* in both sub and upper canopy (Figure 7C). For both species, leaf water content declined linearly with increasing canopy height, and was greater for *T. americana* than *A. saccharum* at all canopy positions (Table 3; Figure 7D).

Overall, both LMA and \( N_{area} \) increased with canopy height and had opposing treatment responses between *A. saccharum* and *T. americana*, whereas, \( N_{mass} \) and %C had no treatment effect and the height response differed between species. Canopy position had a strong positive effect on both *A. saccharum* and *T. americana* LMA and \( N_{area} \) (Figures 8A–D). LMA showed evidence of a \( \sim22\% \) decrease with warming, but only in the upper canopy and only for *T. americana* (near significant 3-way interaction; Table 3; Figure S4B), while *A. saccharum* showed no treatment effect on LMA at any canopy position (Table 3; Figure S4A). Both species showed \( N_{area} \) treatment effects in the upper canopy only, but in opposite directions (Figures S4C,D), a pattern which mirrored LMA (Figures S4A,B). *A. saccharum* upper canopy \( N_{area} \) was greater than sub canopy or understory for both heated and control leaves (Figures 8C,D). *T. americana* control leaf \( N_{area} \) steadily increased from the understory to the upper canopy (Figure 8C), while heated leaf \( N_{area} \) increased from the understory to sub canopy but did not increase from the sub to upper canopy (Figure 7D). This resulted in a 39% higher \( N_{area} \) for *T. americana* upper canopy control leaf than the sub canopy leaf (\( p = 0.001 \), Figure 8B); however, heated sub and upper canopy leaves did not differ in \( N_{area} \) (Figure 8C). Heated upper canopy \( N_{area} \) was slightly greater than control leaf \( N_{area} \) (14%, \( p = 0.019 \)) in *A. saccharum* leaves (Figure S4C). *A. saccharum* \( N_{mass} \) did not change with height, while \( N_{mass} \) of *T. americana* was greatest in the sub canopy (Figures 8E,F). *T. americana* \( N_{mass} \) was significantly greater than *A. saccharum* at all canopy positions (Figures 8E,F). There

| Species          | Max daily heated \( T_{leaf} \) | % Frequency \( \Delta T > 10^\circ C \) |
|------------------|-------------------------------|--------------------------------------|
| *A. saccharum*   | 0.002**                       | 0.907                                |
| *T. americana*   | 0.271                         | 0.296                                |
| Species x Canopy position | <0.001***                   | 0.447                                |

Heated leaf daily maximum leaf temperature and percentage of occurrences where the difference heated and control \( T_{leaf} \) was \( >10^\circ C (\Delta T > 10^\circ C) \) p-value results of treatment, canopy position, and the interaction effect of species and canopy position. **\( p < 0.01 \), ***\( p < 0.001 \). Understory and sub canopy daily maximum \( T_{leaf} \) \( n = 9 \), where \( n \) is the number of days used to calculate the mean. *A. saccharum* upper canopy \( n = 8 \), *T. americana* upper canopy \( n = 10 \).
were no significant treatment effects or treatment interaction effects found for $N_{\text{mass}}$ (Table 3: Figures S4E,F). *A. saccharum* had a higher %C than *T. americana* in the understory and mid canopy control leaves, but not in the heated leaves. %C tended to increase with canopy height for *A. saccharum* and *T. americana* control leaves but was consistent throughout the canopy in the heated leaves (Figures 8G,H). There were also no significant treatment or treatment interaction effects for %C (Table 3: Figures S4G,H).

All photosynthetic leaf gas exchange parameters and leaf traits ($A_{\text{opt}}$ vs. LMA, $N_{\text{area}}$, and $N_{\text{mass}}$) were correlated, while leaf water content and $E_{\text{leaf}}$ intercept was not (Table S5). There were no species differences in the slope response of any gas exchange parameters to any leaf traits; however, there were differences in intercepts (Table S6). *T. americana* had a higher $A_{\text{opt}}$ per LMA intercept ($4.03 \mu\text{mol m}^{-2} \text{s}^{-1}$) than *A. saccharum* ($-0.938 \mu\text{mol m}^{-2} \text{s}^{-1}$; $p = 0.003$). *A. saccharum* had higher $A_{\text{opt}}$ per $N_{\text{mass}}$ intercept ($-1.94 \mu\text{mol m}^{-2} \text{s}^{-1}$) than *T. americana* ($-4.49 \mu\text{mol m}^{-2} \text{s}^{-1}$; $p = 0.032$); however, there were no differences in $A_{\text{opt}}$ per $N_{\text{area}}$ between the two species ($p = 0.485$). $T. americana$ also had a slightly higher leaf water content per $E_{\text{leaf}}$ (intercept) than *A. saccharum* ($p = 0.026$; Figure S5). The only significant treatment interaction occurred for $A_{\text{opt}}$ response to $N_{\text{area}}$ where post-hoc comparison showed that *T. americana*
heated leaf had double the slope of the control leaf \((p = 0.043, \text{ Table S6})\).

