

**Leaf temperature regulation in tree species of the eastern United States: the influence of leaf traits and resource availability**

by

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Temperature regulation, leaf traits, ecophysiology, stomatal conductance

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## Abstract

Temperature has a direct effect on plant function (e.g., photosynthesis, respiration). However, the temperature that plants experience at the leaf level ( $T_{\text{leaf}}$ ) is often decoupled from air temperature ( $T_{\text{air}}$ ). Theoretical and experimental work has indicated that leaf morphology and physiology can influence  $T_{\text{leaf}}$  regulation, defined as  $\beta$  ( $\beta = \Delta T_{\text{leaf}} - \Delta T_{\text{air}}$ ). Yet, our understanding of which traits most strongly influence  $T_{\text{leaf}}$  regulation is limited. Moreover, it is not clear whether variation in leaf traits, across species or resource environments, influences  $T_{\text{leaf}}$  regulation. In this study, seedlings of 10 angiosperm tree species native to the eastern U.S. were grown outdoors for 10 months under a factorial combination of water and nutrient availability. Each month of the peak growing season (May and September), diurnal measurements of canopy  $T_{\text{leaf}}$  and stomatal conductance were made on seedlings of each species growing in each resource environment and temporally matched with  $T_{\text{air}}$  measurements. Leaves were collected to measure leaf dimensions, leaf dry matter content, leaf mass per area, leaf chlorophyll content, and stomatal density. We addressed the following questions: 1) Does  $T_{\text{leaf}}$  regulation ( $\beta$ ) differ among tree species of the eastern U.S. and between different water and nutrient environments? and 2) Do species leaf traits (structural, anatomical, gas-exchange) explain variation in  $T_{\text{leaf}}$  regulation ( $\beta$ )? Averaged across timepoints (month), species, and treatments, we found that seedlings were largely poikilothermic ( $\beta = 1$ ). However, month, species, and water availability each contributed to significant variation in  $\beta$ . High water availability dampened  $\beta$ , although differences between water treatments decreased over time. Among species, four species were found to have  $\beta < 1$ , and six species had  $\beta > 1$ . Nutrient availability had no direct effect on  $\beta$ . Leaf traits exhibited variability across months, species, treatments, or a combination thereof. Across months, species, and resource environments, we found that  $\beta$  tended to be reduced when stomatal conductance, leaf size, and leaf dry matter content were higher, but leaf nitrogen was lower. These results provide new insight into the traits or trait combinations that influence  $T_{\text{leaf}}$  regulation across species and resource environments.

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## List of Abbreviations

$T_{\text{leaf}}$	Leaf temperature	Chl	Leaf chlorophyll content
$T_{\text{air}}$	Air temperature	Leaf N	Leaf nitrogen
LMA	Leaf mass per area	SD	Stomatal density
LDMC	Leaf dry matter content	Rad	Solar radiation
$g_s$	Stomatal conductance	RH	Relative humidity
LS	Leaf size	WS	Wind speed
LW	Leaf width	WD	Wind direction

## 1.0 Introduction

Temperature directly affects many physiological processes in plants including photosynthesis, transpiration, and respiration (Fauset, et al., 2018; Cook, Berry, Milner, & Leigh, 2021; Berry & Bjorkman, 1980; Dong, Prentice, Harrison, Song, & Zang, 2017; Michaletz S. , Weiser, Zhou, Heliker, & Enquist, 2015). Leaf temperature ( $T_{\text{leaf}}$ ) has important implications for physiological processes. Increases in  $T_{\text{leaf}}$  above optimal temperatures cause reduction in photosynthesis (Lin, Medlyn, & Ellsworth, 2012; Farquhar, von Caemmerer, & Berry, 1980; Bernacchi, et al., 2013) via inhibition of Rubisco activase (Salvucci & Crafts-Brandner, 2004) and damage to photosystem II (Wise, Olson, Schrader, & Sharkey, 2004) and may cause downregulation of stomatal conductance (Lloyd & Farquhar, 2008). Models have historically assumed that canopy or leaf temperature and air temperature ( $T_{\text{air}}$ ) are equivalent (Dong, Prentice, Harrison, Song, & Zang, 2017). This assumption presumes that leaves are poikilothermic; such that,  $T_{\text{air}}$  and  $T_{\text{leaf}}$  are perfectly coupled, with a slope ( $\beta$ ) of 1, where  $\beta = \Delta T_{\text{leaf}} - \Delta T_{\text{air}}$  (Blonder & Michaletz, 2018; Blonder, Escobar, Kapas, & Michaletz, 2020). However,  $T_{\text{leaf}}$  and  $T_{\text{air}}$  are often decoupled depending upon prevailing atmospheric conditions as well as variability in leaf morphology or other traits (i.e., stomatal conductance) which may ‘regulate’  $T_{\text{leaf}}$  (Blonder & Michaletz, 2018; Fauset, et al., 2018). This variation in  $T_{\text{leaf}}$  to  $T_{\text{air}}$  has been coined as ‘limited homeothermy’ (Michaletz S. T., et al., 2016). However, our understanding of the relationship between leaf traits and  $T_{\text{leaf}}$  regulation is incomplete. Little is known how the influence of plant resource (i.e., water and nutrient) availability might also impact this decoupling. Identifying factors that drive variation in the  $T_{\text{leaf}} - T_{\text{air}}$  relationship is important for understanding plant performance across ecosystem conditions as well as accurately predicting spatial and temporal variation in ecosystem carbon and water fluxes under current and future climate conditions.

Deviations in  $T_{\text{leaf}}$  from  $T_{\text{air}}$  can be partially explained by environmental conditions such as windspeed and solar radiation (Drake, et al., 2020). When  $T_{\text{air}}$  is high, but windspeed is low,  $T_{\text{leaf}}$  can be several degrees lower than  $T_{\text{air}}$ . Likewise, when  $T_{\text{air}}$  is low, but solar radiation is high,  $T_{\text{leaf}}$  can be several degrees higher than  $T_{\text{air}}$ . Many plants can track solar rays in order to capture more sunlight and increase photosynthetic rates, and this tracking can lead to higher leaf



temperatures (Ehleringer & Forseth, 1980). Environmental factors that affect  $T_{\text{leaf}}$  have been used to create energy balance equations to better understand how individual environmental factors affect  $T_{\text{leaf}}$  (Gates, 1968; Mahan & Upchurch, 1988).

Leaf functional traits could also influence  $T_{\text{leaf}}$ . Reich et al (2003) defined a plant functional trait as “any attribute that has potentially significant influence on establishment, survival, and fitness” (Reich, et al., 2003). These traits have been widely used to understand and predict species growth, biotic and abiotic interactions, and function (Drake, et al., 2020; Fauset, et al., 2018; Hikosaka, Ishikawa, Borjigidai, Muller, & Onoda, 2006; Poorter, Niinemets, Poorter, Wright, & Villar, 2009; Dong, Prentice, Harrison, Song, & Zang, 2017; Michaletz S. , Weiser, Zhou, Heliker, & Enquist, 2015). Most leaf functional traits are considered “pattern” traits that are quickly and easily measured, such as leaf mass per area (LMA) and leaf dry matter content (LDMC). These traits can covary with “process” traits such as photosynthesis, respiration, and stomatal conductance ( $g_s$ ) – all traits measured per unit time. These traits often vary greatly between species and individuals under different growing conditions. Environmental conditions have a strong influence on the expression of leaf traits, both through long-term evolutionary adaptation and short-term acclimation or “phenotypic plasticity” (Reich, et al., 2003). The influence of leaf traits on  $T_{\text{leaf}}$  regulation may be passive (e.g., phenotypic plasticity of leaf dimensions) or active (e.g., change in rate of transpiration) (Drake, et al., 2020). Understanding when and at what magnitude leaf traits are influential will create a broader understanding how  $T_{\text{leaf}}$  decouples from  $T_{\text{air}}$ .

Leaf mass per area is a commonly measured leaf functional trait that varies between species, functional groups, and biomes (Poorter, Niinemets, Poorter, Wright, & Villar, 2009) and provides information about resource investment and return on investment (i.e., resource acquisition) – important attributes of plant species’ life history. Leaves with high LMA are generally thicker or denser and more costly to produce, but typically longer-lived and more damage resistant (i.e., tougher). Low LMA leaves typically have greater area for capturing light per unit leaf mass but lack toughness and are shorter-lived (Poorter & Bongers, 2006; Gutschick & Wiegand, 1988; Wright I. J., et al., 2004). Across species, LMA covaries strongly with mass-based rates of leaf net photosynthesis and respiration; species with high LMA leaves often have

lower rates of photosynthesis and respiration. This general pattern is partly due to tradeoffs between tissue construction and investment of leaf nitrogen between enzymes and structural components of the cell wall. Changes in LMA are often linked to changes in LDMC as LDMC is a function of tissue density (Poorter, Niinemets, Poorter, Wright, & Villar, 2009). LMA can be inferior to LDMC for understanding resource capture and tissue composition when leaves are small, wavy, or curved due to issues in accurately measuring area for LMA (Wilson, Thompson, & Hodgson, 1999). Since LDMC is relatively easy to obtain, coupling this measurement with LMA can give better insight into resource use at the leaf level. LMA and LDMC may influence  $T_{\text{leaf}}$  regulation since thicker leaves are more thermally stable (Curtis, Leigh, & Rayburg, 2012; Michaletz S. , Weiser, Zhou, Heliker, & Enquist, 2015) and dampen  $T_{\text{leaf}}$  increases when leaf transpiration and wind speeds are low (Buckley, John, Scoffoni, & Sack, 2015; Lin, Chen, Zhang, Fu, & Fan, 2017).

