

# Plant heat tolerance characterizes carbon assimilation strategy

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## Abstract

The heat tolerance of photosystem II (PSII) may promote carbon assimilation at higher temperatures and may help explain plant responses to climate change. PSII heat tolerance could lead to 1) increases in the high temperature compensation point ( $T_{\max}$ ); 2) increases in the thermal breadth of photosynthesis (i.e. the photosynthetic  $\Omega$  parameter) to promote a thermal generalist strategy of carbon assimilation; 3) increases in the optimum rate of carbon assimilation  $P_{\text{opt}}$  and promote faster carbon assimilation; and/or 4) increases in the optimum temperature for photosynthesis ( $T_{\text{opt}}$ ). To address these hypotheses, we tested if the  $T_{\text{crit}}$ ,  $T_{50}$  and  $T_{95}$  metrics of PSII heat tolerance were correlated with each carbon assimilation parameter for 21 species. Hypothesis 1 was not supported, but we observed that  $T_{50}$  may estimate the upper thermal limit for  $T_{\max}$  at the species-level, and that community mean  $T_{\text{crit}}$  may be useful for approximating  $T_{\max}$ . The  $T_{50}$  and  $T_{95}$  heat tolerance metrics were positively correlated with  $\Omega$  in support of hypothesis 2. We found no support for hypotheses 3 or 4. Our study shows that high PSII heat tolerance is unlikely to improve carbon assimilation at higher temperatures, but may characterize thermal generalists with slow resource acquisition strategies.

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The heat tolerance of photosystem II (PSII) may promote carbon assimilation at higher temperatures and may help explain plant responses to climate change. PSII heat tolerance could lead to 1) increases in the high temperature compensation point ( $T_{\max}$ ); 2) increases in the thermal breadth of photosynthesis (i.e. the photosynthetic  $\Omega$  parameter) to promote a thermal generalist strategy of carbon assimilation; 3) increases in the optimum rate of carbon assimilation  $P_{\text{opt}}$  and promote faster carbon assimilation; and/or 4) increases in the optimum temperature for photosynthesis ( $T_{\text{opt}}$ ). To address these hypotheses, we tested if the  $T_{\text{crit}}$ ,  $T_{50}$  and  $T_{95}$  metrics of PSII heat tolerance were correlated with each carbon assimilation parameter for 21 species. Hypothesis 1 was not supported, but we observed that  $T_{50}$  may estimate the upper thermal limit for  $T_{\max}$  at the species-level, and that community mean  $T_{\text{crit}}$  may be useful for approximating  $T_{\max}$ . The  $T_{50}$  and  $T_{95}$  heat tolerance metrics were positively correlated with  $\Omega$  in support of hypothesis 2. We found no support for hypotheses 3 or 4. Our study shows that high PSII heat tolerance is unlikely to improve carbon assimilation at higher temperatures, but may characterize thermal generalists with slow resource acquisition strategies.

**Key Words:** (need 5-10) leaf economics spectrum, climate change, photosynthetic temperature response, thermoregulation, mountain passes hypothesis

## Introduction

The heat tolerance of plants' photosystem II (PSII) photochemistry may provide a useful estimate of the upper thermal limit of photosynthesis, and has the potential to explain the physiological mechanisms underlying

some of the ecological responses of plants to climate change (Clark, Piper, Keeling & Clark 2003; Doughty & Goulden 2009; Mau, Reed, Wood & Cavaleri 2018; Pau, Detto, Kim & Still 2018; Feeley, Fadrique, Perez & Zuleta 2020). Higher heat tolerance of PSII photochemistry is generally assumed to allow for improved growth, reproduction, and/or survival in hot environments, presumably by allowing for photosynthesis at higher temperatures (Krause, Winter, Krause & Virgo 2015; Feeley, Martinez-villa, Perez & Duque 2020a; Perez & Feeley 2020; Tiwari *et al.* 2020). However, these assumptions have not been widely tested and it is unclear how PSII heat tolerance integrates with different thermal strategies that may be important for determining the impacts of climate change.

Heat tolerance of PSII is commonly measured using chlorophyll *a* fluorescence. Early studies to adopt the use of chlorophyll fluorescence quantified PSII heat tolerance using the  $F_0$  fluorometric parameter - indicating the number of maximally open reaction centers - and found it was correlated with the temperature that caused carbon assimilation to approach zero ( $T_{max}$ ; Downton, Berry & Seemann 1984; Seemann, Berry & Downton 1984). However,  $F_0$  can provide biased estimates of PSII function during heat treatments that change leaf optical properties (Baker 2008), which has led many researchers to adopt the maximum quantum yield ( $F_V/F_M$ ) fluorometric as a more robust metric for estimating PSII heat tolerance where  $F_V = F_M - F_0$ , and  $F_M$  indicates closed reaction centers in saturating light (Maxwell & Johnson 2000; Baker 2008).