**DISCUSSION**

**Photosynthetic Acclimation and Responses to Warming**

Contrary to our hypotheses, neither study species showed evidence of photosynthetic acclimation (either an increase in \(T_{\text{opt}}\) or \(A_{\text{opt}}\)) at any canopy position. It is possible that 1 week of warming was not enough time to allow for acclimation; although, photosynthetic acclimation has been found to occur after 1 week of experimental warming (e.g., Smith and Dukes, 2017). In addition, our low sample size might have limited our ability to detect statistical significance. Most warming studies measure acclimation response after a set time that leaves are exposed to warmer temperatures, instead of throughout the warming experiment, making it difficult to pinpoint an acclimation timeframe. Gunderson et al. (2000) showed that \(A. \text{saccharum}\) seedlings have the capacity to acclimate to +4°C
warmer temperatures through a shift in $A_{opt}$. While we did not see any positive shifts in $A_{opt}$ or $T_{opt}$ with our experiment (Figure 5), a study conducted on taller trees during a different growing season that was located about 72 kilometers away from our study site (that experienced maximum daily temperatures similar to our study) found $A.\ saccharum$ $T_{opt}$ to be much higher than the leaves in our study site ($\sim$27°C at 12.5 m, compared to our average of 20.2°C) (Mau et al., 2018), suggesting that higher $T_{opt}$ can be found within this species.

It is also possible that thermal acclimation is more likely to occur when experimental warming is applied to entire plants as opposed to individual leaves or that acclimation is more likely to occur when the heated treatment is applied to leaves that are not fully developed. When warming individual leaves as opposed to entire plants, there is a potential to miss important aspects of plant physiological acclimation. For example, stomatal closure is induced through the signaling of the hormone abscisic acid, a reaction often induced by leaf importation of abscisic acid produced in roots (Davies and Zhang, 1991; but not always Sampaio Filho et al., 2018). This suggests a possibility that warming of leaves might not induce the same amount of stomatal closure as whole-plant warming. While there are disadvantages to heating individual leaves instead of whole plants, leaf-level warming studies can give us important information on the physiological responses of forest ecosystem upper canopies that are currently unattainable through in-situ ecosystem-level warming studies. Physiological acclimation has also been found to occur more readily in plant tissues that are not fully developed (Turnbull et al., 1993); however, warming individual plant tissues, instead of whole plants, introduces carbon sink-source interactions that are difficult to parse from one another. Fully-developed leaves lose their capacity to import carbohydrates upon maturation (Turgenev, 2006). As such, mature leaves can only use the carbohydrates they produce themselves for both maintenance and growth. In contrast, developing leaves are able to import carbohydrates from other parts of the plant. Therefore, our leaves were treated as independent units with respect to whole-tree source-sink dynamics, and by warming mature leaves our results were not affected by changes in sink activity of our experimental leaves.

Instead of $A_{opt}$ thermally acclimating to warmer temperatures, we found evidence of photosynthetic decline in the heated leaves (Figure 5B). Other warming studies conducted on $A.\ saccharum$ have found evidence of photosynthetic decline in both seedlings (Filewod and Thomas, 2014) and saplings (Gunderson et al., 2000), even in some cases where the trees show positive $T_{opt}$ acclimation (Gunderson et al., 2000). In addition, the only other leaf-level canopy warming experiment to study photosynthesis also found evidence of photosynthetic decline (Doughty, 2011). In our study, the decline in $A_{opt}$ is likely due to a decline in the functioning of photosynthetic machinery because we did not detect a decline in either $V_{\text{max}}$ or $g_s$ with experimental warming (Table 2; Figures 5C,D). Leaf scorching discovered in our heated leaves shows further evidence of potential photosynthetic apparatus damage. While our study only investigated photosynthetic acclimation and responses at the leaf level, declines in $A_{opt}$ have important implications for larger, plant-scale carbon gain. If CO$_2$ release through respiration does not equally acclimate to the declines in photosynthesis, plant carbon balance could be negatively affected (Drake et al., 2016).

### Potential Resilience to Future Warming: Comparing Species

$T.\ americana$ showed a greater resiliency to warming compared to $A.\ saccharum$, possibly through more efficient thermoregulation. Maximum temperatures of $A.\ saccharum$ heated leaves were higher in the upper canopy, while $T.\ americana$ maximum temperatures were consistent between canopy positions (Figure 4A). $A.\ saccharum$ also had more leaf scorching than $T.\ americana$. Lower leaf temperatures and less evidence of leaf scorch suggests that $T.\ americana$ may have a greater thermoregulation ability than $A.\ saccharum$. This is further supported by species differences in leaf area, stomatal conductance at $T_{opt}$, and evapotranspiration. Smaller leaves can promote convective cooling by allowing higher transpiration rates induced through a thinner boundary layer (Michaletz et al., 2016; Fauset et al., 2018). $T.\ americana$ leaf area is lower in the upper canopy compared with the sub canopy, while $A.\ saccharum$ leaf area is consistent across all canopy positions (Figure 7C).