Other commonly measured functional traits include leaf size (i.e., area, LS), leaf chlorophyll content (Chl), leaf nitrogen (leaf N), and stomatal density (SD). Leaf dimension traits strongly influence  $T_{\text{leaf}}$  regulation given that smaller and narrower leaves have thinner boundary layers than larger or wider leaves, which allows for quicker diffusion and greater evaporative cooling (Yates, Anthony Verboom, Rebelo, & Cramer, 2010; Fauset, et al., 2018; Leigh, Sevanto, Close, & Nicotra, 2017; Parkhurst & Loucks, 1972). Importantly, many leaf traits covary such that no single trait might independently predict variation in  $T_{\text{leaf}}$  regulation. Across species, leaf Chl and leaf N show positive relationships with leaf photosynthetic capacity (Croft, et al., 2017). Leaves with higher photosynthetic capacity also have higher demand for  $\text{CO}_2$ , and therefore have higher SD and  $g_s$  to help sustain  $\text{CO}_2$  uptake (Franks & Beerling, 2009; Schymanski, Or, & Zwieniecki, 2013; Lin, Chen, Zhang, Fu, & Fan, 2017). In this way, higher leaf Chl, N, and SD may be related to stronger  $T_{\text{leaf}}$  regulation. However, the individual and combined influence of these traits on  $T_{\text{leaf}}$  regulation may not be straightforward given that there is evidence that LMA scales positively with SD across some species (Loranger & Shipley, 2010; Blackman, Aspinwall, Resco de Dios, Smith, & Tissue, 2016; Henry, et al., 2019). but scales negatively with leaf N (per unit mass) across species (Wright, Reich, & Westoby, 2003; Wright I. J., et al., 2004; Villar, et al., 2021).

Leaf temperature regulation may be more directly linked with leaf water fluxes driven by  $g_s$  (Fauset, et al., 2018; Parkhurst & Loucks, 1972). Stomatal conductance is important in semi-empirical models and is a function of relative humidity at the leaf surface, assimilation rate, and surface  $CO_2$  concentration (Fauset, et al., 2018). Increasing  $g_s$  during elevated atmospheric temperatures helps reduce leaf temperature and reduce leaf mortality (Blonder & Michaletz, 2018), but closing stomata and decreasing  $g_s$  allows plants to conserve water and reduce risk of hydraulic failure if elevated  $T_{air}$  coincides with drought (Xu & Zhou, 2008; Fauset, et al., 2018). Water availability often dictates rates of  $g_s$  during elevated temperatures. Investigating stomatal behavior (e.g., conductance and density), analyzed through the lens of  $T_{leaf}$  regulation, may lead to greater understanding of functional trait variation between species which may be critical to understanding diverse strategies for  $T_{leaf}$  regulation (Blonder & Michaletz, 2018). This is especially important when species functional traits differ yet  $T_{leaf}$  is the same under the same conditions (Blonder, Escobar, Kapas, & Michaletz, 2020). Additionally, there could be species variation in traits and adaptations that influence  $T_{leaf}$  regulation in offsetting ways such that  $T_{leaf}$  is more or less equivalent to  $T_{air}$ . Instead, multiple traits may alter at varying levels such that the same leaf temperature can be achieved a multitude of ways (Blonder, Escobar, Kapas, & Michaletz, 2020).

All leaf traits that could be involved in  $T_{leaf}$  regulation could change plastically in response to environmental / resource gradients and acclimate towards a community mean more fitted for the current environmental conditions (Michaletz S. , Weiser, Zhou, Heliker, & Enquist, 2015). While several traits have been predicted to influence  $T_{leaf}$ , it is unclear how species variation in leaf traits influences  $T_{leaf}$  regulation, and how resource availability potentially modifies leaf traits for  $T_{leaf}$  regulation. Currently, there is no clear prediction of how leaf functional traits may change with resource availability to maintain  $T_{leaf}$  regulation. High water availability leads to greater efficiency of transpiration, allowing for  $T_{leaf}$  to be maintained to a degree from  $T_{air}$  (Fauset, et al., 2018; Lin, Chen, Zhang, Fu, & Fan, 2017; Dong, Prentice, Harrison, Song, & Zang, 2017). Reduced water availability can force a tradeoff between the maintenance of  $T_{leaf}$  and the maintenance of hydraulic function by closing stomata, triggered by water stress (Fauset, et al., 2018). Leaves with low internal diffusion resistance will have a

higher rate of transpiration and consume more water while leaves with smaller dimensions will have lower water use efficiency during photosynthesis (Fauset, et al., 2018; Gates, 1968).  $T_{\text{leaf}}$  regulation below  $T_{\text{air}}$  may be predicted when  $g_s$  is high and convective resistance is low (Blonder & Michaletz, 2018). With decreasing water availability, LMA tends to increase (Poorter, Niinemets, Poorter, Wright, & Villar, 2009). High LMA leaves grown under low water conditions have thicker cell walls and less cell expansion, allowing for continued function under dry conditions (Poorter, Niinemets, Poorter, Wright, & Villar, 2009; Wright I. J., et al., 2004). Severe drought may lead to reductions in SD, but when drought is only moderate, an increase is possible. The reduction in SD may be an incidental result of inhibited guard cell division during severe drought, while the increase in SD under moderate drought may stabilize or improve  $g_s$  (Xu & Zhou, 2008). The vast connection between leaf traits and processes with water availability underscores the significance of considering each of these factors together in  $T_{\text{leaf}}$  regulation.

Unlike water availability, less is known about the impact that nutrient availability has on trait alterations. Leaf N concentrations have been shown to positively correlate with transpiration rates (Schulze, Kelliher, Korner, Lloyd, & Leuning, 1994) and negatively correlate with LMA (Poorter, Niinemets, Poorter, Wright, & Villar, 2009; Reich, et al., 2003; Wright I. J., et al., 2004). If N increases, higher rates of photosynthesis may be achieved (Niinemets & Sack, 2006) and higher  $g_s$  can be expected in order to supply  $\text{CO}_2$  for photosynthetic enzymes (Poorter, Niinemets, Poorter, Wright, & Villar, 2009).

The purpose of this study was to determine the influence of species and resource availability on  $T_{\text{leaf}}$  regulation, and whether leaf trait variation across species and growth conditions predicts variation in  $T_{\text{leaf}}$  regulation. The study was a factorial experiment with 10 tree species, two water availability treatments (high, low), and two nutrient availability treatments (high, low). We addressed the following questions: 1.) Does  $T_{\text{leaf}}$  regulation ( $\beta$ ) differ amongst diverse tree species of the eastern U.S. and between resource availability? and 2.) Do species leaf traits (structural, anatomical, gas-exchange) explain variation between  $T_{\text{leaf}}$  and  $T_{\text{air}}$ ? We hypothesized that  $\beta$  would differ across species as a result of distinct leaf traits associated with specific adaptations driven by ecological niches commonly occupied by each species. In addition, we expected that resource availability would alter leaf traits via phenotypic plasticity,

and that high water and high nutrient availability would lead to an increase in ability to regulate  $T_{\text{leaf}}$  relative to  $T_{\text{air}}$ , and therefore alter  $\beta$ . Improving our understanding of  $T_{\text{leaf}}$  regulation and what factors drive variation is crucial for understanding plant performance, especially across environmental conditions.

## 2.0 Materials and Methods

### 2.1 Study site and species

This study took place in an open location on the campus of Auburn University in Auburn, AL, USA (32°35'31.2"N, 85°29'17.9"W). Local climate is warm-temperate. Mean annual maximum temperature is 23.4 °C and mean annual minimum air temperature is 13.5 °C. Mean annual precipitation is 101.3 mm. Solar radiation (Rad, W/m<sup>2</sup>), air temperature (T<sub>air</sub>, °C), relative humidity (RH, %), wind speed (WS, m s<sup>-1</sup>), and gust speed (GS, s<sup>-1</sup>) was recorded at the study site during the length of the study (May to September 2022) every 10 minutes using a HOBO U30 USB Weather Station (ONSET; Bourne, MA). Each of these variables were measured every 10 seconds on dates when measurements of leaf temperature and stomatal conductance were carried out.

**Table 1.** Tree species included in this study.

Family	Latin	Common	Brief Description* (growth, shade, habitat)
<b>Sapindaceae</b>	<i>Acer rubrum</i>	Red Maple	Fast, tolerant, broad
<b>Nyssaceae</b>	<i>Nyssa aquatica</i>	Water Tupelo	Slow, partial, southern coastal/swamp
<b>Magnoliaceae</b>	<i>Liriodendron tulipifera</i>	Tulip Poplar	Fast, partial, broad
<b>Altingiaceae</b>	<i>Liquidambar styraciflua</i>	Sweetgum	Fast, intolerant, broad
<b>Fagaceae</b>	<i>Quercus texana</i>	Nuttal Oak	Fast, partial, Mississippi river/bottomlands
	<i>Q. lyrata</i>	Overcup Oak	Slow, intolerant, southern bottomlands
	<i>Q. phellos</i>	Willow Oak	Fast, intolerant, broad/bottomlands
	<i>Q. shumardii</i>	Shumard Oak	Fast, intolerant, broad/bottomlands
	<i>Q. alba</i>	White Oak	Slow, partial, broad
	<i>Q. virginiana</i>	Live Oak	Fast, partial, southern coastal/bottomlands

\*Information for description was obtained from *Silvics of North America* (Burns, 1990).

Ten different species of trees with distributions across the southeastern U.S. were included in this study. The species selected for this study represent 5 different families and vary in their growth rate (e.g., fast or slow), shade tolerance (e.g., tolerant, partial, or intolerant), and general habitat preferences and adaptations within the eastern U.S. (**Table 1**). Two-year old bareroot seedlings of each species were obtained from three nurseries within Georgia and

Alabama (Meeks Nursery in Kite, GA; ArborGen Nursery in Selma, AL; and Georgia Forestry Commission Flint River Nursery in Byromville, GA). Upon arrival (between November 2021 and February 2022), seedlings were stored in a cooler for up to two days then planted in identical 11 L pots filled with topsoil derived from pine fines, hardwood fines, and forest products (Evergreen; Muscle Shoals, AL) with no added fertilizer or amendments (e.g., perlite). Twenty-five mg of 10-10-10 NPK slow-release granular fertilizer (Weaver; Winston Salem, NC) were then added to each pot to prevent nutrient limitations. After planting, tree height and stem diameter at 5 cm above the soil surface were measured for each tree.

