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Carter, Kelsey, "EFFECTS OF IN-SITU LEAF-LEVEL CANOPY WARMING IN A NORTHERN HARDWOOD FOREST", Open Access Master's Thesis, Michigan Technological University, 2017.
<https://doi.org/10.37099/mtu.dc.etdr/412>

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EFFECTS OF IN-SITU LEAF-LEVEL CANOPY WARMING IN A NORTHERN
HARDWOOD FOREST

By

Kelsey R. Carter

A THESIS

Submitted in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

In Applied Ecology

MICHIGAN TECHNOLOGICAL UNIVERSITY

2017

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This thesis has been approved in partial fulfillment of the requirements for the Degree of MASTER OF SCIENCE in Applied Ecology.

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Preface

Chapter 2 of this thesis was made in preparation for publication. The manuscript has not yet been published; therefore, there is no copyright on the document. This thesis was prepared with Molly Cavaleri who conceived and designed the experiment, as well as provided feedback and editing for the thesis manuscript. Kelsey Carter helped design the experiment, collected and analyzed data, and wrote the manuscript.

Acknowledgments

I would like to thank my advisor, Molly Cavaleri, for her guidance, support, and mentorship throughout my pursuit of a Master's degree. Many thanks to my committee members, Robert Froese and Noel Urban for their advice while we were discussing topic selection and for comments and suggests on the thesis. I would like to thank Mark Sloat in Michigan Technological University's Electrical and Computer Engineering Department for designing and helping troubleshoot the warming device. A special thanks to Erik Lilliskov, Joseph DesRoshers, and the USDA Forest Service for the use of their scaffolding, climbing equipment, and environmental data. Also, I would like to thank Jennifer Eikenberry for allowing us to use lab space and for sample analysis. A special thanks to undergraduate assistant, Kaylie Butts, for her ingenuity in the lab and field. I would also like to thank Ben Miller for his assistance in helping troubleshoot and build the warming device. Thanks to undergraduate Elsa Schwartz for her wonderful field and lab assistance. I would also like to thank lab assistants Jack Zwart and Brian Peacock assistance with sample processing. Finally, I would like to thank my husband, Carl Carter, for his encouragement and support throughout the thesis preparation process.

Funding for this project was provided by the National Institute of Food and Agriculture U.S. Department of Agriculture McIntire-Stennis Cooperative Forestry Research Program Grant #1001534 and Department of Energy award DE-SC-0011806. Funding was also provided by the DeVlieg Fellowship and Ecosystem Science Center at Michigan Technological University.

Abstract

Rising mean annual temperatures due to climate change have intensified the need to understand the effects of warming on plant physiological processes. Forest photosynthesis is the most important pathways of terrestrial carbon sequestration, yet continued warming could reduce this important carbon sink. Photosynthesis is highly sensitive to temperature and begins to decline after an optimum temperature (T_{opt}) is reached, leading to reduced carbon uptake. To date, logistical difficulties have limited our ability to test photosynthetic responses to sustained warming in mature forest canopies. In order to understand how elevated temperatures will affect forest ecosystems, we need to be able to test acclimation responses *in-situ*. The two primary aims of this thesis were: (1) to test and describe a leaf-level warming device that can be implemented within a forest canopy, and (2) to investigate the responses of two northern hardwood species to experimental canopy warming. We successfully developed a leaf-level warming device that warmed leaves 3.02 ± 1.86 °C above control leaves within mature forest tree crowns. To examine photosynthetic acclimation response, we heated understory and canopy leaves of *Acer saccharum* and *Tilia americana* for one week at < 2 meters, 6.25 m, and 12.5 m heights. We measured the photosynthetic, stomatal conductance, and leaf trait response to temperature, as well as how responses differed throughout a vertical canopy gradient. We found no evidence of thermal acclimation in plant gas exchange or leaf traits for either species at any height. However, we did find evidence of slight photosynthetic decline, indicating possible damage to photosynthetic

apparatus. T_{opt} was consistently higher than daily maximum temperatures for *T. americana* leaves, while T_{opt} was at or below daily maximum temperatures for *A. saccharum*. This suggests that *T. americana* is less likely to experience photosynthetic decline under climate warming than *A. saccharum*. Future studies should investigate the effects of longer-term warming on northern hardwood canopies.

1. Introduction

Earth's annual mean surface temperature is predicted to increase 2.5– 8 °C by the year 2100 (Cox *et al.* 2000, IPCC 2014). Elevated temperatures can alter plant growth and physiological processes, namely through alterations of plant carbon balance (Way and Oren 2010, Hüve *et al.* 2011, Filewod and Thomas 2014, Teskey *et al.* 2015). Ecosystem carbon (C) balance is determined by the balance of carbon dioxide (CO₂) intake through photosynthesis, and CO₂ release through respiration. Plant respiration increases exponentially with increasing temperature, while photosynthesis increases with temperature until a thermal optimum, T_{opt} , is reached, after which, the photosynthetic rates will decline. Elevated temperatures could shift this balance, potentially reducing net C sequestration and potentially exacerbating climate warming due to increased atmospheric CO₂ (Houghton *et al.* 1998). The balance of photosynthesis and leaf respiration make plants the central controlling component of C cycling in terrestrial ecosystems (Bonan 2008), and understanding how rising temperatures alter plant physiological responses is key in predicting greenhouse gas feedbacks.

1.1 Photosynthetic Response to Temperature

Photosynthesis has been classified as one of the plant processes most sensitive to temperature (Berry and Bjorkman 1980, Ericsson *et al.* 1996). Photosynthesis has a positive response to temperature until it reaches a thermal optimum, T_{opt} , after which, the

photosynthetic rate declines (Berry and Bjorkman 1980, Battaglia *et al.* 1996). Future climate conditions may cause temperatures to rise beyond thermal optima, possibly reducing net photosynthetic uptake (Sage *et al.* 2008). The decline in photosynthesis after T_{opt} can be attributed to a number of direct and indirect mechanisms located in both the light and carbon reactions of photosynthesis (reviewed in Hikosaka *et al.* 2006 and Sage and Kubien 2007).

CO₂ is the principle substrate necessary to fuel the carbon reactions of photosynthesis. High temperatures can limit the availability of CO₂ through stomatal closure. Plant stomata do not directly close in response to high temperatures (Day 2000, Mott and Peak 2010); instead, stomata respond directly to the leaf-atmosphere vapor pressure deficit (VPD) (Ludlow and Jarvis 1971, Day 2000). VPD increases with increasing temperature, and stomatal conductance (g_s) decreases with increasing VPD (Pallardy and Kozlowski 1979); therefore, increasing temperatures can indirectly cause a reduction in g_s (Valladares and Pearcy 1997, Zweifel *et al.* 2006).

Increased temperature can directly affect photosynthetic rates due to the several biochemical limitations on Rubisco fixation of CO₂ to RuBP. First, elevated temperatures increase the rates of photorespiration, which is the fixation of O₂ instead of CO₂ by Rubisco. This increased rate of photorespiration under elevated temperatures occurs through two mechanisms. First, the ratio of leaf internal CO₂:O₂ decreases with increased temperature due to differential solubility, which increases photorespiration (Monson *et al.* 1982, Jordan and Ogren 1984). Secondly, the specificity of Rubisco kinetics changes to

more readily bind O₂ instead of CO₂, further increasing photorespiration and reducing photosynthesis (Ku and Edwards 1978, Kirschbaum and Farquhar 1984).

Rubisco carboxylation can also be inhibited through the thermal sensitivity of Rubisco activase. Rubisco activase is an enzyme responsible for the effective binding of RuBP to Rubisco. Without Rubisco activase, Rubisco binds to RuBP too tightly, preventing carboxylation until Rubisco separates from RuBP (Wang and Portis 1992). Deactivation of Rubisco is associated with high temperatures for two separate reasons. First, Rubisco activase can be limited due to reductions in photosynthetic electron transport at high temperatures. The function of Rubisco activase is dependent on the availability of ATP, which is made available during the light reactions of photosynthesis (Wang and Portis 1992, Zhang *et al.* 2002). Secondly, Rubisco activase has a low thermal threshold, with protein denaturation occurring at lower temperatures than other chloroplast proteins (Salvucci *et al.* 2001).

Photosynthesis at temperatures greater than T_{opt} can also decline due to thermal limitations of the light reactions in photosynthesis. Electron transport thermal limitations primarily affect Photosystem II (PSII) reactions and thylakoid membrane permeability (Berry and Bjorkman 1980, Bukhov *et al.* 1999, Sage and Kubien 2007, Zhang and Sharkey 2009). At temperatures slightly above T_{opt} , higher rates of enzymatic reactions increase the flow of electrons through the electron transport chain (Yamasaki *et al.* 2002). The increased flow of electrons triggers a downregulation of PSII, temporarily uncoupling PSII from electron transport (reviewed in Sage and Kubien 2007). The uncoupling of PSII causes the transport of electrons to turn cyclic where the production

of ATP is primarily reliant on Photosystem I (PSI) (Havaux 1996, reviewed in Egorova and Bukhov 2006). If PSI is unable to maintain the proton gradient, ATP production will decline, eventually causing a reduction in photosynthesis. At temperatures above 46-52°C, photosynthetic electron transport can decline due to increased membrane permeability (Emmett and Walker 1973, Bukhov *et al.* 1999, Zhang *et al.* 2009); although, increased membrane permeability has been found to occur in pima cotton at temperatures as low as 36°C (Schrader *et al.* 2004). A more fluid thylakoid membrane is not able to hold the proton gradient necessary for ATP production, causing a decline in photosynthesis.

Net photosynthetic decline at supraoptimal temperatures can also occur through an increase in the rate of daytime respiration (Peng *et al.* 2013, Schippers *et al.* 2015). The net rate of photosynthesis is calculated as the gross CO₂ assimilation minus the CO₂ respired back into the atmosphere; therefore, to understand the full scope of how photosynthesis is affected by elevated temperatures, the rate of mitochondrial respiration in the light (R_L) must be considered. Respiration increases exponentially with temperature; therefore, if R_L increases due to higher temperatures and gross photosynthetic rate stays the same, the net photosynthetic rate would decrease. Due to light suppression, rates of R_L are lower than respiration in the dark (R_D) (Atkin *et al.* 1997, Ayub *et al.* 2011, Way *et al.* 2015); however, R_L has been shown to be more affected by short term temperature increases compared to R_D (Shapiro *et al.* 2004, Way *et al.* 2015). This makes R_L an important factor in calculation of the net carbon cycle.

1.2 Photosynthetic Acclimation

If plants are able to thermally acclimate, some of the negative effects of elevated temperature could be ameliorated. We know that plants are able to acclimate to warmer temperatures; however, the degree of acclimation is dependent on factors such as leaf traits and growth climate (Berry and Bjorkman 1980, Tjoelker *et al.* 1999).

Photosynthetic thermal acclimation is caused by either a positive shift in T_{opt} , or through an overall increase in the photosynthetic rate at T_{opt} , also called A_{opt} (Berry and Bjorkman 1980, Medlyn *et al.* 2002a). Respiration acclimates through a lowered respiration rate at a given temperature; this occurs either through a decline in slope of the respiration response to temperature or through an overall decrease in the basal respiration rate (Atkin *et al.* 2005). Both plant photosynthesis (Gunderson *et al.* 2000, Cunningham and Read 2002, Sendall *et al.* 2015) and leaf respiration (Gunderson *et al.* 2000, Lee *et al.* 2005) have been shown to acclimate to warmer temperatures in temperate hardwood trees.

Between the two processes of photosynthesis and respiration, respiration has been shown to have a higher acclimation response (Gunderson *et al.* 2000, Yamori *et al.* 2005, Way and Oren 2010), with respiratory acclimation likely, in part, due to lowered substrate availability caused by photosynthetic decline (Dewar *et al.* 1999, Tjoelker *et al.* 1999).

Photosynthetic acclimation can occur in both short and long time scales; however, acclimation occurs more readily in immature plants or developing leaves (Gunderson *et al.* 2000, Niinemets 2007). Although, some level of acclimation does occur in mature leaves and plants (Gunderson *et al.* 2010). In order to make accurate predictions

regarding global carbon storage, thermal acclimation should be included in carbon models (Luo *et al.* 2001, Hanson *et al.* 2005, Smith and Dukes 2013).