Lower $T.\ americana$ leaf area combined with high $E_{leaf}$ could allow higher thermoregulation (Figure 7B). These results are similar to other studies, which have found that *Tilia* species have high leaf morphological plasticity (Lichtenthaler et al., 2007; Legner et al., 2014), leaf area that decreases with canopy height (Koike et al., 2001), and high rates of stomatal conductance (Thomas, 2010). In addition, while not significant, $T.\ americana$ had a trend of higher $T_{opt}$ and greater $T_{opt}$ plasticity, where $T_{opt}$ declined with increasing canopy position. While canopy gradient differences in $T_{opt}$ might be influenced by seasonality, we did not find any differences in $T_{air}$ between our sample months (Table S1). This suggests that ambient temperature variation, or seasonality, likely did not have a strong impact on within-canopy temperature differences.

In addition to canopy gradient plasticity, our results could have been confounded with ontogeny. All of our understory measurements were conducted on saplings, while the canopy warming was implemented on reproductively mature trees. Ontogeny could affect our results because trees that are fully shaded might allocate their resources differently than canopy trees with both sun and shade leaves. Both of our study species have been found to have high photosynthetic capacities and leaf area at intermediate size classes (Thomas, 2010). $A.\ saccharum$ leaf area and photosynthesis have been found to increase with increasing plant size (Sendall et al., 2015a), a result which is similar to what we found in our $A.\ saccharum$ leaves. LMA also tends to increase with increasing plant age, focusing leaf construction toward longer-live leaves with high photosynthetic capacity (Valladares and Niinemets, 2008). Particularly with shade tolerant species, resource allocation differs as trees mature and become less light limited (Sendall et al., 2015a). This suggests that plant age, in combination with canopy position, could have played a role in our results between the understory and canopy trees. While leaf area could have been affected by ontogeny...
and light availability, this is an interaction that occurs in most forest ecosystems, as trees existing in the overstory would be unlikely to develop branches within the understory. In addition, while there is evidence that suggests differing thermoregulation between T. americana canopy positions, it should be noted that T. americana upper canopy leaf area is higher than A. saccharum, and \( T_{\text{LeafMax}} \) does not differ between canopy positions for A. saccharum control leaves (Figure S2D).

### Drivers of Within-Canopy Photosynthetic Rates

Photosynthetic optimization with height differed between the two species, and this optimization was likely driven by different leaf traits. A. saccharum LMA and control leaf \( N_{\text{area}} \) were highest in the upper canopy leaves (Figures 8A–D). High LMA in the upper canopy is a common trend in canopy gradients and is due to higher leaf thickness and/or density which can maximize photosynthetic capacity in high light environment (Niinemets, 1999; Zhang et al., 2011; Coble et al., 2014). Our results are supported by other studies which have found that A. saccharum LMA does increase with height (Ellsworth and Reich, 1993; Coble et al., 2014; Filewod and Thomas, 2014). This is consistent with other studies where \( N_{\text{area}} \) of leaves exposed to high irradiance is predictive of photosynthetic capacity (Meir et al., 2002), and agrees with our results which found positive correlations between \( N_{\text{area}} \) and \( A_{\text{opt}} \) for both of our study species (Figure S5B). A. saccharum \( N_{\text{mass}} \) is distributed evenly throughout the understory and canopy (Figures 7E,F), a result often found in A. saccharum canopies (Ellsworth and Reich, 1993; Niinemets and Tenhunen, 1997; Coble and Cavalieri, 2015). T. americana \( A_{\text{opt}} \) was higher than A. saccharum in the sub and upper canopy (Figure 6B), which can likely be attributed to high \( N_{\text{area}} \), leading to a capacity for high rates of Rubisco carboxylation (\( V_{\text{cmax}} \)), in T. americana leaves (Figures 6C, 8D). This is further supported by the higher \( A_{\text{opt}} \) per \( N_{\text{area}} \) found in T. americana sub and upper canopy leaves (Figure S5B). In contrast to A. saccharum, T. americana photosynthetic rates and \( N_{\text{mass}} \) were maximized in the sub canopy (Figures 6B, 8E,F). In shade tolerant species, leaf nitrogen is prioritized to more shaded leaves to maximize the light harvesting capacity (Niinemets, 1997; Schoettle and Smith, 1999; Koike et al., 2001). Nitrogen is a major component of chlorophyll and photosynthetic enzymes, and the high \( N_{\text{mass}} \) found in the sub canopy likely contributed to high photosynthetic rates in the sub canopy (Evans, 1989) (Figure S5C).