Although  $F_V/F_M$  can reliably measure PSII function under stress treatments and is commonly used to measure PSII heat tolerance,  $F_V/F_M$  may not be a reliable proxy for carbon assimilation under field conditions.  $F_V/F_M$  is only proportional to carbon assimilation under low light conditions and when photorespiration is minimized (Brooks & Farquhar 1985; Baker 2008). These conditions are not met in the field when leaves experience high light and temperatures. Few studies have tested if  $F_V/F_M$  heat tolerance promotes carbon assimilation in hotter environments, but empirical evidence and ecological theory generally support this assumption.

As was shown with heat tolerance estimates that used  $F_0$  (Downton *et al.* 1984; Seemann *et al.* 1984), one way the PSII heat tolerance could promote photosynthesis at higher temperatures is if it is correlated with  $T_{max}$ . Reported values for  $T_{max}$  range from 40.1 to 41.8°C and are comparable to the temperatures that cause the first signs of damage in  $F_V/F_M$  ( $T_{crit}$ ) for tropical species (**Fig. 1a**; Slot *et al.* 2018; Tiwari *et al.* 2020; Perez and Feeley 2020). Coordination between  $T_{crit}$  and  $T_{max}$  would provide support for the hypothesis that PSII heat tolerance fixes the upper limit of carbon assimilation by limiting electron transport (Slot & Winter 2017a).

Another way that PSII heat tolerance could promote carbon assimilation at higher temperatures is by increasing the breadth of temperatures over which carbon assimilation can occur ( $\Omega$ ; **Fig. 1a**; Cunningham S. C. & Read J. 2003; Slot & Winter 2017a). The  $\Omega$  metric can be used to characterize plants as physiological thermal generalists vs. specialists, similar to what is done with animal species (Huey & Hertz 1984; Ghalambor, Huey, Martin, Tewksbury & Wang 2006; Huey 2012). A positive correlation between PSII heat tolerance and  $\Omega$  would be consistent with a thermal generalist strategy of carbon assimilation and would provide a physiological explanation for why thermal specialist plant species are more susceptible to climate change than generalist plants (Janzen 1967; Ghalambor *et al.* 2006; Perez, Stroud & Feeley 2016). Indeed,  $\Omega$  is a key trait linking leaf thermoregulation to the “fast-slow” leaf economic spectrum (Michaletz *et al.* 2015, 2016). Since variation in PSII heat tolerance is driven by high leaf temperature (Perez & Feeley 2020) and ‘fast’ species are expected to have high leaf temperatures and large  $\Omega$  (Michaletz *et al.* 2015, 2016), PSII heat tolerance is expected to be proportional to  $\Omega$ .

Plants with ‘fast’ resource acquisition strategies are characterized in part by their high rates of carbon assimilation (Wright *et al.* 2004; Reich 2014). The plant economic spectrum typically proposes that ‘fast’ strategies are characterized by poor physiological tolerances (Reich 2014), such that the optimum rates of photosynthesis ( $P_{opt}$ , **Fig. 1a**) and PSII heat tolerances may be inversely proportional. Conversely, since ‘fast’ species are also characterized by high leaf temperature (Michaletz *et al.* 2015, 2016), PSII heat tolerance may be positively correlated to  $P_{opt}$ . This expectation is consistent with the idea that high PSII heat tolerance is beneficial for plants growing in hot environments.

The optimum temperature for carbon assimilation ( $T_{\text{opt}}$ , **Fig. 1a**) is another important parameter that describes carbon assimilation as a function of temperature and is potentially coordinated with PSII heat tolerance. For example, species tend to increase in both their PSII heat tolerance and  $T_{\text{opt}}$  when grown in hotter environments (Valladares & Pearcy 1998; Way & Yamori 2014; Zhu *et al.* 2018). High PSII heat tolerance may promote increases in  $T_{\text{opt}}$  by improving electron transport or the availability of ATP and NADH at high temperatures (Genty, Briantais & Baker 1989; Maxwell & Johnson 2000; Baker 2008), in support of the assumption that PSII heat tolerance will facilitate carbon assimilation in hot environments.

In this study we measured three common metrics of PSII heat tolerance that indicate the temperatures that cause an initial, 50%, and 95% decrease in  $F_V/F_M$  ( $T_{\text{crit}}$ ,  $T_{50}$  and  $T_{95}$ , respectively; **Fig. 1b**). We compared these metrics of heat tolerance to  $T_{\text{max}}$ ,  $P_{\text{opt}}$ ,  $T_{\text{opt}}$ , and  $\Omega$  for 21 plant species grown in a quasi-common garden environment (Fairchild Tropical Botanic Garden, Coral gables FL USA; Perez *et al.* 2019). We tested four hypotheses consistent with the assumption that high PSII heat tolerance promotes carbon assimilation in hotter environments. Specifically, we looked at the correlations among the different metrics of heat tolerance and carbon assimilation, after controlling for any potential effect of phylogenetic non-independence, to test the hypotheses that H1)  $T_{\text{max}}$  is constrained by PSII heat tolerance; H2) high PSII heat tolerance is indicative of a thermal generalist strategy of carbon assimilation; H3) high PSII heat tolerance is characteristic of species with “fast” carbon acquisition strategies; and H4) high PSII heat tolerance promotes higher  $T_{\text{opt}}$  (**Fig. 2**).