Thermal acclimation of photosynthesis can occur through several different mechanisms. Thermally induced thylakoid membrane fluidity can be stabilized either through increasing the carotenoid content (Havaux *et al.* 1996) or by shifting the membrane structure to include more saturated fatty acids (Huner 1988). High temperature thermal acclimation can also occur by increasing the rate of water oxidation in the initial step in the electron transport chain, or through increasing the electron transfer rate through the plastoquinone site (Yamasaki *et al.* 2002). Rubisco activase has been shown to acclimate through the production of more thermo-tolerant isoforms (Portis 2003). Alternating isoform production is highly dependent on species (Salvucci *et al.* 2001), and it is possible that species that are only able to produce one isoform could be more susceptible to photosynthetic decline at high temperatures (Sage and Kubien 2007). Yamori *et al.* (2005) found that photosynthetic acclimation can occur through increasing RuBP regeneration and carboxylation rate, further supporting the evidence for Rubisco carboxylase acclimation. Plant leaves can also undergo long-term acclimation to supraoptimal temperatures through morphological changes in their stomata. Elevated temperatures, due either to experimental warming or latitudinal gradient, can increase stomatal density, promoting a rise in A_{opt} (Jin *et al.* 2011, Hill *et al.* 2014).

1.3 Leaf Functional Traits

Leaf functional traits are measureable characteristics of a leaf that relate to functional performance. Useful traits include leaf mass per area (LMA), plant nutrient content (on a mass and area basis), photosynthetic capacity, leaf longevity, as well as leaf water relations such as leaf water content and succulence (Poorter and Bergkotte 1992, 1992, Cornelissen *et al.* 1997, Garnier *et al.* 2001, Cornelissen *et al.* 2003, Uriarte *et al.* 2010). Leaf traits can be used to predict many aspects of leaf economics such as carbon uptake and plant community composition (Reich *et al.* 1997, Wright *et al.* 2004, Asner *et al.* 2016, Guittar *et al.* 2016). Traits vary within tree canopy gradients, where traits higher in the canopy are associated with optimizing photosynthesis, such as high LMA and leaf nitrogen (Niinemets 1999, Koike *et al.* 2001). There is also evidence that plant trait plasticity is key to acclimation to different environments, including thermal acclimation of plant gas exchange (Lee *et al.* 2005, Corlett 2011, dosAnjos *et al.* 2015, Sterck *et al.* 2016). Greater leaf trait plasticity in response to environmental stress may be an indicator of greater acclimation potential (Sterck *et al.* 2016).

LMA is a commonly used leaf trait because of the ease in which it is measured. LMA is often used to predict leaf traits that are logistically difficult to measure, such as photosynthetic capacity (Nicotra *et al.* 2010). Within an individual tree there are factors that influence LMA such as irradiance, seasonality, and xylem water potential (Cavaleri *et al.* 2010, Zhang *et al.* 2011, Coble and Cavaleri 2014, Coble *et al.* 2016). LMA has a positive association with height (Poorter *et al.* 2009); however, depending on whether

leaves are collected early or late in the temperate growing season, light or leaf hydraulics drive the relationship between LMA and height (Coble *et al.* 2016). LMA has also been shown to be an important trait in light acclimation plasticity (Rozendaal *et al.* 2006); however, there is little evidence to suggest that LMA will positively respond to warmer growth temperatures. Some warming experiments have been shown to have a negative or no effect on LMA (Tjoelker *et al.* 1999, Gunderson *et al.* 2010, Cheesman and Winter 2013, Aspinwall *et al.* 2016), while other studies have shown LMA to increase with manipulative growth experiments (Yamori *et al.* 2012, Slot *et al.* 2014). Recently, O'Sullivan *et al.* (2017) found that specific leaf area (the inverse of LMA) not to be predictive of high temperature tolerance. The results of these studies suggest that acclimation of LMA to increased temperature may be species-specific.

Nitrogen (N) is a major component in several important elements in leaf photosynthesis, such as Rubisco and chlorophyll; therefore, leaf N is directly tied with photosynthetic capacity (Evans 1989). Traits associated with photosynthetic capacity are optimized within the canopy for carbon gain (Livingston *et al.* 1998, Niinemets *et al.* 1999, Duursma and Marshall 2006, Legner *et al.* 2014); however, N does not always increase with canopy height (Livingston *et al.* 1998, Weerasinghe *et al.* 2014, Coble *et al.* 2016). The relationship between nitrogen and canopy height are stronger on an area basis as opposed to mass (Weerasinghe *et al.* 2014, Coble and Cavaleri 2015). This is in part due to the correlation between N_{area} and LMA (Bond *et al.* 1999). Stronger associations between height and area is true for many leaf traits; this is especially true in ecosystems

without water limitations (Kenzo *et al.* 2015). Photosynthetic acclimation to light is also an important driver of leaf nitrogen content (Morales *et al.* 2014).

Measures of leaf water traits are commonly used to describe both the potential for plant growth (Poorter and Bergkotte 1992, Hodgson *et al.* 1999) as well as a measure for stress tolerance and longevity (Garnier and Laurent 1994, Cornelissen *et al.* 1997, Weiher *et al.* 2011). Leaf water traits, such as leaf succulence, that quantify stress tolerance between species are found to be higher in plants that perform better under drought conditions (Cornelissen *et al.* 1997, Uriarte *et al.* 2010). Plants tissues that are exposed to higher irradiance environments have lower water contents (Pilegaard *et al.* 2003, Lichtenthaler *et al.* 2007), indicating lower water contents in forest upper canopies compared to forest understories.

1.4 Conclusions

Understanding how plants will respond to increasing temperatures is critical to accurately predicting future carbon balance. Plants have been shown to acclimate to warmer temperatures through many different mechanisms; however, there is still a gap in our understanding of how species exposed to different environmental conditions will respond *in-situ* to climate warming. This is especially true in mature forest canopies where *in-situ* warming studies are logistically difficult to implement. Future studies should focus on investigating how these critical forests will physiologically respond to long-term warming.

2. Northern Hardwood Photosynthetic Response to Leaf-level Warming¹

2.1 Abstract

Northern hardwood forests are already experiencing more extreme heatwaves, and the frequency of extreme weather events is expected to increase in the coming years. Higher temperatures could alter plant physiological processes, possibly causing a decline in forest productivity. Individual plant and ecosystem-level warming experiments have provided valuable insight on how plants respond to elevated temperatures; however, fewer studies have investigated the effects of *in-situ* warming in mature forest canopies. Leaf-level warming studies provide a relatively straightforward method to investigate how warming affects leaf physiological function within a forest canopy. This study implemented *in-situ* leaf-level warming at three canopy positions, understory: < 2 meters, middle canopy: 6.25 m, and upper canopy: 12.5 m, in a northern hardwood forest located in Upper Peninsula Michigan, USA. We investigated the ability of two hardwood species, *A. saccharum* and *T. americana*, to acclimate to 7 days of +3°C warming. We

¹ This document is planned for submission for publication.

measured the photosynthetic and stomatal conductance response to temperature throughout the vertical canopy gradient. Photosynthetic acclimation was assessed by measuring the difference in heated and control leaf optimum temperatures (T_{opt}) and the photosynthetic rates at which T_{opt} occurred (A_{opt}). We also compared how *A. saccharum* and *T. americana* leaf functional traits differed between canopy positions. Functional traits measured were: leaf mass per area (LMA), nitrogen per area (N_{area}), nitrogen per mass (N_{mass}), and leaf water content. The results did not show an increase in heated leaf A_{opt} or T_{opt} at any canopy position, indicating no thermal acclimation. *T. americana* A_{opt} declined at all three canopy positions, suggesting damage to photosynthetic apparatus. A_{opt} was highest in *A. saccharum* upper canopy leaves and the high A_{opt} is likely attributed to increased leaf nitrogen found in the upper canopy. *T. americana* photosynthetic rates were highest in the middle canopy leaves. *T. americana* upper canopy had reduced water content and g_s , attributing decreased rates of A_{net} in the upper canopy to leaf water status. T_{opt} of *T. americana* was 3-7 °C higher than average daily maximum leaf temperature (T_{leaf}), while *A. saccharum* T_{opt} was ± 1 °C from average daily maximum T_{leaf} . Due to higher T_{opt} in *T. americana*, this species is more likely to perform better than *A. saccharum* under future climate regimes.

2.2 Introduction

2.2.1 Effects of Climate Change on Northern Hardwood Forests

Greenhouse gas induced climate change is predicted to give rise to more heat waves in the northern hemisphere compared to the southern hemisphere (Meehl and Tebaldi 2004, Gershunov *et al.* 2009) and northern hardwood forests are already experiencing more extreme temperature events, potentially altering both plant photosynthetic and respiration capacities (Filewod and Thomas 2014). Both the overall elevated temperature and increased number of heat waves could contribute to overall shifts or reduction of species ranges (Thomas *et al.* 2004, Jump *et al.* 2006). There is evidence that upper canopy leaves in mid latitude ecosystems are currently operating near their thermal thresholds (Mau 2015, O'Sullivan *et al.* 2017). Elevated temperatures could negatively impact ecosystem carbon balance through pushing temperatures past the photosynthetic temperature optima, causing a decline in photosynthesis (Berry and Bjorkman 1980). Photosynthetic decline could also be exacerbated in temperate ecosystems due to their characteristically hot, dry summers. Lowered rates of photosynthesis have important implications for ecosystem carbon balance because photosynthesis is the primary form of terrestrial carbon uptake. Reduced photosynthesis decreases CO₂ fixed from the atmosphere, possibly further inducing greenhouse gas induced climate warming (Cowling *et al.* 2004).

2.2.2 Photosynthetic Response to Temperature

Instantaneous rates of photosynthesis initially rise in response to temperature until an optimum temperature (T_{opt}) is reached. Temperatures beyond T_{opt} cause a decline in photosynthesis. The decline in photosynthesis after T_{opt} occurs due to altered reactions in both the photosynthetic light and carbon reactions. Within the light reactions of photosynthesis, supraoptimal temperatures can induce downregulation of light capture in Photosystem II (Yamasaki *et al.* 2002) or, at very high temperatures, through loss of the proton gradient through increased membrane permeability (Bukhov *et al.* 1999, Zhang *et al.* 2009). Thermally induced declines in the photosynthetic carbon reactions occur through direct and indirect interactions with temperature. Indirectly, elevated temperature can induce stomatal closure, limiting CO_2 availability for the photosynthetic carbon reactions (Farquhar and Sharkey 1982). Directly, elevated temperatures can increase photorespiration (Jordan and Ogren 1984, Ku and Edwards 1978, Kirschbaum and Farquhar 1984) and cause reduced CO_2 fixation through Rubisco activase dysfunction (Wang and Portis 1992, Salvucci *et al.* 2001, Zhang *et al.* 2002). Decreased rates of photosynthesis can also be reported because net photosynthesis (A_{net}) is measured as the gross photosynthetic rate minus the rate of daytime mitochondrial respiration. Respiration increases exponentially with temperature; therefore, if daytime respiration increases with elevated temperatures, then A_{net} will be depressed (Peng *et al.* 2013, Schippers *et al.* 2015).

Photosynthetic thermal acclimation could help alleviate some of the negative impacts of supraoptimal temperatures. Photosynthetic acclimation occurs either through a positive shift in T_{opt} , or through higher rates of A_{opt} (photosynthesis at T_{opt}). Mechanisms involved in photosynthetic thermal acclimation include increased membrane stability through physical changes in the thylakoid membrane structure (Huner 1988, Havaux *et al.* 1996), production of more stable isoforms of Rubisco activase (Portis 2003, Salvucci *et al.* 2001), and, over longer time periods, changes in the size and density of plant stomata (Jin *et al.* 2011, Hill *et al.* 2014). Although northern hemisphere ecosystems are expected to experience drastic changes in their temperature regimes, there is convincing evidence that temperate forests will be able to photosynthetically acclimate to moderate climate warming (Gunderson *et al.* 2000, Turnbull *et al.* 2004, Gunderson *et al.* 2010, Way and Oren 2010, Sendall *et al.* 2015). Temperate species are hypothesized to be more capable of acclimation due to the wide range of year-round temperatures that they experience (Cunningham and Read 2002). Seedlings and saplings in temperate hardwoods have been shown to acclimate to moderate temperature increases (Gunderson *et al.* 2000, Cunningham and Read 2002, Gunderson *et al.* 2010, Sendall *et al.* 2015); however, photosynthetic acclimation in a mature temperate hardwood forest is yet to be examined.