A. saccharum upper canopy heated leaf \( N_{\text{area}} \) was higher than the control leaves, while T. americana had lower \( N_{\text{area}} \) in the upper canopy heated leaves. There were no treatment differences for T. americana \( N_{\text{mass}} \) (Table 3); therefore, this pattern was largely driven by a reduction in LMA in the upper canopy heated leaves (Figure 8B). Lowered \( N_{\text{area}} \) and LMA with warming suggests the possibility of lowered substrate availability in T. americana upper canopy leaves. While we did not measure respiration, declined LMA in T. americana upper canopy leaves may be attributed to higher rates of respiration in the heated leaves. If respiration was higher in the heated leaves, non-structural carbohydrates could have been used more quickly, leading to lower leaf mass; although, we had high variability in T. americana upper canopy heated leaf %C and did not find differences between treatments in the upper canopy (Figure 8H). A. saccharum had the opposite trend of \( N_{\text{area}} \) in the upper canopy leaves, where \( N_{\text{area}} \) was higher in the control leaves compared to the heated leaves (Figures S4C). Shifts in \( N_{\text{area}} \) suggest possible acclimation of other physiological processes, such as respiration, which is closely associated with leaf nitrogen (Turnbull et al., 2003) and has been found occur in A. saccharum (Gunderson et al., 2000; Reich et al., 2016). Other studies have found little evidence of \( N_{\text{area}} \) acclimation to experimental warming in temperate trees (Sendall et al., 2015b; Scafaro et al., 2017; Sharwood et al., 2017); however, a tropical leaf warming study found that LMA and \( N_{\text{area}} \) can increase within 1 week of experimental warming (Slot et al., 2014).

In addition to differences in nitrogen optimization, the two species also had different patterns of water-associated leaf traits at different canopy positions. T. americana photosynthesis could be limited in the upper canopy due to hydraulic constraints, as well as stomatal and mesophyll conductance restrictions that occur with increasing canopy height (Niinemets and Tenhunen, 1997; Bond et al., 1999; Ryan et al., 2006; Duursma and Medlyn, 2012; Buckley et al., 2013). Lowered mesophyll conductance can limit \( A_{\text{max}} \) through decreased CO\(_2\) diffusion through cells and through alterations in intercellular membrane structure due to tissue shrinkage (Lawlor and Tezara, 2009). Hydraulic restrictions on \( g_s \) can limit \( A_{\text{max}} \) by decreasing intercellular CO\(_2\) concentrations.

### Table 3: P-value results for mixed effect model comparing treatment, species, canopy position, and the interactions between all three variables, with individual tree as the random effect, for LMA, \( N_{\text{area}}, N_{\text{mass}}, \%C, \) leaf area, and leaf water content.

|                      | LMA     | \( N_{\text{area}} \)   | \( N_{\text{mass}} \) | \%C | Leaf area | Leaf water content |
|----------------------|---------|------------------------|-----------------------|-----|-----------|-------------------|
| Treatment            | 0.516   | 0.007**                | 0.336                 | 0.806 | 0.676     | 0.420             |
| Species              | 0.348   | <0.001***              | <0.001***             | 0.011* | 0.024*    | 0.001**           |
| Canopy position      | <0.001***| <0.001***             | <0.001***             | 0.004** | 0.048*    | 0.001**           |
| Treatment \( \times \) Species | 0.366   | 0.001**                | 0.210                 | 0.684 | 0.301     | 0.301             |
| Treatment \( \times \) Canopy position | 0.695   | 0.015*                 | 0.784                 | 0.730 | 0.386     | 0.622             |
| Species \( \times \) Canopy Position | 0.722   | <0.001***              | 0.002**                | 0.548 | 0.136     | 0.899             |
| Treatment \( \times \) Species \( \times \) Canopy position | 0.062   | <0.001***              | 0.444                 | 0.330 | 0.972     | 0.113             |

*Denotes significance at \( p < 0.05 \). **Denotes significance at \( p < 0.01 \). ***Denotes significance at \( p < 0.001 \). The full names for the symbols are provided in the abbreviations list. \( n = 3 \).
thereby limiting CO₂ fixation in the Calvin cycle (Farquhar and Sharkey, 1982). *T. americana* g, at T_opt and leaf water content were high in the sub canopy (Figures 7A,D); however, leaf water content was low in the upper canopy, which could have limited upper canopy photosynthesis. *T. americana* also had a more negative WUE relationship with temperature compared to *A. saccharum*, which suggests that this species might maintain higher rates of E_leaf even at a detriment to short-term carbon
gain (Table S4). Stomatal conductance was high in A. saccharum upper canopy leaves (Figure 7A), likely contributing to high rates of photosynthesis in the upper canopy. While leaf water content was lower in the upper canopy than in the understory, A. saccharum photosynthetic rates were highest in the upper canopy (Figure 6B). This suggests that leaf water is not a limiting factor for A. saccharum upper canopy leaves.