## **2.2 Water and nutrient availability treatments**

This experiment utilized a randomized complete block design with six blocks and four treatments: high water x high nutrients (HWHN), low water x low nutrients (LWLN), high water x low nutrients (HWLN), and low water x high nutrients (LWHN). With 10 species, this resulted in 24 individuals per species in each block, and 240 seedlings total (10 species x 4 treatments x 6 blocks = 240 individuals). Blocking for this experiment was used to account for potential spatial effects in the field (e.g., effects from nearby mature trees on the north side of the plot, and parking lot on south side of plot). Treatments were randomly assigned to plots within each block, and seedlings of each species were randomly assigned to positions within each treatment plot. The treatments began in April 2022 before the first leaves formed on seedlings. Seedlings in the high-water treatments (HWHN, HWLN) received at least 1.5 L of water every 1-2 days (equivalent of ~550 mm rainfall over entire study period) and received water twice the amount of days per month than seedlings in the low water treatments (LWHN, LWLN). Importantly, given that seedlings were containerized and grown in an outdoor uncovered location, they all received ambient precipitation (~1030.56mm over entire study period; (NOAA, 2022)). LW seedlings were watered before the soil had completely dried and received the same amount of water for half the amount of days that the HW treatments received. Seedlings in both nutrient treatments received 10-10-10 NPK slow-release granular fertilizer once per month. Seedlings in the low nutrient treatments (HWLN, LWLN) received 15 mg of fertilizer per application while seedlings

in the high nutrient treatment (HWHN, LWHN) treatments received 30 mg of fertilizer per application. This range was found to be within the range of other seedling studies with multiple nutrient treatments (Villar-Salvador, Penuelas, Nicolas-Peragon, Benito, & Dominguez-Lerena, 2013; Taylor, Lowenstein, & Chapelka, 2006; Phillips & Fahey, 2008) All seedlings were watered on the same day that fertilizer was applied.

### **2.3 Leaf temperature and stomatal conductance measurements**

Measurements of canopy leaf temperature ( $T_{\text{leaf}}$ ) and stomatal conductance to water vapor ( $g_s$ ) began at the end of May 2022 and continued monthly through September 2022. In each month, measurements were carried out on two or three days, with two to three blocks measured each day.  $T_{\text{leaf}}$  and  $g_s$  were measured once per seedling at three time points per measurement day: Morning (7:00 to 9:00), afternoon (12:00 to 14:00), and evening (17:00 to 19:00). For  $T_{\text{leaf}}$ , an infrared radiometer (MI-230 Ultra-Narrow Field of View Infrared Temperature with Handheld Meter; APOGEE Instruments; Logan, UT) was held flat at 15 cm above each seedling so that  $T_{\text{leaf}}$  was recorded with minimal interference from non-leaf material (e. g., twigs, branches, soil). After  $T_{\text{leaf}}$  was recorded,  $g_s$  was immediately measured using a LI-600 Leaf Porometer (Licor; Lincoln, NE). This device was clamped onto one mature, sunlit leaf in the same canopy area that  $T_{\text{leaf}}$  was recorded with the infrared radiometer. Both  $T_{\text{leaf}}$  and  $g_s$  was measured on a single seedling before moving on to the next seedling. The exact time of  $T_{\text{leaf}}$  measurements was recorded so that  $T_{\text{leaf}}$  measurements could be matched in time with  $T_{\text{air}}$  measurements recorded by the weather station.

### **2.4 Leaf trait measurements**

One sunlit upper canopy leaf was collected from each seedling to determine LDMC, LMA, leaf size (area,  $\text{cm}^2$ ; LS), chlorophyll content, and stomatal density measurements. Leaves were collected each month within a few days of  $T_{\text{leaf}}$  and  $g_s$  measurements. Leaves were placed in sealed plastic bags and stored in a cooler before being transported from the study site to the



Auburn University Tree Physiology Lab. When each leaf was removed from the bag, measurements were taken as follows. First, chlorophyll content (Chl,  $\mu\text{mol m}^{-2}$ ) was recorded using an MC-100 Chlorophyll Concentration Meter (Apogee Instruments; Logan, UT). Next, three leaf discs (3 mm each) were taken from each fresh leaf and placed directly into a 0.5 mL plastic graduated microcentrifuge test tube filled with a 9:1 mixture of 100 % Ethanol and 100 % Glacial Acetic Acid to clear (i.e., remove chlorophyll) overnight at 5 °C. Each leaf was then weighed using the MS104S Analytical Balance (Mettler Toledo; Columbus, OH) to determine fresh mass (FM, g). Next, leaf dimensions (area, length, maximum width, average width) were measured using a LI-3000C area meter (LICOR; Lincoln, NE). Leaves were oven dried at 70 °C to constant weight to obtain dry mass. The following day, leaf discs were added to 90% Ethanol to rehydrate and stored until ready to be processed. Leaf discs were transferred into Visikol® for Plant Biology™ (VISIKOL, Hampton, NJ) up to a week before examination to finish the clearing process and obtain the most transparent samples. Leaf discs were placed under a fluorescence microscope to view the epidermis of the leaf and photographed for stomatal density (SD,  $\text{m m}^{-2}$ ) to be counted.

## 2.5 Analysis

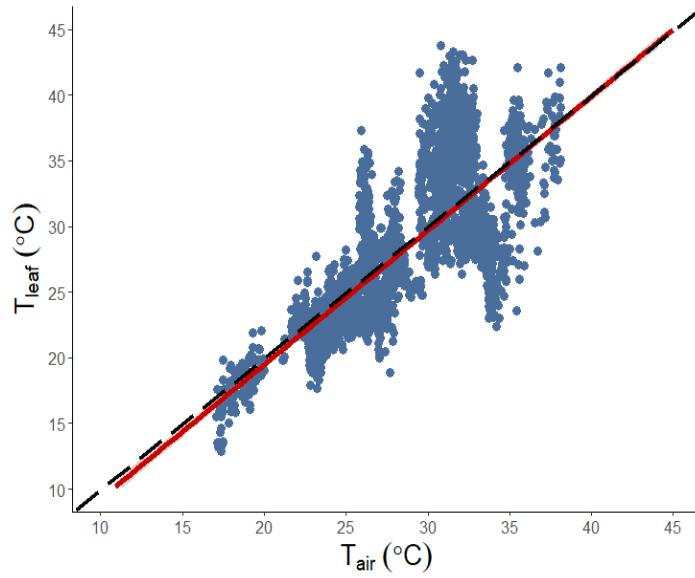
All analysis were conducted using RStudio (R v.4.1.1, RStudio v.2023.3.1.446; R Core Team 2021). We fit a mixed-effect ANCOVA using *lmerTest* package in R (Kuznetsova, Brockhoff, & Christensen, 2017) to test the effects of  $T_{\text{air}}$ , species, month, water, nutrient, and their interactions on  $T_{\text{leaf}}$ . ‘Time’ and ‘block’ were added to the model as crossed random effects. We fit a simple linear regression model to test the effects of mean values of LMA, LDMC, Chl, SD, Leaf N, LS, and  $g_s$  on  $\beta$  parameters for  $T_{\text{leaf}} \sim T_{\text{air}}$ . Mean values of each trait were calculated for each species x month x water x nutrient (e.g., live oaks in May under HW and HN had a mean LMA of 103.80  $\text{g m}^{-2}$ ). Due to the lack of sampling months for Leaf N and SD, we developed three models, one with all leaf traits, one without Leaf N, and one without Leaf N and SD. To create the most parsimonious models, we used stepwise selection with AIC criteria with a critical value of  $P = 0.05$  to ensure only significant variables remained in the model. We also fit a mixed effect

ANOVA to test the effects of species, month, water, nutrient, and their interactions on each leaf trait. ‘Block’ was added as a random effect. Pair-wise analyses with multiplicity adjustments were used for post-hoc analysis to reduce type I error (package *emmeans* for *post-hoc* Tukey pairwise comparisons; Lenth, 2023).

## 3.0 Results

### 3.1 Environmental conditions and leaf temperature variation

Mean ( $\pm$  standard error) values for ambient air temperature, RH, Rad, WS, and GS from days where field observations were made are included in **Table 2**. During field observation days, ambient air temperature ranged from 13 to 38 °C, with the lowest temperature occurring in the mornings of September and highest occurring in the afternoons of June. Relative humidity ranged from 20.2 to 99.9%, with the lowest occurring in the afternoons of September and the highest occurring in the mornings of May, July, and August. Solar radiation ranged from 0.6 to 1159 W m<sup>-2</sup>, with the lowest occurring during the mornings of every month and the highest occurring in the afternoon of all months except September. Wind speed ranged from 0 to 11.25 m s<sup>-1</sup> while GS ranged from 0 to 12.39 m s<sup>-1</sup>, with the highest speed of each occurring in the afternoons of May. Averaged across months, soil VWC was 65% higher for HW treatments ( $20.5 \pm 0.2$  %) than LW treatments ( $12.4 \pm 0.3$  %) on field measurement days. Leaf temperature varied between 12.9 and 45.1 °C and was strongly correlated with T<sub>air</sub>. Averaged across measurement months, species, and resource availability treatments, the relationship between T<sub>air</sub> and T<sub>leaf</sub> was linear with a slope of 1.02, indicating that these seedlings were poikilothermic on average (**Fig. 1**). The difference between T<sub>leaf</sub> and T<sub>air</sub> ranged from -13.8 to 11.9 °C, with most of the variation between -1.4 and 2.6 °C.



**Figure 1.** Relationship between leaf temperature ( $T_{\text{leaf}}$ ) and air temperature ( $T_{\text{air}}$ ) across all species, treatments, and measurement months. Black dashed line represents 1:1 relationship, and red solid line represents line of best fit for data ( $y = -0.94 + 1.02x$ ,  $r^2 = 0.58$ ,  $P < 0.001$ ).

**Table 2.** Mean ( $\pm$  standard error) values for meteorological parameters air temperature ( $T_{\text{air}}$ ), relative humidity (RH), solar radiation (Rad), wind speed (WS), and gust speed (GS) measured on field observation days from May to September 2022.