2.2.2 Review of in-situ Experimental Warming

Temperate forests are among the most commonly studied ecosystems, and most *in-situ* temperate ecosystem warming studies have focused on warming the forest

understory (de Frenne *et al.* 2010, Melillo *et al.* 2011, Fu *et al.* 2013, Jarvi and Burton 2013, Noh *et al.* 2016, also reviewed in Chung *et al.* 2013) or an early successional growth forest (Rollinson and Kaye 2012, Rich *et al.* 2015, Sendall *et al.* 2015). Due to the logistical obstacles, fewer studies have looked at the effects of warming on temperate forest canopies beyond the seedling developmental stage. Bauerle *et al.* (2009) conducted a study on 4-meter-tall 2-year-old trees and controlled canopy temperature through pumping heated air through tubes and into the tree canopy. Gunderson *et al.* (2010) constructed warming chambers where whole saplings were heated +2°C and +4°C above a control chamber. Open top chambers have also been used to passively heat whole branches (Doughty 2011, Yamaguchi *et al.* 2016). There are limitations associated with the passive heating method in that branches are only heated during sunny conditions; however, each of these studies found that branches were heated +2°C and +1°C, respectively.

A barrier to canopy warming studies is the cost and energy that go into mature tree-scale warming. Smaller scale within-canopy warming, is a practical method to investigate the plant physiological effects of warming in forest canopies (Cavaleri *et al.* 2015). Small scale canopy warming has been implemented through branch, leaf level warming, and combined branch and leaf warming (Nakamura *et al.* 2010, Doughty 2011, Slot *et al.* 2014, Nakamura *et al.* 2016). Nakamura *et al.* (2010) used heated cables to warm mature *Quercus crispula* branches +5 °C in Japan above ambient temperature and found that heating extended the growing season as well as increased acorn production. Heating of combined branch and leaf warming has recently been implemented in Japan to investigate physiological changes using infrared heaters placed in a temperate forest birch

(*Betula ermanii*) canopy, which warmed leaves +1°C above ambient temperatures (Nakamura *et al.* 2016). As far as we are aware, there have only been two examples of leaf-level warming in mature forests canopies and both studies have been implemented in tropical ecosystems (Doughty 2011, Slot *et al.* 2014). Doughty (2011) used resistance wires covered in aluminum foil to warm canopy leaves in the Brazilian Amazon for 90 days and found that photosynthesis declined due to thermal damage. Slot *et al.* (2014) used heat rope fixed underneath leaves using infrared reflective frames to heat leaves +3°C during nighttime hours in Panama for 6-8 days. This study found that leaf respiration was able to acclimate to elevated temperatures. These within canopy warming studies have given us important information regarding logistics of setting up smaller-scale warming studies, as well as the rate at which acclimation can occur on a branch or leaf scale. To date, there are no published studies on the effects leaf level warming on temperate forest canopy photosynthetic acclimation.

2.2.3 Objectives and Hypotheses

This study aimed to both test a newly developed leaf warming device and examine the temperature response and acclimation capacity of two N. hardwood species, *Tilia americana* and *Acer saccharum*, when exposed to seven days of leaf-level +3°C warming. We conducted this study across a forest vertical canopy gradient, capturing the variation in understory saplings, middle canopy, and upper canopy leaves. We hypothesized that 1) both species would be able to photosynthetically acclimate to warmer temperatures; 2) leaves in the upper canopy would have a higher capacity to

acclimate than leaves at lower heights. The capacity to acclimate was measured by measuring both A_{opt} and T_{opt} . We hypothesized that 3) stomatal conductance will be similar in heated and control leaves. We also hypothesized that 4) there would be no differences in leaf traits between heated and control leaves because leaf traits are unlikely to shift within one week of treatment.

2.3. Methods

2.3.1 Study Site

This study was conducted in a mature secondary growth northern hardwood stand dominated by white ash (*Fraxinus americana*) but is also comprised of sugar maple (*A. saccharum*), basswood (*T. americana*), and northern red oak (*Quercus rubra*), located at the USDA Forest Service Northern Research Station, Forestry Sciences Laboratory in Houghton, MI, USA (N47° 6' 52.884", W 88°32' 52.332"). In 2013, the basal area was 27 m² ha⁻¹ and the stand density was 2960 trees ha⁻¹, with a stand age from 50-60 years. *A. saccharum* comprises 10% of the stand basal area, while *T. americana* comprises 20% stand basal area. The average air temperature ranges from -13.1 °C to 24.3 °C. Mean annual precipitation is 86.6 cm and mean annual snowfall is 564 cm. The soil is classified as Michigamme-Trimountain sandy loam (NRCS 2017). A more detailed site description can be found in (Potvin and Lilleskov 2017).

Measurements were conducted on canopy scaffolding (Contur Modular Scaffold, BilJax, Archbold, OH, USA). The scaffolding had two canopy access points located at 6.25 meters and 12.5 meters, hereby called upper and middle canopy. One *A. saccharum* tree (height: 12.5 m, dbh:15.6 cm) was accessible from both the upper and middle canopy. Two separate *T. americana* trees were accessible from the middle (7.5 m, 7.0 cm) and upper canopy (14 m, 16.9 cm). Understory measurements were conducted on saplings located in the area directly surrounding the canopy scaffolding on individuals ranging from 0.3 to 2.1 m height.

The experiment was conducted on the understory and middle canopy leaves during mid to late July 2016. Experimental warming and sampling was conducted on the upper canopy leaves late August to early September 2016. Pretreatment measurements for the understory and middle canopy were conducted during early to middle July 2016. Pretreatment measurements for the upper canopy were conducted during late July 2016.

2.3.2 Experimental Design and Leaf Warming

Experimental warming was conducted on two species, *T. americana* and *A. saccharum*, at three tree heights (understory, middle canopy, and upper canopy). Three leaves per species per height were heated 3.0 °C above a nearby control leaf for 7 days (n = 36). Individual leaves were heated using 100 watt, 120VAC silicon heating pads (24100, Kat's, Five Star Manufacturing Group Inc., Springfield, TN). Leaf temperature was monitored using 30 AWG copper-constantan thermocouple wire (TT-T-30

SLE(ROHS), OMEGA Engineering, Inc., Norwalk, CT, USA) wired to a solid-state thermocouple multiplexer (AM25T, Campbell Scientific Inc., Logan, UT, USA) connected to a data logger (CR1000, Campbell Scientific Inc.). Leaf thermocouples were adhered on the abaxial side of the heated leaf and nearby control leaf using breathable medical tape. The thermocouples were extended using 24 AWG copper-constantan thermocouple wire or 20 AWG for any thermocouples extended more than 15 meters (TT-T-20 and TT-T-24 OMEGA Engineering Inc.). The larger and smaller gauge wires were connected using thermocouple connectors (SMPW-CC-T-MF, OMEGA Engineering Inc.). The heating pad temperature was controlled using a 24-380VAC SSR-25 DA solid state relay module wired into digital output module (SSR-25 DA, Fotek Controls Co., Taiwan) (SDM-CD 16D, Campbell Scientific Inc.). The temperature was controlled by turning the relay module, and thus the heating pad, off when the heated leaf temperature reached more than 3 °C above the control leaf temperature. The heating pads were positioned underneath the leaf by attaching the pad to a metal frame that was positioned 7-12 cm below the leaf (Fig. 2.1). Control and heated leaves were selected controlling for height and shade of each individual leaf. This ensured that the heated leaf was exposed to a similar ambient environment to the control leaf. Leaves were heated continuously throughout daytime and nighttime hours.

Understory and middle canopy warming was conducted for seven days from July 14 – 21, 2016. Upper canopy warming was conducted August 23 – 30, 2016. During the warming experiment, one *T. americana* upper canopy heater malfunctioned and the heater was turned off for 3 days. While all the other upper canopy heaters were turned off at the same time on August 30, the previously malfunctioned heater was shut off

September 1 in order to allow the leaf to experience as close to 7 days of warming as possible. The malfunctioning heater heated the leaf for a total of 6 days and 5 hours, 19 hours less than the other heaters in the experiment.

2.3.3 Gas Exchange and Leaf Traits

Gas exchange measurements were conducted using an open-system LI6400XT infrared gas analyzer fitted with a 6400-88 expanded temperature kit (Li-COR Inc., Lincoln, NE, USA). Photosynthetic response to temperature was measured by constructing instantaneous temperature response curves measured at nine temperatures (17, 20, 23, 25, 30, 33, 35, 37 °C). Due to difficulties reaching a low enough temperature to detect the optimum temperature, we added a 15 °C temperature measurement to *A. saccharum* understory and middle canopy measurements. Photosynthetic photon flux density was controlled at 800 $\mu\text{mol m}^{-2} \text{s}^{-1}$ for the understory and middle canopy leaves and 1200 $\mu\text{mol m}^{-2} \text{s}^{-1}$ for the upper canopy leaves. CO₂ concentration was controlled at 400 ppm. Flow was controlled between 200 to 500 $\mu\text{mol m}^{-2} \text{s}^{-1}$ in order to control the vapor pressure deficit between 1 and 2 kPa; although, at temperatures above 33 °C, VPD often reached above 2 kPa. At temperatures below 20 °C, VPD was often slightly below 1 kPa.

After completing daily gas exchange measurements, leaves were collected, placed in a sealed plastic bag, and temporarily stored in an ice cooler. Upon returning to the lab, leaves were weighed for fresh mass and immediately placed in a -20 °C freezer. At the conclusion of the experiment, frozen leaves were thawed and measured for leaf area

using a desktop scanner (HP Deskjet 4480). Leaf dry mass was collected after leaves were placed in a 60°C drying oven for at least 72 hours. Leaf mass per area (LMA) was obtained by dividing the dry mass (g) by total leaf area (cm²) (Cornelissen *et al.* 2003). Leaf area was measured using ImageJ v1.50 image analysis software (Schneider *et al.* 2012). Leaf water content was calculated by subtracting dry mass (g) from wet mass (g) and dividing by dry mass (g). Dry leaves were ground to a fine powder in a ball bearing grinder (8000M Mixer/Mill, Spex Sample Prep, Metuchen, NJ, USA) and analyzed for %carbon and %nitrogen using a combustion analyzer (ESC 4010, Costech Analytical Technologies Inc., Valencia, CA, USA) in Michigan Technological University Forest Ecology Analytical Laboratory. N per area (N_{area}) was calculated by dividing N (g) by leaf area (cm²).

2.3.4 Leaf Scorching

While the heated leaf target temperature was 3 °C above a control leaf, an unavoidable consequence of this leaf warming method is that the temperature of the leaf often increased higher than 3 °C before the digital output module turned the heater off. In order to account for possible damage to photosynthetic apparatus caused by spikes in leaf temperature, percent leaf scorching, defined as the visible leaf necrosis, was measured on all heated leaves by scanning the leaves and then analyzing using ImageJ software. The scorched area on the leaf was traced and extracted from the original leaf picture. Percent area scorched was calculated by dividing the scorched area by the total leaf area and multiplying by 100.

2.3.5 Data Analysis

Warming device efficacy was analyzed between different canopy positions by analyzing differences in heated and control leaf temperatures using a one-way ANOVA. A Student's t-test was used to test for variations in control and heated leaf temperature difference between time of day (daytime or nighttime), month that the leaves were heated (July or August), and differences between species. Temperature spiking was compared by analyzing the mean maximum daily temperature and the percentage of time points where heated leaves were 10 °C higher than control leaves. Both maximum daily temperatures and percentage of temperature spiking between species and canopy positions were compared by using a two-way ANOVA.

Photosynthetic acclimation is denoted by a positive shift in T_{opt} or an increase in A_{opt} , which were determined by fitting individual temperature response curves to the second order polynomial regression model (Cavieres *et al.* 2000):

$$A_{net} = aT_{leaf}^2 + bT_{leaf} + c \quad \text{Equation (1)}$$

where A_{net} is net CO₂ assimilation at leaf temperature (T_{leaf}). The T_{opt} is calculated by taking the 1st derivative of the polynomial equation:

$$T_{opt} = -b/2a \quad \text{Equation (2)}$$

A_{opt} is extracted by setting $T_{leaf} = T_{opt}$ in equation 1 and solving for A_{net} . Extracting T_{opt} from a second order polynomial regression requires that the linear term, b , is positive. There were 9 out of 36 total cases in our data (5 heated leaves and 4 control leaves) where the linear term was negative; all occur in *A. saccharum*. The negative linear term causes an inversion of the polynomial curve. The likely cause of the inversion is that we were unable to reach a low enough temperature to take measurements at or below T_{opt} . For these data, instead of using the polynomial equation from the modeled data, we took the temperature at the maximum A_{net} value from each individual curve and treated this value as T_{opt} . For each species separately, A_{opt} and T_{opt} values between treatment and canopy position were compared using a two-way ANOVA.

