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Investigating within-canopy variation of functional traits and cellular structure of sugar maple (*Acer saccharum*) leaves

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INVESTIGATING WITHIN-CANOPY VARIATION OF FUNCTIONAL TRAITS
AND CELLULAR STRUCTURE OF SUGAR MAPLE (*ACER SACCHARUM*)
LEAVES

By

Adam P. Coble

A DISSERTATION

Submitted in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

In Forest Science

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2015

This dissertation has been approved in partial fulfillment of the requirements for the Degree of DOCTOR OF PHILOSOPHY in Forest Science.

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Preface

Chapter 1 provides an introduction to four chapters that were published in peer-reviewed journals (Chapters 2 and 3) or are currently in preparation for publication in peer-reviewed journals (Chapters 4 and 5). Copyright and author contributions for each chapter are listed below. Chapters published in peer-reviewed journals are acknowledged in a footnote at the beginning of each chapter.

Chapter 2, Light drives vertical gradients of leaf morphology in a sugar maple (*Acer saccharum*) forest: © by the Oxford University Press 2014. Documentation that includes permission to use the copyrighted material is provided in the appendix of this chapter. Adam P. Coble conceived, designed, and performed the experiments, analyzed the data, and wrote the manuscript. Molly A. Cavaleri conceived and designed the experiments and wrote the manuscript.

Chapter 3, Light acclimation optimizes leaf functional traits despite height-related constraints in a canopy shading experiment: © by Springer-Verlag Berlin Heidelberg 2015. Documentation that includes permission to use the copyrighted material is provided in the appendix of this chapter. Adam P. Coble conceived, designed, and performed the experiments, analyzed the data, and wrote the manuscript. Molly A. Cavaleri conceived and designed the experiments and wrote the manuscript.

Chapter 4, Seasonal variation of leaf functional traits within a mature sugar maple (*Acer saccharum*) canopy reveals potential biases in canopy photosynthesis models: in

preparation for submission to the peer-reviewed journal *New Phytologist*. Adam P. Coble conceived and designed the study, collected and analyzed the data, and wrote a majority of the manuscript. Molly A. Cavaleri conceived, designed, and contributed to writing and editing the manuscript. Brittany VanderWall collected and analyzed the data and contributed to writing the manuscript. Alida Mau contributed to data collection.

Chapter 5, Vertical gradients in *Acer saccharum* leaf cellular structure display a trade-off between palisade layer thickness and mesophyll porosity: to be submitted to the peer-reviewed journal *Trees*. Adam P. Coble conceived, designed, and performed the experiments, analyzed the data, and wrote the manuscript. Molly A. Cavaleri conceived and designed the experiments and wrote the manuscript.

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Abstract

Patterns of increasing leaf mass per area (LMA), area-based leaf nitrogen (N_{area}), and carbon isotope composition ($\delta^{13}\text{C}$) with increasing height in the canopy have been attributed to light gradients or hydraulic limitation in tall trees. Theoretical optimal distributions of LMA and N_{area} that scale with light maximize canopy photosynthesis; however, sub-optimal distributions are often observed due to hydraulic constraints on leaf development. Using observational, experimental, and modeling approaches, we investigated the response of leaf functional traits (LMA, density, thickness, and leaf nitrogen), leaf carbon isotope composition ($\delta^{13}\text{C}$), and cellular structure to light availability, height, and leaf water potential (Ψ_1) in an *Acer saccharum* forest to tease apart the influence of light and hydraulic limitations. LMA, leaf and palisade layer thickness, and leaf density were greater at greater light availability but similar heights, highlighting the strong control of light on leaf morphology and cellular structure. Experimental shading decreased both LMA and area-based leaf nitrogen (N_{area}) and revealed that LMA and N_{area} were more strongly correlated with height earlier in the growing season and with light later in the growing season. The supply of CO_2 to leaves at higher heights appeared to be constrained by stomatal sensitivity to vapor pressure deficit (VPD) or midday leaf water potential, as indicated by increasing $\delta^{13}\text{C}$ and VPD and decreasing midday Ψ_1 with height. Model simulations showed that daily canopy photosynthesis was biased during the early growing season when seasonality was not accounted for, and was biased throughout the growing season when vertical gradients in LMA and N_{area} were not accounted for. Overall, our results suggest that leaves acclimate

to light soon after leaf expansion, through an accumulation of leaf carbon, thickening of palisade layers and increased LMA, and reduction in stomatal sensitivity to Ψ_1 or VPD. This period of light acclimation in leaves appears to optimize leaf function over time, despite height-related constraints early in the growing season. Our results imply that vertical gradients in leaf functional traits and leaf acclimation to light should be incorporated in canopy function models in order to refine estimates of canopy photosynthesis.

1. Introduction

1.1. Importance of leaf functional traits

Two key leaf functional traits, leaf mass per area (LMA) and area-based leaf nitrogen (N_{area}), are often strongly and positively correlated with leaf photosynthetic capacity within forest canopies (Ellsworth and Reich 1993, Bond et al. 1999, Jones and Thomas 2007). Within-canopy patterns in leaf traits and photosynthetic capacity allow prediction of leaf-level photosynthesis across a broad range of light conditions and across multiple canopy layers, which may refine canopy-level and global-scale process models that do not integrate spatial details of the canopy profile (Raulier et al. 1999, Hanson et al. 2004, Thornton and Zimmerman 2007). Multi-layer canopy function models that describe vertical variation in canopy structure and function often use LMA and N_{area} to model forest water and carbon balance in response to short- and long-term environmental conditions (Raulier et al. 1999, Luo et al. 2001, Hanson et al. 2004, Medlyn et al. 2004). Consequently, LMA and N_{area} are key parameters for scaling from leaf- to ecosystem- to global-level processes when modeling carbon, water, and nutrient cycling (Reich et al. 1999; Hanson et al. 2004; Wright et al. 2004; Poorter et al. 2009; Ryu et al. 2011). Also, N_{area} and LMA are easier to measure than photosynthetic capacity and are, therefore, powerful traits that can be applied in modeling forest carbon and water balance at multiple scales (Wright et al. 2004, Poorter et al. 2009). Overall, modeling forest carbon and water balance is an important tool in assessing potential forest response to future climate change (Baldocchi and Wilson 2001, Luo et al. 2001, Medlyn et al. 2005).

1.2. Environmental drivers of spatial variation in leaf functional traits

Tree species among a broad range of forest types display a consistent pattern where LMA (g m^{-2}) and N_{area} (g m^{-2}) increase from the bottom to the top of tree canopies and crowns (Hutchison et al. 1986, Ellsworth and Reich 1993, Bond et al. 1999, Niinemets et al. 1999, Marshall and Monserud 2003, Koch et al. 2004, Woodruff et al. 2004, England and Attiwill 2006, Ishii et al. 2008, Mullin et al. 2009, Cavaleri et al., 2010). Early investigations used the terms “sun” and “shade” leaves, which described morphological and anatomical adjustments to increasing irradiance (Nobel 1976, Boardman 1977). Experimental morphological responses to increased light treatments in tree seedlings or small plants commonly included increased leaf and palisade layer thickness, LMA, and greater surface area of mesophyll cells exposed to intercellular air-space, all of which were associated within increasing photosynthetic capacity of leaves (Nobel et al. 1975, Smith and Nobel 1978, Chabot et al. 1979). Increasing palisade layer thickness in response to high light availability has been considered a mechanism that facilitates light deeper into the leaf mesophyll that maximizes light absorption and photosynthesis. Thus, light gradients that occur in tree canopies provided a reasonable explanation for thicker leaves with a greater LMA at the top of the canopy and thinner leaves with a lower LMA at the bottom (Hollinger 1989, Ellsworth and Reich 1993, Bond et al. 1999, Niinemets et al. 1999).

More recently, LMA has been found to sometimes correlate more strongly with height than with measures of light (Cavaleri et al. 2010). The strong association between leaf height and LMA has been considered a direct result of changes in water relations within the canopy (Koch et al. 2004, Woodruff et al. 2004, Cavaleri et al. 2010). Xylem

tension in tree water columns, a key requirement for water transport, is generated by surface tension at the site of evaporation in the leaf mesophyll (Tyree 1997, Tyree 2003). Due to the strong cohesive forces of water, the tension is transmitted through the length of the entire water column and is maintained throughout the night despite little to no transpiration. In tall trees, the xylem tension is not uniform along the length of the water column, but rather tension increases (i.e., water potential decreases) 0.01 MPa per meter increase in height. This linear decrease in water potential has been confirmed by water potential measurements during periods of no transpiration (Scholander et al. 1965, Hellkvist et al. 1974, Bauerle et al. 1999, Woodruff et al. 2004). Cellular osmotic adjustments through stored or photosynthetically derived solute may compensate for low water potential during leaf development (Bahari et al. 1985, Abrams 1988, Kubiske and Abrams 1994, Zhang et al. 2011); however, this was not the case for *Psuedotsuga menziesii* needles growing at high positions in the canopy (Woodruff et al. 2004, Meinzer et al., 2008). During *Psuedotsuga menziesii* needle development, the steeper decline in leaf water potential compared with osmotic potential suggests that reduced turgor pressure may be the limiting factor in tree canopy leaf development (Woodruff et al. 2004). Reduced turgor can limit cell expansion and division leading to smaller, denser leaves with reduced mesophyll air-space (Hsiao 1973). Both leaf density and mesophyll porosity (fraction of intercellular air-space) have been associated with mesophyll conductance (Ishii et al. 2008, Mullin et al. 2009). In addition to reductions in mesophyll conductance at higher heights, stomatal conductance may be constrained, as indicated by increasing $\delta^{13}\text{C}$ with height (Koch et al. 2004, Ishii et al. 2008).

1.3. Temporal variation in leaf functional traits

In addition to spatial variation in leaf functional traits and cellular structure, leaves can experience considerable changes in function, structure, and chemistry throughout the growing season, which can influence LMA and leaf nitrogen. Soon after bud break, cellular expansion and division causes leaves to rapidly expand, which can occur over a period of a few weeks before leaves achieve a final area. During leaf expansion, increasing LMA through time corresponded with increasing photosynthetic capacity in six evergreen broad-leaved species (Miyazawa et al. 1998, Miyazawa and Terashima 2001), while decreasing LMA during leaf expansion was observed in *Acer saccharum* seedlings (Ellsworth and Reich 1992). Despite leaves containing high leaf nitrogen contents, photosynthetic capacity was still low during leaf expansion, likely because the photosynthetic apparatus was not fully functional (Yasamura et al. 2006). Following leaf expansion, photosynthetic capacity has been found to increase (Yasamura et al. 2006), decrease (Kitajima et al. 2002), or not change (Miyazawa and Terashima 2001). Increases in photosynthetic capacity following leaf expansion has been associated with increasing leaf nitrogen and Rubisco (Yasamura et al. 2006), whereas no changes in photosynthetic capacity were likely due to limitations on internal CO₂ conductance through increasing cell wall thickness (Miyazawa and Terashima 2001). Vertical elongation following expansion of palisade cells has been reported for numerous species indicating that leaves continue to thicken after achieving a final area and may contribute to increasing photosynthetic capacity over time (Miyazawa and Terashima 2001, Miyazawa et al. 2003, Yano and Terashima 2004). During senescence, leaves typically decrease in LMA and leaf nitrogen, likely due to resorption of mobile carbon and

nitrogen back into branches (Jurik 1986, Kull et al. 1998, Yasamura et al. 2006, Migita et al. 2007).

1.4. Implications of temporal and spatial variation in leaf functional traits

Greater investments in leaf carbon and nitrogen for leaves growing at higher light availability have been suggested to increase photosynthetic capacity and optimize distributions in leaf carbon and nitrogen that maximize canopy photosynthesis (Field 1983, Gutschick and Wiegel 1988, Niinemets 2012). Thus, spatial distribution and temporal changes in leaf functional traits may influence the optimization of leaf functional traits (Field 1983, Hirose and Werger 1987, Sellers et al. 1992, Amthor 1994, Migita et al. 2007). Optimal distributions in carbon and nitrogen within forest canopies are an important assumption in big-leaf models that are used to estimate canopy photosynthesis. However, sub-optimal distributions in leaf carbon, nitrogen, and photosynthetic capacity are often observed in forest and plant canopies (Dewar et al. 2012, Niinemets 2012, Peltoniemi et al. 2012, Buckley et al. 2013, Osada et al. 2014). Hydraulic limitations to leaf development and function have been implicated as the primary reason for discrepancies between theoretical optimal distributions and observed distributions that are often sub-optimal (Peltoniemi et al. 2012, Buckley et al. 2013). Direct and indirect effects of greater xylem tension on stomatal or mesophyll conductance of CO₂ may cause leaf nitrogen and photosynthetic capacity to saturate at high light availability in the upper canopy, leading to sub-optimal distributions in photosynthetic capacity (Peltoniemi et al. 2012, Buckley et al. 2013).

1.5. Research objectives and approaches to investigating spatial and temporal variation in leaf functional traits

The main objectives of this dissertation were to identify patterns of leaf morphology and associated environmental drivers in a broad-leaved deciduous (*Acer saccharum*) forest (Chapter 2), investigate potential mechanisms behind these patterns (Chapter 3), assess potential biases in canopy function models that do not account for spatial and temporal variation in leaf functional traits (Chapter 4), and assess leaf cellular structure in response to height and integrated light conditions that accounted for seasonal changes in leaf structure (Chapter 5). In order to identify patterns of leaf functional traits and cellular structure, we used an observational approach that involved comparisons in light environment, leaf water potential, and leaf morphological and cellular structure at a closed and exposed locations within the canopy. This approach allowed us to compare leaf morphological and cellular structure at similar heights but different light conditions. In order to further investigate potential mechanisms behind these patterns, we experimentally shaded branches at different heights in the canopy. This approach was useful in teasing apart the effects of leaf water potential and light availability on leaf functional traits. Finally, we investigated detailed temporal changes in leaf functional traits in order to model seasonal changes in canopy photosynthesis. We then compared various canopy photosynthesis models to identify potential biases associated with not accounting for vertical gradients and seasonal changes in leaf functional traits.

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2. Light drives vertical gradients of leaf morphology in a sugar maple (*Acer saccharum*) forest¹

2.1. Abstract

Leaf mass per area (LMA, g m⁻²) is an essential trait for modeling canopy function due to its strong association with photosynthesis, respiration, and leaf nitrogen. LMA, which is influenced by both leaf thickness and density (LMA = Thickness × Density), generally increases from the bottom to the top of tree canopies, yet the mechanisms behind this universal pattern are not yet resolved. For decades, light environment was assumed to be the most influential driver of within-canopy variation in LMA, yet recent evidence has shown hydrostatic gradients to be more important in upper canopy positions, especially in tall evergreen trees in temperate and tropical forests. The aim of the study was to disentangle the importance of various environmental drivers on vertical LMA gradients in a mature sugar maple (*Acer saccharum*) forest. We compared LMA, leaf density, and leaf thickness relationships with height, light, and predawn leaf water potential (Ψ_{Pre}) within a closed and an exposed canopy to assess leaf morphological traits at similar heights but different light conditions. Contrary to our expectations and recent findings in the literature, we found strong evidence that light was the primary driver of vertical gradients in leaf morphology. At similar heights (13 to 23 m), LMA was greater within the exposed canopy than the closed canopy, and light had a stronger influence over LMA compared to Ψ_{Pre} . Light also had a stronger influence over both leaf thickness and

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density compared to Ψ_{Pre} ; however, the increase in LMA within both canopy types was primarily due to increasing leaf thickness with increasing light availability. This study provides strong evidence that canopy structure and crown exposure, in addition to height, should be considered as a parameter for determining vertical patterns in LMA and modeling canopy function.

2.2. Introduction

The physiological function of leaves is strongly related to leaf morphological traits. Leaf mass per area (LMA, g m^{-2}), in particular, is correlated with photosynthetic capacity, respiration, and leaf nitrogen, and reflects overall leaf investment strategies of plants (Reich et al. 1997, Wright et al. 2004). Canopy processes models that predict forest response of carbon and water budgets to short and long-term changes in environmental conditions use within-canopy patterns of LMA to describe 3-dimensional representations of canopy structure and function (Hanson et al. 2004, Medlyn 2004). From landscape to global spatial scales, LMA is used as a model parameter for estimating global gross primary productivity and evapotranspiration (Ryu et al. 2011). At the global scale, LMA is also useful for converting nitrogen on a mass basis to nitrogen on an area basis in numerous models (Schulze et al. 1994, Wright et al. 2004, Ryu et al. 2011). Due to its ubiquity as an input parameter in vegetation dynamics models, LMA represents a powerful linkage between scales in hierarchical model analyses of carbon, nutrients, and water fluxes through forests.

LMA increases with height within tree canopies, but the mechanisms are not yet resolved (Hutchison et al. 1986, Hollinger 1989, Ellsworth and Reich 1993, Bond et al.

1999, Koch et al. 2004, Ishii et al. 2008, Cavaleri et al. 2010). In early experiments on small plants, leaves exposed to greater light intensity were thicker as a result of a thicker palisade mesophyll cell layer (Nobel et al. 1975, Boardman 1977, Nobel 1977, Smith and Nobel 1978, Chabot et al. 1979). In many canopy studies, vertical light gradients in canopies have been implicated as a primary driver for increasing LMA with height (Hutchison et al. 1986, Hollinger 1989, Ellsworth and Reich 1993, Niinemets 1997, Bond et al. 1999, Niinemets et al. 1999b, Niinemets et al. 2001b). Experimental studies that manipulated canopy light environments provided further evidence of adjustments in LMA in response to light environment (Brooks et al. 1994, Jones and Thomas 2007, Ishii and Ohsugi 2011).

Recent studies, however, have attributed the LMA-height gradient to increasing xylem tension with height in tall trees (Marshall and Monserud 2003, Koch et al. 2004, Ishii et al. 2008, Cavaleri et al. 2010). Decreasing water potential with height imposes constraints on leaf turgor pressure (Woodruff et al. 2004, Meinzer et al. 2008), a requirement for leaf cell expansion and division. Reduced turgor pressure could result in smaller, denser leaves with densely packed cells, and subsequently, greater LMA (Hsiao 1973). This mechanism has been suggested for leaves at the tops of tall trees where water must be transported across long distances prior to reaching the upper canopy, whereas LMA lower in the canopy has been found to be primarily limited by light (Koch et al. 2004, Woodruff et al. 2004, Ishii et al. 2008, Cavaleri et al. 2010). More recent field observations and experimental studies of leaf morphological and anatomical traits of *Robinia psuedoacacia* and *Sequoia sempervirens* provide further evidence that vertical

gradients in water potential limit leaf expansion, and therefore LMA (Oldham et al. 2010, Zhang et al. 2011a, 2011b, 2011c).

Factors that dictate vertical gradients in leaf morphology such as light and water stress may be mediated by forest type, tree height, and canopy structure. In temperate needle-leaved forests (Koch et al. 2004, Ishii et al. 2008), temperate and tropical broad-leaved evergreen forests (England and Attiwill 2006, Cavaleri et al. 2010, Coble et al. 2013), and temperate deciduous trees (Zhang et al. 2011c), vertical gradients in leaf morphology have been attributed to hydrostatic constraints on leaf development. Constraints on leaf development via water stress were most apparent in very tall trees such as *S. sempervirens* (Koch et al. 2004, Ishii et al. 2008); however, light and exposure has been suggested to be more influential than hydrostatic constraints in other temperate deciduous tree species (Ellsworth and Reich 1993, Sack et al. 2006). For example, leaves growing on the exterior of the crown of open-grown trees for many temperate deciduous species have significantly greater LMA compared to leaves growing in the interior portions of crown (Sack et al. 2006). Also, stands that are more open (i.e. greater light availability at the forest floor) tend to have more gradual increases in LMA with height compared to closed-canopy conditions, which suggests that canopy structure and crown exposure may determine the limiting factors in leaf development such as light or water (Burgess and Dawson 2007).

Two components of LMA, leaf thickness and density ($LMA = \text{Thickness} * \text{Density}$), have been found to respond independently to environmental conditions and influence mass and area-based photosynthetic capacity (Witkowski and Lamont 1991, Niinemets 1999a). Leaf thickness is a result of the number of layers and length of palisade cells, and

is largely assumed to be controlled by light conditions (Nobel 1977, Smith and Nobel 1978, Chabot et al. 1979). In contrast, leaf density is a result of cell wall thickness, cell size, and the amount of air space between cells, which is likely dictated by rates of cell expansion and division (Hsiao 1973, Witkowski and Lamont 1991). Consequently, structural adjustments at the cellular level, as indicated by leaf density, exert control over cell wall elasticity, an important factor associated with a plant's ability to tolerate water stress (Niinemets 2001a). Due to their potential for independent responses to light and water stress, thickness and density are promising traits that may help tease apart the effects of light and water stress on LMA in tree canopies.

We investigated the primary environmental drivers of vertical patterns in leaf morphology in a mature sugar maple (*Acer saccharum*) forest in Upper Peninsula, Michigan within an exposed canopy and a closed canopy. This allowed for comparisons of leaf morphology at different levels of light availability but similar heights within the canopy. Previous investigations of mechanisms associated with vertical leaf morphological gradients in temperate deciduous forests did not analyze leaf water potential and have primarily focused on canopy light conditions (Hutchison et al. 1986, Hollinger 1989, Ellsworth and Reich 1993, Niinemets et al. 1999b). However, there is increasing evidence that the water potential gradient may be more influential in determining leaf morphology, primarily in upper canopy positions. Furthermore, sugar maple is sensitive to change in water status, as evident by lack of osmotic adjustment and concomitant declines in turgor pressure with decreasing leaf water potential during dry conditions (Bahari et al. 1985, Abrams 1988, Ellsworth and Reich 1992, Kubiske and Abrams 1994). We tested the following hypotheses:

1. Water potential (Ψ_{Pre}) has a stronger influence over LMA compared to the light environment.
2. The response of foliar morphology to light availability is constrained by water relations in the upper canopy and by light availability in the lower canopy.
3. The two components of LMA respond independently to different environmental gradients, such that changes in leaf thickness primarily correspond to variation in light availability, whereas leaf density primarily responds to water stress.

2.3. Material and Methods

2.3.1. Study Site and Sampling Design

The study was conducted at an “uncut control” stand that is part of a study area divided into nine silvicultural experimental trials at the Michigan Technological University Ford Forestry Center near Alberta, Michigan, U.S.A (46.65°N, 88.48°W). Mean annual temperature and precipitation in this region was 4.9 °C and 879 mm, respectively (Burton et al. 2011). The uncut control consisted primarily of sugar maple (*Acer saccharum*) but also includes *Ulmus americana*, *Tilia americana*, *Betula alleghaniensis*, and *Ostrya virginiana*. This forest has had several historical disturbances, including at least two heavy cutovers between 1850 and 1900, fire circa 1910, and a high-grade harvest in 1938. Following the high-grade harvest in 1938, no trees have been cut or removed from the uncut control stand except for *Ulmus americana* trees in the 1980’s as part of a sanitation cut (Campione et al. 2012). The soils in the area are classified as Alouez gravelly coarse sandy loams. In 2009, mean height of the stand was 23.0 m and mean height to live crown was 13.8 m. From 1956 through 1988, the basal area per acre

was 24-31 m² ha⁻¹ and the density was 297-309 trees ha⁻¹ (Erickson et al. 1990). In 2009, the basal area was 33 m² ha⁻¹ and the density was 264 trees ha⁻¹.

At the uncut control stand, three horizontal steel cable zip-lines were installed 15 meters (m) above the ground. The three zip-lines were anchored to three “node” trees 21 to 26 m apart, allowing canopy access at and below the cables (Figure 2.1). Two to three vertical transects were designated to each zip-line, and 6 to 21 sampling locations (dependent on the number of accessible branches) were assigned to each transect. This allowed sampling from the same location throughout the collection period. One to five trees were accessible along transects below the zip-lines. At each cable, Tandem Cable pulleys (Petzl, Crolles, France) were installed on the zip-line and linked with two steel carabiners. A static climbing rope was knotted to the steel carabiners and was used for climbing with an ascender and belay device. An additional rope was attached to the tandem pulley, threaded through a single pulley attached to the node tree, and was accessible at the ground. This system allowed the climber to be moved along a two-dimensional plane below each cable through the canopy by a person from the ground. Arborist-style climbing techniques were used to access the canopy above 15 m up to 30 m for the three node trees and a nearby dominant tree next to the zip-line system. To sample from seedlings and saplings (sample heights ranging from 0.6 to 1.9 m), we established a horizontal transect on the ground through the middle of the zip-line system (Figure 2.1) and selected seedling or sapling leaves located closest to each 2 m interval along the transect. We constructed a 19 m mobile aluminum walk-up tower (Upright, Inc., Selma, California) 65 m north of the zip-line in the same stand (Figure 2.1), to access leaves exposed to greater light conditions at similar heights as the zip-line. Four

trees were sampled from the tower, ranging from 13-24 m tall. The canopy opens considerably above ~14 m where the upper canopy of the smaller trees and the southeast-, south-, and southwest-facing portions of crown of the largest tree are exposed to open sky. The tower and zip-line sites will be herein referred to as the ‘exposed canopy’ and ‘closed canopy’, respectively. Table 2.1 summarizes the number of sampling points, trees, mean tree height, and the range of tree heights within each canopy type. Sampling from more trees at multiple exposed canopies would have made for an ideal comparison; however, we were constrained by time and resources for dismantling and reconstructing the tower at multiple locations.