**Leaf Heating Device Performance**

Overall, the novel heating device worked well for both study species. The leaves were heated successfully +3 ± 0.14°C above ambient leaf temperature across canopy positions, times of day, and sample months (Table S2). Higher heating device efficacy in the understory may be because the understory leaves were less exposed to temperature fluctuations due to direct radiation and sun flecks, allowing fewer spikes in temperature and, therefore, more consistent heating. While the heaters performed well, there was evidence of scorch damage to some of the heated leaves, and slightly more damage in the understory and sub canopy than the upper canopy. This suggests that shaded leaves may be more susceptible to damage at supraoptimal temperatures than upper canopy leaves. Leaves located higher in the canopy are exposed to more severe environments; i.e., high irradiance, temperatures, and wind. Plant acclimation to one type of stress can improve protection from other stresses (Havaux, 1992). Niinemets et al. (1999) found that electron transport in temperate tree leaves acclimated to high light environments is more stable under high temperature conditions. Upper canopy leaves are acclimated to high light conditions, possibly inducing stress acclimation in the upper canopy leaves in our study. The single instance of leaf scorching in the upper canopy occurred in A. saccharum, where the heated $T_{leafMax}$ was 43.5°C. This maximum leaf temperature was at least 5°C higher than maximum temperatures found at all canopy positions for both study species (Figure 4). High temperatures experienced by A. saccharum upper canopy leaves could have contributed to leaf scorching.

In addition, A. saccharum leaves were slightly more affected by scorching than T. americana. The higher scorch damage in A. saccharum could have occurred for several reasons. As mentioned previously, higher $E_{leaf}$ might have allowed greater thermoregulation in T. americana leaves (Figure 7B). T. americana also had higher leaf water content (Figure 7D), which might lead to higher tolerance to heat damage. Additionally, while both species are shade tolerant, T. americana is considered less so than A. saccharum (Crow, 1990; Baltzer and Thomas, 2007; Thomas, 2010). Species with higher shade tolerance have an overall lower plasticity to be able to adapt to high stress environments encountered in upper canopies (Reich et al., 2003). This suggests that A. saccharum could be less tolerant of the temperature fluctuations associated with the heating device. Another possibility for higher leaf scorching in A. saccharum is an artifact of the leaf heating device. T. americana petioles position their leaves so that they are relatively parallel to the ground compared to A. saccharum leaves. This makes T. americana easier to consistently heat over the entire surface of the leaf, likely preventing leaf scorch along leaf margins.

**Conclusions**

- We demonstrated that our novel leaf warming device successfully heated individual leaves $3.02 ± 0.01°C$ above control leaf temperatures; however, there was evidence of leaf scorching, suggesting that there is room for improvement with this method. A simple improvement is to select individual leaves that are positioned parallel to the ground, which helps keep the heater in the correct position below the leaf.
- Our results showed that our two study species were not able to photosynthetically acclimate to 1 week of leaf warming; instead, we found declines in photosynthesis. If neither of these species are able to acclimate to longer-term elevated temperatures, we could see a decline in CO2 sequestration in northern hardwood ecosystems.
- Our study supports our hypothesis that T. americana will likely have greater resiliency to climate warming due to a higher thermoregulation ability and higher trait plasticity between canopy positions of traits associated thermoregulation.
- Canopy position photosynthetic optimization differed between our study species and these differences can, in part, be explained by the species' leaf traits. Higher rates of photosynthesis in A. saccharum upper canopy leaves can be attributed to higher $N_{area}$ and LMA. Higher T. americana photosynthetic rates in the sub canopy can be linked to high $N_{mass}$ and $g_s$ in the sub canopy leaves, as well as hydraulic limitations on leaf mesophyll experienced by the upper canopy leaves. Our results suggest that models that predict canopy photosynthesis based on canopy height or leaf traits, such as LMA, may incorrectly estimate photosynthesis for species that do not optimize photosynthesis in their upper canopy.

**AUTHOR CONTRIBUTIONS**

KC and MC designed the experiment and wrote the manuscript. KC collected data, performed the data analysis, and drafted the manuscript.