Stomatal conductance (g_s) was modeled using simple second order polynomial equation:

$$g_s = aT_{leaf}^2 + bT_{leaf} + c \quad \text{Equation (3)}$$

Leaf traits (N_{mass} , N_{area} , LMA, and leaf water content) were also compared for differences in treatment and canopy position using a two-way ANOVA. Leaf traits were measured separately by species. When there was no interaction between treatment and canopy position, heated and control leaf gas exchange and leaf trait data were combined and analyzed for canopy height differences. All leaf traits were measured on the same leaves used for leaf gas exchange measurements. Tukey's method was used for *post-hoc* analyses unless there was an interaction effect between treatment and canopy position. When there was an interaction effect, planned contrasts were used to exclusively compare

canopy heights within each treatment. Contrasts were then used to compare heated and control leaves at individual canopy positions. Statistical analyses were performed using R Statistical Software (R Development Core Team 2013, R Foundation for Statistical Computing, Vienna, Austria).

2.4. Results

2.4.1 Leaf-level Warming

The effectiveness of the leaf-level warming device was determined by the average difference in the heated and control leaves. The average difference for all species and canopy levels was 3.02 ± 1.86 °C. (mean \pm standard deviation). The difference in heated and control leaf temperatures varied slightly with time of day, measurement month, and canopy location. Daytime difference was 2.95 ± 1.85 °C and average nighttime difference of 3.09 ± 1.87 °C ($p < 0.001$). Average temperatures difference for July and August were 3.07 ± 1.89 °C and 2.91 ± 1.82 °C, respectively ($p < 0.001$). The understory heated leaves were 3.00 ± 1.95 °C warmer than the control leaves. Middle and upper canopy leaves were 3.14 ± 1.80 °C and 2.91 ± 1.82 °C warmer than control leaves, respectively ($p < 0.001$). There were no significant differences between species ($p = 0.242$).

While the average difference in canopy leaves was very close to 3 °C, there was evidence of temperature spiking and leaf scorching (Table 2.1, 2.2, Fig. 2.3). *A. saccharum* showed some degree of scorching in four of the twelve total heated leaves.

One leaf from both the upper canopy and understory had between 5-10% scorching, and two of the three heated middle canopy *A. saccharum* leaves exhibiting less than 1% area scorched. Only one *T. americana* leaf, located in the understory, exhibited scorching, at less than 1% of leaf area.

In order to explain the leaf scorching and the likely accompanying damage that occurs to photosynthetic apparatus, leaf temperature spiking was analyzed in two different ways: mean maximum daily temperature in the heated leaves and the frequency of instances that heated leaf temperature rose 10 °C above the control leaf temperature (Table 2.2). There was an effect of species and the interaction between canopy position and species (Table 2.3). *T. americana* heated daily maximum leaf temperatures ranged from ~32-36 °C, with the lowest maximum leaf temperature occurring in the upper canopy and the highest maximum leaf temperature occurring in the understory (Table 2.2). Because *T. americana* understory maximum leaf temperature was higher than both the middle and upper canopy maximum leaf temperature, spiking was investigated for each individual *T. americana* understory heated leaf. The total frequency of temperature spikes where the difference between the control leaf and heated leaf was greater than 10 °C was 222 occurrences (Table 2.3). Of the 222 occurrences of temperature spiking, 74% of the spiking occurred for one single leaf. This suggests that that leaf was inflating the maximum daily leaf temperature. The data were analyzed without the leaf prone to temperature spiking and the average *T. americana* understory maximum daily temperature decreased from 35.75 ± 5.1 °C to 32.82 ± 3.98 °C. After removing the leaf with temperature spiking, the average maximum daily leaf temperature was highest in the middle canopy, which was consistent with the pattern of the control leaf maximum daily

leaf temperatures (Table 2.1). *A. saccharum* daily maximum leaf temperature ranged from ~34-44 °C, with the lowest maximum leaf temperature occurring in the understory and the highest occurring in the upper canopy (Table 2.3).

The total percent frequency that the temperature between control and heated leaves rose above 10 °C was ~ 0.5 %. There were no effects of species, canopy position, and the interaction between species and canopy position on the frequency of time points where heated leaves rose greater than 10 °C above the control leaves (Table 2.1). The lowest frequency occurred in *T. americana* upper canopy leaves where only 0.01% of recorded measurements of heated and control leaf temperature differences were greater than 10 °C (Table 2.2), making up only ~3 % of the upper canopy temperature spikes. In the understory, ~68% of the occurrence were by *T. americana* leaves. *A. saccharum* had a more instances of temperature differences above 10 °C in the middle canopy (~63%). The total frequency of occurrences above 10 °C between daytime (n = 336) and nighttime (n = 341) temperatures was similar.

2.4.2 Photosynthetic and Stomatal Conductance Response to Temperature

Heated and control temperature response curves were combined and analyzed for differences in temperature response between canopy position. The fit of both A_{net} and g_s modeled using polynomial equations varied depending on species and canopy position (Table 2.4). Both *A. saccharum* middle canopy and understory had weak relationships to T_{leaf} , while the relationships were stronger in the upper canopy (Table 2.4, Fig. 2.4a). *T. americana* A_{net} upper and middle canopy positions both had significant relationships to

T_{leaf} , while the understory did not (Table 2.4, Fig. 2.4b). *A. saccharum* upper canopy and understory g_s had significant relationships to T_{leaf} , while the middle g_s vs T_{leaf} relationship was not significant in the middle canopy (Table 2.4, Fig. 2.4c). *T. americana* upper and middle canopy g_s had a significant relationship with T_{leaf} , while the understory did not (Table. 2.4, Fig 2.4d).

2.4.3 Leaf Temperatures and Leaf Level Acclimation

Control leaf temperatures had little variation between sample month, canopy position, and species. Both *T. americana* and *A. saccharum* control leaf temperature only varied by $\sim \pm 1^\circ\text{C}$ between different canopy positions (Table 2.1). The average daily T_{leaf} between both species at all canopy positions ranged from 20.05 to 21.86 $^\circ\text{C}$ (Table 2.1).

Based on the response of both the optimum temperature of photosynthesis (T_{opt}) and the photosynthetic rate at T_{opt} (A_{opt}) in heated and control leaves, there is no evidence to support acclimation of photosynthesis in either *A. saccharum* or *T. americana* after one week of experimental warming. With a T_{opt} of 20.9 ($\pm 5.1^\circ\text{C}$), there were no significant differences between treatments or canopy height for *A. saccharum* (Fig. 2.6a). There was no significant difference between *A. saccharum* heated and control leaf A_{opt} , although the effect was very close to significant (Table 2.5, Fig. 2.5a). There was an effect of A_{opt} at different heights (Fig 2.6c). At 6.84 ($\pm 1.38 \mu\text{mol m}^{-2} \text{s}^{-1}$) *A. saccharum* upper canopy had an average A_{opt} twice the A_{opt} of the understory and middle canopy levels (3.27 ± 0.73

$\mu\text{mol m}^{-2} \text{s}^{-1}$) (Fig. 2.6c). There was no significant treatment effect in *T. americana* T_{opt} ; however, there was a slightly significant effect in *T. americana* A_{opt} (Table 2.5). There were also significant differences between canopy heights in both *T. americana* A_{opt} and T_{opt} . *T. americana* understory and middle canopy T_{opt} (29.3 ± 1.5 °C) was 6.78 °C higher than the top of the canopy (23.4 ± 2.7 °C) (Fig. 2.6b). There was no significant difference between *T. americana* middle and top canopy levels A_{opt} ; however, the average of the middle and top canopy A_{opt} (11.2 ± 1.5 $\mu\text{mol m}^{-2} \text{s}^{-1}$) was more than double the average understory A_{opt} (5.18 ± 1.3 $\mu\text{mol m}^{-2} \text{s}^{-1}$). A_{opt} slightly declined for both species at all canopy positions with warming; although, the decline in *A. saccharum* was not significant (Fig 2.5c, d)

2.4.4 Leaf Traits

There were no significant differences between heated and control leaves for both *A. saccharum* and *T. americana* for all canopy positions for any traits of interest (Table 2.6). While it is not true for all measured leaf traits, there were some differences between some canopy positions (Table 2.6). There was also an interaction effect for *T. americana* N_{area} (Table 2.6, Fig. 2.8b).

There were no effects of treatment or the interaction between treatment and canopy position for *A. saccharum* or *T. americana* LMA; however, there was an effect of canopy position on LMA for both species (Table 2.6). For both species, upper canopy LMA was significantly greater than the middle canopy and understory; while there was no significant difference between the understory and middle canopy (Fig. 2.7a, b). The

average *A. saccharum* upper canopy LMA ($67.28 \pm 9.02 \text{ g m}^{-2}$) were ~75% greater than the average understory and middle canopy ($38.40 \pm 8.58 \text{ g m}^{-2}$, Fig. 2.6a). *T. americana* upper canopy LMA ($60.72 \pm 10.70 \text{ g m}^{-2}$) was ~62% greater than understory and middle canopy leaves ($37.59 \pm 9.38 \text{ g m}^{-2}$, Fig. 2.6b). There was also an interaction effect for both *T. americana* and *A. saccharum*; however, there were no significant differences between heated and control leaves at the same canopy level.

The Two-way ANOVA showed a significant effect of canopy position on *A. saccharum* N_{area} (Table 2.6). The Two-way ANOVA conducted for *T. americana* showed both effects of canopy position and the interaction between canopy position and treatment on N_{area} (Table 2.6); therefore, a *post-hoc* contrast was used to compare the differences between heated and control N_{area} at each canopy position, as well as the difference between control and heated leaves separately for each canopy position. The contrast between *A. saccharum* heated leaves showed no significant difference between understory ($4.56 \pm 0.82 \text{ g m}^{-2}$) and middle canopy N_{area} ($3.50 \pm 0.76 \text{ g m}^{-2}$) ($p = 0.190$); however, the upper canopy ($8.60 \pm 0.22 \text{ g m}^{-2}$) was significantly greater than the understory and middle canopy control leaf N_{area} ($p < 0.001$). Control leaf understory and middle canopy N_{area} was also not statistically different from one another ($p = 0.225$); however, the upper canopy leaves were significantly greater than the middle canopy and understory N_{area} ($p = 0.020$ and $p = 0.002$ respectively). The ANOVA results showed no significant interaction between canopy position and treatment (though borderline at $p = 0.123$), while the contrast showed that the upper canopy heated leaves had a slightly significantly greater N_{area} than the control leaves ($p = 0.040$, Fig. 2.8a). The *post-hoc* contrast for *T. americana* N_{area} showed that the middle and upper canopy N_{area} was

significantly greater than the understory N_{area} ($p = 0.008$ and $p = 0.022$ respectively). There was no significant difference between upper and middle canopy N_{area} ($p = .599$). When the heated and control leaves were contrasted for each canopy position, the upper canopy control leaves had a significantly greater N_{area} than the heated leaves ($p = 0.031$).

There were no effects of treatment, canopy position, or the interaction between treatment and canopy position for *A. saccharum* N_{mass} (Table 2.6). The mean *A. saccharum* N_{mass} was $11.09 \pm 0.24 \text{ mg g}^{-1}$ (Fig. 2.7a). While there were no treatment effects or effects of the interaction between treatment and canopy position on *T. americana* N_{mass} , there was an effect of canopy position (Table 2.6). *T. americana* N_{mass} was significantly higher in the middle canopy than the understory and upper canopy. There was no significant difference between understory and upper canopy *T. americana* N_{mass} (Fig. 2.7b). Middle canopy N_{mass} was $28.85 \pm 0.52 \text{ mg g}^{-1}$, which was 29% greater than the upper canopy ($22.43 \pm 0.114 \text{ mg g}^{-1}$) and 47% greater than the understory ($19.6 \pm 0.86 \text{ mg g}^{-1}$).