Leaves were sampled from the end of May through mid-August 2012 at the zip-line sampling area (closed canopy) and from mid-August through late-August 2012 at the tower (exposed canopy). We collected five leaves at each sampling location: two leaves were used for predawn leaf water potential (Ψ_{pre}) and morphological measurements (LMA, leaf density), two for morphological measurements only, and one for anatomical (leaf thickness) and morphological measurements. One of the five leaves collected at each sampling location for anatomical analysis was also used to determine relationships between leaf thickness, LMA and density. Individual leaves at each sampling point were collected by cutting near the base of the petiole, but leaves at the tops of trees were collected by cutting small branches with a telescoping pole-pruner because climbing to these leaves was physically impossible.

2.3.2. Height and Light Measurements

At each sampling location, height above the ground was measured using a tape measure, and the light environment or ‘canopy openness’ was measured as diffuse non-interceptance (DIFN %), the fraction of radiation that is transmitted through the canopy (Norman and Welles 1983), using two Plant Canopy Analyzers (LAI-2000 and LAI-2200, Li-Cor, Inc., Lincoln, Nebraska). Studies have found that DIFN as measured by the LAI-2000 was strongly correlated with percent above-canopy photosynthetic photon flux density (PPFD) as measured by quantum sensors (Machado and Reich 1999). Light measurements were made following sunrise until one hour after sunrise or during overcast conditions. Below-canopy measurements were collected with the LAI-2200, while above-canopy measurements were simultaneously collected with the LAI-2000 mounted on a tripod at 30-second intervals in an open field approximately 400 m from the site. Prior to and following the below-canopy measurements, we collected two to three light measurements with the LAI-2200 next to the LAI-2000 in order to calibrate open measurements collected by the LAI-2000. At each sampling point, we collected two light measurements, and used the average for all further analyses. We used a 180° view cap for all measurements to prevent climbing ropes and the tower from obstructing the view. DIFN for each sampling point was estimated by matching open and below canopy readings closest in time using FV2200 software (Li-Cor, Inc., Lincoln, Nebraska). The FV2200 software also was used to adjust the open readings (LAI-2000) based on the two to three measurements collected with the LAI-2200 prior to and after below canopy readings. We assumed a 100% DIFN for leaves collected at the tops of the trees (pole-

pruner collection method) because light measurements using the LAI-2200 were not possible for these leaves.

2.3.3. LMA and Density Measurements

LMA and density measurements were made on all leaves that were collected in this study. Leaves were placed in a sealed plastic bag with a moist paper towel and temporarily stored in an ice chest. Leaves were scanned using a bench-top leaf area meter (Li-3100, Li-Cor, Inc., Lincoln, Nebraska) to estimate leaf surface area. Using Archimede's Principle, we derived leaf volume by immersing fresh leaves in a beaker of water placed on a balance. Each leaf was folded and clamped by a short straw with slits cut into the end. The straw was attached to an articulating stand with an iron base located on the lab bench next to the beaker and balance. The articulating stand was tapped and rotated to remove air bubbles trapped under the leaf. We wanted to capture the volume in substomatal chambers and intercellular air space but not the air bubbles attached to the outside of the leaf. Leaf volume was always adjusted to account for the volume of water displaced by the straw (0.03 cm^3) and the straw was inserted into the water at a consistent depth. Leaves were dried at 65°C for 48 hours and weighed to the nearest 0.1 mg. LMA was calculated as leaf dry mass (g) divided by leaf area (m^2), and density was calculated as the leaf dry mass (g) divided by leaf volume (cm^3).

2.3.4. Leaf Water Potential

Two of the five leaves were collected between 0430 and 0630 hrs for *in situ* measurements of predawn leaf water potential (Ψ_{Pre}) using a pressure chamber (PMS

Instrument, Co, Corvallis, Oregon). Leaves collected for Ψ_{Pre} were cut near the base of the petiole, immediately placed in a sealed plastic bag with a moist paper towel, and stored in an ice chest until measurements were taken. For leaves collected at the tops of trees with a telescoping pole-pruner, Ψ_{Pre} measurements were taken from the woody tissue. It was not possible to cut at the base of the petiole with the telescoping pole fully extended. This method of collection occurred for 0.6% of leaf samples, whereas 99.4% of the leaf samples were cut at the base of the petiole. All leaf water potential measurements were made within an hour of leaf collection. A pilot study found no significant differences in leaf water potential for leaves stored up to 75 minutes (One-way ANOVA, d.f. = 14, $F = 0.872$, $p = 0.513$). After water potential measurements, leaves were stored at 2 °C until LMA and leaf densities were measured for each leaf in the lab as described above.

2.3.5. Leaf Thickness Measurements

For a subset of 76 leaves from the exposed canopy and closed canopy, a small section of leaf (~16 x 8 mm) was cut from the right lobe of the leaves and placed in a formaldehyde-acetic acid-ethanol (FAA) solution prior to volume, area, and dry mass measurements. Each leaf section was cut into two or three equal-sized sections and embedded in paraffin. Using a microtome (Shandon, Finesse 325, Pittsburgh, Pennsylvania), leaf specimens in each block were exposed and each leaf section was cut at 5 μm perpendicular to the adaxial leaf surface. Three transverse cuts on the block were made to produce six to nine leaf transverse sections. Between each leaf section, 200 microns of the block were sliced off. Leaf transverse sections were placed on slides,

stained with hematoxylin and eosin in an automatic stainer (Shandon, Model Linistain GLX, Pittsburgh, Pennsylvania), and covered with a cover-slip. We collected 10 images at 20x magnification from three leaf transverse sections per leaf and randomly selected one image per set of 10 images (three images per leaf). Using ImageJ software (Schneider et al. 2012), we created a grid of lines on each image that produced five evenly spaced vertical lines that were randomly offset. We measured leaf thickness (15 measurements per leaf) at each of the vertical lines and averaged these measurements to obtain one thickness measurement per leaf for 76 leaves. Leaf thickness was measured with image analysis because the resolution achieved (10^{-8} m) by this method was required for this study and was much greater than the resolution offered by digital calipers (10^{-5} m).

We could not measure Ψ_{Pre} and thickness on the same leaves because there was evidence of damage to the anatomical structure of leaves that had experienced high pressures in the pressure chamber. To estimate thickness for leaves where we also measured Ψ_{Pre} , we developed a model for predicting leaf thickness using the parameters leaf density and LMA, all measured on the same leaf ($\text{Thickness} = 37.8 + 1.82(\text{LMA}) - 79.0(\text{Density})$; $R^2 = 0.915$, $p < 0.001$). Previous studies have estimated thickness by dividing LMA by density ($\text{LMA} = \text{Thickness} * \text{Density}$); however, the estimated thickness values using this approach were weakly correlated with thickness measurements using the microtome approach ($R^2 = 0.064$, $p = 0.027$, data not shown). Due the strong predictive power of LMA and density, we calculated thickness ('predicted thickness') using the multiple regression model for leaves collected in the study to compare relationships between predicted thickness and Ψ_{Pre} and partial R^2 values for

relationships between predicted thickness, light, and Ψ_{Pre} . Actual thickness measurements using anatomical methods were used to compare relationships between thickness, height, and light between canopy types and compare correlations and partial R^2 values between LMA, thickness, and density (Tables 3 and 5).

2.3.6. Statistical Analysis

Statistical analyses were conducted using R statistical software (R Development Core Team 2013). Relationships between leaf morphological parameters, predawn water potential, leaf height and light were examined using regression analysis. Comparison of these relationships between the two canopy types (closed canopy and exposed canopy) were conducted using ANCOVA. Within each canopy type, we compared the contribution of light and Ψ_{Pre} to the full model for predicting LMA, leaf thickness, and leaf density using partial R^2 values. We also compared the contribution of leaf thickness and leaf density to the full model for predicting LMA using partial R^2 values. The last analysis mentioned above included leaves where LMA, leaf density, and leaf thickness were all measured on the same leaves at the closed canopy and exposed canopy. For all statistical analysis, light (Tables 2-4), LMA, leaf density, and leaf thickness values (Tables 3-5) were natural log-transformed (\ln). However, these variables were not transformed in the figures because the untransformed data were more easily interpreted. Light was log-transformed because light displayed curved, asymptotic relationships with LMA, leaf density, and leaf thickness and an exponential relationship with height. LMA, leaf density, and leaf thickness were log-transformed because these parameters displayed exponential relationships with height and Ψ_{Pre} . Log-transformation of these parameters

satisfied regression assumptions and allowed us to develop linear models for ANCOVA and partial R^2 analyses. To eliminate timing of sample collection as a possible source of difference in LMA between the two canopy types, we compared a subsample of LMA values collected only in August at the closed ($n = 35$) and exposed ($n = 54$) canopy within heights of 17-21 m using one-way ANOVA.

2.4. Results

2.4.1. Differences in Light and Hydrostatic Environment by Canopy Type

Light increased exponentially with height within both the closed canopy and exposed canopy (Figure 2.2a). We observed marked differences in light environment (DIFN %) between the closed canopy and the exposed canopy at greater heights (Figure 2.2a).

Above 10 m in height, we observed an abrupt increase in light with height at the exposed canopy relative to the closed canopy (Figure 2.2a). The ANCOVA indicated significant height and canopy type effects on $(\ln)\text{light}$ and a significant height \times canopy type interaction (Table 2.2). The intercept of the relationship between $(\ln)\text{light}$ and height was significantly smaller at the exposed canopy compared to the closed canopy ($p < 0.001$).

The relationship between height and Ψ_{Pre} was negatively linear for both canopy types (Figure 2.2b). There was a significant height and canopy type effect on Ψ_{Pre} but the interaction height \times Ψ_{Pre} was not significant ($p = 0.993$, Table 2.2). Predawn water potential declined 0.011 MPa per meter increase in height. The intercept of Ψ_{Pre} -height relationships at the closed canopy (-0.19 MPa) was significantly greater than the intercept at the exposed canopy (-0.29 MPa; $p < 0.001$).

2.4.2. LMA Relationships with Height, Light, and Ψ_{Pre}

LMA increased exponentially with height (Figure 2.3a). Above 10 meters, the trend lines diverge and leaves within the exposed canopy had greater LMA than the closed canopy (Figure 2.3a). The ANCOVA indicated significant height, canopy type, and interaction (height \times canopy type) effects on (ln)LMA (Table 2.3). The relationship between LMA and light was log-linear, and the increase in LMA with increasing light availability was most pronounced at low light (<25% DIFN, Figure 2.3b). Above 25% DIFN, the increase in LMA with light was greater within the exposed canopy than the closed canopy (Figure 2.3b). We observed significant (ln)light, canopy type, and interaction (ln(light) \times canopy type) effects on (ln)LMA (Table 2.3). LMA exponentially decreased with Ψ_{Pre} (Figure 2.3c). There were significant Ψ_{Pre} , canopy type, and interaction ($\Psi_{\text{Pre}} \times$ canopy type) effects on (ln)LMA (Table 2.3). Intercepts were significantly different between canopy types for relationships between (ln)LMA and height, (ln)light, and Ψ_{Pre} ($p < 0.01$). (ln)Light explained more variation in (ln)LMA than for Ψ_{Pre} for the closed canopy and exposed canopy (Table 2.4). Based on partial R^2 values, adding ln(light) to the Ψ_{Pre} models showed 0.15 to 0.44 increase in R^2 over the Ψ_{Pre} model, whereas adding Ψ_{Pre} to the ln(light) model showed only 0.05 increase in R^2 over the ln(light) model (Table 2.4). In our *post-hoc* analysis of LMA values collected in August at both the exposed and closed canopy at 17-21 m, we found that mean LMA at the exposed canopy was significantly greater ($p < 0.001$, $F = 267$) than LMA at the closed canopy.

2.4.3. Thickness and Density

Density displayed similar trends with height, light, and Ψ_{Pre} among canopy types to those observed for LMA (Figure 2.4). Leaf density exponentially increased with height, displayed a log-linear increase with light, and exponentially decreased with Ψ_{Pre} (Figure 2.4). The main effects, (height, $\ln(\text{light})$, Ψ_{Pre} , and canopy type) and interaction terms [(height \times canopy type, $\ln(\text{light}) \times$ canopy type, $\Psi_{\text{Pre}} \times$ canopy type)] were all significant explanatory variables for $\ln(\text{density})$ (Table 2.3). Intercepts were significantly different ($p < 0.001$) for $\ln(\text{density})$ - $\ln(\text{light})$ relationships, but not for height ($p = 0.35$) and Ψ_{Pre} ($p = 0.26$). Partial R^2 for adding $\ln(\text{light})$ (0.08 to 0.36) to the full model for predicting $\ln(\text{density})$ were greater compared to adding Ψ_{Pre} (0.03 to 0.07, Table 2.4).

Thickness and predicted thickness also followed similar trends with height, light, and Ψ_{Pre} to those observed for both LMA and density (Figure 2.5). The interaction terms height \times canopy type and $\Psi_{\text{Pre}} \times$ canopy type were significant, but not the interaction term $\ln(\text{light}) \times$ canopy type (Table 2.3). Intercepts for all relationships were significantly different between canopy types ($p < 0.05$). $\ln(\text{Light})$ explained more variation in predicted leaf thickness than did Ψ_{Pre} (Table 2.4). Partial R^2 values for adding $\ln(\text{light})$ (0.15 to 0.44) to the full model for predicting $\ln(\text{thickness})$ were greater compared to adding Ψ_{Pre} (0.04, Table 2.4).

While both leaf morphological components correlated strongly with LMA, thickness explained more of the variability in LMA than did leaf density (Table 2.5). The partial R^2 values for adding thickness (0.16) to the full model for predicting LMA were greater compared to adding density (0.07, Table 2.5). The high R^2 of the full model ($\text{LMA} = \beta_0 + \beta_1 \text{ Thickness} + \beta_2 \text{ Density}$, $R^2 = 0.96$, Table 2.5) corroborates high precision of the

independent measures of LMA, thickness, and density because $LMA = Thickness \times Density$. However, we provided strong evidence that sugar maple leaf thickness should not be estimated using this equation (see Methods).

2.5. Discussion

2.5.1. Light Controls LMA

If gradients in water potential were driving changes in LMA (Hypothesis 1), we would expect LMA to be more closely correlated with leaf water potential than with light, and we also would expect water potential to explain more of the variation in LMA. Our results did not support our first hypothesis that water potential would have a stronger influence over leaf morphology compared to light, but did support the findings of early canopy research conducted in sugar maple and temperate deciduous forests where light strongly influenced leaf morphological gradients with height (Hutchison et al. 1986; Hollinger 1989; Ellsworth and Reich 1993; Niinemets et al. 1999b). Multiple lines of evidence from this study have revealed that light is driving changes in LMA in the sugar maple stand. The relationships between LMA and height were strikingly different between the closed and exposed canopy and reflect patterns of light-height relationships among canopy types. Despite similar sampling heights at the closed and exposed canopy, LMA was greater at the exposed canopy where light availability was greater. Secondly, our model comparison for each canopy type showed that light explained more variation in LMA than water potential did in the full model ($\ln(LMA) = \ln(light) + \Psi_{Pre}$).

Within forest canopies, a linear increase in LMA with height was observed among mixed conifer (Marshall and Monserud 2003), *Sequoiadendrum giganteum* (conifer),

Eucalyptus diversicolor (broadleaf evergreen; Burgess and Dawson 2007), and tropical evergreen forests (Cavaleri et al. 2010), while other studies have found an exponential increase in LMA with height in *Sequoia sempervirens* (Koch et al. 2004, Burgess and Dawson 2007, Ishii et al. 2008) and *Psuedotsuga menziesii*, both conifers (Woodruff et al. 2004). Burgess and Dawson (2007) discuss differences in height-LMA relationships of two closely related species, *S. giganteum* and *S. sempervirens*, which displayed a linear and an exponential increase in LMA, respectively. Burgess and Dawson (2007) speculate that open stand conditions of the *S. giganteum* resulted in a more gradual change in LMA with height, suggesting that variation in light and exposure (i.e., temperature, wind speed, and humidity) as a result of canopy structure may influence the profile of the LMA-height relationship. In the case of very tall trees, an exponential increase in LMA may be the result of extreme environmental gradients from the lower branches to the top of the trees, coupled with extraordinary gravitational resistances to sap flow. In our study, we did not anticipate that these extreme gradients in water potential because the range of tree heights was 25 to 34 m, whereas the height of *S. sempervirens* trees in other studies was 85 to 113 m (Koch et al. 2004; Ishii et al. 2008). We did, however, observe steeper gradients in LMA with height within the exposed canopy, likely as a result of more dramatic changes in light availability compared to the closed canopy where light availability gradients were more gradual with height. Thus, the relative changes in light availability with height as influenced by canopy structure and crown exposure seems to be critical for determining LMA-height profiles.

2.5.2. Constraints on Leaf Morphology in the Upper Canopy

If the response of LMA to light availability was constrained by water relations in the upper canopy (Hypothesis 2), we would expect no difference in LMA between different light environments at the same heights in the upper canopy. Our results did not support our second hypothesis. In recent studies in temperate needle-leaved and tropical broad-leaved evergreen forests, vertical gradients in LMA have been linked to xylem water potential or height, suggesting that leaf morphology is mostly determined by water relations in upper canopies, while light is important only in low light conditions (Koch et al. 2004; Ishii et al. 2008; Cavaleri et al. 2010). Ishii et al. (2008) found that LMA of *S. sempervirens* leaves did not respond to light above 15% canopy openness, while Cavaleri et al. (2010) reported a threshold of 22% diffuse transmittance in a tropical forest. Contrary to these results, we found that LMA continued to increase at higher light availability, particularly within the exposed canopy, suggesting that morphological adjustments to light was not constrained by low water potential in the upper canopy of this sugar maple forest.

Sugar maple tends to grow at sites with high moisture availability (wet-mesic) and has a lower propensity for osmotic adjustment during drier years relative to species that can tolerate lower moisture availability (Abrams 1988, Bahari et al. 1985, Ellsworth and Reich 1992, Kubiske and Abrams 1994). Limited osmotic adjustment can lead to decreased leaf turgor pressure in wet-mesic species during dry conditions compared to xeric species (Kubiske and Abrams 1994). Contrary to these findings, osmotic adjustment under water-stress conditions was observed for sugar maple seedlings (Kolb et al. 1991). Likewise, our preliminary results show a significant trend of decreasing

osmotic potential at full and zero turgor with height and an increase in turgor pressure with height within the exposed canopy (data not shown). We speculate that lower Ψ_{Pre} values in the upper canopy observed in this study did not impose major water limitations on sugar maple leaf development. Based on trend lines, we observed Ψ_{Pre} values from -0.20 to -0.55 MPa for sugar maple leaves (Fig. 2b), which were similar Ψ_{Pre} values (always > -0.50 MPa) reported by Ellsworth and Reich (1993) for dominant and sub-canopy sugar maple trees. Ellsworth and Reich (1992) observed large declines in both net photosynthesis and stomatal conductance when Ψ_{Pre} reached -1.80 MPa for sugar maple seedlings growing in high light conditions. This value was also near the turgor loss point (-1.87 MPa; Ellsworth and Reich 1992). This provides some indication that sugar maple leaves growing in high light conditions (tops of trees at exposed and closed canopy) in this study were not water-stressed.

2.5.3. Leaf Thickness and Leaf Density Response to Light and Water Stress

If leaf thickness and density responded independently to light and water stress, respectively (Hypothesis 3), we would expect thickness to be more closely correlated to light conditions and density to be more closely correlated to leaf water potential. Our results did not support our third hypothesis, as light appeared to directly influence variation in both leaf thickness and density. In high light conditions, sugar maple leaves were denser and thicker compared to leaves growing in low light conditions. Witkowski et al. (1991) found that leaf thickness and density varied independently in response to resource gradients (light, moisture, and nutrients) and suggested that thickness and density should be considered separately because of these potentially independent

responses and confounding relationships with LMA. Our conclusions for sugar maple leaf thickness and density are not consistent with those species studied by Witkowski et al. (1991). Our findings were consistent with Niinemets et al. (1999b) who found that leaf thickness and density of two temperate deciduous species (*Tilia cordata* and *Populus tremula*) increased with increasing light availability. Our results were inconsistent with findings of an increase in thickness but not density for *Pinus strobus*, *Picea abies*, and *Pinus sylvestris* (Niinemets 1997, Niinemets et al. 2001b, Leal and Thomas 2003). Sugar maple LMA appears to respond primarily to gradients in light through changes in thickness, as indicated by partial R^2 analysis (Table 2.5). Thicker ‘sun leaves’ typically have a two-cell-layered and thicker palisade tissue as a result of periclinal cell division and elongation (Yano and Terashima 2004). Leaves growing in high light intensity also tend to have greater volume occupied by mesophyll cells and less volume of air space (Chabot and Chabot 1977, Chabot et al. 1979), which may explain why sugar maple leaves growing in the upper canopy may have greater density in this study.

2.5.4. Additional Possible Sources of Variation

We showed that light explained much of the variation in LMA, density, and thickness, but other factors may contribute to variation in leaf morphological traits. Timing of collection may be one potential source of variation. Cell wall thickness and total cellulose and hemicelluloses have been found to increase following full leaf expansion (Miyazawa et al. 2003), which may contribute to changes in LMA or density. In fact, LMA has been shown to increase rapidly following leaf expansion in the spring but reaching a constant level after a few weeks to a month (Miyazawa et al. 1998;

Miyazawa and Terashima 2001; Miyazawa et al. 2003). However, we found that LMA was significantly greater at the exposed canopy compared to the closed canopy for leaves collected in August and at similar heights (17 -21 m). This suggests that the greater LMA observed at the exposed canopy relative to those observed at the closed canopy (at similar heights) was not associated with seasonal changes.

While an attempt was made to eliminate any potential sources of error during volume measurements, small air pockets trapped around the leaf's waxy cuticle, lower epidermis, or major and minor veins may have contributed to greater variation observed for density, as this would affect the volume measurements. This may also explain why thickness values as measured by ImageJ were weakly correlated with thickness values estimated with LMA and density.

Other potential sources of variation in LMA between the two canopy types include differences in soil water availability and/or exposure to wind, both of which were not measured in this study. In an extensive review of factors that influence LMA, Poorter et al. (2009) found that LMA moderately increases with decreasing water availability. This finding was likely a consequence of more tightly packed cells and reduced air-space in the mesophyll of leaves growing in water-stress conditions (Poorter et al. 2009). The two sites displayed slight differences in the leaf water potential gradient (different intercepts of the relationship between height and Ψ_{Pre}). However, the increase in LMA with height was greater within the exposed canopy, yet declines in leaf water potential with height were identical among sites. Trees growing in wind-exposed sites have been found to have lower LMA and cell wall mass per unit area (Nagano et al. 2009). In contrast, Niklas (1996) found that the slopes of the relationship between leaf mass and area of

sugar maple at wind-exposed and wind-protected sites were identical, suggesting that LMA was not affected by wind exposure. Future investigation of seasonal, soil water availability, and wind effects on LMA for sugar maple is required, particularly at different levels of light availability. Finally, we recognize that the limited sample size at the exposed canopy could potentially impact the interpretation of our results. With a limited sample size, we may have under- or over-estimated the canopy type and interaction effects. Thus, current understanding of leaf morphological traits along vertical canopy gradients would benefit from a more robust test of differences in leaf morphology at closed and exposed canopy conditions.

2.6. Conclusions and Implications

Recent research suggests that the hydrostatic gradient in trees limits leaf growth and development through reduced turgor pressure. However, our study in a sugar maple stand corroborates studies that attributed vertical gradients in leaf morphology to natural light gradients that exist in tree canopies. For a given height, LMA, leaf thickness, and leaf density were greater within the exposed canopy than within closed-canopy conditions, especially at the highest heights. Also, we observed stronger contributions of light to all leaf morphology models. These results suggest that greater leaf density may not be exclusively linked to water stress, and light may play a major role in determining LMA, leaf density, and leaf thickness. Our results are contrary to recent studies on conifers and tropical trees, suggesting that deciduous broadleaf trees may have different mechanisms at work than either needle-leaf or broadleaf evergreens. Further work that compares the plasticity and biophysical constraints on leaf morphology of evergreen

versus deciduous species will advance our understanding of potential mechanisms that control leaf morphology.