## Materials and Methods

### *Site and species selection*

This study used plants in the living collections of the Fairchild Tropical Botanic Garden (FTBG) in Coral Gables, FL USA. FTBG is located at 25.68N / 80.28W, has a mean annual temperature of 24.1°C, and an average total annual precipitation of 130 cm. We took advantage of the FTBG’s diverse collection by selecting study plants that were mature individuals, had canopies that received direct sunlight for most of the day, were accessible from the ground, and represented different families. We ultimately selected 21 species from 20 different families that exhibited a variety of growth habits (Table S1). We used only one individual per species to measure the parameters describing the relationship between temperature and carbon assimilation, and the three different metrics of heat tolerance.

### *Temperature-assimilation curves*

To estimate the carbon assimilation parameters used in this study (i.e.  $P_{\text{opt}}$ ,  $T_{\text{opt}}$ ,  $T_{\text{max}}$ , and  $\Omega$ ), we first repeatedly measured leaf temperature and net carbon assimilation for each of our study species using a LI-6800 portable photosynthesis system (LICOR, Lincoln, NE, USA). More specifically, we randomly visited each focal plant during sunny days between June 21 and September 1, 2018, and measured carbon assimilation over a range of leaf temperatures within the canopy of each individual following the general methods of Slot and Winter (2017a). Leaf temperature was first measured on a set of randomly selected leaves within the canopy of each individual with a MT6 MiniTemp infrared thermometer (Raytek, Wilmington, NC USA). For each leaf, the LI-6800 cuvette was set to the observed temperature, and the leaf was allowed to acclimate to chamber conditions before its net assimilation was measured. During all measurements, the LI-6800 leaf chamber was maintained at saturating light levels ( $1000 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$ ).

The  $\text{CO}_2$  concentration was maintained at either 400 or 405ppm in the reference chamber (differences due to operator error). Varying  $\text{CO}_2$  reference chamber concentrations can potentially bias estimates of our carbon assimilation parameters. To correct for this, we conducted a separate set of measurements for 17 of our 21 species in which we varied the reference chamber  $\text{CO}_2$  over a range of concentration to measure the effect that this could have on  $\text{CO}_2$  assimilation in the sample chamber. We modeled this effect using the ‘smooth.spline’ function in base R’s ‘stats’ package (Core 2020) to calculate the difference in leaf assimilation rates between sample chamber  $\text{CO}_2$  concentrations of 400 and 405 ppm. This difference was then added to assimilation measurements taken at 400ppm to correct for any potential bias in our results. Even prior to correction,  $\text{CO}_2$  concentrations within the sample chamber were uniformly distributed with a mean  $\text{CO}_2$

concentration of 386ppm and a standard deviation of 8 ppm – a level of variation that is only slightly greater than those observed in leaf chambers of other studies (sd = 6ppm; Slot & Winter 2017a), and is unlikely to have affected our results.

The sample chamber’s relative humidity was set to 50% during sampling, but was automatically varied as needed to prevent moisture condensation within the LI-6800. In order to avoid sensor drift, the LI-6800 reference and sample chambers’ infrared gas analyzers were matched any time the sample chamber’s leaf temperature was changed by  $\sim 5^\circ\text{C}$  since the previous match. We visually assessed stabilization of leaf temperatures, assimilation rates, and stomatal conductances before recording carbon assimilation rate ( $\mu\text{mol m}^{-2} \text{s}^{-1}$ ).

Assimilation was modeled as a function of temperature following the model presented in June, Evans & Farquhar (2004) and adapted by Slot & Winter (2017a):

$$P(T) = P_{\text{opt}} \times e^{-\left(\frac{T_{\text{leaf}} - T_{\text{opt}}}{\Omega}\right)^2} \quad \text{Eq. 1}$$

where  $T_{\text{leaf}}$  is leaf temperature and  $\Omega$  is defined as the difference between the temperatures above and below  $T_{\text{opt}}$  at which assimilation ( $P$ ) is reduced by  $\sim 37\%$  from  $P_{\text{opt}}$  (**Fig. 1a**).