Leaf water status was analyzed using the ratio of leaf wet mass to dry mass (Leaf water content). There were no effects of treatment or the interaction between treatment and canopy position for leaf water content in both species (Table 2.6). With an overall mean of $(1.30 \pm 0.26 \text{ g g}^{-1})$, there were no significant differences between canopy positions for leaf water content in *A. saccharum* (Fig. 2.7i). There were significant differences between leaf water content for *T. americana* canopy positions (Fig. 2.7j). *T. americana* understory and middle canopy leaf water content was not significantly different; however, the mean of understory and middle canopy ($2.09 \pm 0.22 \text{ g g}^{-1}$) was 31% greater than the upper canopy ($1.60 \pm 0.51 \text{ g g}^{-1}$).

2.5 Discussion

2.5.1 Leaf Heating Device Performance

The novel heating device successfully heated the leaves + 3 °C above ambient leaf temperature (Fig. 2.2). Average heated temperature differences were all within ± 0.15 °C of 3.0 °C across canopy positions, time of day, and seasons. There were no significant differences between species or canopy positions and the percentage of time points where the heated leaf was 10 °C above the associated control leaf, suggesting that the heating device does not control temperature differently under high light or shaded conditions (Table 2.1).

While the heaters performed well, there was evidence of scorch damage to some of the heated leaves, and slightly more damage in the lower and mid canopy levels than the upper canopy. This suggests that there is a difference in traits that make shaded leaves more susceptible to damage at supraoptimal temperatures than upper canopy leaves. This is aligned with leaf traits that are usually associated with upper canopy environments. Leaves located higher in the canopy are exposed to more severe environments; i.e. high irradiance, temperatures, and wind (Wright *et al.* 2004). Plant acclimation to one type of stress can improve protection from other stressors (Havaux 1992). Niinemets *et al.* (1999) found that electron transport in temperate trees leaves that are more acclimated to high light environments is more stable under high temperature conditions. Upper canopy

leaves are acclimated to high light conditions, possibly inducing stress acclimation in the upper canopy leaves in our study. The single instance of leaf scorching in the upper canopy occurred in *A. saccharum*, where the average maximum daily leaf temperature was 43.5 °C. This maximum leaf temperature was at least 5 °C higher than maximum temperatures found in all *T. americana* canopy positions, as well as *A. saccharum* maximum temperatures in the middle canopy and understory. High maximum temperatures experienced by *A. saccharum* upper canopy leaves could have contributed to leaf scorching.

Between species, *A. saccharum* leaves were slightly more affected by scorching than *T. americana*. The higher resistance to heat damage in *T. americana* could have occurred for several reasons. While both species are shade tolerant, *T. americana* is less so than *A. saccharum* (Crow 1990, Baltzer and Thomas 2007, Thomas 2010). Species with higher shade tolerance have an overall lower plasticity to be able to adapt to high stress environments encountered in upper canopies (Reich *et al.* 2003). This suggests that *A. saccharum* would be less tolerant of the temperature fluctuations associated with the heating device. Leaf traits associated with stress avoidance (e.g. leaf water content) were also higher in *T. americana* (Fig. 2.7 e, f). The average *T. americana* leaf water content was 49% higher than *A. saccharum*, respectively, possibly leading to higher heat tolerance.

2.5.2 Photosynthetic Response to Temperature

Interestingly, both *A. saccharum* understory and middle canopy photosynthetic (A_{net}) response to temperature did not show the positive polynomial response that typically occurs. *A. saccharum* understory A_{net} response to temperature was almost linear, which was likely, in part, due to the very low stomatal conductance (g_s) (Fig. 2.4c). If stomatal conductance is low, then there is a deficiency in CO_2 available for photosynthesis, causing lowered A_{net} (Farquhar and Sharkey 1982). The lack of fit for the middle canopy and understory can be attributed to the wide spread in the magnitude of the photosynthetic response (Fig. 2.4a). When modeled with a 2nd order polynomial, the linear variable of the *A. saccharum* middle canopy is negative (Table 2.3). This makes the temperature response curve inverted suggesting a rise in A_{net} at high and low temperatures (Fig. 2.4a). The increase in A_{net} could be attributed to a rise in g_s , as g_s also responded to T_{leaf} via an inverted polynomial curve (Fig. 2.4c). A rise in g_s at high temperatures commonly occurs to increase transpirational cooling at high leaf temperatures (Radin *et al.* 1994, Way and Sage 2008, Slot *et al.* 2016). While the rise in photosynthesis at higher temperatures could be attributed to elevated g_s rates, the regression response is not significant; therefore, the occurrence of transpirational cooling as a cause for the rise in A_{net} is speculative. *T. americana* understory had low rates of stomatal conductance, possibly contributing to the weak photosynthetic response to temperature (Fig. 2.4b, d).

Within the scope of this study, we did not investigate the specific causes of photosynthetic decline after T_{opt} . The only possible mechanism for photosynthetic decline

that was measurable was the g_s response to temperature. Apart from two curves, all g_s response to T_{leaf} curves declined at high temperatures. This suggests that the declines in photosynthesis for these species/ canopy position are in part due to a decline in g_s (Farquhar and Sharkey 1982). Other than *A. saccharum* middle canopy, the only other g_s curve with a negative response to temperature was *T. americana* upper canopy. Unlike *A. saccharum* middle canopy, *T. americana* upper canopy A_{net} did not increase at high temperatures (Fig 2.4b). This suggests that, in this case, A_{net} decline after T_{opt} is due to a factor other than g_s , such as Rubisco activase or declined rates of electron transport (Sage and Kubien 2007).

2.5.3 Photosynthetic Acclimation and Responses to Warming

Based on T_{opt} and A_{opt} values, there is no evidence of photosynthetic acclimation for either *A. saccharum* or *T. americana* at any canopy position; however, there is slightly significant evidence of A_{opt} decline in *T. americana* heated leaves (Fig. 2.5). For *A. saccharum*, there were no differences in T_{opt} between canopy positions (Fig. 2.5a). This is similar to Mau (2015), who found that, while there was a height gradient associated with *A. saccharum* T_{opt} , it was not significant. T_{opt} is correlated with growth temperature (Berry and Bjorkman 1980) and, since *A. saccharum* average daily leaf temperatures only varied $\sim 1^\circ\text{C}$ for all canopy positions, it is expected that T_{opt} would be similar at all heights (Table 2.1).

T. americana T_{opt} did change with height, with the upper canopy having a slightly lower T_{opt} than the understory and middle canopy (Fig. 2.6b). These results could have been due to our study design. Our warming experiment was set-up so that the upper canopy was warmed approximately one month after the understory and middle canopy leaves. This may have confounded time and height. Average *T. americana* daytime control T_{leaf} for the understory and middle canopy were higher during the July campaign than the upper canopy T_{leaf} during the August campaign (Table 2.4). Other studies that measure T_{opt} throughout a growing season have found that T_{opt} shifts with temperature, acclimating to their current growing season temperature (e.g. Sendall *et al.* 2015); although, the seasonal shift in T_{opt} is often less than the magnitude of seasonal temperatures changes (Battaglia *et al.* 1996, Medlyn *et al.* 2002b). The lower temperatures experienced during the August campaign could have influenced T_{opt} . The high variation between heights in *T. americana* T_{opt} compared with the low variation daytime average T_{leaf} suggests that *T. americana* T_{opt} was determined by more than just the average daytime temperature (Table 2.1, Fig. 2.6b). *T. americana* T_{opt} was only 1°C from the average daily maximum in the understory and middle canopy levels and ~ 5°C lower than average daily maximum in the upper canopy (Table 2.1, Fig. 2.6b). *A. saccharum*, on the other hand, had an average T_{opt} approximately 10°C to 12°C below the average maximum temperature at the three canopy positions and was ~ 1°C below the average daytime T_{leaf} . This suggests that *T. americana* T_{opt} could be controlled more by maximum daily temperatures, whereas *A. saccharum* is more influenced by average daily temperatures.

Instead of A_{opt} thermally acclimating to warmer temperatures, we found a slight decline in *T. americana* A_{opt} at all canopy positions (Fig. 2.5d). A decline in A_{opt} suggests some degree of thermal damage to the photosynthetic apparatus. Photosynthetic apparatus damage is usually associated with thylakoid membrane dissociation and protein denaturation that occurs at temperatures of 46°C or higher (Berry and Bjorkman 1980, Sage and Kubien 2007, Hüve *et al.* 2011). The only instance of temperatures higher than 46 °C occurred at one time point in *A. saccharum* upper canopy (data not shown). To our knowledge, there have been no studies that investigate the enzymatic and membrane thermal threshold of leaf temperatures in either of our study species; although, a study investigating the effects of heatwaves on *A. saccharum* displayed photosynthetic damage in leaves where air temperature was ~31.5 °C (Filewod and Thomas 2014).

It is a possibility that one week of warming was not enough time to allow photosynthetic acclimation. Photosynthetic acclimation has been found to occur in as little as one day to several weeks (Berry and Bjorkman 1980, Kattge and Knorr 2007). Most warming studies measure acclimation response after a set time that leaves are exposed to warmer temperatures instead of throughout the warming experiment, making it difficult to pinpoint a set timeframe for acclimation. Gunderson *et al.* (2000) showed that *A. saccharum* seedlings have the capacity to acclimate to +4 °C warmer temperatures; however, acclimation occurred with a shift in A_{opt} instead of T_{opt} . Mau (2015) examined the optimum temperature of *A. saccharum* in a northern hardwood forest with a higher canopy (23 m) but in close proximity to our experiment. Mau (2015) found *A. saccharum* to have a much higher T_{opt} (~27 °C at 12.5 m) than our study. T_{opt} for the Mau (2015) study was found to be higher than maximum daily leaf temperature at

12.5 m (~25 °C); however, unlike our current study, maximum daily leaf temperature did increase with canopy height.

Species distribution models have predicted that the abundance of *A. saccharum* will decline in northern Michigan (Iverson and McKenzie 2013, Peters *et al.* 2013), and the overall distribution of *T. americana* is also expected to decline (Iverson and Prasad 1998). Gunderson *et al.* (2000) found that while *A. saccharum* is able to acclimate to warmer temperatures, populations from different climate regions had similar acclimation potential. The results of our study suggest that, while neither *T. americana* or *A. saccharum* acclimated under warmer temperatures, the higher T_{opt} found in *T. americana* would cause it to be less likely to experience photosynthetic decline under elevated temperatures. The proximity of *A. saccharum* to the average daytime temperature also suggests that, if this species does not acclimate, climate induced warming would cause *A. saccharum* to be operating above the temperature optimum more often than not.

2.5.4 Drivers of Canopy Photosynthetic Rates

There were differences in photosynthetic rates and leaf traits at different canopy heights and these differences varied by species (Table 2.6, Fig. 2.7, 2.8). Based on the trait differences at different canopy positions, we can discuss how these two species allocate resources and how beneficial these specific leaf traits are predicted to be under the future climate.

Leaf traits associated with high photosynthetic capacities are highest in *A. saccharum* upper canopy leaves. *A. saccharum* LMA does not differ between the

understory and middle canopy; however, it is higher in the upper canopy (Fig. 2.7c). High LMA in the upper canopy is due to higher leaf thickness and density which maximizes photosynthetic capacity in the high light environment (Niinemets 1999, Zhang *et al.* 2011, Coble and Cavaleri 2014). Our results are supported by other studies which have found that *A. saccharum* LMA does increase with height (Ellsworth and Reich 1993, Coble and Cavaleri 2014, Filewod and Thomas 2014). We also found that *A. saccharum* N_{area} , a functional trait associated with LMA, was also highest in the upper canopy leaves (Fig. 2.8a). Our *A. saccharum* higher N_{area} results supports other studies where N_{area} of leaves exposed to high irradiance is predictive of photosynthetic capacity (Meir *et al.* 2002). This is also supported by the higher rates of photosynthesis found in the upper canopy leaves (Fig. 2.4a). The relationship between N_{area} and LMA, however, should not be discounted. N_{area} is derived from LMA; therefore, the significant relationship in upper canopy leaves and N_{area} can be largely explained by LMA (Ellsworth and Reich 1992, 1993). *A. saccharum* N_{mass} is distributed evenly throughout the understory and canopy (Fig. 2.7a). This is consistent with other studies on *A. saccharum* (Ellsworth and Reich 1993, Niinemets and Tenhunen 1997, Coble and Cavaleri 2015). Studies have found that leaf water traits decrease with increasing height when calculated on a mass basis (Pilegaard *et al.* 2003, Lichtenthaler *et al.* 2007). We did not find this in our study. Based on leaf water content, *A. saccharum* leaf water stayed consistent throughout the forest canopy (Fig. 2.7e). These results suggest that leaf water is not a limiting factor for upper canopy leaves.