Adjustments in leaf structure in response to increasing light may affect the capacity of leaves to assimilate CO₂. Previous work in sugar maple forests showed that LMA was strongly and positively correlated with area-based maximum photosynthetic capacity and leaf nitrogen (Ellsworth and Reich 1993). Thus, our findings of greater LMA within the exposed canopy suggest that trees growing adjacent to canopy gaps with exposed crowns are likely more productive due to greater light availability and morphological adjustments to maximize CO₂ assimilation. Since modeling of whole-stand carbon gain must account for height-related variation in leaf morphology, this study will broaden our understanding of the factors that contribute to variation in leaf morphology. Through the application of LiDAR (light detection and ranging), attributes of canopy structure such as canopy height and cover across a large spatial scale can be estimated with a high degree of confidence (Ritchie et al. 1995, Lefsky et al. 2002). Thus, field based measurements that identify relationships between physiological traits, LMA, and height linked with LiDAR estimations of stand structure may allow for modeling of forest productivity across larger spatial and temporal scales.

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2.10. Tables and Figures

Table 2.1. Number of sampling points and trees, mean tree height, and tree height range by canopy type.

Canopy Type	Number of Sampling Points	Number of Trees	Mean Tree Height (m)	Tree Height Range (m)
Closed Canopy	131	21	12.2 ± 2.6	0.6 – 34.7
Exposed Canopy	28	4	16.3 ± 2.5	12.7 – 23.8

Table 2.2. Summary of ANCOVA results for test of height, canopy type (CT), and height \times canopy type effects on light ((ln) %DIFN) and Ψ_{Pre} (MPa). Degrees of freedom (df), mean square, F-Ratio, and level of significance are listed for main, interaction, and error terms. Analysis included two canopy types (closed and exposed canopy). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Source	(ln)Light			Ψ_{Pre}		
	df	Mean Square	F-Ratio	df	Mean Square	F-Ratio
Height	1	52.10	419.63***	1	1.56	305.01***
Canopy Type (CT)	1	4.87	39.20***	1	0.61	119.15***
Height \times CT	1	12.80	103.07***	1	0.00	0.00
Error	155	0.12		314	0.01	

Table 2.3. Summary of ANCOVA results for test of: height, canopy type (CT), and height \times canopy type effects on LMA (g m^{-2}), density (g cm^{-3}), and thickness (μm); light, canopy type, and light \times canopy type effects on LMA, density, and thickness; and Ψ_{Pre} , canopy type, and $\Psi_{\text{Pre}} \times$ canopy type effects on LMA, density, and thickness. Degrees of freedom (df), mean square, F-Ratio, and level of significance are listed for main, interaction, and error terms. Analysis included two canopy types (closed and exposed canopy). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; †Predicted values were used for ANCOVA of Ψ_{Pre} and interaction effects on thickness.

Source	(ln)LMA			(ln)Density			(ln)Thickness		
	df	Mean Square	F-Ratio	df	Mean Square	F-Ratio	df	Mean Square	F-Ratio
Height	1	77.94	2571.38***	1	44.78	874.95***	1	3.59	256.69***
Canopy Type (CT)	1	13.78	454.46***	1	33.19	648.47***	1	0.32	23.18***
Height \times CT	1	9.45	311.58***	1	6.89	134.60***	1	0.57	41.28***
Error	895	0.04		889	0.05		72	0.01	
(ln)Light	1	92.92	2702.99***	1	67.43	1380.06***	1	4.18	240.69***
Canopy Type	1	4.02	116.79***	1	19.12	391.41***	1	0.02	1.03
(ln)Light \times CT	1	0.60	17.35***	1	0.36	7.45**	1	0.05	2.76
Error	895	0.03		889	0.05		72	0.02	
Ψ_{Pre}	1	12.92	258.07***	1	10.24	203.57***	1	5.57	231.62***†
Canopy Type	1	3.39	67.76***	1	8.24	163.92***	1	0.81	33.77***†
$\Psi_{\text{Pre}} \times$ CT	1	0.51	10.09**	1	0.47	9.36**	1	0.29	12.08***†
Error	314	0.05		314	0.05		314	0.02	

Table 2.4. For each canopy type, sample size, regression coefficients, and R^2 values are displayed for the log-log regressions between leaf morphological parameters (LMA, density, and predicted thickness) and light, log-linear relationships between leaf morphological parameters and Ψ_{Pre} , and multiple regressions including both terms. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Response Variable	Canopy type	n	(ln)Light Only				Ψ_{Pre} Only				(ln)Light and Ψ_{Pre}			
			β_0	β_1	R^2		β_0	β_1	R^2		β_0	β_1	β_2	R^2
(ln)LMA	Closed Canopy	262	4.4***	0.32***	0.60		3.1***	-1.8***	0.49		4.0***	0.23***	-0.82***	0.64
	Exposed Canopy	55	4.6***	0.36***	0.81		2.7***	-2.9***	0.41		4.0***	0.30***	-1.1***	0.85
(ln)Density	Closed Canopy	262	-0.84***	0.24***	0.36		-1.8***	-1.4***	0.31		-1.3***	0.16***	-0.72***	0.40
	Exposed Canopy	55	-0.43***	0.28***	0.74		-2.0***	-2.4***	0.45		-1.0***	0.23***	-1.1***	0.81
(ln)Predicted Thickness	Closed Canopy	262	5.0***	0.21***	0.58		4.2***	-1.2***	0.47		4.7***	0.15***	-0.53***	0.62
	Exposed Canopy	55	5.1***	0.25***	0.79		3.8***	-2.0***	0.39		4.7***	0.22***	-0.72***	0.83

Table 2.4. (continued)

Partial R^2 for adding:	
(ln)Light	Ψ_{pre}
<i>0.15</i>	<i>0.04</i>
<i>0.44</i>	<i>0.05</i>
<i>0.08</i>	<i>0.03</i>
<i>0.36</i>	<i>0.07</i>
<i>0.15</i>	<i>0.04</i>
<i>0.44</i>	<i>0.04</i>

Table 2.5. For both canopy types pooled together, sample size, regression coefficients, and R² values are displayed for the linear regressions between LMA, thickness, and density and multiple regressions including both terms. * $p<0.05$, ** $p<0.01$, *** $p<0.001$

Response	n	Thickness Only [†]			Density Only [‡]			Thickness and Density [§]			Partial R ² for adding:	
		β_0	β_1	R ²	β_0	β_1	R ²	β_0	β_1	β_2	Thickness	Density
LMA	75	-18***	0.66***	0.89	6.6***	132***	0.80	-15***	0.43***	62***	0.16	0.07

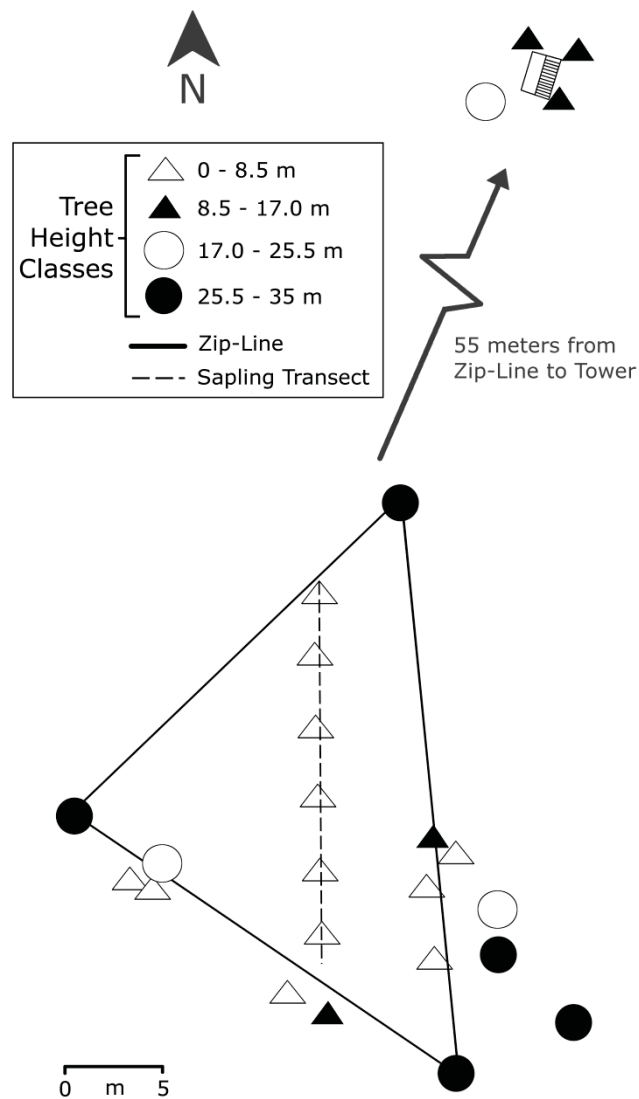


Figure 2.1. Map of study site that includes the zip-line system, tower, sampled trees, and the sapling transect. The sampled trees were grouped into four height classes and were designated a symbol for each height class. The size of the symbols does not correspond with stem or crown diameter, and the distance from the zip-line to the tower is not drawn to scale.

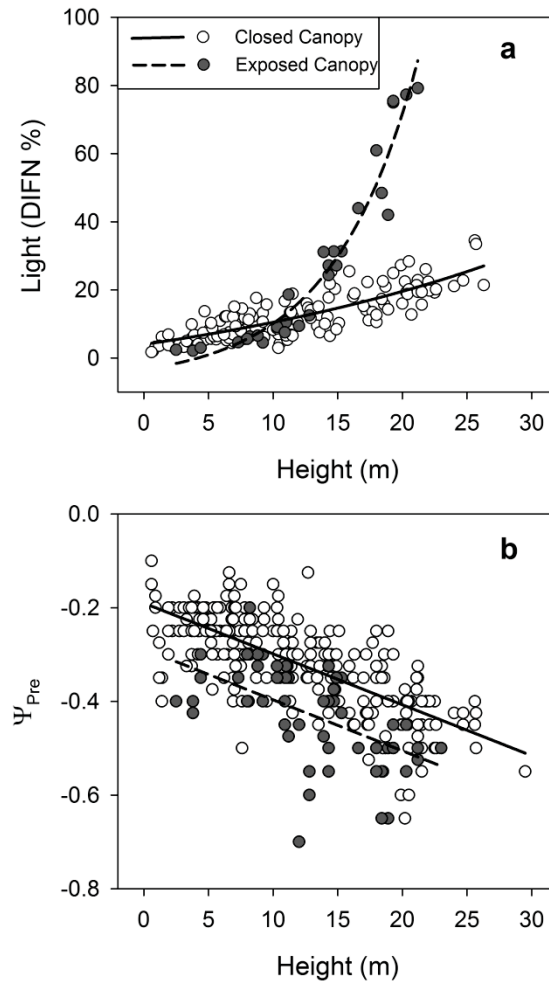


Figure 2.2. Relationships between light and height (a) and Ψ_{Pre} and height (b) at two canopy types: closed canopy and exposed canopy. Regression models: (a) closed canopy, $DIFN = -0.124 + 0.164 \times \exp^{(0.033 \times \text{Height})}$; exposed canopy, $DIFN = -0.071 + 0.038 \times \exp^{(0.151 \times \text{Height})}$, and (b) closed canopy, $\Psi_{Pre} = -0.190 - 0.011 \times \text{Height}$; exposed canopy, $\Psi_{Pre} = -0.288 - 0.011 \times \text{Height}$.

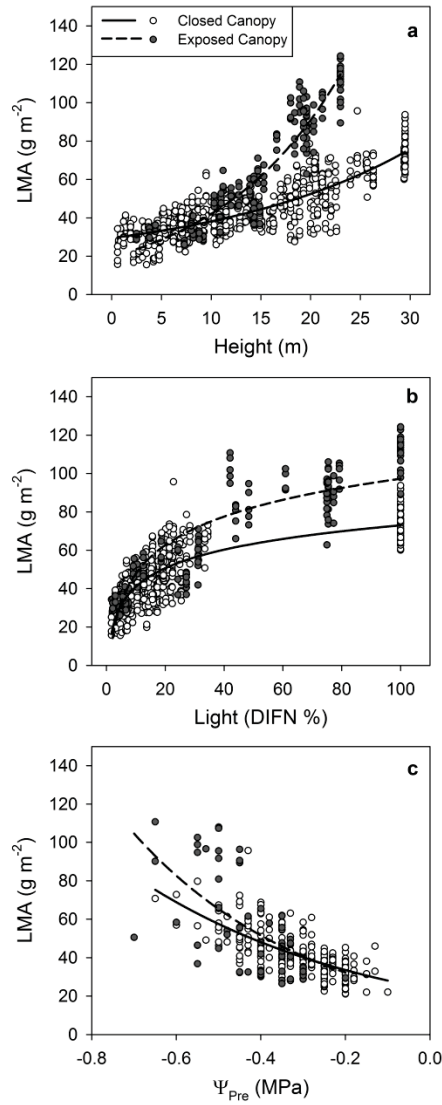


Figure 2.3. Relationships between LMA and height (a), light (b), and Ψ_{Pre} (c) at closed canopy and exposed canopy. Regression models: (a) closed canopy, $\text{LMA} = 15.919 + 13.564 \times \exp^{(0.050 \times \text{Height})}$; exposed canopy, $\text{LMA} = 0.539 + 19.077 \times \exp^{(0.078 \times \text{Height})}$, (b) closed canopy, $\text{LMA} = 73.004 + 13.889 \times (\ln)\text{DIFN}$; exposed canopy, $\text{LMA} = 97.334 + 21.521 \times (\ln)\text{DIFN}$, and (c) closed canopy, $\text{LMA} = 23.425 \times \exp^{(-1.796 \times \Psi_{\text{Pre}})}$; exposed canopy, $\text{LMA} = 20.114 \times \exp^{(-2.354 \times \Psi_{\text{Pre}})}$.

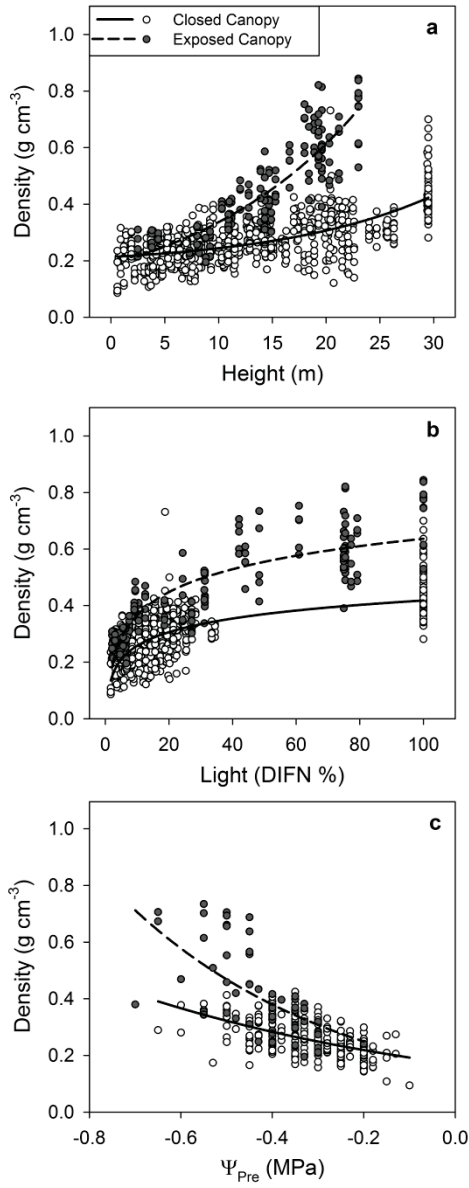


Figure 2.4. Relationships between leaf density and height (a), light (b), and Ψ_{Pre} (c) at closed canopy and exposed canopy. Regression models: (a) closed canopy, $\text{Density} = 0.182 + 0.031 \times \exp^{(0.069 \times \text{Height})}$; exposed canopy, $\text{Density} = 0.008 + 0.178 \times \exp^{(0.061 \times \text{Height})}$, (b) closed canopy, $\text{Density} = 0.418 + 0.069 \times (\ln)\text{DIFN}$; exposed canopy, $\text{Density} = 0.637 + 0.118 \times (\ln)\text{DIFN}$, and (c) closed canopy, $\text{Density} = 0.169 \times \exp^{(-1.288 \times \Psi_{\text{Pre}})}$; exposed canopy, $\text{Density} = 0.164 \times \exp^{(-2.097 \times \Psi_{\text{Pre}})}$.

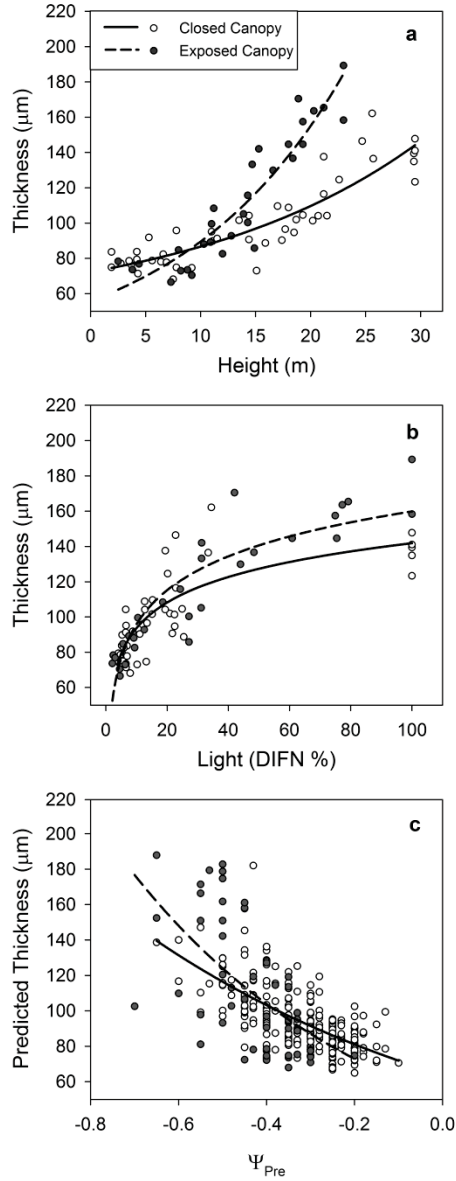


Figure 2.5. Relationships between leaf thickness and height (a), light (b), and Ψ_{Pre} at closed canopy and exposed canopy. Regression models: (a) closed canopy, Thickness = $48.427 + 23.893 \times \exp^{(0.047 \times \text{Height})}$; exposed canopy, Thickness = $19.924 + 35.906 \times \exp^{(0.066 \times \text{Height})}$, (b) closed canopy, Thickness = $141.865 + 20.928 \times (\ln)\text{DIFN}$; exposed canopy, Thickness = $159.936 + 27.809 \times (\ln)\text{DIFN}$, and (c) closed canopy, Thickness = $63.716 \times \exp^{(-1.206 \times \Psi_{Pre})}$; exposed canopy, Thickness = $51.190 \times \exp^{(-1.770 \times \Psi_{Pre})}$.

3. Light acclimation optimizes leaf functional traits despite height-related constraints in a canopy shading experiment²

3.1. Abstract

Within-canopy gradients of leaf functional traits have been linked to both light availability and vertical gradients in leaf water potential. While observational studies can reveal patterns in leaf traits, within-canopy experimental manipulations can provide mechanistic insight to tease apart multiple interacting drivers. Our objectives were to disentangle effects of height and light environment on leaf functional traits by experimentally shading branches along vertical gradients within a sugar maple (*Acer saccharum*) forest. Shading reduced leaf mass per area (LMA), leaf density, area-based leaf nitrogen (N_{area}), and carbon:nitrogen (C:N) ratio, and increased mass-based leaf nitrogen (N_{mass}), highlighting the importance of light availability on leaf morphology and chemistry. Early in the growing season, midday leaf water potential (Ψ_{mid}), LMA, and N_{area} were driven primarily by height, while later in the growing season, light became the most important driver for LMA and N_{area} . Carbon isotope composition ($\delta^{13}\text{C}$) displayed strong, linear correlations with height throughout the growing season, but did not change with shading, implying that height is more influential than light on water use efficiency and stomatal behavior. LMA, leaf density, N_{mass} , C:N ratio, and $\delta^{13}\text{C}$ all changed seasonally, suggesting that leaf ageing effects on leaf functional traits are equally as important as microclimatic conditions. Overall, our results indicate that: i) stomatal

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sensitivity to vapor pressure deficit or Ψ_{mid} constrains the supply of CO_2 to leaves at higher heights, independent of light environment and; ii) LMA and N_{area} distributions become functionally optimized through morphological acclimation to light with increasing leaf age despite height-related constraints.

3.2. Introduction

Across biomes, leaf functional traits are important for predicting leaf and ecosystem functioning (Wright et al. 2004; Poorter et al. 2009). Coupled with large-scale relationships developed for plant albedo and leaf nitrogen (Ollinger et al. 2008, Hollinger et al. 2010), these patterns in leaf functional traits (leaf nitrogen per unit leaf area (N_{area}), leaf mass per area (LMA), and photosynthetic capacity) have been used to estimate gross primary productivity from local to global scales (Ryu et al. 2011). Vertical patterns in LMA and carbon:nitrogen (C:N) ratio have been incorporated into canopy process and land surface component models that predict carbon flux and forest response to changes in environmental conditions (Gutschick and Wiegel 1988; Raulier et al. 1999; Hanson et al. 2004; Medlyn 2004; Thornton and Zimmermann 2007). Consequently, leaf functional traits are useful for scaling from leaf- to ecosystem- to global-level processes when modeling carbon, water, and nutrient cycling.

A central theme when modeling forest canopy photosynthesis is the assumption that structural carbon and leaf nitrogen concentrations are optimally distributed with respect to light to maximize carbon gain (Field 1983; Hirose and Werger 1987; Sellers et al. 1992; Amthor 1994). During acclimation to high light availability, greater investment of carbon into leaf construction (high LMA) and nitrogen into RUBISCO and thylakoid

proteins (high N_{area}) often results in higher rates of light-saturated photosynthesis and greater carbon isotope composition ($\delta^{13}C$) (Evans 1989; Hollinger 1989; Ellsworth and Reich 1993; Berry et al. 1997; Niinemets 1997; Livingston et al. 1998; Bond et al. 1999; Niinemets et al. 1999; Niinemets et al. 2001; Sack et al. 2006; Duursma and Marshall 2006). Thus, leaf functional traits (LMA, N_{area} , and photosynthetic capacity) and $\delta^{13}C$ are often coordinated and scale with light within tree crowns and forest canopies, corroborating optimal patterns derived from models (Hirose and Werger 1987, Livingston et al. 1998; Ellsworth and Reich 1993; Duursma and Marshall 2006).

However, optimal patterns in photosynthetic capacity with respect to light may be constrained by other environmental factors, resulting in a decline in photosynthetic capacity per unit of irradiance often observed in field studies (Hollinger 1996, Bond et al. 1999, Niinemets and Valladares 2004, Buckley et al. 2013). For example, in tall *Sequoia sempervirens* and *Pseudotsuga menziesii* (e.g., up to 113 m; Koch et al. 2004; Woodruff et al. 2004; Burgess and Dawson 2007; Ishii et al. 2008) and in shorter tropical (e.g., up to 45 m; Cavaleri et al. 2010) and temperate deciduous trees (e.g. up to 18 m; Zwieniecki et al. 2004; Zhang et al. 2011b), gravity and the length of the hydraulic pathway result in water potential gradients with height, potentially limiting leaf development as indicated by LMA. Hydraulic limitation on leaf structure can increase leaf tissue density and cell wall thickness and reduce mesophyll air-space, potentially restricting mesophyll conductance to CO_2 and photosynthesis, as indicated by increasing $\delta^{13}C$ with canopy height (Koch et al. 2004, Niinemets et al. 2004, Mullin et al. 2009, Oldham et al. 2010). The height at which water potential is limiting to leaf development is likely to vary among species depending on their hydraulic characteristics. Leaf N_{area} measured along

vertical gradients has been linked to changes in LMA primarily due to the conversion of N_{mass} to N_{area} through LMA and the constant values of N_{mass} (Ellsworth and Reich 1993; Bond et al. 1999). Therefore, any constraints on LMA are likely to have similar constraints on N_{area} . Consistent with these observations, model-based approaches have attributed the discrepancy between theoretical patterns (optimal) and actual patterns (suboptimal) in photosynthetic capacity and leaf nitrogen to hydraulic constraints (Peltoniemi et al. 2012, Buckley et al. 2013). When hydraulic constraints are considered, leaf nitrogen and photosynthetic capacity are not directly proportional to light, likely due to the direct and indirect effects of greater xylem tension on either stomatal or mesophyll conductance to CO_2 (Peltoniemi et al. 2012, Buckley et al. 2013). Evidence also suggests that the discrepancy among theoretical and actual patterns in leaf nitrogen may be an artifact of light models that do not account for diffuse and direct light (Hikosaka 2014).