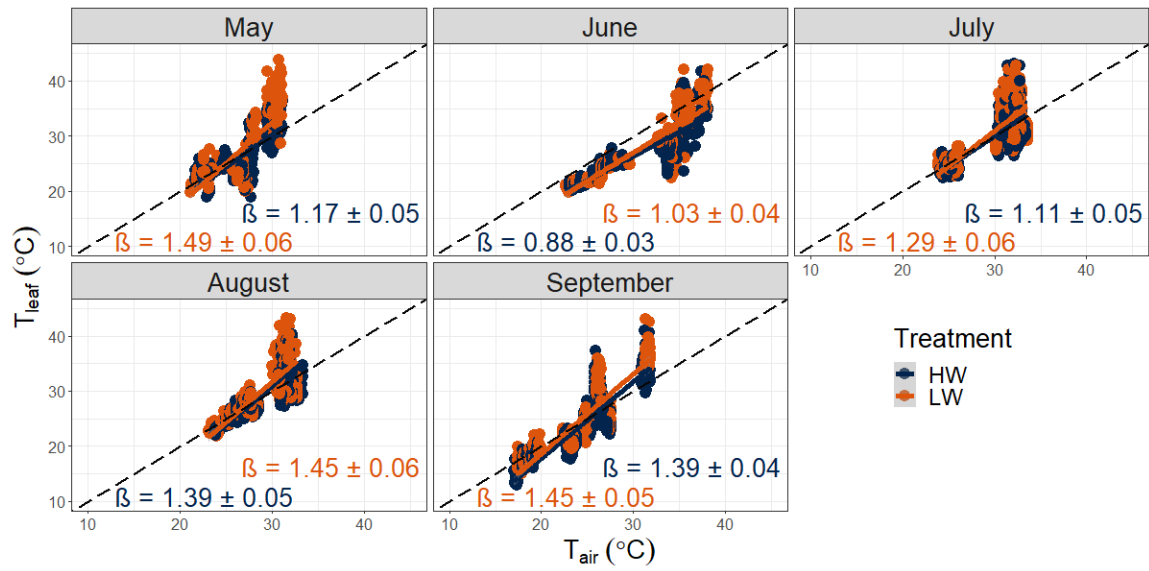
Month	Time of Day*	$T_{\text{air}}$ (°C)	RH (%)	Rad ( $\text{W m}^{-2}$ )	WS ( $\text{m s}^{-1}$ )	GS ( $\text{s}^{-1}$ )
May	Morning	$22.6 \pm 0.2$	$91.9 \pm 0.8$	$175 \pm 24$	$3.5 \pm 0.2$	$5.1 \pm 0.2$
	Afternoon	$28.8 \pm 0.2$	$60.5 \pm 1.0$	$702 \pm 37$	$5.9 \pm 0.3$	$7.3 \pm 0.3$
	Evening	$27.9 \pm 0.4$	$60.6 \pm 1.1$	$159 \pm 28$	$3.2 \pm 0.3$	$4.5 \pm 0.3$
June	Morning	$26.7 \pm 0.6$	$66.9 \pm 1.2$	$259 \pm 35$	$0.1 \pm 0.1$	$0.3 \pm 0.1$
	Afternoon	$35.7 \pm 0.2$	$42.1 \pm 0.6$	$876 \pm 17$	$0.7 \pm 0.1$	$1.6 \pm 0.2$
	Evening	$34.7 \pm 0.3$	$44.1 \pm 1.0$	$307 \pm 37$	$0.5 \pm 0.1$	$1.1 \pm 0.2$
July	Morning	$25.0 \pm 0.3$	$90.6 \pm 0.9$	$218 \pm 26$	$0.5 \pm 0.1$	$0.7 \pm 0.1$
	Afternoon	$31.7 \pm 0.1$	$61.4 \pm 0.6$	$783 \pm 34$	$1.3 \pm 0.1$	$1.7 \pm 0.1$
	Evening	$31.8 \pm 0.2$	$57.6 \pm 0.8$	$312 \pm 31$	$1.0 \pm 0.1$	$1.7 \pm 0.1$
August	Morning	$24.8 \pm 0.3$	$88.8 \pm 1.1$	$225 \pm 24.0$	$0.2 \pm 0.1$	$0.5 \pm 0.1$
	Afternoon	$31.5 \pm 0.1$	$62.1 \pm 0.6$	$723 \pm 34.3$	$1.6 \pm 0.2$	$2.4 \pm 0.2$
	Evening	$29.9 \pm 0.3$	$67.6 \pm 1.3$	$176 \pm 23.1$	$0.8 \pm 0.2$	$1.3 \pm 0.2$
September	Morning	$18.9 \pm 0.5$	$60.2 \pm 2.0$	$224 \pm 24.5$	$0.1 \pm 0.0$	$0.3 \pm 0.1$
	Afternoon	$27.8 \pm 0.3$	$28.6 \pm 0.6$	$817 \pm 6.9$	$0.7 \pm 0.2$	$1.2 \pm 0.2$
	Evening	$24.4 \pm 0.4$	$32.8 \pm 0.8$	$175 \pm 26.1$	$0.3 \pm 0.1$	$0.6 \pm 0.2$

\*Note: Morning (07:00 to 09:00), afternoon (12:00 to 14:00), and evening (17:00 to 19:00)

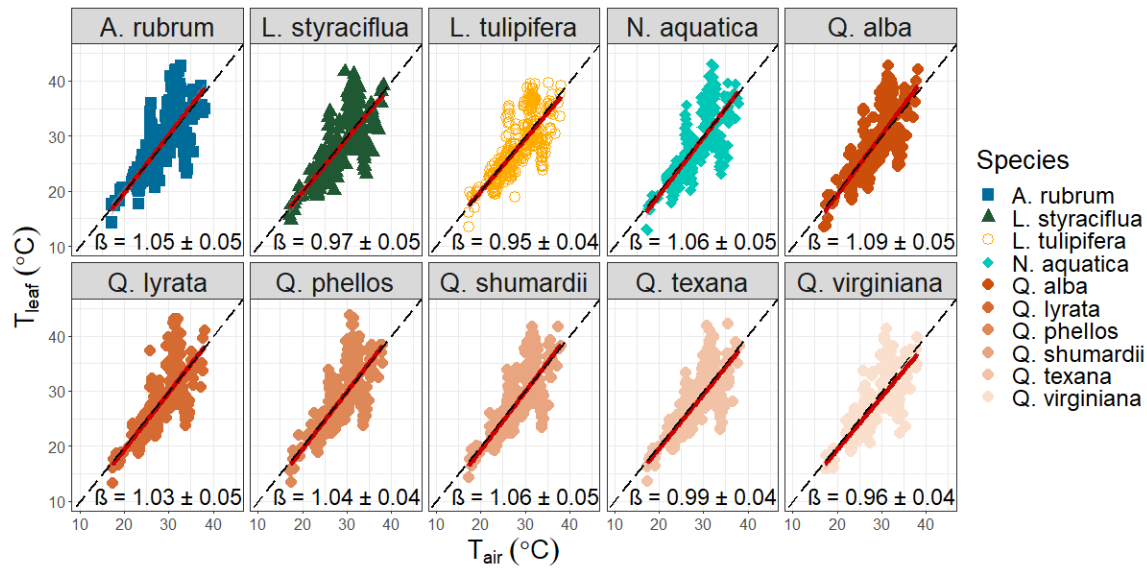
### 3.2 Does $\beta$ differ amongst diverse tree species and resource availability?

While the relationship between  $T_{\text{air}}$  and  $T_{\text{leaf}}$  was generally poikilothermic, we found that interactions between  $T_{\text{air}}$  and species as well as interactions between  $T_{\text{air}}$ , measurement month, and water influenced  $T_{\text{leaf}}$  (**Table 3**). The HW treatment dampened the increase in  $T_{\text{leaf}}$  per unit increase in  $T_{\text{air}}$  (i.e.,  $\beta$ ) compared to LW in the same month (**Fig 1**). However, the magnitude of effect of water availability differed between months ( $T_{\text{air}} \times M \times W$ ; **Table 3, Fig 2**). The variance in  $\beta$  between HW and LW treatments was greatest in May, and with each successional month, the variance in  $\beta$  between the two treatments became less, such that by August and September, there was no significant difference in  $\beta$  between the two treatments.

The relationship between  $T_{\text{air}}$  and  $T_{\text{leaf}}$  also differed between species ( $T_{\text{air}} \times S$ ; **Table 3, Fig 3**). For *Q. virginiana*, *Q. texana*, *L. styraciflua*, and *L. tulipifera*, the slope of the relationship between  $T_{\text{leaf}}$  and  $T_{\text{air}}$  (i.e.,  $\beta$ ) was less than 1 ( $0.95 \pm 0.04$  to  $0.99 \pm 0.04$ ), indicating that leaves of these species were generally cooler than  $T_{\text{air}}$ . For *A. rubrum*, *N. aquatica*, *Q. lyrata*, *Q. phellos*, *Q. shumardii*, and *Q. alba*,  $\beta$  was greater than 1 ( $1.03 \pm 0.05$  to  $1.09 \pm 0.05$ ), indicating that leaves of these species were generally warmer than  $T_{\text{air}}$  (**Fig 3**). We found no evidence of nutrient availability directly influencing the relationship between  $T_{\text{leaf}}$  and  $T_{\text{air}}$ .



**Figure 2.** Relationship between air temperature ( $T_{\text{air}}$ ) and leaf temperature ( $T_{\text{leaf}}$ ) for month by water interaction. The black dashed line reflects a 1:1 relationship.  $\beta$  represent lines of best fit of all data for high water (HW) and low water (LW) each month.



**Figure 3.** Relationship between air temperature ( $T_{air}$ ) and leaf temperature ( $T_{leaf}$ ) for species. The black dashed line reflects a 1:1 relationship. Red solid line and  $\beta$  represent lines of best fit of all data for each species. Shapes used to denote different families.

**Table 3.** Mixed-effect ANCOVA of  $T_{air}$ , species, month, water, nutrient, and their interactions on  $T_{leaf}$ . P-values with ‘\*’, ‘\*\*’, and ‘\*\*\*’ are significant at  $P < 0.05$ ,  $P < 0.01$  and  $P < 0.001$ , respectively. 4- and 5- way interactions were not shown and were insignificant.