We estimated  $T_{\text{max}}$  and additional values of  $T_{\text{opt}}$  and  $P_{\text{opt}}$  following the model from Cunningham S. C. & Read J. (2003), which provides better fits for asymmetrical temperature-assimilation curves:

$$P = \{b(T_{\text{leaf}} - T_{\text{min}}) \times [1 - e^{c(T_{\text{leaf}} - T_{\text{max}})}]\}^2 \quad \text{Eq. 2}$$

where  $b$  and  $c$  are constants,  $P$  is the assimilation rate,  $T_{\text{min}}$  is the theoretical low-temperature compensation point and  $T_{\text{max}}$  is the theoretical high-temperature compensation point (**Fig. 1a**).

The  $P_{\text{opt}}$ ,  $T_{\text{opt}}$  and  $\Omega$  parameters of Eq. 1, and the  $b$ ,  $c$ ,  $T_{\text{min}}$  and  $T_{\text{max}}$  parameters from Eq. 2 were estimated based on the fits of logistic non-linear least squares (nls) functions in R’s base ‘stats’ package (Core 2020). We bootstrapped the parameter estimates for each model and species by randomly resampling our leaf temperature and assimilation dataset 1000 times with replacement. We present the bootstrapped means for  $P_{\text{opt}}$ ,  $T_{\text{opt}}$  and  $\Omega$  from Eq 1, and  $T_{\text{max}}$ ,  $P_{\text{opt}}$ , and  $T_{\text{opt}}$  from Eq. 2.

#### *Determining $T_{\text{crit}}$ , $T_{50}$ , and $T_{95}$ heat tolerances*

At the end of the study period, we collected random leaves from each focal individual and brought them to nearby laboratory facilities at the University of Miami. Depending on the size of the leaves, between 3 and 66 leaves were collected from each individual and used to determine the heat tolerances. Random leaflets from different leaves were sampled if species had compound leaves. Once in the lab, we used a hole punch to cut  $\sim 1.9$  cm diameter disks from the leaves. We placed six leaf disks from each individual in Miracloth fabric to prevent anaerobiosis during heat treatments (Krause *et al.* 2010); one layer of Miracloth was placed on the abaxial leaf surface and three layers of Miracloth were placed on the adaxial leaf surface. We then placed the Miracloth-enclosed leaves into waterproof plastic bags with air removed and submerged in water baths maintained at room temperature ( $\sim 23^\circ\text{C}$ ), 38, 40, 42, 44, 46, 48, 50, 52, 54, or  $60^\circ\text{C}$  with circulating heaters. Immediately following 15-minutes of heat treatment, we removed the leaf pieces from water baths, placed them into petri dishes lined with moist paper towels, and allowed them to recover for 24 hours at room temperature under low light ( $\sim 1\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ). Following this recovery period, we dark-adapted the leaf pieces for 20 minutes before measuring their maximum quantum yield ( $F_v/F_m$ ) with an OS30p<sup>+</sup> handheld fluorometer (Opti-Science, Hudson, NH USA).

To estimate each species’  $T_{\text{crit}}$  and  $T_{50}$ , we modeled the relationship of  $F_v/F_m$  versus treatment temperature for each plant using the ‘nls’ function in base R’s ‘stats’ package (Core 2020). We calculated  $T_{\text{crit}}$  by finding the temperature where the slope of the  $F_v/F_m$  vs. temperature relationship reached 15% of its most extreme value. We calculated  $T_{50}$  and  $T_{95}$  by predicting the temperature that caused a 50% or 95% reduction in  $F_v/F_m$  compared to the control treatment as:

$$\text{heat tolerance} = \frac{\log(\frac{\theta_a}{x} - \theta_b)}{\theta_c} \text{ (eq. 3)}$$

where  $\theta_a$  is the asymptote of the heat treatment-response variable relationship,  $\theta_b$  is a constant,  $x$  represents 50% or 95% reduction in  $F_v/F_m$  compared to control treatments, and  $\theta_c$  is the decay parameter. The  $\theta$  parameters were optimized and fit to the temperature-response relationship using R's 'nls' function following

$$y = \frac{\theta_a}{1 + e^{-(\theta_b + \theta_c T)}} \text{ (eq. 4)}$$

where  $T$  is the heat treatment temperature (R Core Team, 2018). We generated bootstrapped means for  $T_{\text{crit}}$ ,  $T_{50}$ , and  $T_{95}$ , by randomly resampling data and fitting a new model for each species 100 times (**Fig. 1b**). We present the mean bootstrapped values for  $T_{\text{crit}}$ ,  $T_{50}$ , and  $T_{95}$ .

### Data Analysis

We generated a phylogenetic tree for our study species to help control for any potential phylogenetic non-independence in our dataset before testing our hypotheses H1-H4. We created a phylogeny from the trimmed R20120829 mega-tree (Gastauer & Meira-Neto 2016) for our study species using the 'brranching' R package's 'phylomatic' function (Chamberlain 2018). We assigned fossil-calibrated branch lengths for our tree using the ph.bladj function in the 'phylocomr' R package (Ooms & Chamberlain 2018). Any polytomies were randomly resolved using the 'multi2di' function in the 'ape' R package (Paradis & Schliep 2018).