A major difficulty in understanding the effects of light conditions on leaf functional traits is confounding water potential and light gradients with height. Experimental shading can be a useful approach in teasing apart the effects of light and leaf water potential, testing mechanistic hypotheses, and providing insight into inter-related leaf traits and chemistry. Branch-level shading has shown that leaf function (i.e. photosynthesis, respiration, and leaf nitrogen) can acclimate to shading despite no structural changes (Brooks et al. 1994) and that leaf age has similar effects on photosynthetic capacity as shading (Brooks et al. 1996). Manipulation of branch-level and whole-plant light availability has also provided insight into leaf functional and morphological acclimation to light (Goulet and Bellefleur 1986; Naidu and DeLucia 1998; Bloor and Grubb 2004; Jones and Thomas 2007; Ishii and Ohsugi 2011), branch

autonomy (Yamamoto et al. 1999; Brooks et al. 2003; He and Dong 2003; Lacointe et al. 2004; Kawamura 2010), and light effects on branch growth and carbon allocation (Claussen 1996; Henriksson 2001). However, little is known about shading effects on leaves in the presence of gravitational water potential gradients in tall trees.

The main objectives of this study were to identify the effects of shading on leaf functional traits at various heights within a sugar maple (*Acer saccharum*) canopy to tease apart the effects of height (hydraulic limitation) and light environment on leaf functional traits and shoot growth. Using an experimental approach, we tested the following hypotheses for *A. saccharum*: 1) N_{area} is optimally distributed within *A. saccharum* tree crowns as a result of the strong control of light on LMA (but not on N_{mass}); 2) Shading reduces environmental stress on leaves (i.e., reduced light and leaf temperature) resulting in reduced stomatal closure and lower leaf $\delta^{13}\text{C}$; 3) Leaves growing in higher light availability experience greater increases in N_{area} through time due to changes in LMA with no changes in N_{mass} ; 4) Leaf $\delta^{13}\text{C}$ increases through time due to stomatal sensitivity to drier conditions that develop during the mid- to late-summer.

3.3. Methods

3.3.1. Site and experimental design

The study was conducted in closed-canopy sugar maple (*Acer saccharum* Marshall) forest at the Michigan Technological University Ford Forestry Center near Alberta, Michigan, U.S.A (46.65°N, 88.48°W). Mean annual temperature and precipitation at the Ford Forestry Center are 4.8 °C and 810 mm, respectively (NOAA, WS ID 15608). This stand consisted mostly of *Acer saccharum*, but also included *Betula alleghaniensis*,

Ostrya virginiana, *Tilia americana*, and *Ulmus americana*. In 2009, the mean height of the stand was 23.0 m, the basal area was 33 m² ha⁻¹, and the density was 267 trees ha⁻¹. The tree density of *Acer saccharum* was 259 tree ha⁻¹ or 97% of tree density. A cable zip-line system (Coble and Cavaleri 2014) provided crown access along a two-dimensional plane below three 15 m high cables, and arborist-style climbing techniques were used to access the canopy above 15 m. More information about the site history and methodology can be found in Coble and Cavaleri (2014).

Prior to bud burst in the spring of 2013, shade structures were installed at four heights (1-3, 7-9, 12-14, and 17-20 m) along three vertical transects (Appendix A). Seven trees were used in the overall design, and each vertical transect contained two to three trees. Shade structures were constructed with PVC pipe to form a 0.8 × 0.8 m frame. Shade cloth (50%) was draped over the frames and tightly fastened using zip-ties. The shade structures were suspended from aluminum bars, which were either screwed into or clamped onto large branches with a stainless steel padded repair clamp. Branches below the shade structure and paired branches next to shade structures were flagged for leaf sampling and for branch measurements after leaf senescence.

3.3.2. Light, leaf water potential, and morphology measurements

We measured light conditions as percent photosynthetic photon flux density (%PPFD) for paired shaded and unshaded branches in June and August of 2013 using a ceptometer (Sunfleck PAR Ceptometer, Decagon Devices, Pullman, WA). We collected ten measurements above paired shaded and unshaded branches between 1200 and 1500 hours in June and August. Ten open sky measurements were collected prior to light

measurements in a nearby open field to estimate %PPFD (mean below canopy PPFD divided by mean above canopy PPFD times 100). Previous studies have used a similar technique where open sky measurements were temporally and spatially offset from below canopy measurements (Martens et al. 1993, Knapp et al. 2008). In order to minimize error associated with temporal changes in open sky measurements, below-canopy and open sky measurements were made under uniform overcast or cloudless conditions.

Immediately following light measurements (1200-1500 hrs) in June and August, three leaves from shaded and unshaded branches were cut near the base of the petiole, placed in sealed plastic bags with moist paper towels, and stored in an ice chest until measurements were taken. Midday leaf water potential (Ψ_{mid}) measurements were made using a pressure chamber (PMS Instrument, Co., Corvallis, OR, USA) within an hour of leaf collection. Leaves were then stored at 2 °C until leaf morphology measurements were made.

Leaves were scanned into digital format using an Epson Expression 10000XL flatbed color image scanner (Seiko Epson Corporation, Nagano, Japan), and images were digitally analyzed for one-sided leaf area using ImageJ v1.44j (Schneider et al. 2012). Using Archimede's Principle, we derived leaf volume by immersing fresh leaves in a beaker of water placed on a balance (Coble and Cavaleri 2014). Leaves were dried at 65 °C for 48 hours and weighed to the nearest 0.1 mg. LMA was calculated as leaf dry mass (g) divided by leaf area (m^2), and density was calculated as the leaf dry mass (g) divided by leaf volume (cm^3).

3.3.3. Leaf nitrogen and carbon isotope composition

Leaf samples used to estimate leaf nitrogen and carbon isotope composition were processed at the Michigan Technological University Forest Ecology Stable Isotope Laboratory. The set of three leaves collected from each of the shaded and unshaded branches from each month were combined and ground to a fine powder (8000 M Mixer/Mill, Spex SamplePrep LLC, Metuchen, NJ, USA). Leaf nitrogen on a mass-basis (N_{mass} , mg g^{-1}) was determined using a Costech Elemental Combustion System 4010 connected to a Thermo Finnigan ConFloIII Interface and Delta+ Continuous Flow-Stable Isotope Ratio Mass Spectrometer (Thermo Scientific, Waltham, MA, USA). Leaf N_{area} was determined as the product of N_{mass} and LMA divided by 100. Leaf $\delta^{13}\text{C}$ was calculated as $\delta^{13}\text{C} = 1000(R_{\text{sample}}/R_{\text{standard}} - 1)$ (‰) where R_{sample} was the $^{13}\text{C}/^{12}\text{C}$ of the sample, and R_{standard} was the ratio for a standard.

3.3.4. Data Analysis

We conducted an ANCOVA for shading, month, and height effects (independent variables) on light (%PPFD), Ψ_{mid} , leaf mass, leaf area, LMA, leaf density, N_{area} , N_{mass} , C:N ratio, and $\delta^{13}\text{C}$ (dependent variables). Height (1 – 21m) was the covariate, and the categorical variables, shading and month, both had two levels (shaded-unshaded; June-August). Regression analysis was used to determine significant relationships between height and light, morphology, and leaf nitrogen parameters as described above. We used three approaches to separate out the effects of light and height on LMA, N_{area} , and $\delta^{13}\text{C}$. First, we plotted LMA, N_{area} , and $\delta^{13}\text{C}$ vs. height by month within a narrow band of light conditions (1-3%). Second, we plotted the residuals of LMA, N_{area} , and $\delta^{13}\text{C}$ vs. height

against light and the residuals of LMA, N_{area} , and $\delta^{13}C$ vs. light against height. Calculation of residuals accounted for month effects by plotting within each month. Third, we compared the contribution of light and height to the full model ($y = \beta_0 + \beta_1 \text{light} + \beta_2 \text{height}$) for predicting LMA, N_{area} , and $\delta^{13}C$ using partial R^2 values. The interaction terms ($\text{height} \times \text{light}$) were not significant and were not included in the full models. Light values were natural log-transformed (\ln) for relationships with LMA to satisfy regression assumptions and to develop linear models for ANCOVA and partial R^2 analyses. All statistical analyses were conducted using R statistical software (R Development Core Team 2013). The 'lm' and 'anova' R-functions were used to define the linear model and to produce the ANCOVA output, respectively.

3.4. Results

3.4.1. Shading and seasonal effects on environmental drivers and leaf functional traits

Light availability decreased as a result of shading, did not change from June to August, and increased exponentially with height for both shaded and unshaded leaves (Fig. 3.1a-b). Leaf midday water potential (Ψ_{mid}) was unaffected by shading, was lower in August compared with June, and decreased linearly with height for June (Table 3.1, Fig. 3.1c-d). Total cumulative precipitation was 9.0 cm the month prior (May 18-Jun 18) to June measurements and 0.4 cm the month prior (Jul 13-Aug 13) to August measurements (NOAA, WS ID 15608).

Leaf mass and area, two components of LMA, both decreased under shading but displayed opposite trends with height (Table 3.1, Fig. 3.2). Leaf mass increased linearly with height for unshaded leaves and was greater at higher heights compared with shaded

leaves (Table 3.1, Fig. 3.2a). Leaf mass also increased linearly with height for leaves collected in June but not for August, and leaf mass was greater for leaves collected in August (Table 3.1, Fig. 3.2b). Leaf area was lower for shaded leaves, decreased linearly with height for shaded leaves only, and did not change with height within June and August (Table 3.1, Fig. 3.2c-d),

Both LMA and density decreased in response to shading, but shading did not affect branch growth (Table 3.1, Fig. 3.2e-h). LMA increased linearly with height among unshaded and shaded leaves, but the slope of the LMA-height relationship was greater for unshaded leaves (Fig. 3.2e). In contrast, slopes were similar for LMA-height relationships in June and August (Fig. 3.2f). Similar to leaf mass, leaf density increased linearly with height for unshaded leaves and for leaves collected in June (Table 3.1, Fig. 3.2g-h). Finally, height, shade treatment, and height \times shade treatment interaction did not have an effect on 2013 branch growth (cm yr^{-1} ; Table 3.1).

Leaf nitrogen among shaded and unshaded branches along vertical gradients was measured to identify potential shading effects at different heights. Mean N_{mass} of shaded leaves was greater than unshaded leaves (Table 3.1, Fig. 3.3a), and N_{mass} of leaves collected in June was greater than for leaves collected in August (Table 3.1, Fig. 3.3b). N_{area} decreased under shaded treatment, particularly at higher heights (Table 3.1, Fig. 3.3c). N_{area} increased linearly with height for unshaded and shaded leaves (Fig. 3.3c) and for leaves collected in June and August (Fig. 3.3d). The C:N ratio decreased under shaded treatment and increased from June to August (Table 3.1, Fig. 3.3e-f).

Leaf $\delta^{13}\text{C}$ gradients were compared between shaded and unshaded branches to identify acclimation responses to light, but leaf $\delta^{13}\text{C}$ did not change under shaded

treatment (Table 3.1, Fig. 3.3g). Leaf $\delta^{13}\text{C}$ decreased from June to August at all heights (Table 3.1, Fig. 3.3h). Leaf $\delta^{13}\text{C}$ increased linearly with height for unshaded and shaded leaves and for leaves collected in June and August (Fig. 3.3g-h).

3.4.2. Teasing apart light and height on leaf functional traits

We used a three-step approach (see Methods for full description) to further tease apart the effects of light and height on three important variables: LMA, N_{area} , and $\delta^{13}\text{C}$. Within a narrow range of light conditions (1-3 %PPFD), LMA increased with height in June but not in August (Fig. 3.4a). The residuals of LMA vs. (ln)light increased linearly with height, and the residuals of LMA vs. height increased non-linearly with height (Fig. 3.4b-c). The partial regression analysis showed that height contributed more to the full model for predicting LMA in June, but light contributed more to the full model in August (Table 3.2).

N_{area} increased with height in both June and August within the narrow range of light conditions (Fig. 3.4d). The residuals of N_{area} vs. light increased linearly with height and the residuals of N_{area} vs. height increased linearly with height (Fig. 3.4e-f). Partial regression analysis showed that height contributed more to the full model for predicting N_{area} in June, and light contributed more in August (Table 3.2).

Leaf $\delta^{13}\text{C}$ increased linearly with height in June within a narrow range of light conditions, but not in August (Fig. 3.4g). The residuals of $\delta^{13}\text{C}$ vs. light increased linearly with height, and the residuals of $\delta^{13}\text{C}$ vs. height did not change with light (Fig. 3.4h-i). The partial regression analysis showed that height contributed more to the full for predicting $\delta^{13}\text{C}$ in both June and August (Table 3.2).

3.5. Discussion

3.5.1. Optimization of leaf functional traits

Our results provide partial support for our first hypothesis that N_{area} , but not N_{mass} , would be optimally distributed within the canopy as a result of the strong control of light on LMA. Experimentally-reduced light availability resulted in lower leaf mass, density, LMA, and N_{area} , all most apparent in upper canopy positions, whereas N_{mass} showed the opposite trend. Even though N_{mass} increased with shading, a relatively larger decrease in LMA with shading resulted in a decrease in N_{area} . In a previous observational study of *A. saccharum*, Coble and Cavaleri (2014) concluded that changes in LMA, density, and thickness with height were primarily driven by light. Leaves that develop under high light availability tend to be thicker as a result of thicker palisade mesophyll cell layers, which maximize light capture, (Oguchi et al. 2005; Zhang et al. 2011a) and denser leaf tissues (Niinemets et al. 1999; Coble and Cavaleri 2014), both of which lead to greater LMA. For *A. saccharum*, leaves growing in high light with greater LMA also have greater N_{area} and photosynthetic capacity (Ellsworth and Reich 1992a; Ellsworth and Reich 1993; Jones and Thomas 2007), likely due to the critical role of leaf nitrogen in protein-pigment complexes in the thylakoid membrane and in RUBISCO (Evans 1989). Leaves acclimated to high light also maintain higher leaf hydraulic conductance in *A. saccharum* (Sack et al. 2003), other temperate deciduous trees (Aasamaa et al. 2004; Lemoine et al. 2002; Sellin and Kupper 2007; Sellin et al. 2008) and conifer trees (Jerez et al. 2004; Burgess et al. 2006), suggesting that light availability, leaf hydraulic conductance, N_{area} , LMA, and photosynthetic capacity have co-optimal patterns with

light. Overall, adjustments in LMA and N_{area} to shading and along light gradients as reported in this study provide evidence that the distributions of LMA and N_{area} become optimized through morphological acclimation to light over the course of the growing season.

3.5.2. Constraints on leaf functional traits

Height effects on LMA and N_{area} were most apparent early in the growing season (June) when leaf water potential showed linear decreases with height. Height has been found to directly drive LMA gradients in forest canopies, where height effects have been detected under saturating light conditions (Burgess and Dawson 2007; Ishii et al. 2008; Cavaleri et al. 2010; Coble et al. 2014). Height-related limitations to leaf development in tall conifer trees (*Psuedotsuga menziesii* and *Sequoia sempervirens*) include water potential gradients (Koch et al. 2004; Burgess and Dawson 2007; Ishii et al. 2008) and subsequent reductions in turgor pressure (Woodruff et al. 2004; Meinzer et al. 2008). Reduced leaf water potential can constrain cell turgor pressure if no osmotic adjustments are made, which may result in denser leaf tissue because turgor pressure is necessary for cell expansion and division (Lockhart 1965; Hsiao 1973). However, recent evidence suggests that leaf water storage in tall trees may compensate the limitations of hydraulic transport (Ishii et al. 2014). In temperate deciduous species such as *Robinia psuedoacacia*, greater turgor pressure in water-stressed leaves of seedlings can be achieved by osmotic adjustment through most of the day, but midday depressions in turgor pressure that fall below the yield pressure of cell wall extension can lead to reduced leaf expansion (Zhang et al. 2011b). If hydraulic constraints are accounted for

(e.g., low leaf hydraulic conductance), simulated optimal N_{area} distribution is not proportional to light (Peltoniemi et al. 2012), which may explain why field-based observations of photosynthetic capacity saturates at high light (Buckley et al. 2013). Decreasing leaf hydraulic conductance with height often occurs in conifer or evergreen species (e.g., Ryan et al. 2006), but has been found to increase with height in deciduous species, *Tilia cordata* and *Betula penula* (Sellin and Kupper 2007, Sellin et al. 2008). Also, sun leaves in *A. saccharum* can maintain higher leaf hydraulic conductance compared with shade leaves (Sack et al. 2003). Thus, biophysical limitations on leaf growth associated with lower leaf water potentials early in the growing season may constrain optimal distributions of LMA which, in turn, likely constrains optimal N_{area} distributions given the relative insensitivity of N_{mass} to vertical environmental gradients.

Our results do not support our second hypothesis that shading reduces environmental stress resulting in lower $\delta^{13}\text{C}$. In the same stand as this study, Coble and Cavaleri (2014) found that predawn water potential decreased linearly with height, which was likely due to the gravitational component of water potential (Scholander 1965), since transpiration is negligible at night. Thus, leaves at the top of the canopy maintain greater tension in the water column due to height alone. When trees are transpiring, however, this underlying gravitational tension in the water column is amplified by a combination of frictional resistances and greater evaporative demand, potentially leading to decreases in stomatal aperture (Bauerle et al. 1999, Koch et al. 2004, Niinemets et al. 2004, Ishii et al. 2008). In addition to gravitational potential gradients observed in this stand, VPD increased with height during the same study period (data not shown). Stomatal conductance of *A. saccharum* is particularly sensitive to leaf water potential and high

VPD under high light conditions (Ellsworth and Reich 1992b). Collectively, these studies indicate that gradients in leaf water potential and/or VPD may impose constraints on stomatal conductance, resulting in greater $\delta^{13}\text{C}$ higher in the canopy. In contrast, Duursma and Marshall (2006) attributed vertical gradients in $\delta^{13}\text{C}$ to fractionation in the conductance from intercellular air space to the chloroplast, which generally scales with photosynthetic capacity. We suspect this was not the case in our study because N_{area} and LMA both strongly correlate with photosynthetic capacity in *A. saccharum* (Ellsworth and Reich 1993; Jones and Thomas 2007) and N_{area} and LMA both responded to shading, whereas $\delta^{13}\text{C}$ did not.

3.5.3. Seasonal effects on optimization and constraints of leaf function

Our results do not fully support our third hypothesis that leaves growing in higher light would experience a greater increase in N_{area} through time due to changes in LMA. Due to a simultaneous increase in LMA and decrease in N_{mass} , the combination of opposite changes through time neutralized any effects of time on N_{area} . Despite no effect of time on N_{area} at any height, we found that height was more important earlier in the growing season and light was more important later in the growing season. Migita et al. (2007) suggested that N_{area} in *Quercus serrata* is optimized both spatially and temporally where optimal distributions occurred later in the growing season. However, these conclusions were based on data collected only during the late growing season (September) through leaf senescence (November). Extending the work of Migita et al. (2007), we present multiple lines of evidence indicating that N_{area} distribution was

constrained by height early in the growing season and became functionally optimized later in the growing season through acclimation of LMA to light conditions.

Seasonal increases in leaf mass, LMA, leaf density, and C:N and decreases in N_{mass} and $\delta^{13}\text{C}$ suggest that leaves accumulate carbon-rich structural compounds or other compounds low in nitrogen, which may also indirectly affect the seasonal progression toward N_{area} optimization with light. First, studies have suggested that seasonal changes in LMA corresponded with an accumulation of structural proteins (Yasumura et al. 2006) and calcium and silicon (Kitajima et al. 2002), indicating that seasonal patterns in LMA may be due to changes in cell wall structure and chemical composition. Calcium is particularly important in the deposition of lignin and non-cellulosic polysaccharides in cell walls (Eklund and Eliasson 1990). Second, decreasing N_{mass} through time has been observed in other studies and was attributed to accumulation of carbon (Reich and Walters 1994), which is further supported by the increase in the C:N ratio through time in this study. Previous investigations into N_{mass} have found that across and within species, thicker, denser leaves tend to have lower N_{mass} (Reich and Walters 1994; Niinemets 1999, Wright et al. 2004). Consistent with this finding, LMA and density in this study were both negatively correlated with N_{mass} ; however, density explained 54% of the variation in N_{mass} , whereas LMA explained only 23% of the variation in N_{mass} (data not shown). These studies and our experiment indicate a greater investment in cell wall structure or lignification through time, possibly allowing leaves to tolerate low Ψ_{mid} later in the growing season. We hypothesize that early season constraints of leaf water potential on leaf morphology and nitrogen are more apparent because leaf cell wall thickening and lignification are not fully developed. Later in the growing season, however, leaves in

high light conditions are able to invest more into cell wall structure, thus offsetting constraints associated with gradients in leaf water potential later in the growing season (i.e., increasing importance of light through time).

Contrary to our expectations (Hypothesis 4), we observed a decrease in $\delta^{13}\text{C}$ from June to August despite drier conditions in July and August. Seasonal declines in $\delta^{13}\text{C}$ have been reported in other studies and generally showed similar patterns among upper and lower canopy leaves (Damesin et al. 1997; Niinemets et al. 1999; Helle and Schleser 2004; Damesin and Lelarge 2003). During drier conditions, leaves tend to become more enriched in ^{13}C as stomatal aperture decreases and as $^{12}\text{CO}_2$ become depleted in substomatal chambers (Farquhar et al. 1989). However, the decline in $\delta^{13}\text{C}$ and precipitation during the growing season indicates that soil water availability was not a limitation to photosynthesis in July and August since $\delta^{13}\text{C}$ represents the integrated photosynthetic activity (Dawson et al. 2002). We would also expect midday declines in stomatal conductance with decreasing leaf water potential as previously observed in *A. saccharum* seedlings growing in high light, (Ellsworth and Reich 1992b). However, $\delta^{13}\text{C}$ values were lower in August despite the lower Ψ_{mid} observed in August compared with June, suggesting that the Ψ_{mid} was not low enough to initiate midday stomatal closure. Overall, we speculate that leaf acclimation to light during leaf maturation reduced stomatal sensitivity to reduced leaf water potential.

3.5.4. Conclusions

We show that multiple, interacting effects (light, height, and time) play a role in both optimizing and constraining distributions of leaf functional traits in *A. saccharum*.

An underlying assumption behind current ‘big leaf’ models that integrate leaf photosynthesis and functional traits over the canopy is that leaf nitrogen distribution is optimal with respect to light. Based on our results, we contend that constraints on leaf functional traits should be accounted for when integrating leaf functional traits with these models. Early season constraints on leaf functional traits appear to be associated with gradients in leaf water potential. We show that LMA, N_{mass} , C:N ratio, and $\delta^{13}\text{C}$ can change substantially over the growing season, suggesting that highly regulated processes inherent during leaf maturation involve a combination of cell wall thickening and carbon and calcium accumulation. We speculate that cell wall thickening or lignification associated with leaf maturation contributes to the optimization of N_{area} and LMA with respect to light. Overall, our results indicate that light acclimation with increasing leaf age optimized leaf functional traits of a broad-leaf deciduous tree, despite the underlying height-related constraints that were more pronounced in the early growing season.