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REFERENCES

Baltz, J. L., and Thomas, S. C. (2007). Physiological and morphological correlates of whole-plant light compensation point in temperate deciduous tree seedlings. Oecologia 153, 209–223. doi: 10.1007/s00442-007-0722-2

Bastos, A., Gouveia, C. M., Trigo, R. M., and Running, S. W. (2014). Analysing the spatio-temporal impacts of the 2003 and 2010 extreme heatwaves on plant productivity in Europe. Biogosciences 11, 3421–3435. doi: 10.5194/bg-11-3421-2014

Bauerle, W. L., Bowden, J. D., Wang, G. G., and Shahba, M. A. (2009). Exploring the importance of within-canopy spatial temperature variation on transpiration predictions. J. Exp. Bot. 60, 3665–3676. doi: 10.1093/jxb/erp206

Bernacchi, C. J., Singsaa, E. L., Pimentel, C., Portis, A. R., and Long, S. P. (2001). Improved temperature response functions for models of Rubisco-limited photosynthesis. Plant Cell Environ. 24, 253–259. doi: 10.1046/j.1365-3040.2001.00668.x

Berry, J., and Bjorkman, O. (1980). Photosynthetic response and adaptation to temperature in higher plants. Annu. Rev. Plant Physiol. 31, 491–543. doi: 10.1146/annurev.pp.31.060180.002423

Bond, B. J., Farmsworth, B. T., Coulombe, R. A., and Winner, W. E. (1999). Foliation characteristics and biochemistry in response to radiation gradients in conifers with varying shade tolerance. Oecologia 120, 183–192.

Buckley, T. N., Cescatti, A., and Farquhar, G. D. (2013). What does optimization theory actually predict about crown profiles of photosynthetic capacity when models incorporate greater realism? Plant Cell Environ. 36, 1547–1563. doi: 10.1111/j.1365-3040.2012.02208.x

Bukhov, N. G., Wiese, C., Neimanis, S., and Heber, U. (1999). Heat sensitivity of chloroplasts and leaves: Leakage of protons from thylakoids and reversible activation of cyclic electron transport. Photosynth. Res. 59, 81–93. doi: 10.1023/A:1006149317411

Carretero, C., Míguez-Méndez, E., Schreiber, S., McMillan, M. S., and Tomasselli, M. (2013). Proximal leaf temperatures are highly correlated with forest photosynthesis of tropical montane forest canopies. Ecol. Lett. 16, 850–863. doi: 10.1111/j.1461-0248.2013.01831.x

Carter and Cavaleri Northern Hardwood Experimental Warming

Crow, T. R. (1990). “Tilia americana L. American basswood,” in Technical Coord. Silvics North Am. Vol. 2. Hardwoods. Agric. Handb. 654, eds R. M. Burn, B. and H. Honda (Washington, DC: U.S. Department Agric. For. Serv.), 784–791.

Cunningham, S., and Read, J. (2002). Comparison of temperate and tropical rainforest tree species: photosynthetic responses to growth temperature. Oecologia 133, 112–119. doi: 10.1007/s00442-002-1034-1

Davies, W., and Zhang, J. (1991). Drying soil regulation of growth. Annu. Rev. Plant Physiol. 42, 55–76. doi: 10.1146/annurev.pp.42.060691.000415

de Frenne, P., de Schrijver, A., Graae, B. J., Gruwez, R., Tack, W., Vandeloo, F., et al. (2010). The use of open-top chambers in forests for evaluating warming effects on herbaceous understorey plants. Ecol. Res. 25, 163–171. doi: 10.1007/s11284-009-0640-3

De Kauwe, M. G., Lin, Y. S., Wright, I. J., Medlyn, B. E., Crous, K. Y., Ellsworth, D. S., et al. (2016a). A test of the “one-point method” for estimating maximum carboxylation capacity from field-measured, light-saturated photosynthesis. New Phytol. 210, 1130–1144. doi: 10.1111/nph.13815

De Kauwe, M. G., Lin, Y. S., Wright, I. J., Medlyn, B. E., Crous, K. Y., Ellsworth, D. S., et al. (2016b). Corrigendum. New Phytol. 210, 1130–1144. doi: 10.1111/nph.14172

Doughty, C. E. (2011). An in situ leaf and branch warming experiment in the Amazon. Biotropica 43, 658–665. doi: 10.1111/j.1744-7429.2010.00746.x

Drake, J. E., Tjoelker, M. G., Aspinwall, M. J., Reich, P. B., Barton, C. V., Medlyn, R. E., et al. (2016). Does physiological acclimation to climate warming stabilize the ratio of canopy respiration to photosynthesis? New Phytol. 211, 850–863. doi: 10.1111/nph.13978

Drake, J. E., Tjoelker, M. G., Vårhammars, M., Medlyn, B. E., Reich, P. B., Leigh, A., et al. (2018). Trees tolerate an extreme heatwave via sustained transpirational cooling and increased leaf thermal tolerance. Glob. Chang. Biol. 24, 2390–2402. doi: 10.1111/gcb.14037

Duursma, R. A., and Medlyn, B. E. (2012). MAESPA: a model to study interactions between water limitation, environmental drivers and vegetation function at tree and stand levels, with an example application to [CO2] drought interactions. Geosci. Model Dev. 5, 919–940. doi: 10.5194/gmd-5-919-2012

Ellsworth, D. S., and Reich, P. B. (1993). Canopy structure and vertical patterns of photosynthesis and related leaf traits in a deciduous forest. Oecologia 96, 169–178. Available online at: http://www.jstor.org/stable/4220518%5Cnhttp://about.jstor.org/terms

Evans, J. R. (1989). Photosynthesis and nitrogen relationships in leaves of C3 plants. Oecologia 78, 9–19.