We used our phylogenetic tree to compute a phylogenetic variance-covariance (VCV) matrix to test each of our four a priori hypotheses. Our VCV was calculated using the 'phytools' R package and its 'phyl.vcv' function (Revell 2012). This approach assumes traits (in our case the carbon assimilation and heat tolerance metrics) followed a Brownian model of evolution and that trait variance was proportional to branch lengths between two species and their most recent common ancestor. We divided the product of the inverse VCV and the observed trait values by the sum of the inverse VCV matrix to calculate the ancestral trait value at the root of our phylogeny (Blomberg, Garland & Ives 2003; Swenson 2014). These root trait values were used to calculate a phylogenetically corrected covariation matrix among traits that was rescaled to compute Pearson's  $r$  using the 'cov2cor' function in R's base 'stats' package (Core 2020). The t-statistic and an  $\alpha = 0.05$  were used to test for significant trait correlations.

Below, we present the phylogenetically corrected correlations among traits as phylogenetically independent contrasts (PIC) for graphical purposes only. PICs were computed as the difference between two daughter nodes standardized by the square root of the sum of branch lengths (Felsenstein 1985), and performed using the 'pic' function in the 'ape' R package (Paradis & Schliep 2018) which results in  $n - 1$  contrasts where ' $n$ ' is the number of species in the phylogeny. The correlations and PICs are calculated differently, but provide effectively analogous results. We present only the statistics from the phylogenetically corrected correlations for simplicity in our figures. All analyses were conducted using R version 4.0.2 (Core 2020).

### Results

The final dataset that we used to model carbon assimilation as a function of temperature contained between 17 and 52 assimilation measurements per each of 21 plant species. Changes in the sample chamber concentrations from 400 to 405ppm caused no more than a  $0.27 \mu \text{ mol m}^{-2} \text{ s}^{-1}$  increase in carbon assimilation (**Fig. S1**). The fits of Eq. 1 and Eq. 2 to our temperature-assimilation data are presented in **Figure S2**, and the carbon assimilation parameters ( $T_{\text{max}}$ ,  $P_{\text{opt}}$ ,  $T_{\text{opt}}$ , and  $\Omega$ ) estimated from these models are provided in **Table S1**. The modeled changes in  $F_v/F_m$  in response to heat treatments used to calculate  $T_{\text{crit}}$ ,  $T_{50}$ , and  $T_{95}$  heat tolerances are presented in **Figure S3** and provided in **Table S1**. Below we present only values of  $T_{\text{opt}}$  and  $P_{\text{opt}}$  estimated using Eq. 1 because they were highly correlated with their respective estimate from Eq. 2. Results for hypotheses 3 and 4 using  $P_{\text{opt}}$  and  $T_{\text{opt}}$  from Eq. 2 are provided in **Figure S4**.

**Figure 3** summarizes the mean  $T_{\text{opt}}$ ,  $T_{\text{max}}$ ,  $T_{\text{crit}}$ ,  $T_{50}$ , and  $T_{95}$  relative to one another for each species and the entire dataset. The mean trait values for the entire dataset show that  $T_{\text{max}}$  is encompassed within the range of temperatures represented by the mean  $T_{\text{crit}}$  and  $T_{50}$ . This was not the case for species-level data as  $T_{\text{crit}}$  exceeded  $T_{\text{max}}$  for 7 species, but  $T_{\text{max}}$  never exceeded  $T_{50}$ . The only significant correlations we

observed among carbon assimilation parameters involved the  $\Omega$  parameter, which describes the breadth of the temperature-assimilation curves. We observed that  $\Omega$  was significantly correlated to  $T_{\max}$  ( $r = 0.567$ ,  $p = 0.007$ ; **Fig. 4A**), and negatively correlated to  $T_{\text{opt}}$  ( $r = 0.489$ ,  $p = 0.024$ ; **Fig. 4B**). No correlations were observed between  $T_{\text{opt}}$  and  $P_{\text{opt}}$  estimated with either Eq. 1 or 2.

**Figure 5** depicts the phylogenetically controlled correlations between different metrics of heat tolerance for PSII photochemistry and each parameter that describes carbon assimilation as a function of temperature.  $T_{\text{crit}}$  was negatively correlated to  $T_{95}$  ( $r = -0.486$ ,  $p = 0.025$ ) and not correlated to  $T_{50}$  ( $r = -0.089$ ,  $p = 0.700$ ). Our estimates of  $T_{50}$  and  $T_{95}$  were highly correlated ( $r = 0.91$ ,  $p < 0.01$ ) and exhibited similar relationships with the carbon assimilation parameters.