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3.9. Tables and Figures

Table 3.1. Summary of ANCOVA results for test of height (Ht), shade treatment (ST), month (M), and all 2-way interaction effects on light (%PPFD), Ψ_{mid} , leaf morphological parameters (mass, area, LMA, density), shoot growth, area and mass-based leaf nitrogen (N_{mass} and N_{area}), and $\delta^{13}\text{C}$. Coefficients (\pm Standard Error) for each independent variable in the model are listed in the last seven columns. Model equation: $y = \beta_0 + \beta_1\text{Ht} + \beta_2\text{ST} + \beta_3\text{M} + \beta_{12}\text{Ht*ST} + \beta_{13}\text{Ht*M} + \beta_{23}\text{ST*M}$, ST = 1 if shaded and -1 if unshaded, M = 1 if June and -1 if August; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

Response variable	Independent Variables					
	Ht	ST	M	Ht \times ST	Ht \times M	ST \times M
(ln)Light	***	***	ns	ns	ns	ns
Ψ_{mid}	*	ns	**	ns	ns	ns
Mass (g)	**	***	**	*	ns	ns
Area (cm ²)	*	*	ns	ns	ns	ns
LMA (g m ⁻²)	***	***	***	**	ns	ns
Density (g cm ⁻³)	***	***	***	ns	ns	ns
Shoot Growth (cm yr ⁻¹)	ns	ns	-	ns	-	-
N_{mass} (mg g ⁻¹)	ns	***	***	ns	ns	ns
N_{area} (g m ⁻²)	***	**	ns	**	ns	ns
C:N	ns	***	***	*	ns	ns
$\delta^{13}\text{C}$ (‰)	***	ns	***	ns	ns	ns

Table 3.1. (continued)

Coefficients \pm Standard Error						
β_0	β_1	β_2	β_3	β_{12}	β_{13}	β_{23}
0.42 \pm 0.28	0.07 \pm 0.02	-1.42 \pm 0.36	0.31 \pm 0.35	0.01 \pm 0.03	-0.01 \pm 0.03	-0.04 \pm 0.33
-0.92 \pm 0.14	-0.01 \pm 0.01	0.04 \pm 0.18	0.28 \pm 0.18	-0.00 \pm 0.01	-0.00 \pm 0.01	0.01 \pm 0.16
0.23 \pm 0.03	0.01 \pm 0.00	-0.05 \pm 0.04	-0.09 \pm 0.04	-0.01 \pm 0.00	-0.00 \pm 0.00	0.06 \pm 0.03
83.06 \pm 7.32	-0.55 \pm 0.58	-7.28 \pm 9.17	-11.8 \pm 9.13	-0.66 \pm 0.66	0.26 \pm 0.66	8.81 \pm 8.45
26.32 \pm 1.43	1.15 \pm 0.11	-4.00 \pm 1.78	-5.82 \pm 1.78	-0.40 \pm 0.13	-0.19 \pm 0.13	3.31 \pm 1.64
0.27 \pm 0.01	0.00 \pm 0.00	-0.03 \pm 0.02	-0.08 \pm 0.02	-0.00 \pm 0.00	0.00 \pm 0.00	0.01 \pm 0.02
3.83 \pm 0.80	0.05 \pm 0.07	-0.44 \pm 1.13	-	-0.03 \pm 0.09	-	-
20.00 \pm 1.20	0.07 \pm 0.09	4.75 \pm 1.50	5.41 \pm 1.49	-0.20 \pm 0.11	0.02 \pm 0.11	0.63 \pm 1.38
0.51 \pm 0.04	0.03 \pm 0.00	0.04 \pm 0.06	-0.00 \pm 0.05	-0.01 \pm 0.00	0.00 \pm 0.00	0.05 \pm 0.05
22.87 \pm 0.88	-0.08 \pm 0.07	-4.12 \pm 1.10	-4.34 \pm 1.10	0.17 \pm 0.08	-0.00 \pm 0.08	0.33 \pm 1.02
-31.54 \pm 0.42	0.13 \pm 0.03	-0.67 \pm 0.52	0.89 \pm 0.52	0.02 \pm 0.04	0.02 \pm 0.04	0.26 \pm 0.48

Table 3.2. Partial regression analysis of LMA and N_{area} vs height and light. †LMA = $\beta_0 + \beta_1 \ln(\text{light})$, $N_{\text{area}} = \beta_0 + \beta_1 \text{light}$, $\delta^{13}\text{C} = \beta_0 + \beta_1 \text{light}$; ‡LMA = $\beta_0 + \beta_1 \text{height}$, $N_{\text{area}} = \beta_0 + \beta_1 \text{height}$; §LMA = $\beta_0 + \beta_1 \ln(\text{light}) + \beta_2 \text{height}$, $N_{\text{area}} = \beta_0 + \beta_1 \text{light} + \beta_2 \text{height}$, $\delta^{13}\text{C} = \beta_0 + \beta_1 \text{light} + \beta_2 \text{height}$. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

Response variable	Month	<i>n</i>	Light only†			Height only‡			Light and Height§			Partial <i>R</i> ² for adding:		
			β ₀	β ₁	<i>R</i> ²	β ₀	β ₁	<i>R</i> ²	β ₀	β ₁	β ₂	<i>R</i> ²	Light	Height
LMA	June	24	25.1***	4.67***	0.54	20.2***	0.751***	0.64	20.1***	3.17***	0.563***	0.85	0.21	0.30
	August	24	31.0***	6.58***	0.65	24.4***	0.936***	0.57	25.7***	4.73***	0.594***	0.83	0.26	0.18
N _{area}	June	24	0.696***	0.029*	0.23	0.557***	0.021***	0.67	0.543***	0.012	0.019***	0.71	0.03	0.48
	August	24	0.615***	0.051***	0.63	0.533***	0.021***	0.59	0.523***	0.035***	0.013***	0.80	0.21	0.17
δ ¹³ C	June	24	-29.7***	0.154	0.11	-30.9***	0.155***	0.61	-30.9***	0.019	0.152***	0.62	0.00	0.51
	August	24	-30.8***	0.148	0.11	-31.9***	0.135***	0.53	-31.9***	-0.034	0.143***	0.53	0.00	0.42

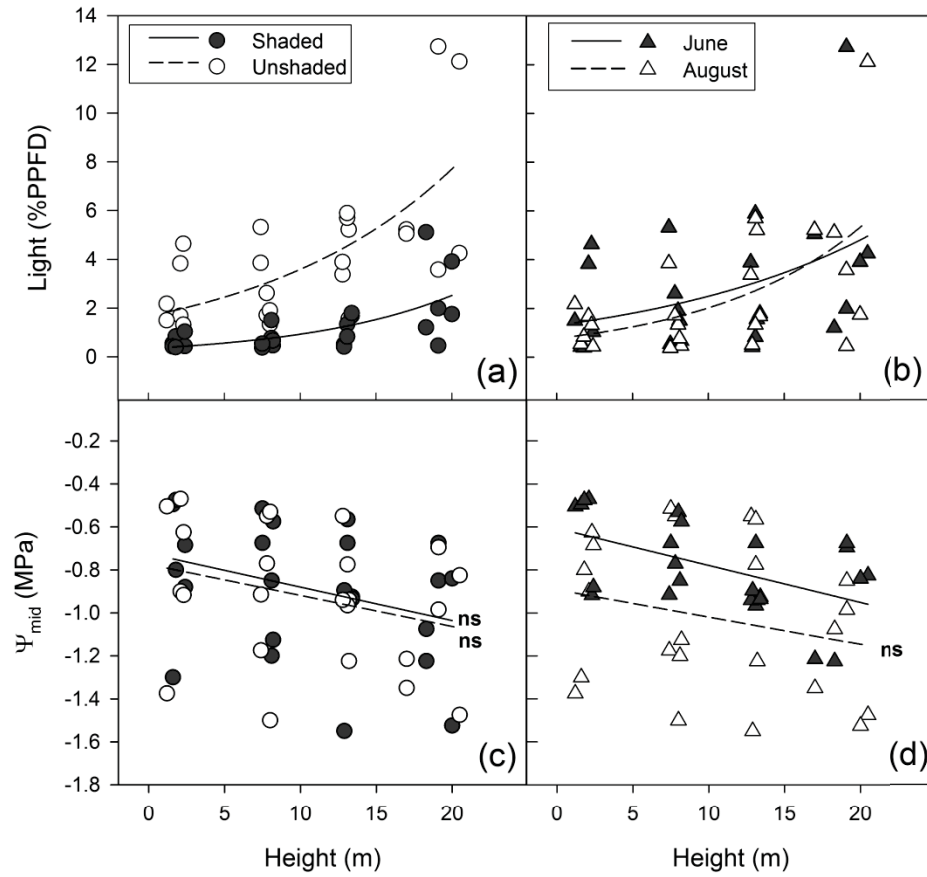


Figure 3.1. Relationships between height and light (a, b) and Ψ_{Mid} (c, d) for shaded and unshaded leaves (a, c) and for leaves collected in June and August (b, d). Non-significant relationships ($p > 0.05$) with height are indicated with the symbol 'ns'.

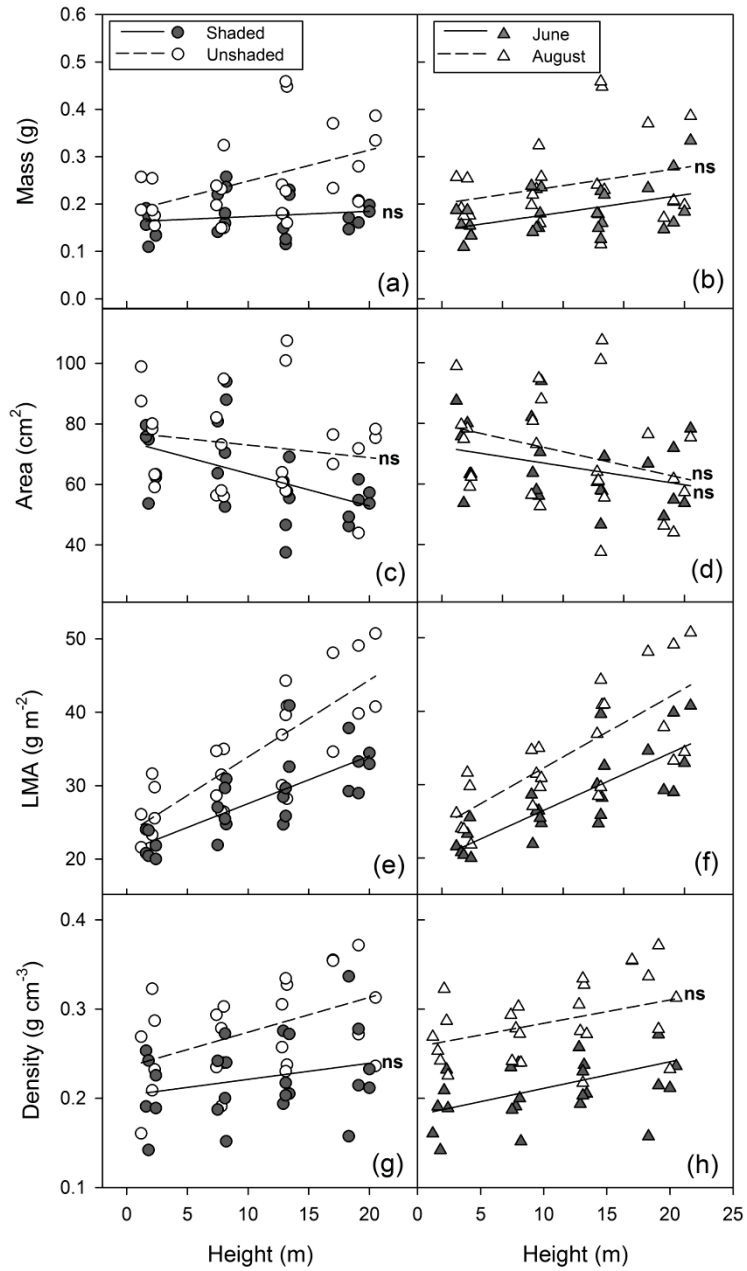


Figure 3.2. Relationships between height and leaf mass (a, b), area (c, d), LMA (e, f) and density (g, h) for shaded and unshaded leaves (a, c, e, g) and for leaves collected in June and August (b, d, f, h). Non-significant relationships ($p > 0.05$) with height are indicated with the symbol 'ns'.

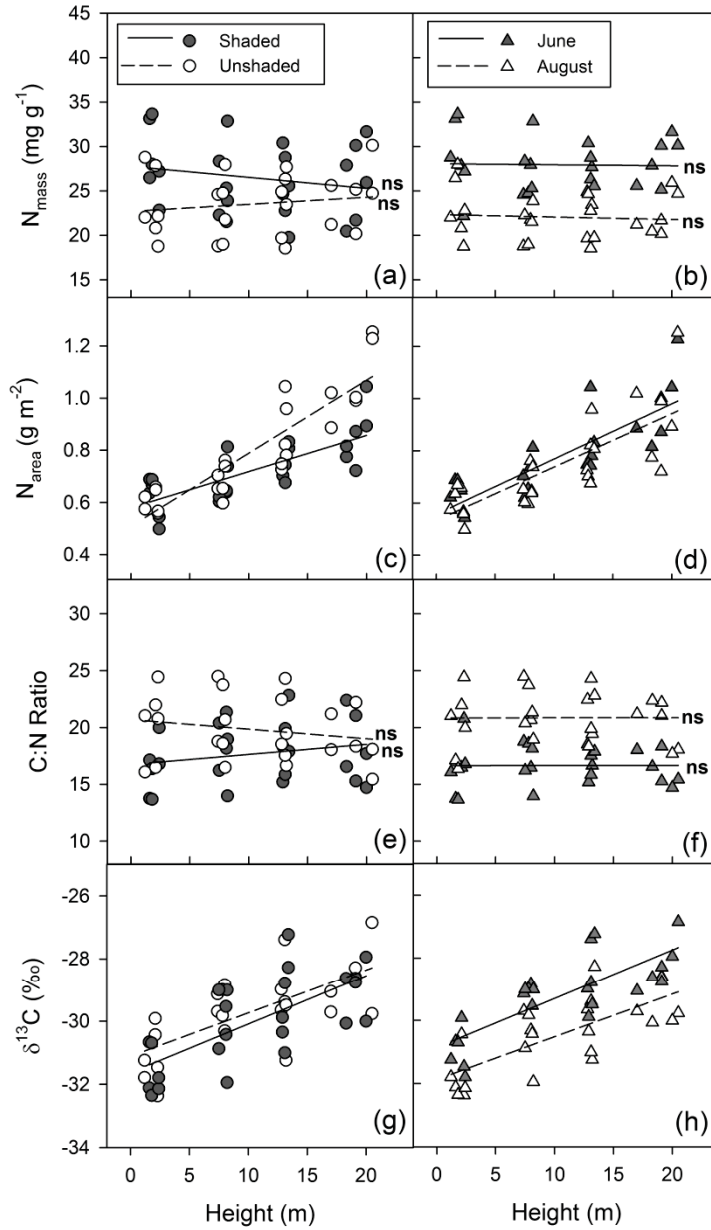


Figure 3.3. Relationships between height and N_{mass} (a, b), N_{area} (c, d), C:N ratio (e, f), and $\delta^{13}\text{C}$ (g, h) for shaded and unshaded leaves (a, c, e, g) and for leaves collected in June and August (b, d, f, h). Non-significant relationships ($p > 0.05$) with height are indicated with the symbol 'ns'.

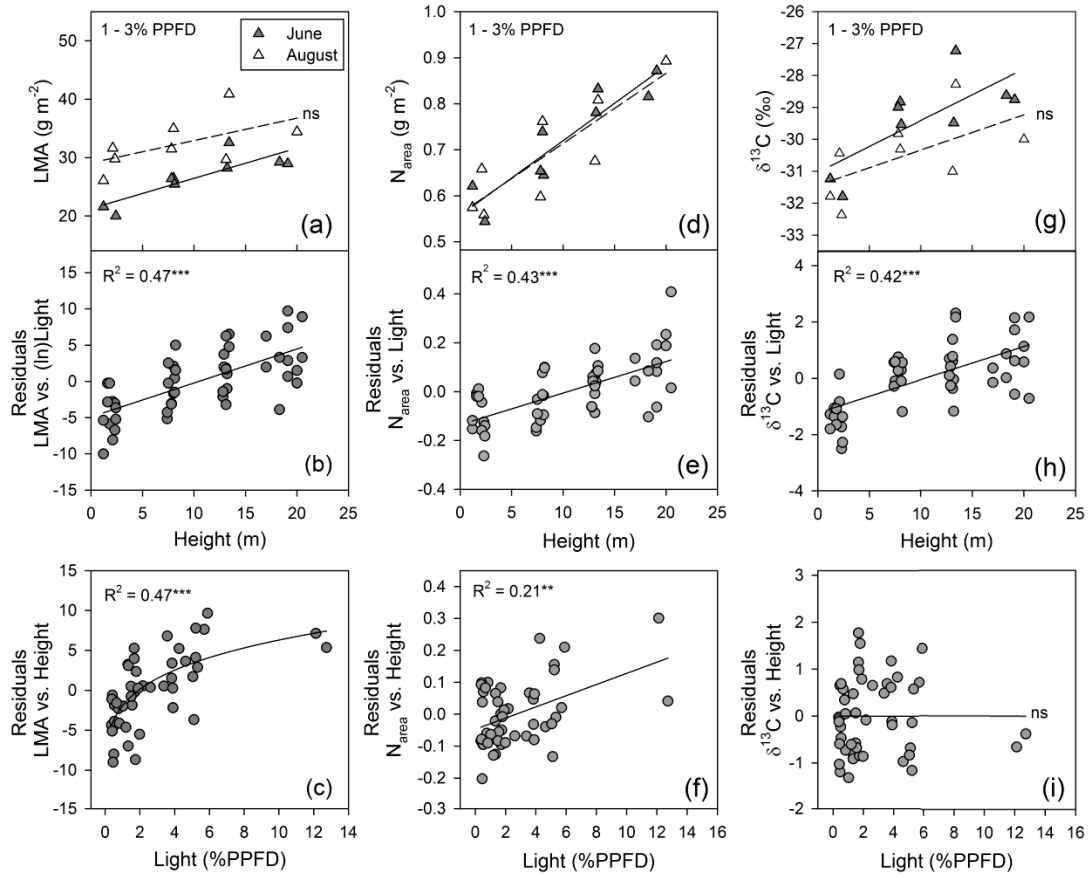


Figure 3.4. Relationship between height and LMA, N_{area} , and $\delta^{13}\text{C}$ for leaves growing within a narrow range of light conditions (1 – 3 %PPFD; a, d, g). Residuals of LMA, N_{area} , and $\delta^{13}\text{C}$ vs. light (%PPFD) plotted against height (b, e, h) and residuals of LMA, N_{area} , and $\delta^{13}\text{C}$ vs. height plotted against light (c, f, i). Note that residuals were calculated from models developed for each month (June and August). Non-significant relationships ($p > 0.05$) with height are indicated with the symbol 'ns'.

4. Seasonal variation of leaf functional traits within a mature sugar maple (*Acer saccharum*) canopy reveals potential biases in canopy photosynthesis models

4.1. Summary

- Leaf functional traits are used in modeling canopy photosynthesis (A_c) due to the strong correlations between photosynthetic capacity (A_{max}), leaf mass per area (LMA), and leaf nitrogen per area (N_{area}). Temporal changes in the vertical distributions of these traits may occur throughout the growing season as a result of light acclimation, which may in turn affect A_c over time.
- We assessed the dependence of within-canopy N_{area} and LMA variation with height and light throughout the growing season to identify potential constraints on both leaf functional traits. We implemented big-leaf and multi-layer models with and without spatial and temporal variation of LMA and N_{area} in order to assess the influences of seasonal constraints on A_c .
- Following springtime leaf expansion, vertical distributions of LMA and N_{area} became optimized through time, primarily through increasing LMA under greater light conditions. Canopy photosynthesis models that did not incorporate seasonal changes in LMA and N_{area} overestimated A_c early in the growing season. However, biases associated with spatial variation in both LMA and N_{area} were consistently greater compared with biases associated with temporal variation.
- Overall, we show that early season constraints on A_c diminish through time, likely through morphological acclimation to light.

4.2. Key words

Acer saccharum, canopy photosynthesis, leaf mass per area, leaf nitrogen, osmotic potential, predawn water potential, turgor pressure

4.3. Introduction

Global patterns in leaf functional traits represent a broad spectrum in investment strategies of carbon and nitrogen (Reich *et al.*, 1999; Wright *et al.*, 2004) and have provided further insight into large-scale patterns in biogeochemical cycling (Cornwell *et al.*, 2008), community composition (Kraft *et al.*, 2008), and productivity of terrestrial ecosystems (Kattge *et al.*, 2009). Two leaf functional traits, leaf nitrogen (N_{area}) and leaf mass per area (LMA), are strong predictors of leaf photosynthetic capacity across biomes and within species and are used in modeling canopy photosynthesis (A_c) and gross primary productivity (GPP) from landscape to global scales (Kull & Jarvis, 1995; dePury & Farquhar, 1997; Raulier *et al.*, 1999; Ryu *et al.*, 2011). The most appealing aspect of LMA and N_{area} is that they are both relatively easy to measure compared with leaf-level gas exchange parameters such as photosynthesis (Cornelissen *et al.*, 2003).

Given the expected rise in global temperatures (IPCC 2014), there has been considerable investigation into the consequences of growing season length on forest CO_2 exchange. Longer growing seasons increase carbon uptake (Baldocchi & Wilson, 2001; Churkina *et al.*, 2005; Piao *et al.*, 2007), particularly during the beginning of the growing season (Black *et al.*, 2000; Jarvis & Linder, 2000; Euskirchen *et al.*, 2006; Richardson *et al.* 2009). In temperate deciduous forests, the growing season begins with bud break at a

critical temperature-based threshold, often assessed using thermal time such as cumulative degree days (McMaster & Wilhelm, 1997; Campbell & Norman, 1998; Raulier & Benier, 2000; Richardson *et al.*, 2006). Within forest canopies, increasing air temperature with height (Niinemets *et al.*, 1999a; Niinemets & Valladares, 2004) may potentially give upper canopy leaves a ‘head-start’ at the beginning of the growing season.

As leaves acclimate to light availability following leaf expansion, leaf nitrogen and LMA are often found to be optimally distributed (Ellsworth & Reich, 1993; Chen *et al.*, 1995; Bond *et al.*, 1999; Niinemets *et al.*, 1998, 1999b; Jones & Thomas, 2007; Coble & Cavaleri, 2014, 2015). However, sub-optimal distributions in photosynthetic capacity, LMA, and leaf nitrogen have been observed in tree crowns, where these traits tend to saturate at high light (Kull *et al.*, 2002; Niinemets, 2012; Buckley *et al.* 2013; Coble *et al.* 2014). Recent studies suggest that sub-optimal distributions may be due to height constraints on leaf hydraulics or stomatal conductance (Peltoniemi *et al.* 2012, Buckley *et al.* 2013). In tall western conifers and tropical trees, leaf water potential gradients may constrain leaf development and stomatal conductance at higher canopy positions, as indicated by increasing LMA and $\delta^{13}\text{C}$ with height (Koch *et al.*, 2004; Woodruff *et al.*, 2004; Ishii *et al.*, 2008; Cavaleri *et al.*, 2010; Kenzo *et al.*, 2014). As stomatal aperture decreases and $^{12}\text{CO}_2$ becomes depleted in substomatal chambers, leaves become more enriched in ^{13}C (Farquhar *et al.* 1989). The lack of osmotic adjustments and subsequent declines in leaf turgor pressure in tall *Psuedotsuga menziesii* trees may be one mechanism behind constraints on leaf development and greater LMA higher in the canopy (Woodruff *et al.*, 2004; Meinzer *et al.*, 2008). Reduced turgor can result in

smaller, denser leaves because turgor pressure facilitates the expansion and division of cells (Hsiao 1973). Studies have also indicated that reduced leaf expansion may be associated with cell wall extensibility, which decreases in response to lower leaf water potential (Nonami & Boyer 1990a,b; Zhang *et al.* 2012a, b). Overall, limitations to leaf development may constrain leaf photosynthesis due to the negative effects of reduced intercellular air space on mesophyll conductance of CO₂ (Flexas *et al.* 2008, Mullin *et al.* 2008; Oldham *et al.* 2010).

Carbon exchange of forests can be estimated using canopy photosynthesis models, which vary in terms of spatial and temporal complexity (Thornley & Johnson, 1990; Kull & Jarvis, 1995; Raulier *et al.*, 1999; Hanson *et al.*, 2004; Medlyn 2004). At larger scales, the ‘big-leaf’ model, which does not take into account spatial variability in canopy structure and function, is quite versatile due to the lower number of necessary parameters; whereas the multi-layer model requires information about vertical variation in canopy structure and function. A critical assumption behind the ‘big-leaf’ modeling approach is that photosynthetic capacity of leaves is optimally distributed with respect to light in order to maximize canopy photosynthesis (Field, 1983; Hirose & Werger, 1987; Gutschick & Wiegel, 1988; Kull & Jarvis, 1995). Thus, models that assume optimal distributions in photosynthetic capacity, LMA, and N_{area} can potentially overestimate canopy photosynthesis if sub-optimal distributions actually exist. Also, models that assume a fixed photosynthetic capacity of leaves through time can also generate biased estimates of leaf-level and canopy photosynthesis (Muraoka *et al.*, 2010). In addition to spatial variation observed for leaf functional traits, studies show that LMA (Jurik, 1986; Poorter *et al.*, 2009; Coble & Cavaleri 2015), leaf nitrogen (Ellsworth & Reich, 1992;

Kull *et al.*, 1998; Migita *et al.*, 2007), photosynthetic capacity (Miyazawa *et al.*, 1998; Miyazawa & Terashima, 2001; Kitajima *et al.*, 2002; Yasumura *et al.*, 2006), and leaf carbon isotope composition ($\delta^{13}\text{C}$; Damesin *et al.* 1997; Damesin & Lelarge 2003; Helle & Schleser 2004) can change considerably throughout the growing season. This temporal variability may, in turn, influence the optimization of leaf functional traits (Migita *et al.* 2007, Coble & Cavaleri 2015) and introduce modeling biases.

The objective of this study was to identify seasonal changes in leaf functional traits in a deciduous broad-leaved tree species (*Acer saccharum*), in addition to assessing potential consequences for not accounting for seasonal changes in leaf function when modeling canopy photosynthesis. We tested the following hypotheses: 1) bud break occurs sooner higher in the canopy due to greater temperatures, giving upper canopy leaves a ‘head-start’ on leaf development; 2) final leaf area following leaf expansion is reduced at greater heights due to greater hydrostatic tension and reduced turgor higher in the canopy; 3) area-based leaf nitrogen (N_{area}) distribution is not always optimal with respect to light due to seasonal changes in LMA, and requires time to ‘optimize’ after leaf expansion through acclimation to available light; 4) as distributions in N_{area} and LMA become more optimized during light acclimation, stomatal sensitivity to drier mid- and late-summer conditions declines as indicated by a concomitant decline in $\delta^{13}\text{C}$ over time; and 5) modeled early-season canopy photosynthesis is over-estimated when light acclimation and subsequent changes in leaf functional traits are not accounted for, and this bias is equally as important as not accounting for vertical gradients in leaf functional traits.