Farquhar, G. D., and Sharkey, T. D. (1982). stomatal conductance and photosynthesis. Annu. Rev. Plant Physiol. 33, 317–345.

Fassett, S., Freitas, H. C., Galbraith, D. R., Sullivan, M. J. P., Aidar, M. P. M., Joly, C. A., et al. (2018). Differences in leaf thermoregulation and water-use strategies between three co-occurring Atlantic forest tree species. Plant Cell Environ. 41, 1618–1631. doi: 10.1111/pce.13208

Filewod, B., and Thomas, S. C. (2014). Impacts of a spring heat wave on canopy processes in a northern hardwood forest. Glob. Chang. Biol. 20, 360–371. doi: 10.1111/gcb.12354

Fu, Y. H., Campioli, M., Deckmyn, G., and Janssens, I. A. (2013). Sensitivity of leaf unfolding to experimental warming in three temperate tree species. Agric. For. Meteorol. 181, 125–132. doi: 10.1016/j.agrformet.2013.07.016

Gershunov, A., Cayan, D. R., and Iacobellis, S. F. (2009). The great 2006 heat wave over California and Nevada: signal of an increasing trend. J. Clim. 22, 6181–6186, 6186, 6190–6192, 6194–6195, 6197–6203. doi: 10.1175/2009JCLI2645.1

Gunderson, C. A., Norby, R. J., and Wullschleger, S. D. (2000). Acclimation of photosynthesis and respiration to simulated climatic warming in northern and southern populations of Acer saccharum: laboratory and field evidence. Tree Physiol. 20, 87–96. doi: 10.1093/treephys/20.2.87

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/ffgc.2018.00011/full#supplementary-material
Rollinson, C. R., and Kaye, M. W. (2012). Experimental warming alters spring phenology of certain plant functional groups in an early successional forest community. Glob. Chang. Biol. 18, 1108–1116. doi: 10.1111/j.1365-2486.2011.02612.x

Ryan, M. G., Phillips, N., and Bond, B. J. (2006). The hydraulic limitation hypothesis revisited. Plant Cell Environ. 29, 367–381. doi: 10.1111/j.1365-3040.2005.01478.x

Sage, R. F., and Kubien, D. S. (2007). The temperature response of C3 and C4 photosynthesis. Plant Cell Environ. 30, 1086–1106. doi: 10.1111/j.1365-3040.2007.01682.x

Salvucci, M. E., Osteryoung, K. W., Crafts-Brandner, S. J., and Vierling, E. (2001). Exceptional sensitivity of Rubisco activase to thermal denaturation in vitro and in vivo. Plant Physiol. 127, 1053–1064. doi: 10.1104/pp.010357

Sampaio Filho, I. de J., Jardine, K. J., de Oliveira, R. C. A., Gimenez, B. O., Cobello, I. O., Piva, L. R. O., et al. (2018). Below versus above ground plant sources of abscisic acid (ABA) at the heart of tropical forest response to warming. Int. J. Mol. Sci. 19:E2023. doi: 10.3390/ijms19072023

Sendall, K. M., Lusk, C. H., and Reich, P. B. (2015a). Becoming less tolerant with age: sugar maple, shade, and ontogeny. Oecologia 179, 1011–1021. doi: 10.1007/s00442-015-3428-x

Sendall, K. M., Reich, P. B., Zhao, C., Jihua, H., Wei, X., Stefanski, A., et al. (2015b). Acclimation of photosynthetic temperature optima of temperate and boreal tree species in response to experimental forest warming. Glob. Chang. Biol. 21, 1342–1357. doi: 10.1111/gcb.12781

Sharwood, R. E., Crous, K. Y., Whitney, S. M., Ellsworth, D. S., and Ghannoum, O. (2017). Linking photosynthesis and leaf N allocation under future elevated CO2 and climate warming in Eucalyptus globulus. J. Exp. Bot. 68, 1157–1167. doi: 10.1093/jxb/erw484

Slot, M., Garcia, M. A., and Winter, K. (2016). Temperature response of CO2 exchange in three tropical tree species. Funct. Plant Biol. 43, 468–478. doi: 10.1071/FP15320