	SS	DF	F value	$P(>F)$
$T_{air}$	11089.418	1	2750.047	<0.001***
Species (S)	89.175	9	2.457	0.009**
Month (M)	383.175	4	23.756	<0.001***
Water (W)	114.622	1	28.425	<0.001***
Nutrient (N)	0.027	1	0.007	0.935
$T_{air}$ X S	133.541	9	3.680	<0.001***
$T_{air}$ X M	1058.238	4	65.608	<0.001***
$T_{air}$ X W	192.571	1	47.755	<0.001***

T <sub>air</sub> x N	0.082	1	0.020	0.886
S x M	129.658	36	0.893	0.652
S x W	33.806	9	0.932	0.496
S x N	19.050	9	0.525	0.858
M x W	70.163	4	4.350	0.002**
M x N	18.018	4	1.117	0.347
W x N	7.218	1	1.790	0.181
T <sub>air</sub> x S x M	166.042	36	1.144	0.256
T <sub>air</sub> x S x W	44.310	9	1.221	0.277
T <sub>air</sub> x S x N	25.261	9	0.696	0.713
T <sub>air</sub> x M x W	73.515	4	4.558	0.001***
T <sub>air</sub> x M x N	19.831	4	1.229	0.296
T <sub>air</sub> x W x N	5.383	1	1.335	0.248
S x M x W	35.124	36	0.242	1.000
S x M x N	53.245	36	0.367	1.000
S x W x N	19.793	9	0.545	0.842
M x W x N	12.555	4	0.778	0.539

### 3.3 Leaf trait variation

We analyzed each leaf trait to understand differences between species, measurement months, and resource treatments (**Table 4**). Interactions and significant main effects for each trait are described below. Species mean ( $\pm$  standard error) for each trait across measurement months and treatments is provided in **Table 5**.

#### Leaf mass per area (LMA)

Species varied in LMA, but species differences depended upon interactions between month and nutrient treatment (S x M x N), and interactions between water and nutrient treatments (S x W x N; **Fig. 4**). For *A. rubrum*, *L. styraciflua*, *L. tulipifera*, *Q. lyrata*, and *Q. virginiana*, LMA variability between months was greater under HN. For *N. aquatica* and *Q. shumardii*, variability



between months was greater under LN. For *Q. alba*, LMA increased each month under HN, but under LN this trend only continued through August, and LMA decreased in September. This was true for *Q. phellos*, except under LN, LMA was more variable, and no trend was detected. For *Q. texana*, nutrient availability did not appear to have an effect on LMA differences between months.

LMA was higher under HW than LW regardless of nutrient availability for *L. styraciflua* ( $89.4 \pm 19.0$  and  $51.4 \pm 10.3$  g m<sup>-2</sup>), *N. aquatica* ( $68.7 \pm 11.3$  and  $63.6 \pm 11.4$  g m<sup>-2</sup>), *Q. alba* ( $99.6 \pm 24.1$  and  $83.6 \pm 17.9$  g m<sup>-2</sup>), and *Q. shumardii* ( $93.9 \pm 15.0$  and  $86.6 \pm 20.8$  g m<sup>-2</sup>). For *A. rubrum*, LMA was lower under HW than LW ( $71.2 \pm 15.9$  and  $79.9 \pm 13.6$  g m<sup>-2</sup>), regardless of nutrient availability. For *L. tulipifera*, *Q. lyrata*, *Q. phellos*, and *Q. texana*, LMA was higher under HW when nutrient availability was low, but when nutrient availability was high, LMA was higher under LW. For *Q. virginiana*, LMA was higher under HW when nutrient availability was high, but when nutrient availability was low, LMA was higher under LW.

#### **Leaf dry matter content (LDMC)**

Species varied in LDMC, but species differences depended upon interactions between month and nutrient treatment (S x M x N), and interactions between water and nutrient treatments (S x W x N; **Fig 4**). For *L. styraciflua*, *Q. shumardii*, and *Q. virginiana*, LDMC continued to increase each month until August and then decreased in September. This trend was consistent between nutrient treatments, however, for *Q. virginiana* the range in LDMC within each month was greater under HN and for *L. styraciflua* the range in LDMC within each month was greater under LN. For *A. rubrum* and *Q. texana*, no clear patterns existed between any months under either nutrient treatment. For *N. aquatica*, *Q. alba*, *Q. lyrata*, and *Q. phellos*, LDMC decreased between May and June, increased until August, and then decreased in September. This trend was the same regardless of nutrient availability for *Q. lyrata* and *Q. phellos*. For *N. aquatica* the range in LDMC within each month was greater under HN than LN, and for *Q. alba* the range in LDMC within each month was greater under LN than HN.

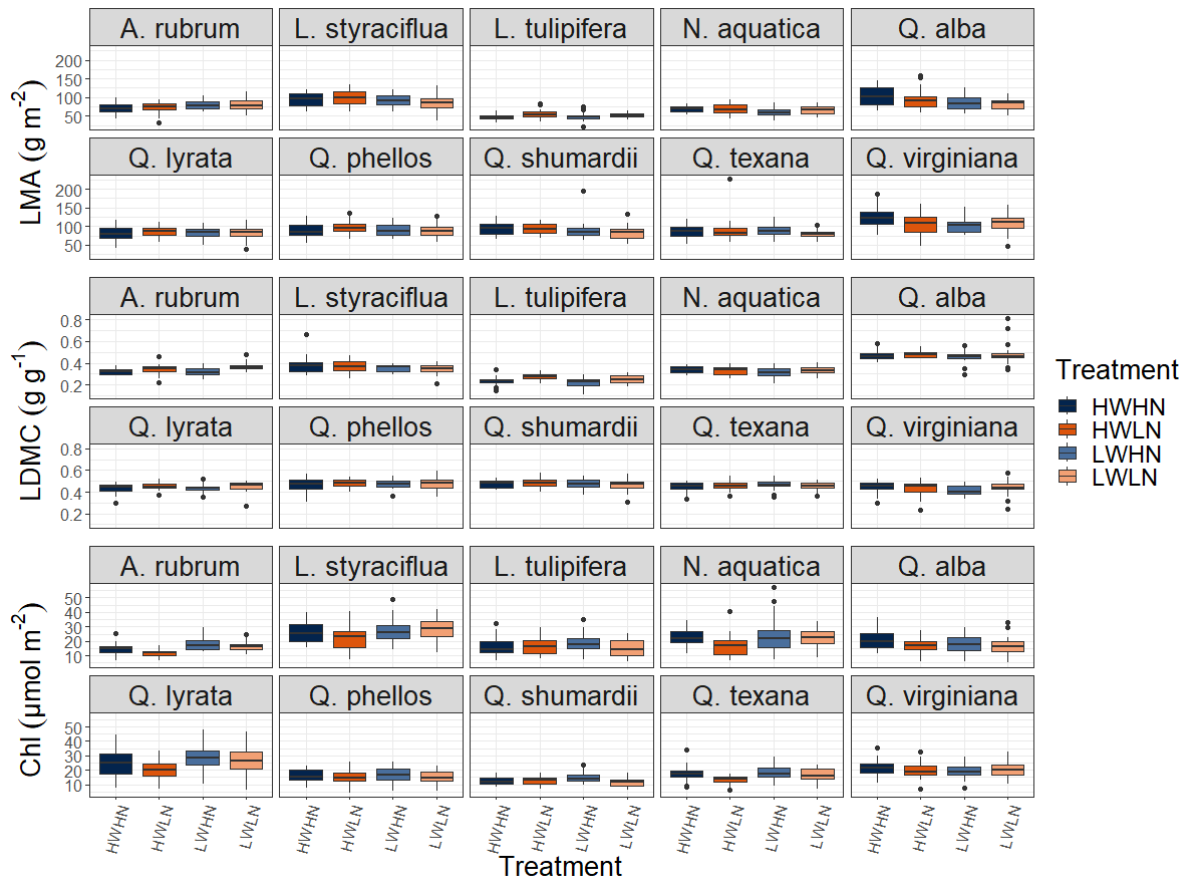
LDMC was higher under HW than LW regardless of nutrient availability for *L. styraciflua* ( $0.37 \pm 0.06$  and  $0.34 \pm 0.03$  g g<sup>-1</sup>), *L. tulipifera* ( $0.25 \pm 0.03$  and  $0.23 \pm 0.04$  g g<sup>-1</sup>), *N.*

*aquatica* ( $0.33 \pm 0.03$  and  $0.32 \pm 0.04$  g g<sup>-1</sup>), *Q. alba* ( $0.47 \pm 0.03$  and  $0.46 \pm 0.07$  g g<sup>-1</sup>), *Q. shumardii* ( $0.48 \pm 0.03$  and  $0.47 \pm 0.05$  g g<sup>-1</sup>), and *Q. virginiana* ( $0.44 \pm 0.05$  and  $0.43 \pm 0.05$  g g<sup>-1</sup>). For *A. rubrum* and *Q. lyrata*, LDMC was higher under HW when nutrient availability was high, but when nutrient availability was low, LDMC was higher under LW. For *Q. phellos*, HN led to a greater range in LDMC under HW, and LN led to a greater range in LDMC under LW. For *Q. texana*, LDMC was higher under LW when nutrient availability was high, but when nutrient availability was low there was nominal difference between LDMC between water treatments.

### **Chlorophyll content (Chl)**

Species varied in LMA, but species differences depended upon interactions between water and nutrient treatments (S x W x N; **Fig 4**) as well as month of measurement (S x M; **Fig 5**). For *A. rubrum* ( $17.2 \pm 3.9$  and  $13.2 \pm 3.6$ ), *L. styraciflua* ( $27.5 \pm 8.1$  and  $24.2 \pm 8.0$ ), and *Q. lyrata* ( $27.7 \pm 8.7$  and  $22.6 \pm 9.0$ ), Chl was higher under LW regardless of nutrient availability. For *L. tulipifera* and *Q. shumardii*, Chl was higher under LW when nutrient availability was high, but when nutrient availability was low, Chl was higher under HW. For *N. aquatica* and *Q. virginiana*, Chl was high when both water and nutrient availability were low. For *N. aquatica*, when nutrient availability was high there was no difference in Chl between high and low water availability. For *Q. virginiana*, when nutrient availability was high Chl was higher under HW than LW. For *Q. alba*, Chl was higher under HW regardless of nutrient availability. For *Q. phellos*, Chl did not differ between either water or nutrient availability. For *Q. texana*, Chl did not differ between water availability under HN, but when nutrient availability was low, Chl was higher under LW.