4.4. Methods

4.4.1. Study site

This study was conducted at the Michigan Technological University Ford Center and Forest in Alberta, MI (46.65°N, 88.48°W). Mean annual temperature and precipitation at the Ford Forestry Center are 4.8 °C and 810 mm, respectively (NOAA, WS ID 15608). Sugar maple (*A. saccharum*) contributed 97% of tree density of this stand, which also included *Ulmus americana* L., *Tilia americana* L., *Betula alleghaniensis* Britton, and *Ostrya virginiana* (Mill.) K. Koch. The mean height of the stand was 23.0 m and the mean height to live crown was 13.8 m. Additional details of this stand are described in Coble and Cavaleri (2014).

4.4.2. Study design and timing of environmental and leaf measurements

We constructed a 19-m mobile aluminum walk-up tower (Upright, Inc., Selma, CA, USA) that provided crown access to a total of five trees. Sampling occurred in 2013 and along ten tower platforms that were spaced approximately 1.8 m apart. Light availability measurements (details below) were made prior to bud break (April 25), during leaf expansion (June 1), and in two sampling periods following leaf expansion (July 22, August 20), which were averaged to represent light conditions following leaf expansion in subsequent analysis. Air temperature at nine height intervals (details below) was measured from April 5 to October 29. The percentage of buds that achieved bud break were estimated for two to three branches per height interval (10 height intervals) per tree on 10 days from May 6 to June 7. During leaf expansion from May 29 to June 24, leaf area of the same leaves was measured on seven days using the same branches as

used for the bud phenology sampling. Toward the end of leaf expansion (June 17), two leaves at every other height interval (12 leaves total) were collected for measurements of pre-dawn leaf water potential (Ψ_{Pre}) and derived estimates of osmotic potential and turgor pressure (detailed methods below).

From leaf expansion to senescence, we used a combination of whole-leaf collection and hole-punches for leaf trait measurements to minimize the impact of repeated whole-leaf collection from the same tower locations and trees. For LMA measurements, we collected two to three hole-punches from two to three leaves (May 26, 29; June 1, 4, 7, 10, 17, 24; July 1, 8, 22; August 20) and two to three whole leaves (May 26; June 1, 7, 17; July 1, 22; August 20; Sept 14, 30; Oct 4) at each height interval per tree. Using a subset of the whole leaves, we measured leaf N_{area} , N_{mass} , C:N ratio, and $\delta^{13}\text{C}$ at seven to 11 height intervals on eight to nine dates throughout the growing season. Leaf photosynthetic capacity was estimated at three height intervals (0, 11.5, 20.3 m) on 4 separate days (June 6, 16; July 7, 8) for a total of two to three leaves at each height interval (detailed methods below).

4.4.3. Environmental measurements

The light environment was measured as percent diffuse non-interceptance (DIFN%; e.g., ‘canopy openness’) using two plant canopy analyzers (LAI-2000 and LAI-2200, LI-COR, Inc., Lincoln, NE, USA) either in the hour following sunrise or in the hour preceding sunset. The LAI-2000 was mounted on a tripod in an open field (~400 m from the tower) to collect “above canopy” measurements at 30-s intervals, and simultaneous below canopy measurements along the vertical tower transect were

measured using an LAI-2200 with a 180° view cap to prevent the tower from obstructing the view. “Above canopy” measurements were not taken from the top of the tower because it was shorter adjacent trees. Air temperature was measured along the vertical tower transect using a temperature sensor at 10 minute intervals (HOBO U23 ProV2, Onset Computer Corporation, Bourne, MA).

Photosynthetic photon flux density (PPFD, $\mu\text{mol m}^{-2} \text{s}^{-1}$) in the open field (described above) was measured at 10 minute intervals using a photosynthetically active radiation (PAR) light sensor (Onset Computer Corporation, Bourne, MA) mounted on a post. In 2013, PPFD data were only available from July 16 to September 22, but a more complete data set was available in 2012 (May 11 to October 19). We assumed that 2012 was a representative year in daily and seasonal variation in PPFD, thus we merged the 2012 data with the existing 2013 data to develop a more complete data set of PPFD for canopy photosynthesis modeling.

4.4.4. Leaf phenology and morphology

The percentage of buds that achieved bud break (i.e., when leaves became visible, Figure 4.1) was calculated by dividing the number of incidences of bud break on each sample date for each sampled branch by the final number of buds that achieved bud break as of June 7th and multiplied by 100.

For leaf area measurements, three expanding leaves per branch were marked with one to three dots with an acid-free permanent paint marker (Sharpie, Newell Rubbermaid, Oak Brook, IL) in order to identify leaves for repeated measurements. Leaves were placed next to a ruler between two sheets of plexi-glass, and black felt was placed under

each leaf to enhance contrast along leaf margins. Photographs were taken when the plexi-glass was perpendicular to the line of sight of the camera and analyzed for leaf area using ImageJ v1.44j (Schneider *et al.*, 2012). A few leaves were excluded from the analysis due to branch dieback.

Samples used for LMA measurements were placed in sealed plastic bags immediately following hole-punch and whole leaf collections. Areas of leaf discs were measured with digital calipers. Whole-leaf area was measured by digitally scanning leaves using an Epson Expression 10000XL flatbed scanner (Seiko Epson Corporation, Nagano, Japan), and analyzing images using ImageJ v1.44j (Schneider *et al.*, 2012). Leaves were dried at 65 °C for 48 hours and weighed to the nearest 0.1 mg for calculating LMA [leaf dry mass (g) / leaf area (m²)]. For a subset of leaf samples, LMA of hole punches was strongly correlated ($R^2 = 0.99$) with LMA of the same whole leaves, but were consistently greater by 2.41 g m⁻². Thus, we adjusted LMA of hole-punches by adding 2.41 g m⁻².

4.4.5. Leaf water relations

Leaves were collected between 0430 and 0530 hrs for *in situ* predawn leaf water potential (Ψ_{Pre}) measurements using a pressure chamber (PMS Instrument, Co., Corvallis, OR, USA). Leaves were cut near the base of the petiole, placed in sealed plastic bags until Ψ_{Pre} measurements were taken near the base of the tower (within 1 hour), and stored in plastic bags until initial fresh weight (g) measurements were taken in the lab within 10-20 min of the Ψ_{Pre} measurements.

To estimate leaf osmotic potential (Ψ_{π}) and turgor pressure (Ψ_P), we developed pressure-volume curves by plotting relative water content (RWC, %) against the inverse of leaf water potential as leaves dehydrated on the laboratory bench (Tyree & Hammel, 1972; Turner, 1981). Leaf water potential (Ψ_L) and fresh weight measurements were collected approximately every 2 hours for each leaf until Ψ_L values exceeded -3.0 MPa. The saturated leaf weight, necessary for estimates of relative water content, was estimated by plotting leaf fresh weight by Ψ_L and extrapolating to zero Ψ_L (Kubiske & Abrams, 1990). Ψ_{π} was estimated by first developing linear regression equations [$-1/\Psi_L = \beta_1(100\text{-RWC}) + \beta_0$] that included the linear portion of the pressure-volume curve below the turgor loss point. Using these regression equations, Ψ_{π} was estimated as the $-1/\Psi_L$ value at the corresponding RWC values from the initial measurements in the lab, and Ψ_P was estimated as the difference between *in situ* Ψ_{Pre} and Ψ_{π} (Woodruff *et al.*, 2004; Zhang *et al.*, 2011b).

4.4.6. Leaf chemistry and photosynthesis

Leaf nitrogen, carbon, and stable carbon isotope composition for sampled leaves were measured at the Michigan Technological University Forest Ecology Stable Isotope Laboratory. Dried whole-leaves collected from each tower section from each day from each tree were combined (~3-4 leaves) and ground to a fine powder (8000 M Mixer/Mill, Spex SamplePrep LLC, Metuchen, NJ, USA). Leaf nitrogen on a mass-basis (N_{mass} , mg g⁻¹) was determined using a Costech Elemental Combustion System 4010 connected to a Thermo Finnigan ConfloIII Interface and Delta+ Continuous Flow-Stable Isotope Ratio Mass Spectrometer (Thermo Scientific, Waltham, MA, USA). Leaf N_{area} (g N m⁻²) was

determined as the product of N_{mass} and LMA divided by 100. Leaf $\delta^{13}\text{C}$ was calculated as $\delta^{13}\text{C} = 1000(R_{\text{sample}}/R_{\text{standard}} - 1)$ (‰) where R_{sample} was the $^{13}\text{C}/^{12}\text{C}$ of the sample, and R_{standard} was the ratio for a standard.

Photosynthesis measurements were taken with a LI- 6400XT (LI-COR Biosciences, Lincoln, NE) infra-red gas analyzer. Relative humidity was kept between 50 and 60%, and CO_2 concentrations were at 400 ppm. Prior to measurements, leaves were allowed to equilibrate to maximum light conditions of $2000 \mu\text{mol m}^{-2} \text{s}^{-1}$ until photosynthesis and stomatal conductance were stable. We measured net photosynthesis at the following photosynthetic photon flux density values: 1800, 1600, 1400, 1200, 1000, 800, 600, 400, 200, 100, and $0 \mu\text{mol m}^{-2} \text{s}^{-1}$. Leaf photosynthetic capacity (A_{max} , $\mu\text{mol m}^{-2} \text{s}^{-1}$), dark respiration (R_d , $\mu\text{mol m}^{-2} \text{s}^{-1}$), and light compensation point (I_c , $\mu\text{mol m}^{-2} \text{s}^{-1}$) were estimated from light curves using equation 1 in Notes S1.

4.4.8. Canopy photosynthesis models

We assessed the potential effects of seasonal changes in leaf functional traits on canopy photosynthesis using four models: a big-leaf (BigL) and multi-layer model (Mult) that did not account for seasonal changes in leaf functional traits, and a big-leaf (BigL-Seas) and multi-layer model (Mult-Seas) that accounted for seasonal changes (Seas for seasonal changes; Raulier *et al.* 1999). The mathematical equations and approaches to modeling leaf and canopy photosynthesis (A_c) are described in detail in Notes S1.

Briefly, the big-leaf model predicts leaf-level net photosynthesis (A_n) for the topmost leaves and scales photosynthesis to the canopy level using the LAI of sunlit leaves. The multi-layer model predicts A_n at multiple canopy layers using height-specific leaf-level

photosynthetic parameters and LAI values of each canopy layer, then all the canopy layers are summed to estimate A_c . The key physiological parameter for all models, photosynthetic capacity (A_{max} , $\mu\text{mol m}^{-2} \text{s}^{-1}$), was predicted using relationships developed between LMA, N_{area} , and A_{max} across multiple studies of *A. saccharum*, including data from this study. The key driving environmental parameter, irradiance at the leaf surface (I_a , $\mu\text{mol m}^{-2} \text{s}^{-1}$), was modeled at multiple canopy layers over time for the Mult model (Table 4.1). Models that accounted for seasonal changes in leaf traits allowed A_{max} and other physiological parameters to vary based on seasonal changes in leaf traits, whereas models that did not account for seasonal changes used a single A_{max} value that was estimated based on leaf trait values at one point in time in the growing season (July 22).

4.4.9. Data analysis

Mean percentages of buds achieving budbreak within upper, mid, and lower canopies were plotted as a function of time to display differences in bud break timing by canopy position. Differences in timing of bud break among canopy positions were assessed using Tukey's Honest Significant Difference on three separate dates.

Cumulative degree days (CDD) during the time of budbreak for each canopy position were estimated using the equation:

$$CDD = \sum \left[\frac{T_{max} + T_{min}}{2} \right] - T_{base}, \quad (1)$$

where T_{max} and T_{min} are the daily maximum and minimum air temperature, respectively, and T_{base} is the base temperature of 10 °C (McMaster & Wilhelm, 1997). When $(T_{max} + T_{min})/2$ was less than T_{base} , the value for that day was equal to zero.

Leaf area expansion rates along the vertical gradient were assessed by fitting a 3-parameter asymptotic sigmoid function for leaf area as function of days following bud break (Day):

$$Leaf\ area = \frac{Final\ Leaf\ Area}{1 + e^{-b*(Day-c)}}; \quad (2)$$

where *Final Leaf Area* (cm²) represents the leaf area value at the asymptote as the denominator ($1 + e^{-b*(Day-c)}$) approaches 1. Parameters *b* and *c* are coefficients that describe the steepness of the curve and the number of days to reach 50% of final leaf area (Days_{50% Area}), respectively. Using this equation, we estimated the number of days to reach 95% of final leaf area (Days_{95% Area}) by setting leaf area divided by final leaf area equal to 0.95 and solving for *Day*. We plotted mean leaf area as a function of days after bud break within canopy positions using Equation 2 in order to display general patterns in leaf expansion among canopy positions. We used regression analysis to develop relationships between height and final leaf area, Days_{50%Area}, and Days_{95%Area}, which were averaged across leaves within each height.

We separated seasonal changes in leaf functional traits into four groups during distinct seasonal phases during the growing season: leaf expansion (May 26-June 9), early growing season (June 10-July 7), late growing season (July 8-September 29), and senescence (September 30-October 4). We used linear and non-linear regression analysis to develop relationships between the independent variables, height and light, and the response variables, LMA, N_{area}, N_{mass}, C:N ratio, and $\delta^{13}C$. We used t-tests to determine significance among slopes and intercepts and to identify seasonal changes. R-statistical software was used for all statistical analysis and model simulations (R Development Core

Team, 2013), and PlotDigitizer software was used to extract data from previous studies (Free Software Foundation, Inc., Boston, MA).

4.5. Results

4.5.1. Bud phenology, leaf area expansion, and leaf water relations

The percentage of buds that achieved bud break substantially increased from May 17th-19th at the middle and upper canopy positions, but not at branches lower in the canopy (Figure 4.2a). On May 19th, the percentages of buds reaching bud break at the lower, mid, and upper canopies were 13, 80, and 87%, respectively. Similarly, cumulative degree days during this time were greater at the upper compared with the lower canopy position (Figure 4.2b). The increase in leaf area following bud break displayed a sigmoid function at each canopy position (Figure 4.3a), and final leaf area did not change with height (Figure 4.3a-b). However, the number of days to reach 50% and 95% of final leaf area was greater at higher heights (Figure 4.3a and 4.3c). Predawn water potential (Ψ_l) and osmotic potential (Ψ_π) linearly decreased with height, while Ψ_p linearly increased with height (Figure 4.4).

4.5.2. Seasonal changes in leaf morphology and chemistry with light and height

Light availability decreased during leaf expansion, but did not differ between July and August measurements following leaf expansion (Figure 4.5). LMA decreased during the leaf expansion phase followed by an increase (Figure 4.6a), with the upper canopy displaying the largest increase in LMA and the greatest maximum LMA (Figure 4.6a), while the mid- and lower canopy showed gradual increases in LMA following leaf

expansion. LMA decreased during leaf senescence, which began prior to the last two leaf collection periods (Sept. 30 and Oct. 4). LMA non-linearly increased (power function, $LMA = a \cdot DIFN^b$) with light (Figure 4.6b), and the slope of this relationship increased from leaf expansion through the late season, but did not change during senescence (Table 4.2). The intercept of this relationship increased from the early to late growing season (Table 4.2). LMA linearly increased with height during leaf expansion and exponentially increased with height for the remainder of the growing season (Figure 4.6c). The slope of this relationship also increased from leaf expansion through the late season, but the intercept did not change over time (Table 4.2).

N_{area} followed slightly different patterns with time, height, and light than observed for LMA (Figure 4.7a-c). Leaf N_{area} decreased during the leaf expansion phase and senescence at all canopy positions (Figure 4.7a). Following leaf expansion and prior to senescence, leaf N_{area} steadily increased for the upper canopy, did not change over time for mid-canopy, and decreased in the lower canopy (Figure 4.7a). N_{area} non-linearly increased ($N_{area} = a \cdot DIFN^b$) with light during the early and late season, but N_{area} did not change with light during leaf expansion and senescence (Figure 4.7b). The slope of this relationship did not change through time, while the intercept decreased from leaf expansion through leaf senescence (Table 4.2). N_{area} exponentially increased with height during the early and late growing season, but did not change with height during leaf expansion and senescence (Figure 4.7c). The slope and intercept of this relationship increased and decreased, respectively, over time (Table 4.2). Leaf N_{mass} decreased at all canopy positions during the first half of the growing season (Figure 4.7d). Leaf N_{mass} linearly decreased with light and height throughout most of the growing season during

the early and late growing season and did not change with light or height during leaf expansion and senescence (Figure 4.7e-f). The slope of the relationships between N_{mass} , light, and height did not change throughout the growing season, but the intercepts decreased from leaf expansion through leaf senescence (Table 4.2).

Leaf C:N ratio increased at all canopy positions during the first half of the growing season (Figure 4.8a). Leaf C:N ratio non-linearly ($C:N = a \cdot \text{DIFN}^b$) increased with light (Figure 4.8b) and linearly increased with height (Figure 4.8c) during the late and early growing season, but did not change with light and height during leaf expansion and senescence. The slope and intercept of the relationship between height and C:N ratio increased from leaf expansion to the early and late growing season (Table 4.2). Leaf $\delta^{13}\text{C}$ decreased through time at all canopy positions (Figure 4.8d), and was consistently greater and more variable in the upper canopy position. Leaf $\delta^{13}\text{C}$ non-linearly increased with light (Figure 4.8e), and linearly increased with height throughout the growing season (Figure 4.8f). The slope of the relationship between height and $\delta^{13}\text{C}$ did not change through time, and the intercept decreased following leaf expansion (Table 4.2).

4.5.3. Modeling leaf functional traits and canopy photosynthesis

Our model simulations were conducted to assess potential effects of seasonal changes of LMA and N_{area} on daily canopy photosynthesis (A_c). Both LMA and N_{area} from *A. saccharum* seedlings and mature trees were strongly correlated with A_{max} , and displayed log-linear relationships using data from our site pooled together with data reported in other studies (Figure 4.9a-b).

We compared big-leaf models that did and did not account for seasonal changes in LMA and N_{area} to assess any bias for not accounting for seasonal variation in LMA and N_{area} . For models that estimated net photosynthesis using LMA and N_{area} , seasonal variations in A_c were comparable among the BigL (big-leaf, no seasonal changes) and BigL-Seas (big-leaf, seasonal changes) models except for June (Figure 4.10a-c). For the LMA models, the largest difference in A_c between the two models was $-0.18 \text{ mol CO}_2 \text{ m}^{-2} \text{ day}^{-1}$ (50% difference) (Figure 4.10b). The difference in A_c approached 0 near the corresponding time of the reference LMA on July 22nd (Figure 4.10b). For the N_{area} models, differences among the models due to seasonal changes in N_{area} were also most noticeable in June (Figure 4.10d), but were smaller compared with differences due to changes in LMA (Figure 4.10b). The largest difference in A_c due to seasonal changes in N_{area} was $-0.05 \text{ mol CO}_2 \text{ m}^{-2} \text{ day}^{-1}$ (10% difference; Figure 4.10d). The differences among models had approached 0 by late June for the N_{area} model (Figure 4.10d).

We next compared big-leaf and multi-layer models to assess any bias for not accounting for details of the canopy profile. The general patterns in A_c among the big-leaf and multi-layer models for both LMA and N_{area} were similar (Figure 4.11a-c). However, A_c as estimated with the multi-layer (Mult) models was consistently greater than A_c as with the big-leaf (BigL) models, particularly during days with high A_c (Figure 4.11a-c). Overall, differences among the models due to vertical gradients in LMA and N_{area} (Figure 4.11b-d) were consistently greater than differences due to seasonal changes in LMA and N_{area} , except during the spring, when seasonal bias was greatest (Figure 4.10b-d).

We assessed the leaf area distribution and contribution of each canopy layer to total A_c throughout the growing season using the Mult-Seas model. LAI was lower during leaf expansion, did not markedly change following leaf expansion in July and August, and was generally greatest in the middle of the canopy (Figure 4.12a). In contrast, the contribution to total A_c was greater higher in the canopy (Figure 4.12b). In fact, the upper 25% of leaf area contributed 57% to total A_c in August, and the contribution to A_c increased over time for upper canopy leaves beginning in June but decreased for mid-canopy leaves (Figure 4.12b).

4.6. Discussion

4.6.1. Vertical gradients in bud and leaf phenology

Our results provided partial support our first hypothesis, that bud break occurs sooner for branches higher in the canopy due to higher temperatures. The delayed bud break for lower canopy leaves was likely due to lower cumulative degree days. Our results were consistent with other studies that observed a strong control of degree days on bud break (Raulier & Bernier, 2000; Richardson *et al.*, 2006), but we are unaware of other studies that assessed differences in bud break and degree days at different canopy heights. Within-canopy variation in degree day and subsequent effects on bud break may help refine phenological components to canopy process models because annual carbon assimilation among canopy positions may differ. Due to the strong control of degree days on bud break, future increases in temperature will likely initiate an early start to the growing season, potentially increasing total carbon assimilation of forests (Baldocchi & Wilson, 2001).

Our results do not support our second hypothesis that final leaf area would be reduced higher in the canopy due to greater hydrostatic tension and reduced turgor pressure (Ψ_P). The decrease in osmotic potential (Ψ_π) with height indicates that osmotic adjustments and subsequent increases in Ψ_P can occur in mature *A. saccharum* crowns. Our findings are inconsistent with a few studies that suggest that *A. saccharum* is limited in osmotic adjustments (Bahari *et al.*, 1985; Abrams, 1988; Ellsworth & Reich, 1992b; Kubiske & Abrams, 1994), but consistent with one study that found osmotic adjustments for *A. saccharum* (Kolb *et al.*, 1991). Despite osmotic adjustments and subsequent increases in Ψ_P with height, leaf expansion appeared to be constrained as indicated by the longer times required to 95% final leaf area higher in the canopy (Figure 4.4c). Leaf expansion may be considered a process dependent on osmotic adjustments and subsequent changes in turgor pressure. However, biophysical properties of cells must be considered due to their influences on the force required to irreversibly expand cells (Lockhart 1965; Cosgrove 2000), and high turgor pressure does not always result in faster expansion. For example, Zhang *et al.* (2011b) found that water-stressed *Robinia* seedlings required a longer time to achieve 95% leaf area compared with well-watered seedlings, despite adjustments in osmotic potential and higher Ψ_P in water-stressed seedlings. Limitations to leaf expansion were primarily due to reduced cell wall extensibility (irreversible cell expansion) and higher yield turgor threshold (force required to expand cells; Zhang *et al.* 2011b). Similarly, Nonami & Boyer (1990a, b) observed reduced cell wall extensibility in response to soil drying and reduced leaf water potential in *Glycine max* seedlings. We suspect that a similar phenomenon is occurring in this stand, but the mechanisms associated with biophysical limitations to leaf

expansion require further investigation. Another interpretation of why expansion was slower in the upper canopy is that upper canopy leaves were thicker, so each unit of area expansion likely required more carbon and nitrogen (i.e., more mass needs to be moved into leaves).

4.6.2. Seasonal constraints and optimization of leaf functional traits

Our results provide partial support for our third hypothesis that N_{area} distributions become functionally optimized over time through acclimation to light. Investigations into the optimality of leaf nitrogen distributions have been gaining attention due to the potential application to large-scale canopy process modeling (Niinemets *et al.*, 2012). Within-crown or canopy distributions of leaf photosynthesis are required for modeling canopy photosynthesis, and leaf nitrogen distributions are often used because leaf N strongly correlates with photosynthetic capacity, yet it is easier to measure, especially in mature tree canopies. When integrating photosynthesis over the entire crown or canopy using big-leaf models, a critical assumption is that leaf nitrogen is optimally distributed with available light in order to maximize canopy photosynthesis (Field, 1983; Kull & Jarvis, 1995; Kull *et al.*, 1998). While many studies have found a strong correlation between N_{area} and light within canopies, there is increasing evidence of suboptimal N_{area} distributions at the branch, whole-plant, and canopy levels (Hollinger, 1996; Anten *et al.*, 1998; Niinemets, 2012; Dewar *et al.*, 2012; Peltoniemi *et al.*, 2012; Osada *et al.*, 2014). In this study, we show that the leaf nitrogen distribution is sub-optimal during leaf expansion and senescence, when leaf N_{area} was not correlated with light, but became functionally optimized during the middle of the season (Jun 10 – Sept 29, Figure 4.7b).

Seasonal changes in N_{area} optimization have been reported in other studies where early-season leaf development and senescence corresponded with sub-optimal distributions (Migita *et al.* 2007; Coble & Cavaleri, 2015). Early season sub-optimal distributions are likely associated with biophysical constraints on LMA (Coble & Cavaleri, 2015), whereas sub-optimal distributions during senescence are likely due to nitrogen resorption (Migita *et al.*, 2007).