T. americana LMA was greatest in the upper canopy. This is similar to other studies, which have found that *Tilia* species have high leaf morphological plasticity, and

that LMA increases with canopy height (Koike *et al.* 2001, Lichtenthaler *et al.* 2007, Legner *et al.* 2014). N_{area} was not significantly different between the upper and middle canopy (Fig. 2.8b). The lack of relationship of N_{area} with increasing height is curious, because, due to the strong correlation between N_{area} and LMA, N_{area} generally increases with increasing height (Ellsworth and Reich 1992, 1993). N_{mass} was higher in the middle canopy than in the upper canopy (Fig. 2.7b). Studies suggest that leaf traits associated with high photosynthetic capacities, such as leaf nitrogen on a mass basis, have a stronger relationship with increasing light capture instead of linearly increasing with height (Niinemets 1997, Johnson *et al.* 2010, Legner *et al.* 2014). In shade tolerant species, leaf nitrogen is prioritized to lower light leaves to maximize the light harvesting capacity (Niinemets 1997, Schoettle and Smith 1999, Koike *et al.* 2001). This suggests that *T. americana*, a shade-tolerant species, invests N resources to lower canopy leaves to optimize light capture in more shaded environments.

Photosynthetic rates were also higher in *T. americana* middle canopy leaves (Fig. 2.4b). N is a major component of chlorophyll and photosynthetic enzymes, and the high N found in the middle canopy likely contributed to high photosynthetic rates in the middle canopy (Evans 1989). The lack of photosynthetic optimization with height could have several explanations. Photosynthesis is not always maximized with increasing irradiance or height. Canopy photosynthetic optimization can be limited due to hydraulic constraints as well as stomatal and mesophyll conductance restrictions that occur with increasing canopy height (Niinemets and Tenhunen 1997, Bond *et al.* 1999, Ryan *et al.* 2006, Duursma and Medlyn 2012, Buckley *et al.* 2013). Lowered mesophyll conductance can limit A_{net} through decreased CO_2 diffusion through cells and through alterations in

intercellular membrane structure due to tissue shrinkage (Lawlor and Tezara 2009). Hydraulic restrictions on g_s can limit A_{net} by decreasing intercellular CO_2 concentrations, thereby limiting CO_2 fixation in the Calvin cycle (Farquhar and Sharkey 1982). Based on the low rates of stomatal conductance (Fig. 2.4d), along with lowered leaf water content found in the upper canopy (Fig. 2.7f), leaf water status and low stomatal conductance could have limited *T. americana* A_{net} in the upper canopy. Another contributing factor to lower photosynthetic rates in the upper canopy than the middle canopy could be our experimental design. Due to limited accessibility from the canopy scaffolding, separate trees were used for the middle and upper canopy warming; therefore, differences could have been due to individual tree variation. While the leaves measured in the upper and middle canopies only represent two trees, higher variation consistently occurs between canopy position than between individuals (Bassow and Bazzaz 1997), and our sample population consists of canopy leaves rather than individual trees.

2.6. Conclusions

The aim of this study was to test the efficacy of a novel leaf warming device as well as examine the acclimation response of two prominent northern hardwood species when exposed to one week of 3 °C warming. The leaf warming device successfully heated individual leaves 3.02 ± 1.86 °C above control leaf temperatures at all understory and canopy positions. While the warming device did successfully heat the leaves, there was evidence of leaf scorching, suggesting that there is room for improvement with the

warming method. Part of the reason for the leaf scorching is likely due to the sensitivity of understory leaves. While leaf sensitivity is outside of the control of our experiment, in the future, scorching could be minimized by ensuring that the leaf is consistently positioned far enough away from the leaf heater. Leaves are rarely arranged horizontally parallel to the ground; therefore, it is unlikely that all areas of the leaf surface are uniformly heated. Scorching could also be minimized by ensuring that the thermocouple is placed at the point on the leaf that is closest to the heater. This would help prevent temperature spiking in the sensitive areas of the leaf that are near the leaf heater.

We found no evidence of thermal acclimation for either species at any canopy height; however, we did find a slight decline in *T. americana* A_{opt} . The decline in A_{opt} suggests that there was some type of damage to the photosynthetic apparatus associated with leaf heating. *A. saccharum* T_{opt} was similar for all canopy positions; however, A_{opt} was highest in the upper canopy. Higher rates of photosynthesis in *A. saccharum* upper canopy leaves can be attributed to higher N availability for photosynthetic machinery. *T. americana* T_{opt} was lower in the upper canopy, possibly due to lower temperatures experienced during upper canopy warming. Surprisingly, *T. americana* photosynthetic rates were highest in the middle canopy. Lowered rates of photosynthesis in *T. americana* upper canopy can likely be attributed to hydraulic limitations on leaf mesophyll and stomatal conductance. While this study did not show evidence of acclimation at different canopy positions, it did provide useful information for modeling within canopy photosynthesis for our two study species. Our results suggest that models that predict canopy photosynthesis based on canopy height or leaf traits, such as LMA, may incorrectly estimate photosynthesis for species that do not optimize photosynthesis in

their upper canopy. Models should consider species differences in photosynthetic rates across canopy gradients.

Drivers of T_{opt} seem to differ between our two studied species. Based on their close proximity, *A. saccharum* T_{opt} seems to be driven by average daily temperature. *T. americana* T_{opt} was variable between the three canopy positions, while the average daily temperature was not. This suggests that *T. americana* T_{opt} is driven by a factor other than average daily temperature, i.e. maximum daily temperature. Even though no acclimation occurred in any of our studied species, higher T_{opt} found in *T. americana* suggests that this species will perform better under warmer temperatures than *A. saccharum*. The lack of acclimation has important implications for future carbon cycling. While our study may have not been long enough to detect photosynthetic acclimation, if neither of these species are able to acclimate to elevated temperatures, we could see a decline in CO_2 sequestration in N. hardwood ecosystems. On a community composition scale, decreased photosynthesis could cause species to shift distribution away from climates where temperatures often exceed thermal thresholds. Future studies should investigate the acclimation response of these two species after longer term warming.

2.7. Tables and Figures

Table 2.1 Summary of average and maximum control T_{leaf} . Average daytime control T_{leaf} (mean \pm stdev) and maximum daily control T_{leaf} (mean \pm stdev) for each sample month, canopy position, and species.

Sample Month	Canopy Position	Species	Average Daytime T_{leaf} (°C)	Average Maximum Daily T_{leaf} (°C)
August	Upper	A. saccharum	20.74 (\pm 5.0)	32.57 (\pm 6.1)
		T. americana	20.05 (\pm 3.6)	28.37 (\pm 3.5)
July	Middle	A. saccharum	21.86 (\pm 4.9)	28.01 (\pm 5.8)
		T. americana	21.47 (\pm 4.8)	31.87 (\pm 5.8)
	Understory	A. saccharum	21.46 (\pm 4.5)	31.22 (\pm 6.8)
		T. americana	20.89 (\pm 4.4)	28.14 (\pm 6.2)

Table 2.2 Summary of heated leaf temperature spiking. Daily maximum temperature (mean \pm stdev), frequency of occurrences where heated T_{leaf} was greater than 10 °C above control T_{leaf} , and percentage of time points where heated T_{leaf} was greater than 10 °C above control T_{leaf} for *A. saccharum* and *T. americana* at each canopy position.

Canopy Position	Species	Average Daily Max Temp.	Freq. heated T_{leaf} >10°C than control T_{leaf}	% heated T_{leaf} >10°C than control T_{leaf}
Upper	<i>A. saccharum</i>	43.56 (\pm 3.0)	79	0.53%
	<i>T. americana</i>	32.31 (\pm 2.7)	2	0.01%
Middle	<i>A. saccharum</i>	37.66 (\pm 5.9)	169	1.10%
	<i>T. americana</i>	33.87 (\pm 5.2)	98	0.67%
Understory	<i>A. saccharum</i>	34.36 (\pm 4.3)	107	0.70%
	<i>T. americana</i>	35.75 (\pm 5.1)	222	1.43%

Table 2.3 Two-way ANOVA results of heated leaf temperature spiking. Heated leaf daily maximum leaf temperature and percentage of occurrences where the difference heated and control Tleaf was greater than 10 °C. p-value results of treatment, canopy position, and the interaction effect of species and canopy position.

* $p < .1$, ** $p < .01$, *** $p < .001$

	Daily Max T (Heated)	Percent T diff. greater than 10 °C
Species	0.001**	0.862
Canopy Position	0.297	0.334
Species × Canopy Position	0.001***	0.435

Table 2.4 Summary of simple 2nd order polynomial regression applied to *A. saccharum* and *T. americana* A_{net} and g_s at the three studied canopy positions. Regression equation, residual standard error, r^2 , p-value. Analysis includes heated and control leaves combined. * $p < .1$, ** $p < .01$, *** $p < .001$

Measurement Type	Species	Canopy Position	Simple polynomial regression	Residual Standard Error	r^2	p-value
A_{net} ($\mu\text{mol m}^{-2} \text{ s}^{-1}$)	A. saccharum	Upper	$y = 4.140 + 0.266x - 0.007x^2$	1.719	0.0953	0.044*
		Middle	$y = 1.451 + 0.108x - 0.002x^2$	0.934	-0.0430	0.857
	T. americana	Understory	$y = 5.936 - 0.241x + 0.004x^2$	0.883	0.0168	0.244
		Upper	$y = 2.714 + 0.647x - 0.015x^2$	1.605	0.1446	0.013*
		Middle	$y = -2.747 + 1.036x - 0.018x^2$	1.300	0.1106	0.024*
		Understory	$y = -2.213 + 0.488x - 0.008x^2$	1.314	0.0414	0.128
g_s ($\mu\text{mol m}^{-2} \text{ s}^{-1}$)	A. saccharum	Upper	$y = 0.219 - 5.44e^{-3}x + 1.51e^{-5}x^2$	0.043	0.2299	0.001**
		Middle	$y = 0.078 - 3.16e^{-3}x + 4.46e^{-5}x^2$	0.015	0.0516	0.134
	T. americana	Understory	$y = 0.145 - 7.84e^{-3}x + 1.32e^{-4}x^2$	0.013	0.2936	< 0.001***
		Upper	$y = 0.236 - 3.81e^{-3}x - 2.32e^{-5}x^2$	0.039	0.3130	< 0.001***
		Middle	$y = 0.485 - 0.021x + 3.07e^{-4}x^2$	0.043	0.1726	0.004**
		Understory	$y = -0.003 + 0.006x - 1.08e^{-4}x^2$	0.036	-0.0275	0.750

Table 2.5 Two-way ANOVA p-value results for tests of treatment and location on T_{opt} and A_{opt} . * $p < .1$, ** $p < .01$, *** $p < .001$.

	Species	T_{opt} (°C)	A_{opt} ($\mu\text{mol m}^{-2} \text{s}^{-1}$)
Treatment	A. saccharum	0.756	0.139
	T. americana	0.263	0.082*
Location	A. saccharum	0.935	0.001***
	T. americana	0.001***	0.001***
Treatment \times Location	A. saccharum	0.685	0.954
	T. americana	0.809	0.837

Table 2.6 Two-way ANOVA p-value results of treatment, canopy position, and the interaction between treatment and canopy position for each species on N_{area} , N_{mass} , LMA, and leaf water content * $p < .1$, ** $p < .01$, *** $p < .001$

	Species	N_{area} (g m^{-2})	N_{mass} (mg g^{-1})	LMA (g m^{-2})	Leaf Water Content
Treatment	A. saccharum	0.424	0.703	0.577	0.319
	T. americana	0.341	0.234	0.534	0.929
Canopy Position	A. saccharum	0.001***	0.193	0.001***	0.114
	T. americana	0.001**	0.001***	0.002**	0.052*
Treatment \times Location	A. saccharum	0.123	0.330	0.102	0.343
	T. americana	0.060*	0.665	0.156	0.373



Fig. 2.1 Example of a leaf heater positioned underneath an understory *T. americana* leaf.