For *A. rubrum*, *L. tulipifera*, *Q. phellos*, *Q. shumardii*, *Q. texana*, and *N. aquatica*, Chl did not significantly vary between months. For *L. styraciflua*, *Q. alba*, *Q. lyrata*, and *Q. virginiana*, Chl did not significantly vary until August, when leaves began to exhibit increased Chl through September.



**Figure 4.** Box plot of mean values for leaf mass per area (LMA), leaf dry matter content (LDMC), and chlorophyll content (Chl) for each species by treatment combination of high water (HW) or low water (LW) and high nutrient (HN) or low nutrient (LN). The boxes represent the interquartile range (25th–75th percentile). Within each box, horizontal black line denotes median value; dots denote observations outside the 10th and 90th quartile.

### Stomatal density (SD)

Species varied in SD, but species differences were dependent upon month (S x M; **Fig. 5**).

Variation between species was highest in July. We found that only *Q. alba* varied significantly in SD between months, and all other species had minimal variation in SD between measurement months. We found that species belonging to the *Fagaceae* family had the highest SD. *Q. alba* had the highest SD ( $997 \pm 293$  mm<sup>2</sup>) while *L. tulipifera* had the lowest ( $306 \pm 82$  mm<sup>2</sup>).

Resource availability did not appear to have an effect on SD.

### **Stomatal conductance ( $g_s$ )**

Species varied in  $g_s$ , but this variation was dependent on water availability (S x W). Seedlings under HW had 31.8% higher rates of  $g_s$  than LW ( $0.15 \pm 0.09$  and  $0.11 \pm 0.08$  mol m<sup>-2</sup> s<sup>-1</sup>, respectively). Greater variation between species also occurred under HW than LW.

The variation between species was also dependent upon month (S x M; **Fig 5**). For *Q. virginiana*,  $g_s$  was lowest in May and increased through August. For *L. styraciflua*,  $g_s$  was consistent in May and June and then decreased through September. All other species maintained consistent levels of  $g_s$  each month. The greatest variation in  $g_s$  occurred in August, while the least variation between species occurred in May. High nutrient availability also resulted in lower rates of  $g_s$  than LN ( $0.12 \pm 0.09$  and  $0.14 \pm 0.1$  mol m<sup>-2</sup> s<sup>-1</sup>, respectively).

### **Leaf size (LS)**

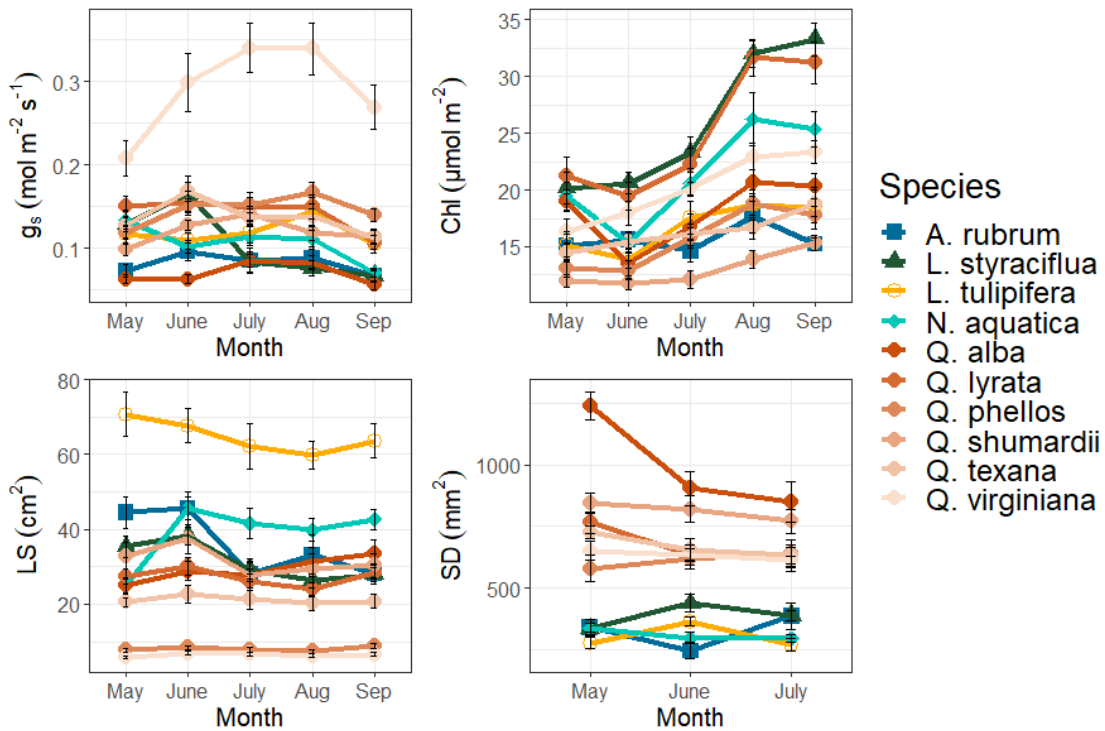
Species varied in LS, but this variation was dependent on water availability (S x W), measurement month (S x M; **Fig 5**), and nutrient availability (S x N). Regardless of water or nutrient availability, *Q. alba* and *Q. phellos* were significantly smaller than all other species, while *L. tulipifera* was significantly larger than all other species (**Table 5**).

Seedlings under HW had generally larger LS ( $31.7 \pm 23.3$  cm<sup>2</sup>) than LW ( $26.5 \pm 18.0$  cm<sup>2</sup>), with the greatest variation between species occurring under HW. Seedlings under HN had generally lower LS ( $28.9 \pm 20.4$  cm<sup>2</sup>) than seedlings under LN ( $29.3 \pm 21.4$  cm<sup>2</sup>), with the greatest variation between species occurring under LN. Species variation in LS was highest in July and lowest in May.

### **Leaf N**

Species varied in Leaf N, but this variation was dependent on nutrient availability (S x N). *L. tulipifera* had significantly higher levels of Leaf N than any other species regardless of nutrient availability (**Table 5**). *L. styraciflua* and *Q. phellos* had significantly lower levels of Leaf N compared to most species (**Table 5**).

Averaged across species and nutrient treatments, water availability also influenced Leaf N (**Table 4**). Leaf N levels were 17.3% higher under LW ( $2.57 \pm 0.52$  %) than HW ( $2.16 \pm 0.63$  %). Leaf N was only determined for leaves collected in July, and therefore further analysis by month could not be conducted.



**Figure 5.** Species means ( $\pm$  standard error) for leaf size (LS), chlorophyll content (Chl), stomatal conductance ( $g_s$ ), and stomatal density (SD) by month. Lines of best fit are included for each species. Shapes used to denote different families.

**Table 4.** Analysis of variance of water, nutrient, species, month, and their interactions on leaf mass per area (LMA), leaf dry matter content (LDMC), chlorophyll content (Chl), stomatal density (SD), stomatal conductance ( $g_s$ ), leaf size (LS), and leaf nitrogen (Leaf N). Leaf traits with (\*) indicate significance in models from Table 6. F-values with ‘\*’, ‘\*\*\*’, and ‘\*\*\*\*’ are significant at  $P < 0.05$ ,  $P < 0.01$  and  $P < 0.001$ , respectively.

	LMA		LDMC*		Chl*		SD		$g_s^*$		LS*		Leaf N*	
	DF	F Value	DF	F Value	DF	F Value	DF	F Value	DF	F Value	DF	F Value	DF	F Value
<b>Species</b>	9	142.994****	9	460.645****	9	74.667****	9	87.487****	9	104.99****	9	160.839****	9	13.151****
<b>Month</b>	4	120.256****	4	97.693****	4	65.981****	2	5.506**	4	12.142****	4	7.284****		
<b>Water</b>	1	22.362****	1	6.939**	1	30.303****	1	2.378	1	114.289****	1	37.234****	1	48.513****
<b>Nutrient</b>	1	0.407	1	22.685****	1	44.72****	1	0.008	1	14.765****	1	0.131	1	29.025****
<b>S x M</b>	36	4.163****	36	3.772****	36	3.929****	18	3.395****	36	3.165****	36	2.567****		
<b>S x W</b>	9	6.157****	9	2.994**	9	4.947****	9	0.930	9	8.216****	9	3.141****	9	1.697
<b>M x W</b>	9	2.43**	4	5.183****	4	7.802****	2	0.192	4	1.236	4	0.575		
<b>S x N</b>	4	2.511*	9	4.024****	9	0.954	9	0.620	9	0.737	9	2.036**	9	3.403****
<b>M x N</b>	4	0.236	4	1.738	4	0.871	2	0.791	4	0.071	4	1.680		
<b>W x N</b>	1	0.169	1	0.569	1	7.298**	1	0.953	1	0.177	1	1.126	1	0.114
<b>S x M x W</b>	36	0.744	36	1.347	36	0.795	18	0.649	36	1.313	36	0.528		
<b>S x M x N</b>	36	1.6*	36	1.449*	36	0.805	18	0.754	36	0.694	36	0.736		
<b>S x W x N</b>	9	3.449****	9	2.237*	9	2.184*	9	0.638	9	0.427	9	1.290	9	1.51
<b>M x W x N</b>	4	1.525	4	2.334	4	1.142	2	1.014	4	0.239	4	0.857		
<b>S x M x W x N</b>	36	1.102	36	0.947	36	0.796	18	1.234	36	0.802	36	0.609		

**Table 5.** Species mean ( $\pm$  standard error) values for leaf mass per area (LMA), leaf dry matter content (LDMC), chlorophyll content (Chl), stomatal density (SD), stomatal conductance ( $g_s$ ), leaf size (LS), and leaf nitrogen (Leaf N).