Even more pronounced than changes in N_{area} were seasonal changes in LMA and C:N ratio. Changes in LMA were likely contributing to seasonal changes in N_{area} due to the strong link between LMA and N_{area} ($N_{\text{area}} = \text{LMA} * N_{\text{mass}}$). Considering that leaf area does not change much following leaf expansion, seasonal changes in LMA reflected similar changes in leaf mass. Seasonal changes in LMA have corresponded with an accumulation of structural proteins (Yasumura *et al.*, 2006) and calcium (Kitajima *et al.*, 2002), which are particularly important in the deposition of carbon-rich compounds such as lignin and non-cellulosic polysaccharides in cell walls (Eklund & Eliasson, 1990). Thus, seasonal patterns in LMA and C:N ratio may be due to changes in cell wall structure and composition. We speculate that these changes may be contributing to improved tolerance of lower water potentials of *A. saccharum* typically observed from mid-to late-summer (Ellsworth & Reich, 1992b; Coble & Cavaleri, 2015).

Our results support our fourth hypothesis that stomatal sensitivity to drier conditions declines as N_{area} and LMA become more functionally optimized. Precipitation at this site declined considerably in July and August, yet $\delta^{13}\text{C}$ decreased through time, suggesting that there were no water-related constraints on photosynthesis. In drier conditions, leaf $\delta^{13}\text{C}$ typically increases due to decreasing stomatal aperture or full

midday stomatal closure to reduce water-loss (Farquhar et al. 1989). Seasonal declines in $\delta^{13}\text{C}$ have been reported in other studies and generally showed similar patterns for upper and lower canopy leaves (Damesin *et al.*, 1997; Niinemets *et al.*, 1999b; Helle & Schleser, 2004; Damesin & Lelarge, 2003). Decreases in $\delta^{13}\text{C}$ through time may be due to a transition to carbon autonomy (non-reliance on reserves) of leaves, accumulation of structural compounds (lipids and lignin), or accumulation of epidermal cuticular waxes low in $\delta^{13}\text{C}$ (Damesin & Lelarge, 2003; Bai *et al.*, 2008). High leaf $\delta^{13}\text{C}$ in the spring may be a result of carbon fractionation during sugar export from the leaves to stems during the previous growing season, which is then imported back to the leaves in the spring (Damesin & Lelarge, 2003). Overall, the decline in $\delta^{13}\text{C}$ suggests that soil water availability and cell wall thickening or lignification associated with leaf development were not limiting to photosynthesis and that stomatal sensitivity to lower leaf water potential decreased over time as leaves accumulated carbon.

4.6.3. Effects of temporal and spatial variation of leaf functional traits on canopy photosynthesis

Our modeling results partially supported our fourth hypothesis that model estimates of A_c are biased when not accounting for seasonal changes in leaf functional traits. The increase in LMA with increasing height through time appeared to have a greater effect on A_c compared with seasonal changes in N_{area} with height. In particular, this bias is likely to occur early in the growing season when LMA is rapidly increasing in upper canopy positions. Due to the dynamic nature of LMA over the course of the growing season, we show that the use of a constant LMA (as derived from mid-summer)

will likely overestimate canopy photosynthesis early in the growing season. Similarly, Muraoka *et al.* (2011) found that the use of a constant photosynthetic overestimated GPP early in the growing season at the stand-level. Bauerle *et al.* (2012) also showed that global NPP was overestimated with the use of constant photosynthetic capacity in Earth Systems Models. Overall, our results suggest that accounting for seasonal changes in leaf functional traits may refine canopy process models.

Biases associated with not accounting for vertical variability in leaf functional traits were greater than biases associated with models that did not account for seasonal changes in traits. Consistent with our findings, Raulier *et al.* (1999) and Bond *et al.* (1999) found that big-leaf models underestimated daily A_c for *A. saccharum* and *Pseudotsuga menziesii*, respectively, relative to the multi-layer model and suggested that this was primarily due to the invalid assumption that A_{max} was proportional to light availability. In this study, LMA and N_{area} increased with DIFN more rapidly in lower light conditions (lower in the canopy), suggesting that A_{max} was not directly proportional with light. While relative irradiance and DIFN are different measures of light availability, both have found to have a 1:1 relationship when relative irradiance is expressed on percentage basis (Machado & Reich, 1999). Overall, our results indicate that the assumption that A_{max} is directly proportional to relative irradiance is invalid for big-leaf models.

By incorporating vertical and seasonal components to A_c , we were able to assess the contribution of leaves to total canopy photosynthesis along the canopy profile. We showed that the upper quarter of canopy leaf area contributed over half of total net canopy photosynthesis. Earlier in the growing season, leaves higher in the canopy

contributed less to total A_c prior to morphological acclimation to light for upper canopy leaves, when height was the primary limitation to leaf traits. However, as upper canopy leaves acclimate to light environments, their contribution to total A_c increases. Our results are consistent with other studies that have detected large contributions to modeled canopy photosynthesis from leaves in the uppermost canopy (Acock *et al.*, 1978; Ellsworth & Reich, 1993; Bond *et al.*, 1999). Thus, understanding limitations to photosynthesis for upper canopy leaves is crucial in assessing seasonal and environmental constraints on canopy CO_2 exchange.

4.6.4. Conclusions

This study describes a series of environmental limitations to bud and leaf phenological phases that ultimately influence canopy photosynthesis. Due to the strong control of temperature on bud break, rising temperatures will likely initiate early bud break, extending the growing season and influencing annual carbon assimilation especially for leaves growing higher in the canopy. As leaves are expanding, limitations on cell wall extensibility likely influence gradients in LMA with height. During the acclimation period, N_{area} and LMA distributions with respect to light become functionally optimized. Consequently, models that do not account for seasonal changes in N_{area} or LMA overestimate daily A_c early in the growing season. However, biases associated with models that do not account for canopy profiles in leaf structure and function are much greater throughout the majority of the growing season.

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4.10. Tables and Figures

Table 4.1. List of model parameters symbols, their definitions, derivation of values, and relevant citations.

Symbol	Definition	Derivation of values	Citations
A_c	Canopy photosynthesis ($\mu\text{mol CO}_2 \text{ m}^{-2} \text{ ground s}^{-1}$)	Big-leaf model: Equation 5; Multi-layer model: Equation 6	Raulier et al. 1999
A_{max}	Maximum photosynthetic capacity ($\mu\text{mol CO}_2 \text{ m}^{-2} \text{ leaf s}^{-1}$)	Pooled data: $A_{max} = 4.42(\ln)LMA - 11.97$; A_{max} $= 4.14(\ln)N_{area} + 5.33$ (Figure 4.2a)	Reich et al. 1991, Ellsworth and Reich 1992, Ellsworth and Reich 1993, Liu et al. 1997, Raulier et al. 1999, Jones and Thomas 2007, This study
A_{max0}	Maximum photosynthetic capacity ($\mu\text{mol CO}_2 \text{ m}^{-2} \text{ leaf s}^{-1}$) of topmost unshaded leaves	A_{max} of leaves corresponding with LMA and N_{area} at 95% DIFN or 20.6 m on July 22, 2013. Same equation as used for predicting A_{max} .	Reich et al. 1991, Ellsworth and Reich 1992, Ellsworth and Reich 1993, Liu et al. 1997, Raulier et al. 1999, Jones and Thomas 2007, This study
A_n	Leaf net photosynthesis ($\mu\text{mol CO}_2 \text{ m}^{-2} \text{ leaf s}^{-1}$)	Equation 3	Hanson et al. 1987
b_{LMA}	Exponential coefficient of LMA vs. Ht	Equation 7, Figure 4.6c	This study
b_N	Exponential coefficient of N_{area} vs. Ht	Equation 8, Figure 4.7c	This study
Ht	Height above the ground (m)	Tape measure	This study
I	PAR Irradiance ($\mu\text{mol m}^{-2} \text{ s}^{-1}$) below the considered LAI	Equation 4	
I_c	Light compensation point ($\mu\text{mol m}^{-2} \text{ s}^{-1}$)	Pooled data: $I_c = -28.59R_d - 2.12$	Ellsworth and Reich 1993, Kloeppel and Abrams 1995, Raulier et al. 1999, This study

Table 4.1. Cont.

Symbol	Definition	Derivation of values	Citations
$I_{c,0}$	Light compensation point ($\mu\text{mol m}^{-2} \text{s}^{-1}$) of topmost unshaded leaves	I_c of leaves corresponding with LMA and N_{area} at 95% DIFN or 20.6 m on July 22, 2013. Same equation as used for predicting I_c .	Ellsworth and Reich 1993, Kloeppel and Abrams 1995, Raulier et al. 1999, This study
I_0	PAR Irradiance above the canopy ($\mu\text{mol m}^{-2} \text{s}^{-1}$)	PAR sensor (2012 and 2013 continuous measurements at 10 minute interval)	This study
k	Extinction coefficient	$k = 0.5$	Raulier et al. 1999
L^*	Stand LAI (m^{-2} leaf m^{-2} ground)	LAI-2200 and LAI-2000	This study
LMA	Leaf mass per area (g m^{-2})	balance (mass) and scanner and ImageJ (area)	This study
LMA_{min}	Minimum leaf mass per area (g m^{-2})	Equation 7, Figure 4.6c	This study
N_{area}	Leaf nitrogen per area (g N m^{-2})	Equation 8, Figure 4.7c	This study
$N_{area,min}$	Minimum leaf nitrogen per area (g N m^{-2})		
R_d	Leaf dark respiration ($\mu\text{mol CO}_2 \text{m}^{-2} \text{leaf s}^{-1}$)	Pooled data: $R_d = -0.074A_{max} - 0.095$	Ellsworth and Reich 1993, Kloeppel and Abrams 1995, Raulier et al. 1999
$R_{d,0}$	Leaf dark respiration ($\mu\text{mol CO}_2 \text{m}^{-2} \text{leaf s}^{-1}$) of topmost unshaded leaves	R_d of leaves corresponding with LMA and N_{area} at 95% DIFN or 20.6 m on July 22, 2013. Same equation as used for predicting R_d .	This study
α	Quantum yield (mol CO_2 (mol quanta absorbed) $^{-1}$)	$\alpha = 0.041$	Ellsworth and Reich 1993, Kloeppel and Abrams 1995, Raulier et al. 1999
			This study

Table 4.2. Summary of slopes and intercepts for the relationships among height, DIFN%, LMA, N_{area}, N_{mass}, C:N ratio, and $\delta^{13}\text{C}$ for four parts to the growing season: leaf expansion, early, late, and senescence (See methods for full description). Different letters indicate significant differences in slopes or intercepts within each row among the four seasons. Asterisks indicate that the slope or intercept is significantly different from zero. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Response variable	Independent variable	Slopes of relationship (\pm SE)			
		<i>Intercepts of relationships (\pm SE)</i>			
		Season:		Late	
		Expansion	Early		Senescence
(ln)LMA (g m ⁻²)	(ln)DIFN (%)	***0.14 \pm 0.01 ^a	***0.23 \pm 0.01 ^b	***0.27 \pm 0.01 ^c	***0.24 \pm 0.01 ^{bc}
	Height (m)	***2.98 \pm 0.03 ^a	***2.98 \pm 0.02 ^a	***3.09 \pm 0.02 ^b	***3.06 \pm 0.02 ^{ab}
		***0.03 \pm 0.00 ^a	***0.06 \pm 0.00 ^b	***0.07 \pm 0.00 ^c	***0.06 \pm 0.00 ^{bc}
(ln)N _{area} (g m ⁻²)	(ln)DIFN (%)	0.09 \pm 0.09 ^a	***0.14 \pm 0.02 ^a	***0.19 \pm 0.01 ^a	*0.15 \pm 0.07 ^a
	Height (m)	-0.05 \pm 0.26 ^c	***-0.42 \pm 0.04 ^{bc}	***-0.53 \pm 0.03 ^b	***-0.80 \pm 0.16 ^a
		0.02 \pm 0.01 ^a	***0.03 \pm 0.00 ^{ab}	***0.05 \pm 0.00 ^b	**0.05 \pm 0.02 ^b
N _{mass} (mg g ⁻¹)	DIFN (%)	-0.01 \pm 0.17 ^c	***-0.52 \pm 0.05 ^b	***-0.67 \pm 0.04 ^b	***-1.05 \pm 0.19 ^a
		-0.07 \pm 0.07 ^a	***-0.13 \pm 0.03 ^a	***-0.11 \pm 0.02 ^a	-0.12 \pm 0.06 ^a
	Height (m)	***44.93 \pm 2.39 ^d	***30.42 \pm 1.03 ^c	***25.04 \pm 0.55 ^b	***20.65 \pm 1.72 ^a
		-0.19 \pm 0.28 ^a	***-0.67 \pm 0.12 ^a	***-0.47 \pm 0.08 ^a	-0.31 \pm 0.28 ^a
		***45.36 \pm 3.52 ^d	***35.18 \pm 1.46 ^c	***28.06 \pm 0.94 ^{ab}	***21.89 \pm 3.41 ^a

Table 4.2. Cont.

Response variable	Independent variable	Slopes of relationship (\pm SE) <i>Intercepts of relationships (\pm SE)</i>				
		Season:	Expansion	Early	Late	Senescence
(ln)C:N	(ln)DIFN (%)		-0.00 \pm 0.06 ^a	***0.10 \pm 0.02 ^{ab}	***0.09 \pm 0.01 ^{ab}	0.12 \pm 0.06 ^b
	Height (m)		***2.45 \pm 0.17 ^a	***2.66 \pm 0.04 ^a	***2.84 \pm 0.03 ^b	***3.03 \pm 0.12 ^b
$\delta^{13}\text{C}$ (‰)			-0.00 \pm 0.01 ^a	***0.02 \pm 0.00 ^b	***0.02 \pm 0.00 ^b	0.02 \pm 0.02 ^{ab}
			***2.45 \pm 0.11 ^a	***2.59 \pm 0.06 ^{ab}	***2.80 \pm 0.04 ^{bc}	***3.04 \pm 0.22 ^c
	(ln)DIFN (%)	***2.10 \pm 0.30 ^c		***1.16 \pm 0.10 ^a	***1.26 \pm 0.09 ^a	***1.10 \pm 0.15 ^a
		***-33.28 \pm 0.90 ^a		***-31.11 \pm 0.25 ^b	***-32.05 \pm 0.23 ^a	***-32.09 \pm 0.39 ^{ab}
	Height (m)	***0.30 \pm 0.04 ^a	***0.28 \pm 0.02 ^a	***0.30 \pm 0.02 ^a	***-32.66 \pm 0.30 ^a	***0.26 \pm 0.04 ^a
		***-30.51 \pm 0.56 ^b	***-31.71 \pm 0.30 ^a		***-32.61 \pm 0.54 ^a	

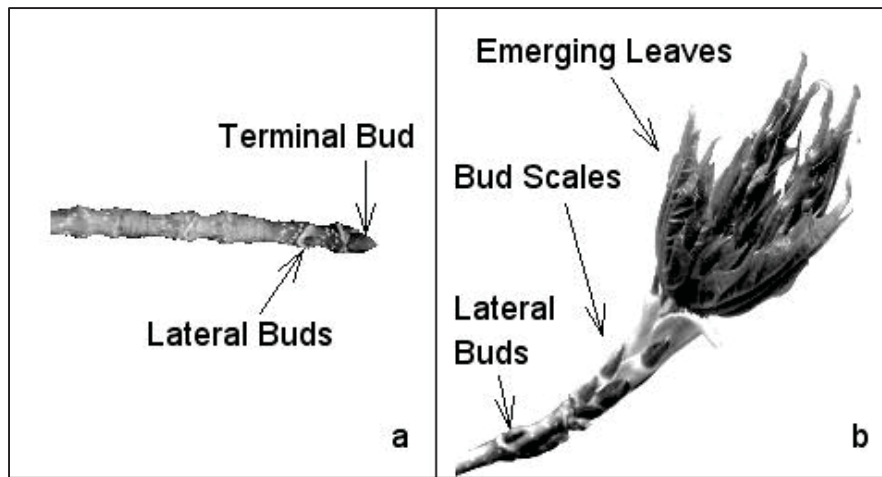


Figure 4.1. Images of terminal and lateral buds prior to budbreak (a) and emerging leaves from terminal bud immediately after budbreak (b).

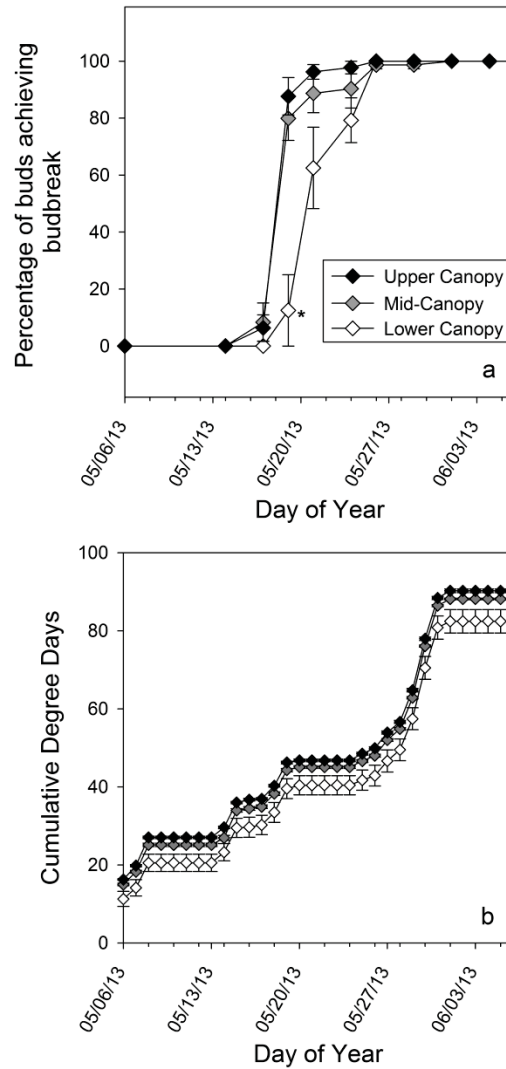


Figure 4.2. Percentage of buds that achieved budbreak by day of year for each canopy position (a). The asterisk indicates that the percentage of buds achieving bud break at the lower canopy position was significantly (Tukey's HSD; $p < 0.05$) lower than at the mid- and upper canopy positions on 5/17/2013. Significant differences among canopy positions were not detected at any other dates. Cumulative degree days by day of year at each canopy position (b). Cumulative degree days was significantly greater at the upper canopy position compared with the lower canopy position for all dates (Tukey's HSD; $p < 0.05$).

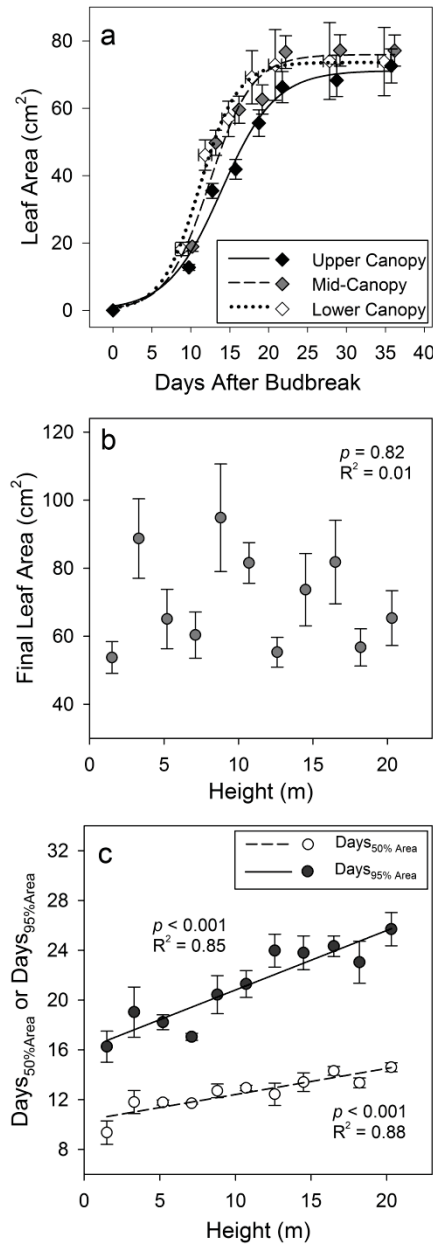


Figure 4.3. Relationship between leaf area and days after bud break for three canopy positions (lower, mid-, and upper canopy) fit with a sigmoid function (a). Relationships between height and final leaf area (b), Day_{50% Area} (Number of days to achieve 50% of final leaf area), and Day_{95%Area} (Number of days to achieve 95% of final leaf area; c).

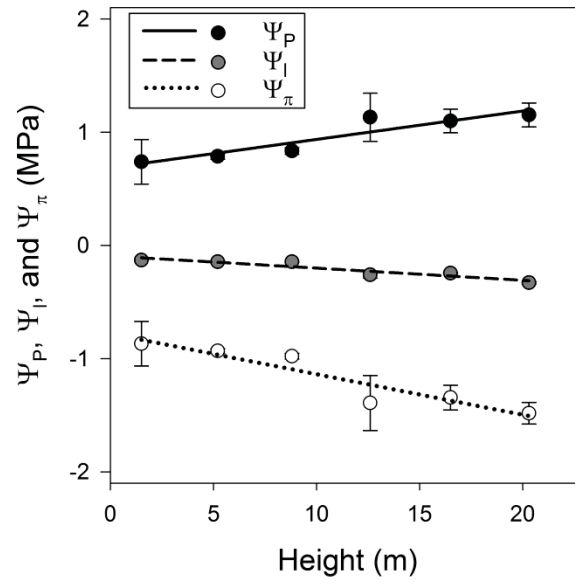
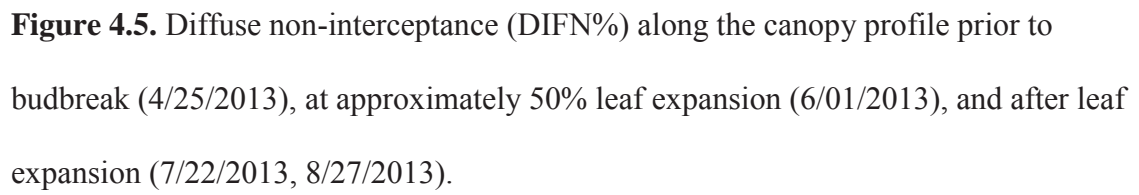


Figure 4.4. Relationships between height and predawn turgor pressure (Ψ_P), leaf water potential (Ψ_l), and osmotic potential (Ψ_π , MPa). R^2 and p -values: Ψ_P , $R^2 = 0.86$, $p < 0.01$; Ψ_l , $R^2 = 0.87$, $p < 0.01$; Ψ_π , $R^2 = 0.87$, $p < 0.01$.



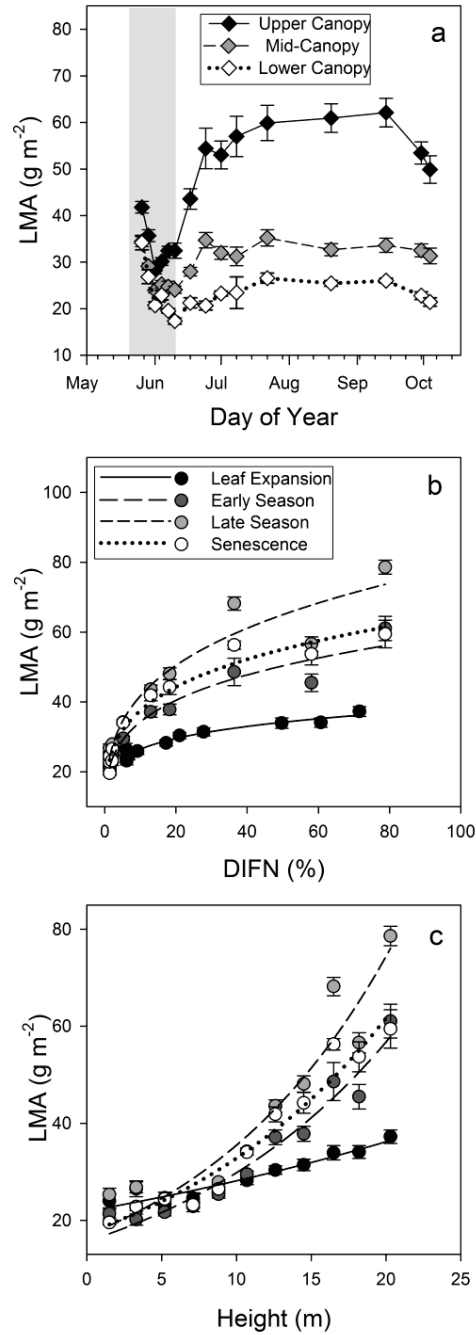


Figure 4.6. Changes in LMA (g m^{-2}) at three canopy positions beginning on May 26 and ending on day Oct 4 (a). The gray bar represents the leaf expansion phase. Relationships between light and LMA (b) and height and LMA (c) during four phases of the growing season.