We found that  $T_{\max}$  was not correlated with  $T_{\text{crit}}$ ,  $T_{50}$ , or  $T_{95}$  (**Fig. 5A-C**;  $r = -0.334$ ,  $p = 0.138$ ;  $r = 0.270$ ,  $p = 0.237$ ;  $r = 0.372$ ,  $p = 0.256$ ), which does not support our hypothesis H1.  $T_{\text{crit}}$  was not correlated with  $\Omega$  (**Fig. 5D**;  $r = -0.190$ ,  $p = 0.409$ ), but in support of hypothesis H2 we found that  $T_{50}$  and  $T_{95}$  were positively correlated with  $\Omega$  (**Fig. 5E-F**;  $r = 0.581$ ,  $p = 0.006$ ;  $r = 0.590$ ,  $p = 0.005$ ). Our hypothesis H3 was not supported since we found that  $T_{\text{crit}}$  was not correlated with  $P_{\text{opt}}$  (**Fig. 5G**;  $r = 0.211$ ,  $p = 0.359$ ), but  $T_{50}$  and  $T_{95}$  were negatively correlated with  $P_{\text{opt}}$  (**Fig. 5H-I**;  $r = -0.495$ ,  $p = 0.022$ ;  $r = -0.521$ ,  $p = 0.015$ ). Similar results were obtained using assimilation estimates from Eq. 2 (**Fig. S4A-C**). Our hypothesis H4 was not supported as we observed no correlation between  $T_{\text{crit}}$  and  $T_{\text{opt}}$  from Eq.'s 1 or 2 (**Fig. 5J**;  $r = 0.193$ ,  $p = 0.401$ ; **Fig. S4D**). Furthermore, we observed that  $T_{50}$  exhibited a marginally significant negative correlation to  $T_{\text{opt}}$  from Eq. 1 (**Fig. 5K**;  $r = -0.432$ ,  $p = 0.051$ ), and a significant negative correlation to  $T_{\text{opt}}$  from Eq. 2 (**Fig. S4E**). We found  $T_{95}$  that was negatively correlated to  $T_{\text{opt}}$  from Eq. 1 (**Fig. 5L**,  $r = -0.452$ ,  $p = 0.039$ ), but not from Eq. 2 (**Fig. S4F**). Two notable patterns among these relationships are that 1) correlations between  $T_{\text{crit}}$  and each carbon assimilation parameter were in the opposite direction as those observed for  $T_{50}$  and  $T_{95}$ , and 2) heat tolerances that signify greater PSII impairment ( $T_{95} > T_{50} > T_{\text{crit}}$ ) tend to be more strongly correlated with carbon assimilation parameters, with the exception of  $T_{\text{opt}}$  from Eq. 2 (**Fig. S4D-F**).

When heat tolerances and carbon assimilation traits were not corrected for phylogenetic non-independence, the only significant correlation that persisted was between  $\Omega$  and  $T_{\text{opt}}$ . **Figure S2** suggests Eq. 2 provided a poor fit for our *Hamelia patens* data. We excluded this species due to a potentially erroneous estimation of  $T_{\max}$ , but exclusion of this species did not change our results, so it remained in our final results. We also log- and square root-transformed our estimates of  $T_{\text{opt}}$  and  $T_{\text{crit}}$ , respectively to improve assumptions of normality before our phylogenetic corrections, but this had no effect on our results.

Given the poor coordination between  $T_{\max}$  and our predefined estimates of PSII heat tolerance, we wanted to know if there was a predictable level of damage in  $F_V/F_M$  equal to  $T_{\max}$ . We used Eq. 3 to predict the  $F_V/F_M$  at the temperature equal to  $T_{\max}$  for each species. This estimate of  $F_V/F_M$  was then divided by the mean  $F_V/F_M$  values observed for our control treatment temperatures. The mean  $F_V/F_M$  damage represented by  $T_{\max}$  was 0.07 with a range of  $<0.0$  to 0.45. As a point of comparison, our estimate of  $F_V/F_M$  damage at  $T_{\text{crit}}$  was 0.02 (0.00-0.08, 95% C.I.) damage. Based on this information, we re-calculated a heat tolerance equivalent to the temperature that caused  $F_V/F_M$  to decrease by 7% ( $T_{07}$ ) for each species. After performing the phylogenetic correction explained above, we found that  $T_{07}$  was only correlated with  $T_{\text{crit}}$  ( $r = 0.70$ ,  $p < 0.01$ ).