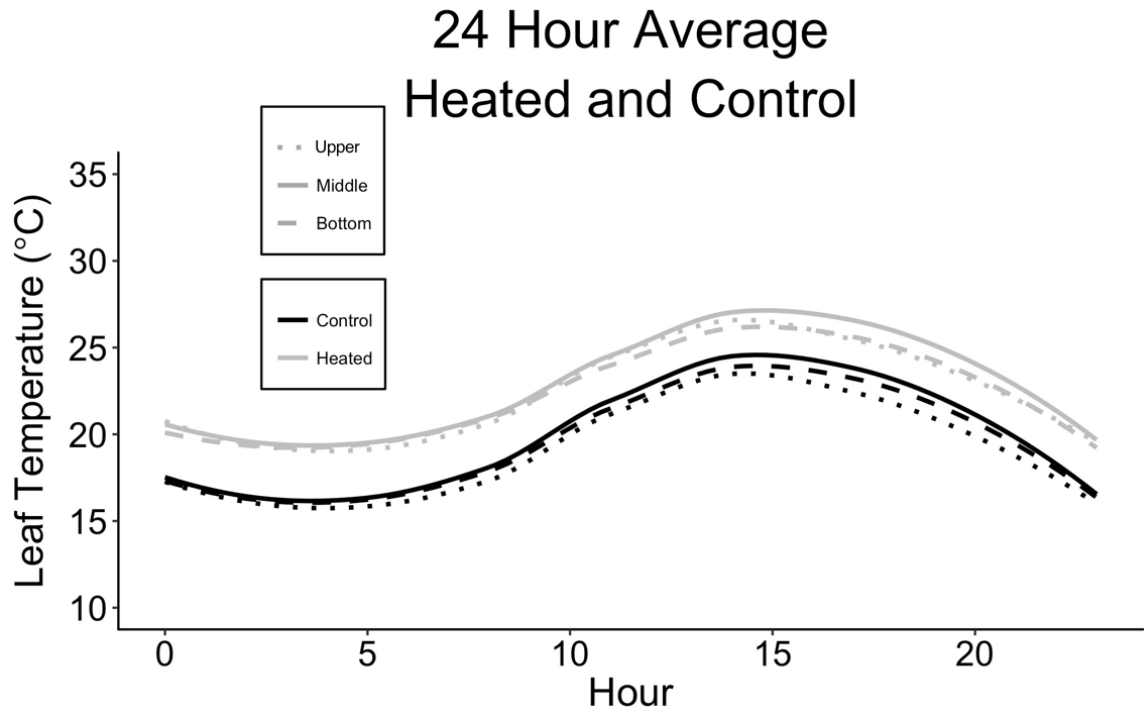


Fig. 2.2 Average of heated and control leaves at the three canopy positions. Data displayed are *A. saccharum* and *T. americana* T_{leaf} combined. Control T_{leaf} is depicted by the black lines and heated T_{leaf} is depicted by grey lines. Upper canopy is represented by dotted lines, middle canopy is depicted by solid lines, and understory (also called bottom) is depicted by dashed lines.

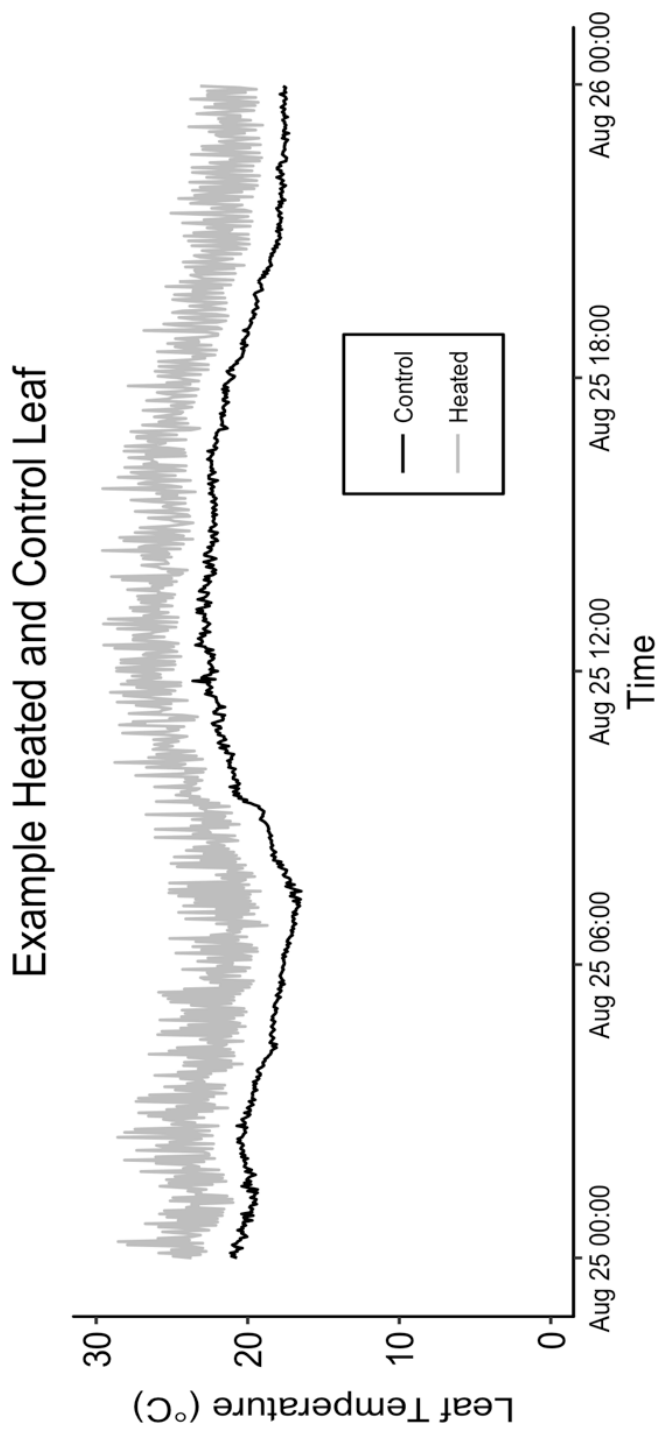


Fig. 2.3 Example of the variation in one heated and control leaf over 24 hours. The leaf temperature for one *T. americana* upper canopy heated leaf (grey line) and the associated *T. americana* control leaf (black line) for 24 hours on August 25, 2016.

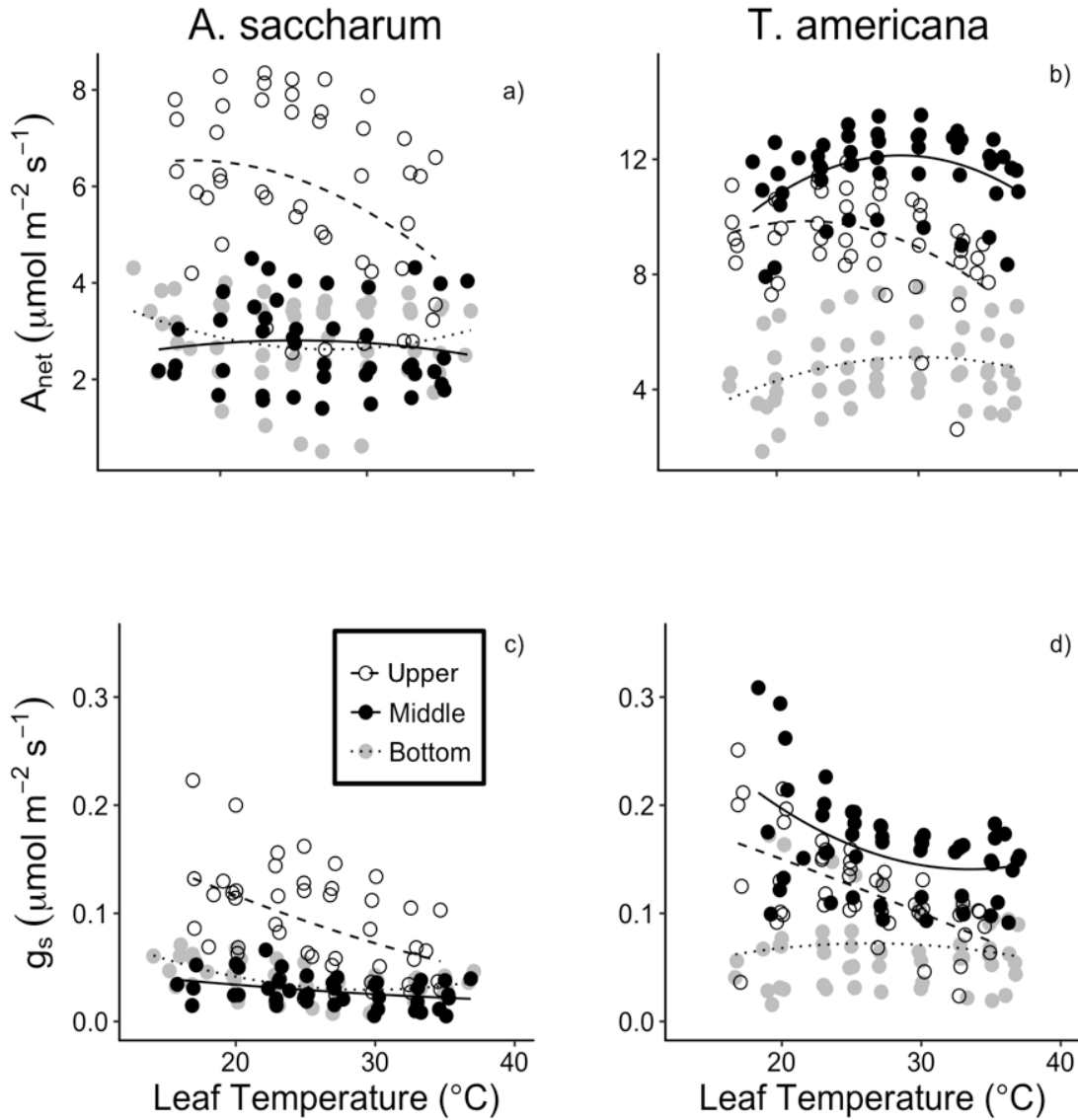


Fig. 2.4 Photosynthetic and stomatal conductance response to leaf temperature fit to a 2nd order polynomial regression. Response of upper canopy, middle canopy, and understory a) *A. saccharum* A_{net} to leaf temperature b) *T. americana* A_{net} to leaf temperature c) *A. saccharum* g_s to leaf temperature d) *T. americana* g_s to leaf temperature. Individual data points (white- upper, black- middle, grey- understory) depict individual measurements. Heated and control leaves are pooled into their respective canopy positions. Lines depict the polynomial regression for the upper canopy (dashed), middle canopy (solid), and the understory (also called bottom: dotted).