Slot, M., Rey-Sánchez, C., Gerber, S., Lichstein, J. W., Winter, K., and Kitajima, K. (2014). Thermal acclimation of leaf respiration of tropical trees and lianas: Response to experimental canopy warming, and consequences for tropical forest carbon balance. Glob. Chang. Biol. 20, 2915–2926. doi: 10.1111/gcb.12563

Smith, N. G., and Dukes, J. S. (2017). Short-term acclimation to warmer temperatures accelerates leaf carbon exchange processes across plant types. Glob. Chang. Biol. 23, 2783–2800. doi: 10.1111/gcb.13566

Schneider, C. A., Rasband, W. S., and Eliceiri, K. W. (2012). NIH image to ImageJ: 25 years of image analysis. Nat. Methods 9, 671–675. doi: 10.1038/nmeth.2089

Schoettle, A. W., and Smith, W. K. (1999). Interrelationships among light, photosynthesis and nitrogen in the crown of mature Pinus contorta ssp. latifolia. Tree Physiol. 19, 13–22. doi: 10.1093/treephys/19.1.13

Turnbull, M. H., Tissue, D. T., Murthy, R., Wang, X., Sparrow, A. D., and Griffin, K. L. (2004). Nocturnal warming increases photosynthesis at elevated CO2 partial pressure in Populus deltoides. New Phytol. 161, 819–826. doi: 10.1111/j.1469-8137.2003.00994.x

Turnbull, M. H., Whitehead, D., Tissue, D. T., Schuster, W. S. F., Brown, K. J., and Griffin, K. L. (2003). Scaling foliar respiration in two contrasting forest canopies. Funct. Ecol. 17, 101–114. doi: 10.1046/j.1365-2435.2003.07013.x

Urban, J., Ingwers, M. W., McGuire, M. A., and Teskey, R. O. (2017). High temperature opens stomata and decouples net photosynthesis from stomatal conductance in Populus taeda and Populus deltoides × nigra. J. Exp. Bot. 68, 1757–1767. doi: 10.1093/jxb/err052 1.5

Valladares, F., and Niinemets, Ü. (2008). Shade tolerance, a key plant feature of complex nature and consequences. Annu. Rev. Ecol. Evol. Syst. 39, 237–257. doi: 10.1146/annurev.ecolsys.39.110707.173506

Vogel, S. (1970). Convective cooling at low airspeeds and the shapes of broad leaves. J. Exp. Bot. 21, 91–101.

Walker, K. V., Davis, M. B., and Sugita, S. (2002). Climate change and shifts in potential tree species range limits in the Great Lakes Region. J. Great Lakes Res. 28, 555–567. doi: 10.1016/S0380-1330(02)70605-9

Wang, Z. Y., and Portis, A. R. (1992). Dissociation of ribulose-1,5-bisphosphate bound to ribulose-1,5-bisphosphate carboxylase/oxygenase and its enhancement by ribulose-1,5-bisphosphate carboxylase/oxygenase activase-mediated hydrolysis of ATP. Plant Physiol. 99, 1348–1353. doi: 10.1104/pp.99.4.1348

Way, D. A., and Oren, R. (2010). Differential responses to changes in growth temperature between trees from different functional groups and biomes: a review and synthesis of data. Tree Physiol. 30, 669–688. doi: 10.1093/treephys/tqp015

Way, D. A., and Yamori, W. (2014). Thermal acclimation of photosynthesis: On the importance of adjusting our definitions and accounting for thermal acclimation of respiration. Photosynth. Res. 119, 89–100. doi: 10.1007/s11120-013-0143-9

Yamaguchi, D. P., Nakaji, T., Hiura, T., and Hikosaka, K. (2016). Effects of seasonal change and experimental warming on the temperature dependence of photosynthesis in the canopy leaves of Quercus serrata. Tree Physiol. 36, 1283–1295. doi: 10.1093/treephys/twp021

Zhang, N., Kallis, R. P., Ewy, R. G., and Portis, A. R. (2002). Light modulation of Rubisco in Arabidopsis requires a capacity for redox regulation of the larger Rubisco activase isoform. Proc. Natl. Acad. Sci. U.S.A. 99, 3330–3334. doi: 10.1073/pnas.022529999

Zhang, R., Cruz, J. A., Kramer, D. M., Magallanes-Lundback, M. E., Dellapenna, D., and Sharkey, T. D. (2009). Moderate heat stress reduces the pH component of the transthalikaid proton motive force in light-adapted, intact tobacco leaves. Plant Cell Environ. 32, 1538–1547. doi: 10.1111/j.1365-3040.2009.01812.x

Zhang, Y., Eiquiza, M. A., Zheng, Q., and Tyree, M. T. (2011). Factors controlling plasticity of leaf morphology in Robinia pseudoacacia: III. biophysical constraints on leaf expansion under long-term water stress. Physiol. Plant. 143, 367–374. doi: 10.1111/j.1399-3054.2011.01 504.x

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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