## Discussion

We investigated the assumption that heat tolerances promote carbon assimilation at higher temperatures. We did not find support for our first hypothesis (H1) that PSII heat tolerance is coordinated with  $T_{\max}$ . One reason that  $T_{\max}$  may not be directly correlated with PSII heat tolerance is because it and the  $T_{\min}$  parameter used to fit Eq. 2 are purported to have no physiological significance (Cunningham S. C. & Read J. 2003), and in many cases  $T_{\min}$  is unrealistically low (Slot & Winter 2017a). It is also likely that stomatal closure ceases carbon assimilation before the actual thermal limits of plant biochemistry (i.e. electron transport or NADPH and ATP generation) are reached (Slot & Winter 2017a b). While PSII heat tolerance and

$T_{\max}$  were not correlated we did find limited support for our hypothesis H1 that PSII heat tolerance provides a conservative high-temperature limit for  $T_{\max}$ . Our estimates of  $T_{\max}$  corresponded to the temperatures that caused between 0 and 45% damage to  $F_v/F_m$ , indicating that  $T_{50}$  may provide a reasonable upper bound for estimates of  $T_{\max}$ . On the other hand, our estimates of  $T_{\text{crit}}$ , which corresponded to temperatures causing ~2% damage to  $F_v/F_m$ , were still higher than  $T_{\max}$  for one third of our study species.

The positive correlations we observed between  $T_{50}$ ,  $T_{95}$  and  $\Omega$  support our second hypothesis (H2) that PSII heat tolerance is characteristic of thermal generalists. This is a notable result given that it is one of the only examples in plants providing an explicit physiological mechanism for the macroecological hypothesis that greater thermal variability should select for broader physiological tolerance (Janzen 1967; Perez *et al.* 2016). Specifically, our results showed that species with the greatest thermal ranges for photosynthesis also tend to have the highest PSII heat tolerances. These results are generally consistent with the predictions of leaf thermoregulatory theory. Deviations from this expectation may occur if there are acclimatory shifts of the PSII heat tolerance or photosynthetic traits away from their optimal values for which the leaf thermoregulatory theory was developed. However, these deviations from theoretical trait relationships are unlikely given our results.

The negative correlations that we observed between  $T_{50}$ ,  $T_{95}$  and  $P_{\text{opt}}$  does not support our hypothesis H3 as proposed in accordance with leaf thermoregulatory theory. Instead these results are consistent with the prediction that species with low carbon assimilation rates are likely to exhibit greater stress tolerance (Wright *et al.* 2004; Reich 2014). Indeed, maintenance of PSII heat tolerance imposes a large metabolic cost that ‘fast’ species may not be able to incur (see below).

Our final hypothesis (H4) posited that if PSII heat tolerance promoted greater carbon assimilation at higher temperatures, it should correspond to higher  $T_{\text{opt}}$ . However, our results suggest that high PSII heat tolerance may actually reduce  $T_{\text{opt}}$ . This counterintuitive relationship may be explained by the metabolic cost of maintaining high PSII heat tolerance. PSII heat tolerance is linked to increased production of heat shock proteins (Wahid, Gelani, Ashraf & Foolad 2007), isoprenoids (Logan & Monson 1999), photoprotective pigments (Krause *et al.* 2015), membrane-fortifying solutes (Hüve, Bichele, Tobias & Niinemets 2006), and the saturation of lipid bilayers (Zhu *et al.* 2018). The production of some of these metabolites may deplete the pools of NADPH and ATP that are available for carbon fixation as they are redirected to PSII thermoprotection (Süss & Yordanov 1986; Gershenzon, 1994; Wahid *et al.* 2007; Taylor, Smith, Slot & Feeley 2019; Voon & Lim 2019), explaining why both  $T_{\text{opt}}$  and  $P_{\text{opt}}$  decrease as PSII heat tolerance increases.

An important assumption we made was that our data were phylogenetically non-independent before we tested our hypotheses. Given that we measured species from a diverse set of families and clades (i.e., 21 species in 20 families), the topology and branch lengths of our phylogenetic tree are likely to provide a reasonable hypothesis of species relatedness. However, our assumption of phylogenetic non-independence could be violated if plasticity in PSII heat tolerance and carbon assimilation actually caused our trait estimates to be unrepresentative of each species (Way & Yamori 2014; Sastry, Guha & Barua 2018). That said, our results currently suggest that there is strong covariation between some PSII heat tolerances and carbon assimilation parameters within phylogenies. Regardless of any phylogenetic correction, we confirmed that at the species-level  $T_{\max}$  occurs at lower temperatures than  $T_{50}$  but not  $T_{\text{crit}}$ , and that a community’s mean  $T_{\text{crit}}$  may provide a reasonable approximation for  $T_{\max}$ ; however we found little evidence to support the assumption that heat tolerance promotes carbon assimilation at high temperatures.

According to our phylogenetically corrected results, the only way that PSII heat tolerance may promote carbon assimilation at higher temperatures is by expanding  $\Omega$ , but this benefit may be offset by concomitant decreases in  $T_{\text{opt}}$  and  $P_{\text{opt}}$ . This is potentially explained by high PSII heat tolerance promoting electron transport or the production of NADPH and ATP at high temperatures (Genty *et al.* 1989; Baker 2008). We noted that the heat tolerances that signify greater PSII impairment (i.e., greater  $F_v/F_m$  damage) tended to have stronger correlations with carbon assimilation parameters. This is consistent with the hypothesis that larger reductions in the quantum yield have a greater effect on plant carbon economics, and may explain why  $T_{\text{crit}}$  heat tolerance was not correlated with any metric of carbon assimilation (Perez & Feeley 2020).