<b>Species</b>	<b>LMA</b> ( $\text{g m}^{-2}$ )	<b>LDMC</b> ( $\text{g g}^{-1}$ )	<b>Chl</b> ( $\mu\text{mol m}^{-2}$ )	<b>SD</b> ( $\text{mm}^2$ )	<b><math>g_s</math></b> ( $\text{mol m}^{-2} \text{s}^{-1}$ )	<b>LS</b> ( $\text{cm}^2$ )	<b>Leaf N</b> (%)
<i>A. rubrum</i>	76.4 $\pm$ 15.2	0.34 $\pm$ 0.04	15.6 $\pm$ 4.3	327 $\pm$ 134	0.08 $\pm$ 0.03	36.7 $\pm$ 20.5	2.57 $\pm$ 0.49
<i>L. styraciflua</i>	92.7 $\pm$ 19.2	0.36 $\pm$ 0.06	25.8 $\pm$ 8.2	386 $\pm$ 97	0.10 $\pm$ 0.04	31.2 $\pm$ 11.3	1.71 $\pm$ 0.42
<i>L. tulipifera</i>	50.8 $\pm$ 9.9	0.25 $\pm$ 0.04	16.5 $\pm$ 6.3	306 $\pm$ 82	0.12 $\pm$ 0.03	65.1 $\pm$ 23.5	2.99 $\pm$ 0.94
<i>N. aquatica</i>	66.2 $\pm$ 11.6	0.33 $\pm$ 0.04	21.4 $\pm$ 8.3	311 $\pm$ 65	0.11 $\pm$ 0.02	38.8 $\pm$ 16.2	2.42 $\pm$ 0.44
<i>Q. alba</i>	91.6 $\pm$ 22.6	0.47 $\pm$ 0.06	18.1 $\pm$ 6.0	997 $\pm$ 293	0.07 $\pm$ 0.02	29.3 $\pm$ 14.8	2.28 $\pm$ 0.43
<i>Q. lyrata</i>	83.3 $\pm$ 16.3	0.44 $\pm$ 0.04	25.2 $\pm$ 9.2	672 $\pm$ 136	0.14 $\pm$ 0.04	27.2 $\pm$ 10.0	2.51 $\pm$ 0.54
<i>Q. phellos</i>	91.4 $\pm$ 17.2	0.47 $\pm$ 0.05	15.6 $\pm$ 4.6	612 $\pm$ 155	0.15 $\pm$ 0.02	8.0 $\pm$ 2.7	2.53 $\pm$ 0.67
<i>Q. shumardii</i>	89.9 $\pm$ 18.8	0.47 $\pm$ 0.05	13.0 $\pm$ 3.3	811 $\pm$ 148	0.12 $\pm$ 0.02	31.6 $\pm$ 14.8	2.24 $\pm$ 0.50
<i>Q. texana</i>	85.8 $\pm$ 20.1	0.46 $\pm$ 0.04	16.3 $\pm$ 4.9	673 $\pm$ 137	0.14 $\pm$ 0.02	21.1 $\pm$ 9.6	2.25 $\pm$ 0.49
<i>Q. virginiana</i>	111.6 $\pm$ 26.5	0.44 $\pm$ 0.06	20.2 $\pm$ 5.4	635 $\pm$ 121	0.29 $\pm$ 0.08	6.4 $\pm$ 2.5	2.25 $\pm$ 0.25

### 3.4 Do leaf traits and their variations explain differences in $\beta$ ?

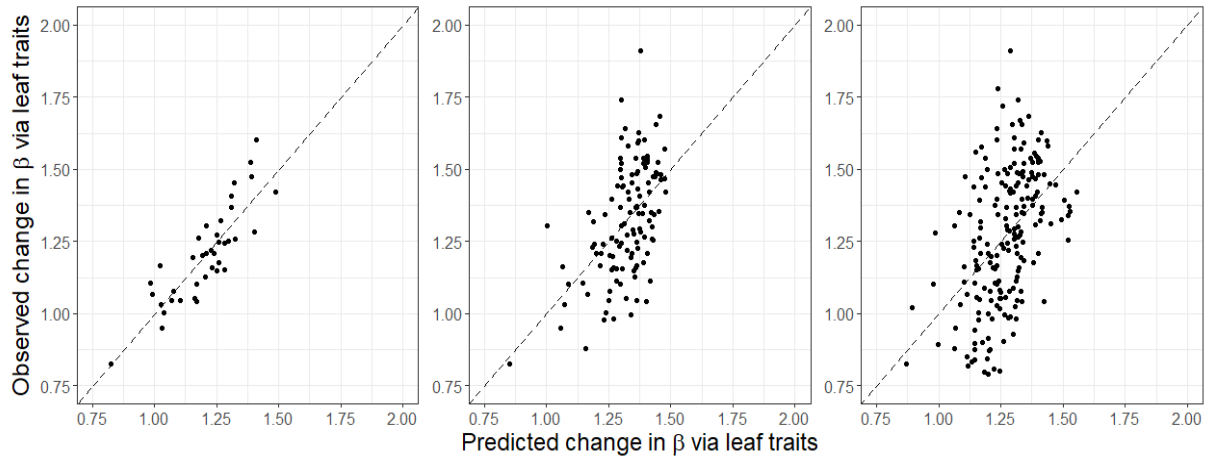
We tested whether variation in the relationship between  $T_{\text{leaf}}$  and  $T_{\text{air}}$  was explained by variation in leaf traits. We performed three separate stepwise regression models based on mean leaf trait values for different months. Model 1 analyzed all data from the month of July, when Leaf N observations were present. Model 2 analyzed all mean leaf trait data from the months of May, July, and September, when SD observations were present and excluding Leaf N. Model 3 analyzed all mean leaf trait data from all measurement months, excluding Leaf N and SD. In model 1, ( $n = 40$ ; **Table 6**), we found that LS,  $g_s$ , and LDMC were negatively associated with  $\beta$  (reduced  $T_{\text{leaf}}$  relative to  $T_{\text{air}}$ ), while leaf N was positively associated with  $\beta$  (increased  $T_{\text{leaf}}$  relative to  $T_{\text{air}}$ ). In order of importance (based on t-value),  $g_s$  was most important followed by leaf N, LS, LDMC. Overall, this model explained 72% of the variation in  $\beta$  ( $P = <0.001$ ). In model 2 ( $n = 120$ ; **Table 6**), we found that LS,  $g_s$ , and LDMC were each negatively associated with  $\beta$ . This model explained 28% of the variation in  $\beta$  ( $P < 0.001$ ). In model 3 ( $n = 200$ ; **Table 6**) we found that Chl became a significant factor, positively influencing  $\beta$ , and that LS and  $g_s$  remained significant by negatively influencing  $\beta$ . This model explained 23% of the variation in  $\beta$  ( $P < 0.001$ ). We conclude that  $\beta$  variability is most responsive to  $g_s$ , followed by Leaf N (when included) and LS.



**Table 6.** Summary of stepwise multiple linear regression analyses used to determine the relationship between leaf traits (independent variables) and leaf temperature to air temperature relationship (dependent variable;  $\beta$ ). Only significant variables are included in the table. Leaf size (LS), leaf dry matter content (LDMC), stomatal conductance ( $g_s$ ), leaf nitrogen (Leaf N), and chlorophyll content (Chl) were found to be significant in at least one of the models.

<b>Model</b>	<b>Variable</b>	<b>Parameter Estimate (<math>\pm</math> SE)</b>	<b><i>t</i>-value</b>	<b><i>p</i>-value</b>	<b>Model <math>r^2</math></b>	<b>Model <i>p</i>-value</b>
<b>Model 1</b>	Intercept	1.524 $\pm$ 0.222	6.85	<0.001		
<i>Only July</i> <i>n = 40</i>	LS	-0.006 $\pm$ 0.001	-4.104	<0.001		
	LDMC	-0.793 $\pm$ 0.315	-2.517	0.017		
	$g_s$	-1.303 $\pm$ 0.204	-6.385	<0.001		
	Leaf N	0.151 $\pm$ 0.034	4.504	<0.001	0.7273	<0.001
<b>Model 2</b>	Intercept	1.85 $\pm$ 0.137	13.542	<0.001		
<i>May, July, Sept.</i> <i>n = 120</i>	LS	-0.004 $\pm$ 0.001	-3.445	0.001		
	LDMC	-0.488 $\pm$ 0.247	-1.974	0.051		
	$g_s$	-1.626 $\pm$ 0.245	-6.635	<0.001	0.284	<0.001
<b>Model 3</b>	Intercept	1.4 $\pm$ 0.073	19.159	<0.001		
<i>All measurement months</i> <i>n = 200</i>	$g_s$	-1.486 $\pm$ 0.227	-6.55	<0.001		
	Chl	0.009 $\pm$ 0.003	3.494	0.001		
	LS	-0.003 $\pm$ 0.001	-3.591	<0.001	0.2373	<0.001

**Figure 6.** Relationship between the predicted vs observed response of  $\beta$  to leaf traits for the linear regression models in **Table 6**. Left panel includes all leaf traits ( $n = 40$ ), middle panel includes all leaf traits except leaf N ( $n = 120$ ), and right panel includes all leaf traits except Leaf N and SD ( $n = 200$ ).



## 4.0 Discussion

Our study examined how species leaf traits and resource availability affected the relationship (i.e., slope) between leaf temperature and air temperature ( $\beta$ ). Previous discussions on  $T_{\text{leaf}}$  primarily focused on whether  $T_{\text{leaf}}$  differs from  $T_{\text{air}}$ , and this research aimed to deepen our understanding of the underlying morphological, anatomical, or physiological mechanisms driving variation in  $\beta$ . Across all timepoints, species, and treatments, we found that  $\beta$  was close to 1, suggesting that these trees were largely poikilothermic. However, we found that species, water availability, and to some extent timing (month) influenced  $\beta$  to change significantly. Consistent with our hypothesis, species variation in  $\beta$  was in part due to species variation in a few key leaf traits. Specifically, stomatal conductance and leaf size significantly influenced  $\beta$ , while leaf N content, LDMC and chlorophyll content influenced  $\beta$  at varying levels. While few studies have looked at how leaf traits influence  $T_{\text{leaf}}$  (Guo, et al., 2022; Fauset, et al., 2018; Cook, Berry, Milner, & Leigh, 2021; Leigh, Sevanto, Close, & Nicotra, 2017), even fewer have looked at particular traits that we measured in this study (e.g., Chl, leaf N). Species were chosen to reflect the diversity of eastern U.S. forests, and species leaf traits were expected to influence  $\beta$  at varying degrees as a reflection of environmental adaptation to each species niche. The results of this study give new insight into drivers of  $T_{\text{leaf}}$  regulation and improves our understanding of plant performance within diverse forests and across environmental conditions.