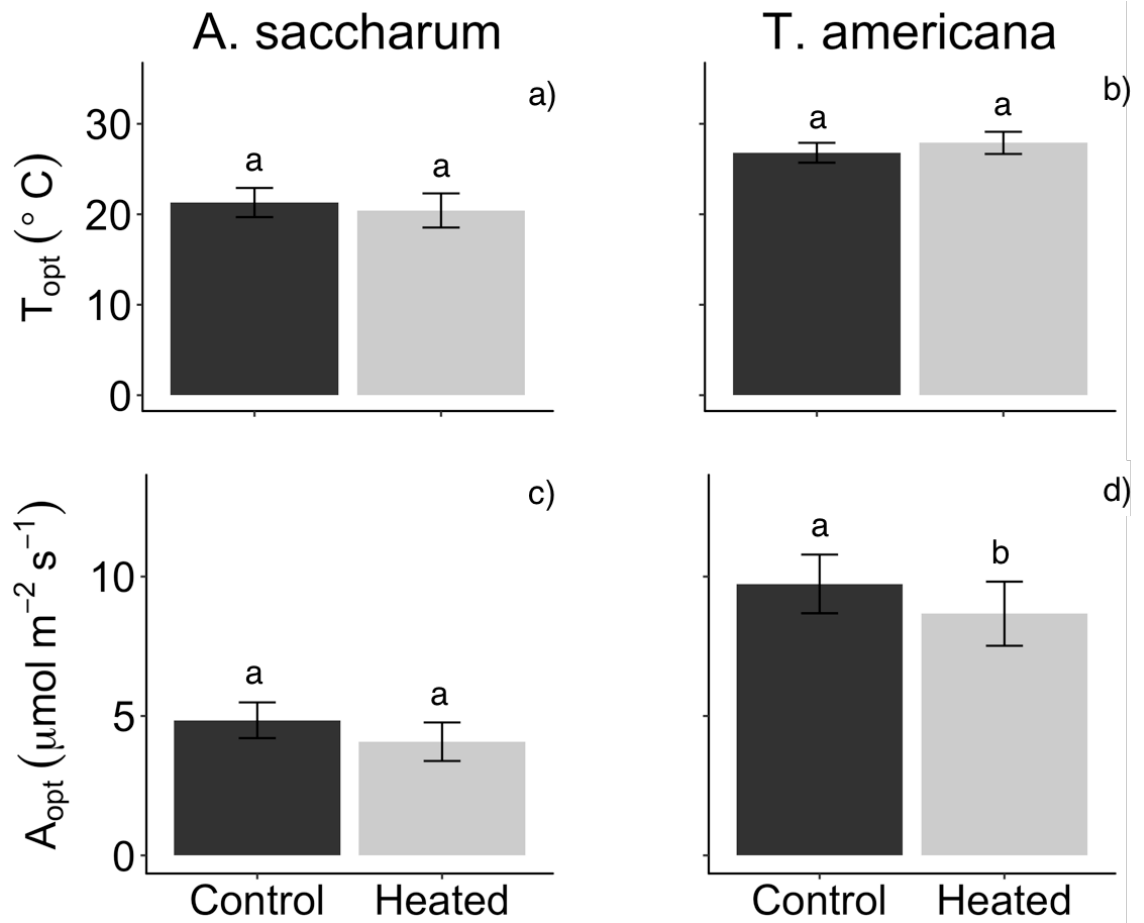


Fig. 2.5 T_{opt} and A_{opt} for the heated and control leaves. a) T_{opt} for control (black) and heated (grey) *A. saccharum* leaves b) T_{opt} for *T. americana* c) A_{opt} for *A. saccharum* and d) A_{opt} for *T. americana*. Heated and control T_{opt} and A_{opt} are averaged across all canopy positions. Letters above bars denote results of the *post-hoc* Tukey test.

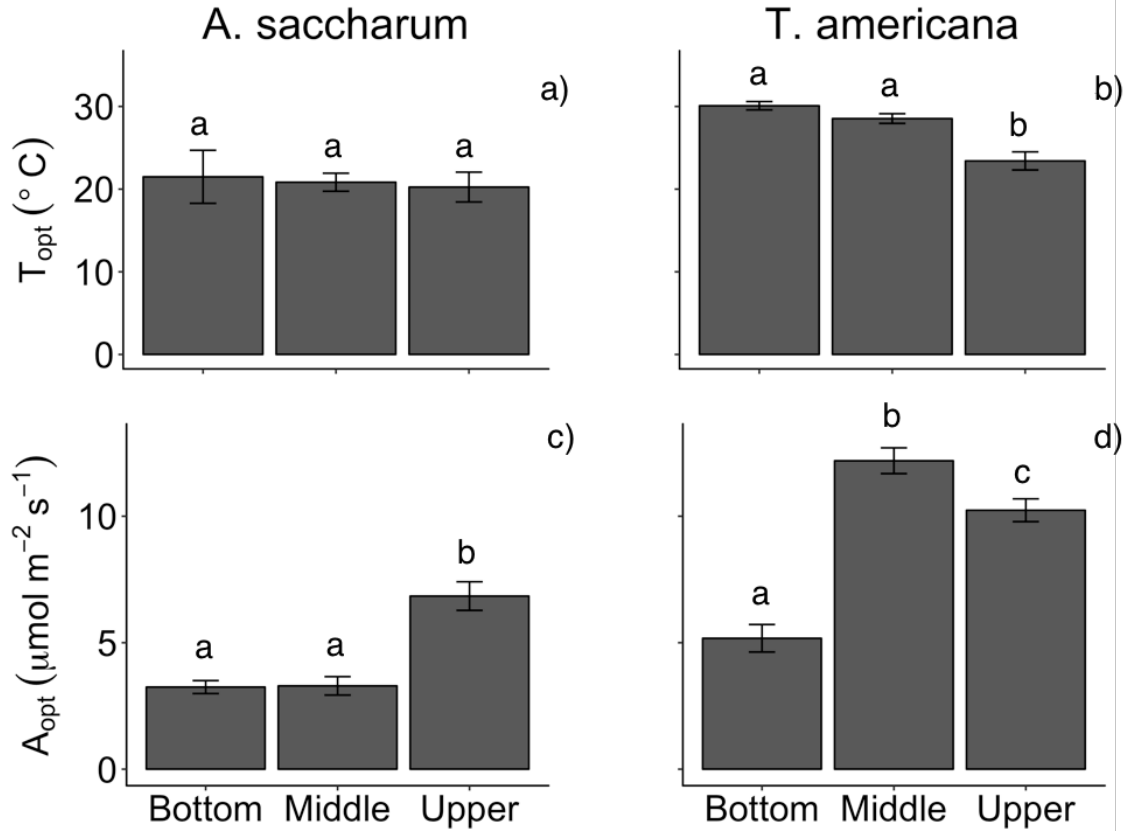


Fig. 2.6 T_{opt} and A_{opt} at each canopy position. a) T_{opt} for *A. saccharum* leaves for understory (bottom), middle canopy, and upper canopy leaves b) T_{opt} for *T. americana* leaves c) A_{opt} for *A. saccharum* leaves and d) A_{opt} for *T. americana* leaves. T_{opt} and A_{opt} for each canopy position includes both heated and control leaves. Letters above bars denote results of the *post-hoc* Tukey test.

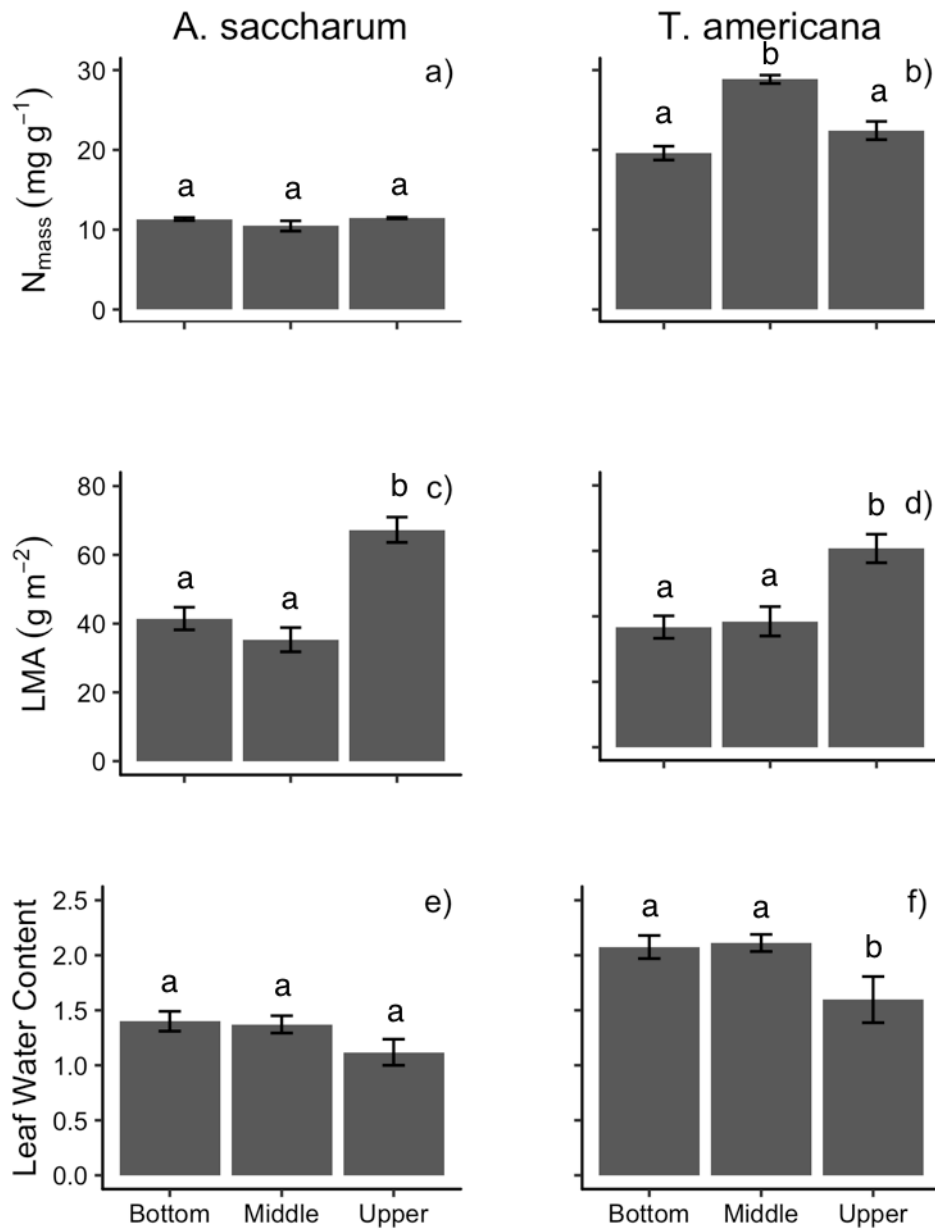


Fig. 2.7 Leaf traits at each canopy position for each species. A) *A. saccharum* N_{mass} b) *T. americana* N_{mass} c) *A. saccharum* LMA d) *T. americana* LMA e) *A. saccharum* leaf water content f) *T. americana* leaf water content. Letters above bar graphs denote results of *post-hoc* Tukey test.

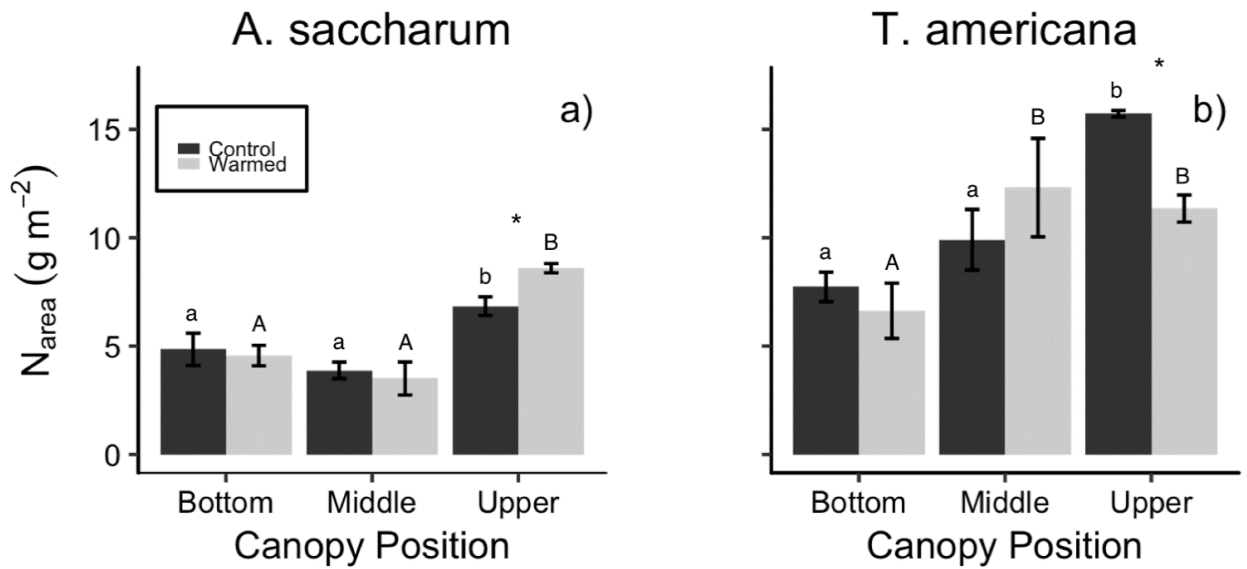


Fig. 2.8 N_{area} at each canopy position for a) *A. saccharum* and b) *T. americana* control (black) and heated (grey) leaves. Lower case letters above bar graphs denote results of post-hoc planned contrasts between control leaves at each canopy position. Upper case letters above bar graphs denote results of post-hoc planned contrasts between heated leaves at each canopy position. Asterisks (*) above bar graphs denote significant difference for post-hoc contrast between heated and control leaves at individual canopy positions. * $p < .1$, ** $p < .01$, *** $p < .001$

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