Consequently,  $T_{95}$  may characterize plant thermal ecological strategies more effectively than  $T_{50}$ , but provide overestimates of  $T_{max}$ .

Our results suggest that the heat tolerances of PSII measured with dark-adapted quantum yield ( $F_v/F_m$ ) are not ideal proxies for carbon assimilation. Heat tolerances estimated with light-adapted quantum yield ( $F_q'/F_m'$ ) may be better proxies for assimilation (although these heat tolerances estimates are also subject to biases; Baker 2008). Importantly, we show that  $T_{50}$  provides an upper bound for  $T_{max}$ . We also show that high PSII heat tolerance is characteristic of thermal generalist plant species with ‘slow’ carbon acquisition strategies. These results increase our understanding of the high temperature limits of photosynthesis and can potentially be used to explain macroecological patterns in plant responses to climate change. More specifically, since PSII heat tolerance can characterize thermal specialization, it may prove as a useful tool for predicting the thermal specialists and generalists that are hypothesized to be most and least vulnerable to climate change, respectively (Perez & Feeley 2020).

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## Figure Legends:

Figure 1 A): Illustrates carbon assimilation as a function of leaf temperature for a single exemplar species, *Cynophalla flexuosa* . The black and gray colours indicate Eq. 1 and Eq. 2 model fits to the diamond-shaped

data points, respectively. Eq. 1 was used to calculate  $T_{\text{opt}}$ ,  $P_{\text{opt}}$ , and  $\Omega$ . Eq. 2 was used to calculate  $T_{\text{opt}}$ ,  $P_{\text{opt}}$ , and  $T_{\text{max}}$ ; B) Illustrates the quantum yield ( $F_V/F_M$ ) as a function of temperature treatment used to calculate the  $T_{\text{crit}}$ ,  $T_{50}$ , and  $T_{95}$  heat tolerance (represented as shaded vertical lines) using Eq.'s 3 and 4 for *Cynophalla flexuosa*. The curved shaded lines show 1 of 100 bootstrapped iterations

Figure 2: We address the assumption that PSII heat tolerance can promote greater carbon assimilation at higher temperature. Figure the change in a given trait between a species with a low heat tolerance (solid line) and high heat tolerance (dashed line). Hypothesis 1 (H1) proposes  $T_{\text{max}}$  is constrained by PSII heat tolerance, which could be associated with an increase in  $\Omega$  (short dashed line), an increase in  $T_{\text{max}}$  (long dashed line), or a combination of the two; H2 is that high PSII heat tolerance promotes greater  $\Omega$  indicative of a thermal generalist strategy of carbon assimilation; H3 that high PSII heat tolerance promotes higher  $P_{\text{opt}}$  characteristic of species with “fast” carbon acquisition strategies; and 4) PSII heat tolerance promotes higher  $T_{\text{opt}}$

Figure 3: (top) The mean  $T_{\text{opt}}$  (circles),  $T_{\text{crit}}$  (left edge of the box),  $T_{\text{max}}$  (triangles),  $T_{50}$  (vertical line in center of box), and  $T_{95}$  (right edge of box) for our dataset. The coloured boxes depict the thermal safety margins of PSII heat damage. Dotted lines connect the carbon assimilation traits; (middle) Box plots of temperatures that correspond to the heat tolerance and carbon assimilation parameters of each species; (bottom) The relative temperatures of  $T_{\text{opt}}$ ,  $T_{\text{crit}}$ ,  $T_{\text{max}}$ ,  $T_{50}$ , and  $T_{95}$  for each species arranged from highest to lowest  $T_{\text{max}}$

Figure 4: Here we illustrate the significant correlations among A)  $\Omega$  and  $T_{\text{max}}$ , and B)  $\Omega$  and  $T_{\text{opt}}$  photosynthetic traits after correcting for phylogenetic independence. Each figure shows the phylogenetically independent contrasts for each trait with the phylogenetically corrected Pearson's correlation coefficient and significant in the bottom left corner. Solid lines within plots indicate significant correlations

Figure 5: Here we illustrate the correlations among PSII heat tolerances and photosynthetic traits after correcting for phylogenetic independence. The top rows panels A-C correspond to hypothesis 1, panels D-F correspond to hypothesis 2; panels G-I correspond to hypothesis 3; and panels J-L correspond to hypothesis 4. Each figure shows the phylogenetically independent contrasts for each trait with the phylogenetically corrected Pearson's correlation coefficient and p-value in the bottom left corner. No line within a plot indicates no significant correlation, solid lines indicate significant correlations, and the dashed line indicates a marginally insignificant correlation