### 4.1 Influence of diverse species leaf traits

Higher rates of  $g_s$  leads to greater evaporative cooling (Urban, Ingwers, McGuire, & Teskey, 2017), and therefore is unsurprising that  $g_s$  had the greatest impact on  $T_{\text{leaf}}$ . We found that *Quercus* individuals had higher rates of  $g_s$  than other species in our study, which is likely because the individuals that we studied are primarily from bottomland habitats. These *Quercus* species use higher amounts of water at the leaf scale because they are adapted to environments where water limitation rarely occurs (Oren & Pataki, 2003). *N. aquatica* is also well-adapted to swamps and bottomlands but did not show as high of rates of  $g_s$ , which may reflect this species' slow-growing nature. We found no significant correlations between  $g_s$  and the other leaf traits

measured in this study, and therefore conclude that  $g_s$  regulates  $T_{\text{leaf}}$  independent of variations in other traits.

We found larger LS correlated with lower  $\beta$ . However, we expected that larger LS may result in higher absorbed radiation, and previous studies supported that smaller and narrower leaves have thinner boundary layers that allow for quicker diffusion and greater evaporative cooling (Yates, Anthony Verboom, Rebelo, & Cramer, 2010; Fauset, et al., 2018; Leigh, Sevanto, Close, & Nicotra, 2017; Parkhurst & Loucks, 1972). Our findings can be attributed to the thinner and lower LMA of the large leaves we studied compared to the smaller leaves (Leigh, Sevanto, Close, & Nicotra, 2017). We found that LS was negatively correlated with LMA and LDMC, and this aligns with past studies showing a trade-off between thermal stability and leaf longevity (Loucks, 1972). Additionally, LS offers high variation via phenotypic plasticity which may buffer  $T_{\text{leaf}}$  while maximizing photosynthesis (Michaletz et al 2015). *Q. shumardii* and *L. tulipifera* were found to have the highest LS, and both species are found in broad habitat ranges which may allow for greater phenotypic plasticity in many environments and conditions (Richards, Bossdorf, Muth, Gurevitch, & Pigliucci, 2006; Gratani, 2014). *Q. phellos* and *Q. virginiana* had the smallest LS, but also had higher density (i.e., LMA) than other species. We conclude that the variation in LS between species partially explains the difference in species  $\beta$ , and that high LS can lead to lower  $\beta$  when species have lower LMA and LDMC.

We also found an inverse relationship existed between leaf N and  $g_s$  in regard to  $T_{\text{leaf}}$  regulation; high leaf N led to higher  $\beta$ -values, while high  $g_s$  lead to lower  $\beta$ -values, yet no significant correlation existed between the two. This contradicts a previous study that showed that leaf N positively correlated with  $g_s$  (Schulze, Kelliher, Korner, Lloyd, & Leuning, 1994). Instead, our result is in line with more recent work done by Cramer et al. (2009) which found that a complex and varying trade-off exists between water and nutrient fluxes. Higher levels of leaf N are indicative of higher rates of photosynthesis and greater photosynthetic stability (Croft et al., 2017), which are found to have an inverse relationship with thermal stability (Michaletz S., Weiser, Zhou, Heliker, & Enquist, 2015). However, higher  $T_{\text{leaf}}$  related to higher rates of photosynthesis may be offset by the influx of latent cooling caused by higher levels of  $g_s$ ,

creating a balance between the maintenance of  $T_{\text{leaf}}$  near optimum photosynthetic levels (Friend, 1991; Blonder & Michaletz, 2018). It is important to recognize that leaf N measurements were only recorded for the month of July. Obtaining additional data could potentially reveal patterns or correlations that were not apparent in the limited dataset. However, we conclude that higher leaf N leads to warmer  $T_{\text{leaf}}$ , and that future  $T_{\text{leaf}}$  regulation studies should include leaf N to further capture the relationship between leaf N and  $T_{\text{leaf}}$ .

We found that LDMC had a negative relationship with  $\beta$ . As LDMC increased, leaves tended to be cooler and therefore had a smaller  $\beta$ . This aligns with studies that have previously hypothesized that an increase in LDMC would dampen  $T_{\text{leaf}}$  increases under certain conditions and increase thermally stability (Curtis, Leigh, & Rayburg, 2012; Lin, Chen, Zhang, Fu, & Fan, 2017; Michaletz S., Weiser, Zhou, Heliker, & Enquist, 2015). Notably, *Quercus* species had higher LDMC than other species in this study and again this may be due to the rich bottomland environments that they are adapted to living in. We conclude that larger LDMC will lead to cooler  $T_{\text{leaf}}$  regulation.

We also found that Chl had a positive relationship with  $\beta$ , with higher Chl correlating with warmer  $T_{\text{leaf}}$ . Although no previous studies have been conducted on the effects of Chl on  $T_{\text{leaf}}$ , Chl is generally correlated with high light absorption (Vogelmann & Evans, 2002) and photosynthetic capacity (Croft, et al., 2017) and therefore is unsurprising that higher concentrations would lead to greater  $T_{\text{leaf}}$  and  $\beta$ . Chlorophyll content did not correlate with any other traits and varied greatly between species. Therefore, we conclude that Chl is an independent trait that influences  $T_{\text{leaf}}$  regardless of the degree of variance between other traits.

#### **4.2 Influence of resource availability and season**

We found evidence that  $T_{\text{leaf}}$  was influenced by resource availability both directly and indirectly. Water availability had a direct effect on  $T_{\text{leaf}}$ , with high water availability leading to lower  $T_{\text{leaf}}$  and  $\beta$  values. Water availability also affected all leaf traits except SD; of the traits that influenced  $\beta$ , high water availability led to higher  $g_s$  and LS, while low water availability led to higher leaf N. This indicates that the relationship between these traits and  $\beta$  is made stronger when water supply is adequate. Our results regarding water availability are both intuitive and consistent with

other work (Marchin, et al., 2021; Dong, Prentice, Harrison, Song, & Zang, 2017; Cook, Berry, Milner, & Leigh, 2021). We also found that over the length of the study, differences between water treatments became minimal, such that by August and September the influence of water on  $\beta$  was the same between treatments. This shows a degree of acclimation amongst the trees to the environmental conditions they are subject to.

Although we found no direct evidence of nutrient availability on  $T_{\text{leaf}}$ , we found the majority of leaf traits were variable between nutrient treatments and that changes in leaf traits had an effect on  $\beta$ . Therefore, nutrient availability may have an indirect effect on  $T_{\text{leaf}}$  regulation. This has been observed in studies such as Ordonez et al (2010) which found that the effect of soil fertility levels on leaf traits was present and variable for each trait, implying that regulation of traits may differ at different nutrient levels. Interestingly, we did not identify patterns between traits for any species under different nutrient availability treatments. Understanding the impacts of resource availability on both  $T_{\text{leaf}}$  and leaf traits is important for creating a better understanding of the direct and indirect effects that growth conditions have on plant physiological processes. It is evident that water and nutrient availability play a strong role in the relationship between leaf physiology and temperature regulation. Trade-offs between traits may be strengthened under distinct levels of resource availability (Cramer, Hawkins, & Verboom, 2009), further allowing complex combinations of traits at varying levels to influence  $T_{\text{leaf}}$  beyond what we have observed (Blonder, Escobar, Kapas, & Michaletz, 2020).

In addition, we found that all species leaf traits varied by month, and it may be important to consider time of year and leaf development when discussing the effects that traits have on  $T_{\text{leaf}}$  regulation (Reich P. B., 2014; Yang, et al., 2016). Reich et al (1990) found that leaves are not fully functionally developed for efficient photosynthetic rates early in the growing season, but later in the season the relationship between Leaf N, LS, and LMA changes, leading to increased photosynthetic rates (Reich, Walters, & Ellsworth, 1990). As leaf traits change seasonally, and as relationships between traits shift, we can expect that individual traits influence on  $T_{\text{leaf}}$  will also change. Additionally, further research into the acclimation of leaf traits to environmental conditions may provide more understanding for the balance between  $T_{\text{leaf}}$  regulation and other

leaf physiological processes (e.g., photosynthesis, photorespiration). Our study was limited to months where leaves were fully developed, and therefore environments where seasonality is less prevalent may find less impact of measurement month on  $T_{\text{leaf}}$  regulation.

The lack of greater observed difference between  $T_{\text{leaf}}$  and  $T_{\text{air}}$  may have been influenced by the environmental constraints we imposed during the study. Our measurements were taken on clear sunny days without stressful events (e.g., heatwave), and our low resource availability treatments were not meant to simulate extreme stressed environments such as prolonged drought or severe nutrient deficiencies (Guo et al., 2023). The cost to regulate  $T_{\text{leaf}}$  may be greater than the risk of senescence when ambient air temperatures are within leaf safety margins (Drake et al 2020). Although avoiding high temperatures via leaf temperature regulation may be theoretically advantageous to a plant's growth and survival (Henn, et al., 2018), the mechanisms that could drive thermoregulation may not be stronger than the intense role that environmental factors have been observed to have on  $T_{\text{leaf}}$  (Drake, et al., 2020). When we modeled leaf trait influences on  $\beta$  with the maximum number of observations, we found that the fit of the model was low, indicating much was unaccounted for. Further research with a broader range of traits (e.g., leaf thickness, vapor pressure deficit) and controlled experimental conditions could provide a more comprehensive understanding of the factors influencing  $T_{\text{leaf}}$  regulation.

Nonetheless, our findings support that seedlings are largely poikilothermic, and that resource availability, species variability in leaf traits, and changes across months affect the relationship between  $T_{\text{leaf}}$  and  $T_{\text{air}}$ . High water availability contributes to lower  $\beta$  and greater variability in many leaf traits. Variations in leaf traits have influence on the relationship between  $T_{\text{leaf}}$  and  $T_{\text{air}}$ , and certain species exhibit better adaptations for  $T_{\text{leaf}}$  regulation. Additionally, leaf traits and the influence of resource availability can be variable throughout a single season of growth. These results broaden our understanding of plant physiological responses under changing climates and will allow for better predictions of individual species and overall forest responses in the future.

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