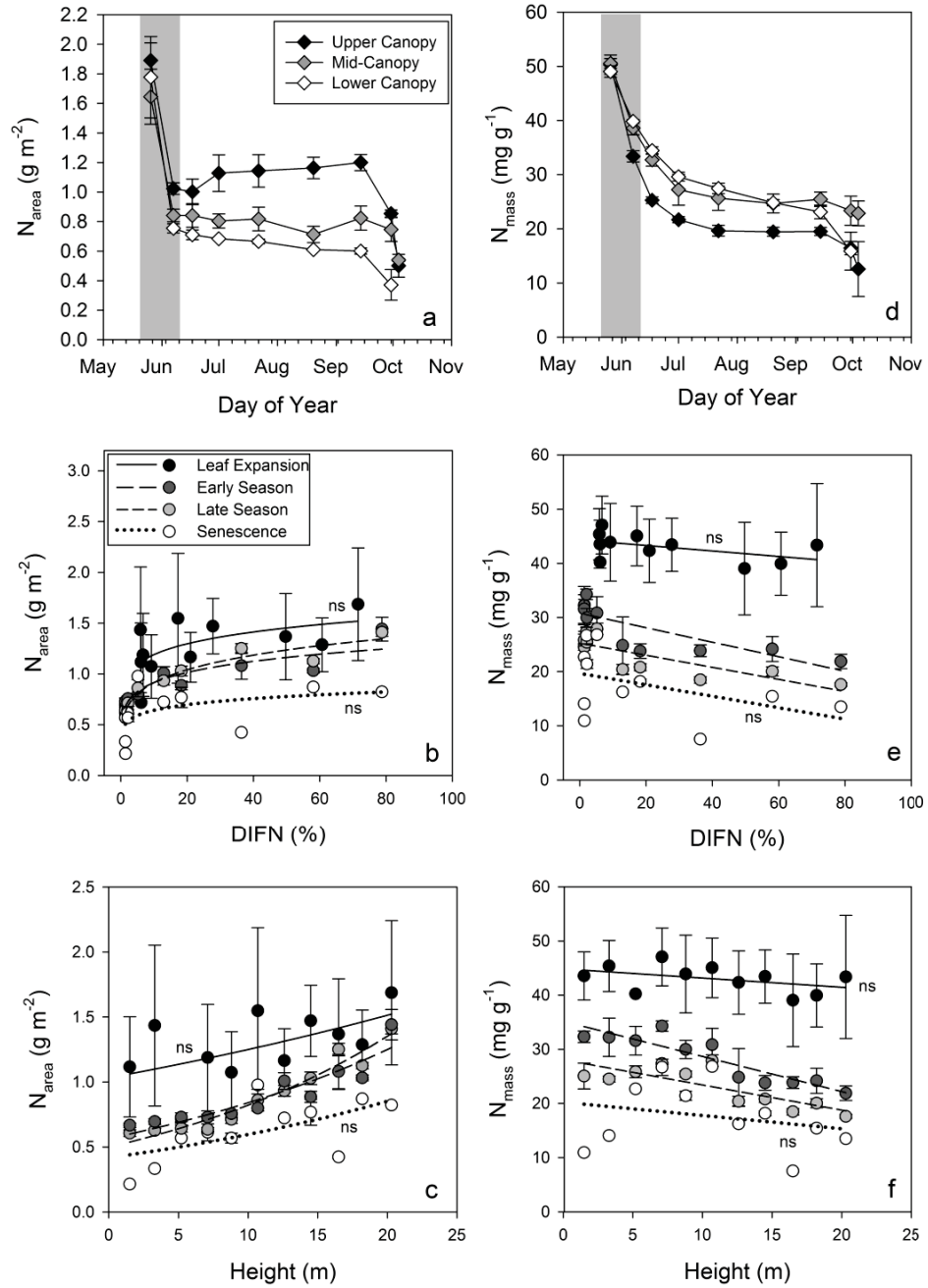


Figure 4.7. Changes in N_{area} (g m^{-2}) and N_{mass} (mg g^{-1}) at three canopy positions beginning on May 26 and ending on day Oct 4 (a, d). The gray bar represents the leaf expansion phase. Relationships between light, N_{area} , and N_{mass} (b, e) and height, N_{area} , and N_{mass} (c, f) during four phases of the growing season.

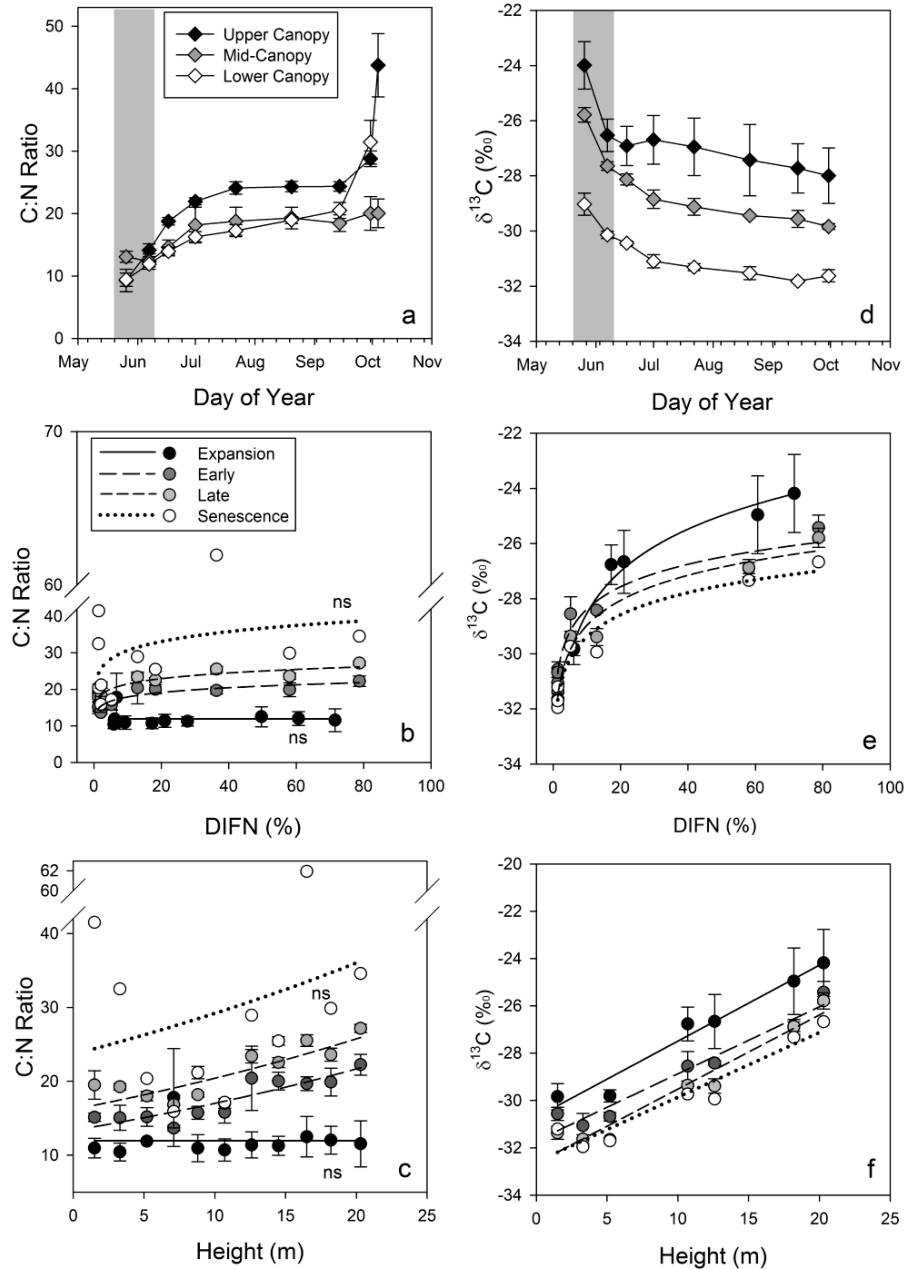


Figure 4.8. Changes in C:N (carbon: nitrogen) ratio and $\delta^{13}\text{C}$ (‰) at three canopy positions beginning on 5/26/13 and ending on day 10/04/13 (a, d). The gray bar represents the leaf expansion phase. Relationships between light, C:N ratio, and $\delta^{13}\text{C}$ (b, e) and height, C:N ratio, and $\delta^{13}\text{C}$ (c, f) during four phases of the growing season.

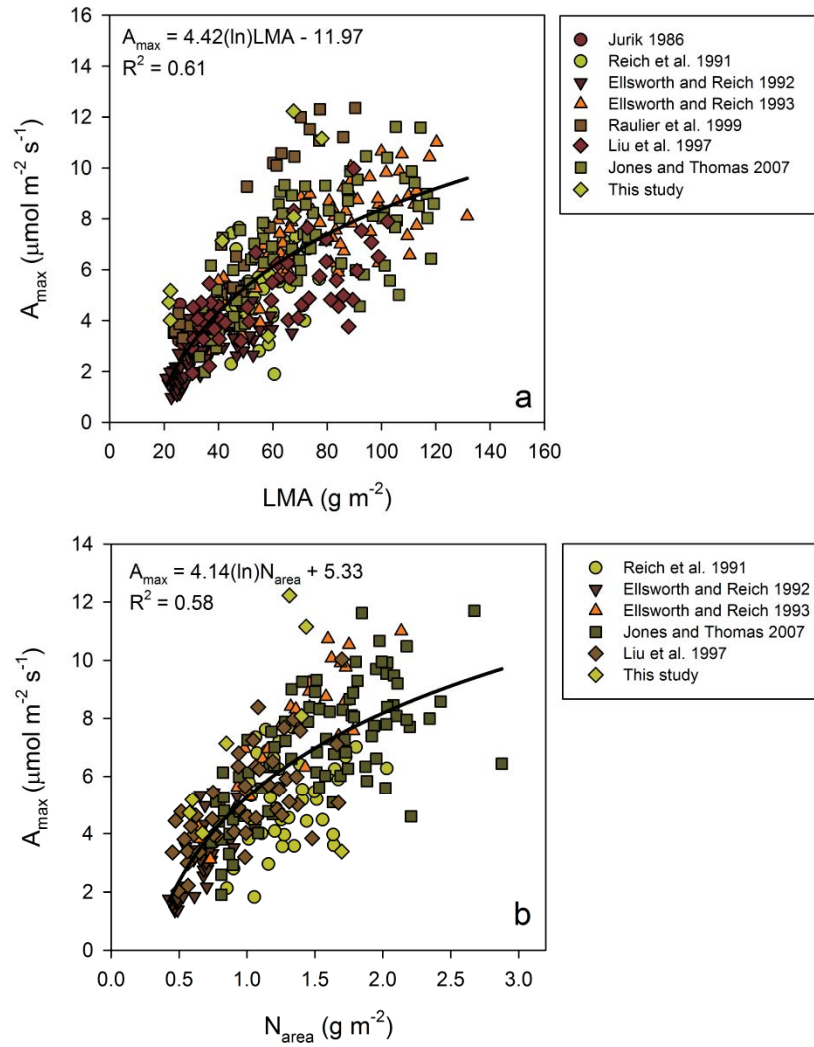


Figure 4.9. Relationships among A_{\max} and LMA (a) and A_{\max} and N_{area} (b) for multiple studies that included *A. saccharum* mature trees and seedlings. The coefficients of the regression equations listed in each panel were used in the model simulations.

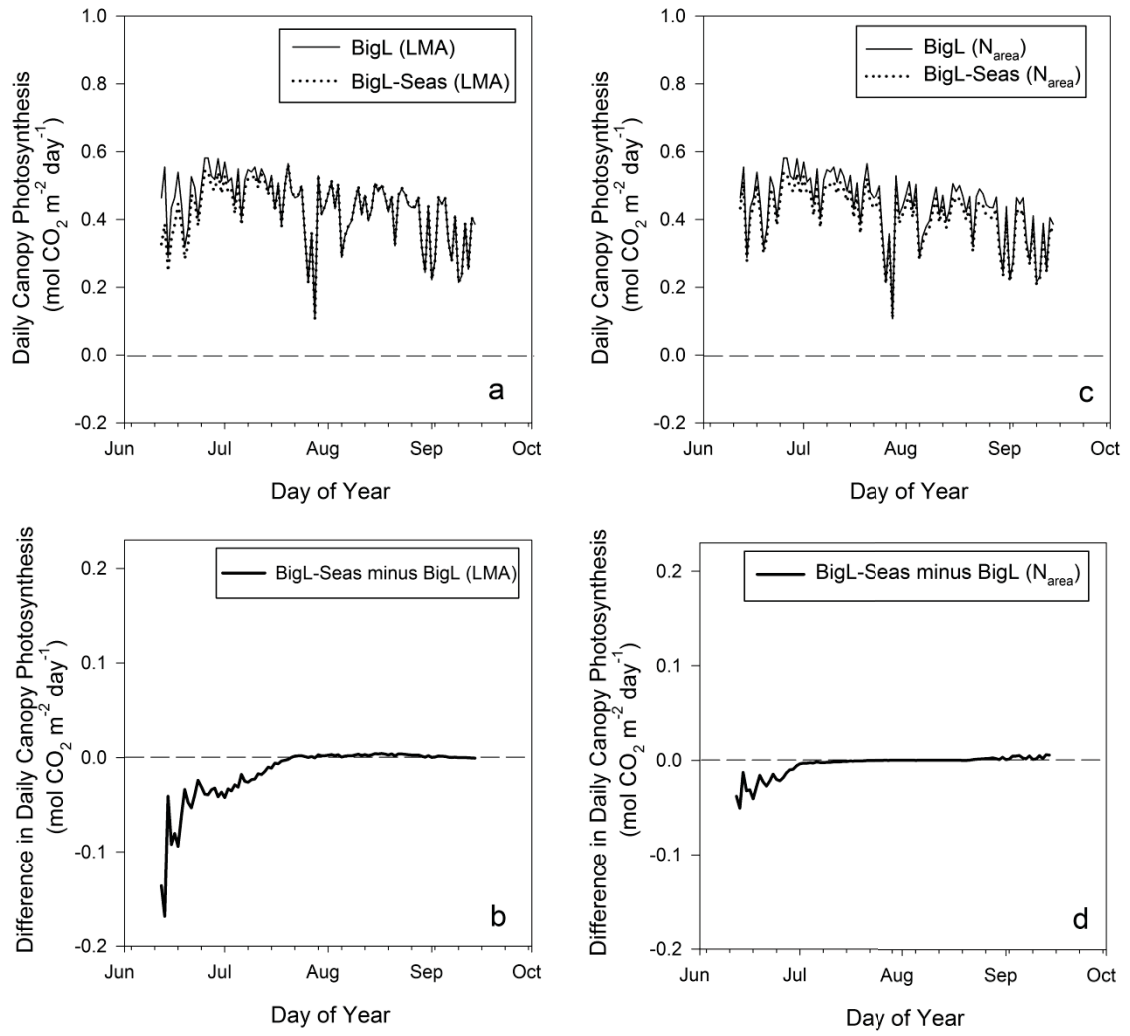


Figure 4.10. Simulated daily canopy photosynthesis (mol CO₂ m⁻² day⁻¹) using a big-leaf model that did (BigL-Seas) and did not account for seasonal changes in LMA (BigL) using LMA (a) and N_{area} (c). Differences in daily canopy photosynthesis using big-leaf models that did (BigL-Seas) and did not (BigL) account for seasonal changes in LMA (b) and N_{area} (d). The time period investigated does not include the period of leaf expansion and senescence. Differences between models (b and d) show bias when seasonal variation is not considered.

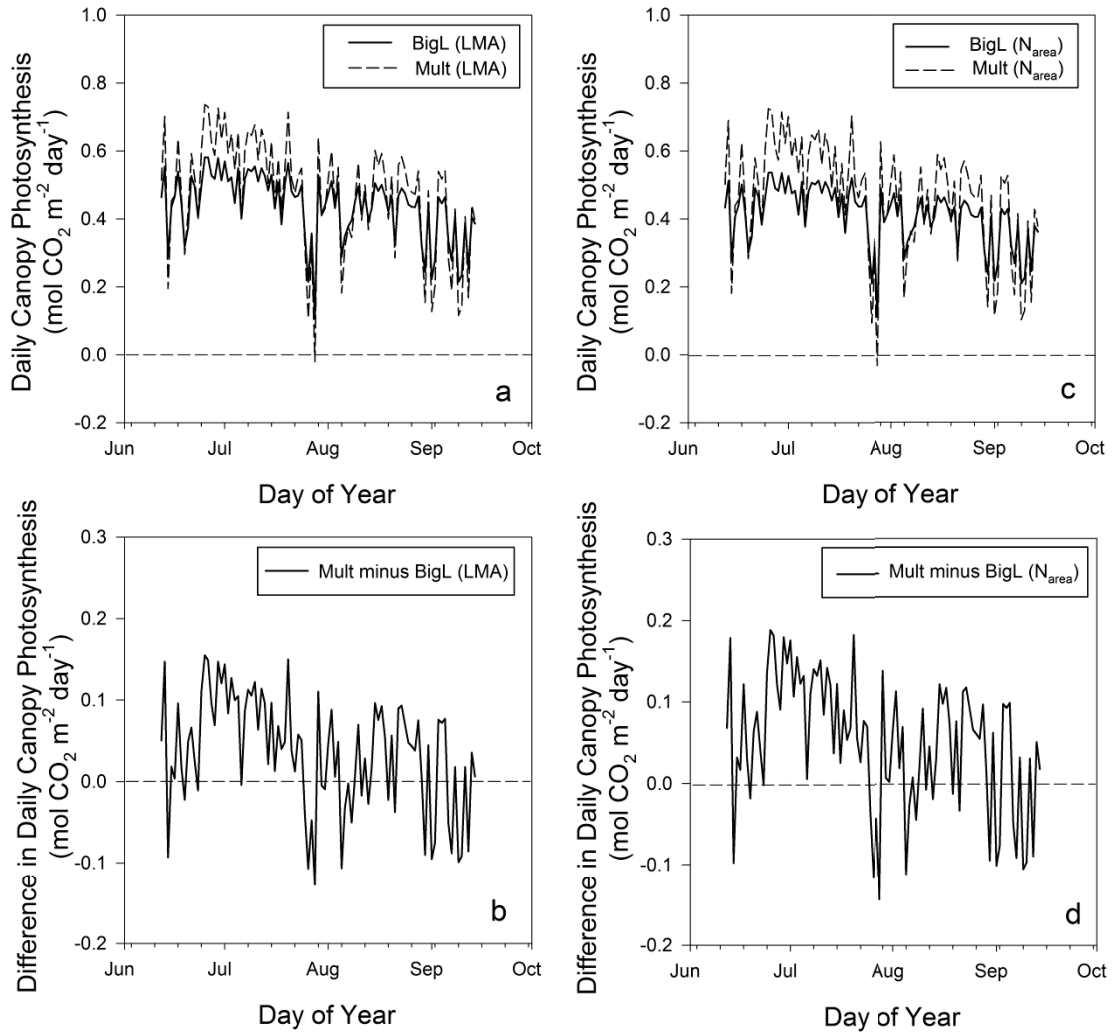


Figure 4.11. Simulated daily canopy photosynthesis (mol CO₂ m⁻² day⁻¹) using a model that did (Mult) and did not (BigL) account for vertical gradients in LMA (a) and N_{area} (c). Differences in daily canopy photosynthesis using models that did (Mult) and did not (BigL) account for vertical gradients in LMA (b) and N_{area} (d). The time period investigated does not include the period of leaf expansion and senescence. Differences between models (b and d) show bias when intra-canopy spatial variation is not considered.

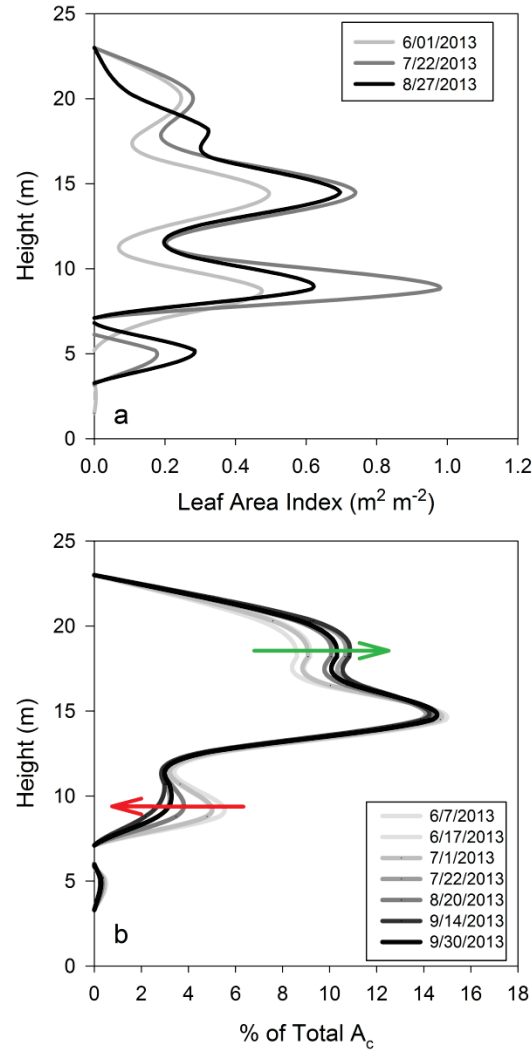


Figure 4.12. Distribution of leaf area index ($\text{m}^2 \text{m}^{-2}$) along the canopy profile at approximately 50% leaf expansion (6/01/2013), and after leaf expansion (7/22/2013, 8/27/2013; a). Distribution of the percentage (%) of total canopy photosynthesis (A_c) along the canopy profile throughout the entire growing season beginning at the end of leaf expansion and prior to leaf senescence as estimated from the Mult-Seas model (b). The green arrow indicates that the contribution to total A_c for leaves higher in the canopy is increasing over time, whereas the red arrow indicates that the contribution to total A_c for leaves lower in the canopy is decreasing over time.

4.11. Supporting Information

Leaf-level net photosynthesis (A_n) was modeled using a non-rectangular hyperbola (Hanson *et al.*, 1987):

$$A_n = A_{max} \left[1 - \left(1 - \frac{R_d}{A_{max}} \right)^{1 - \frac{I_a}{I_c}} \right], \quad (3)$$

Where A_{max} is the maximum photosynthetic capacity ($\mu\text{mol m}^{-2} \text{s}^{-1}$), I_a is the irradiance ($\mu\text{mol m}^{-2} \text{s}^{-1}$) at the leaf surface, R_d is leaf dark respiration ($\mu\text{mol m}^{-2} \text{s}^{-1}$), and I_c is the light compensation point ($\mu\text{mol m}^{-2} \text{s}^{-1}$) (Table 4.1).

An underlying assumption to the big-leaf model is that relative irradiance decreases with cumulative LAI (leaf area index, $\text{m}^2 \text{leaf m}^{-2} \text{ground}$):

$$I = I_0 e^{-kL^*}, \quad (4)$$

Where I is irradiance at the leaf surface ($\mu\text{mol m}^{-2} \text{s}^{-1}$), I_0 is irradiance above the canopy ($\mu\text{mol m}^{-2} \text{s}^{-1}$), k is the extinction coefficient, and L^* is the stand leaf area index (LAI). We used a big-leaf model (Raulier *et al.*, 1999) that integrates over the entire canopy in order to estimate canopy photosynthesis (A_c):

$$A_c = A_{max.0} \left[1 - \left(1 - \frac{R_{d.0}}{A_{max.0}} \right)^{1 - \frac{kI_0}{I_{c.0}}} \right] \frac{1 - e^{-kL^*}}{k}. \quad (5)$$

$A_{max.0}$, $R_{d.0}$, I_0 , and $I_{c.0}$ are A_{max} , R_d , I , and I_c , respectively, at the topmost unshaded leaves. We modified the multi-layer model as presented by Raulier *et al.* (1999) that scales leaf-level photosynthesis (Equation 3) to canopy photosynthesis using leaf area index at a given canopy layer (L_i):

$$A_c = \sum_i L_i A_n, \quad (6)$$

Where A_n is estimated using Equation 3.

For each sampling date, we fit a two-parameter exponential fit to LMA and N_{area} vs. height:

$$LMA = LMA_{min}e^{b_{LMA}Ht}, \quad (7)$$

$$N_{area} = N_{area.min}e^{b_N Ht}, \quad (8)$$

Where LMA_{min} and $N_{area.min}$ are the minimum LMA and N_{area} , b_{LMA} and b_N are the exponential coefficients that describe how LMA and N_{area} change with height, and Ht is height from the ground. This procedure was executed in order to assess changes in LMA_{min} , $N_{area.min}$, and the exponential coefficients that were used in modeling canopy photosynthesis throughout the growing season.

In order to predict A_{max} (required for equations 3 and 5), we incorporated Equations 7 and 8 with previously developed relationships between A_{max} , LMA, and N_{area} (Table 4.1):

$$A_{max} = \beta_0 + \beta_1(LMA_{min}exp^{b_{LMA}Ht}), \quad (9)$$

$$A_{max} = \beta_2 + \beta_3(N_{area.min}exp^{b_N Ht}); \quad (10)$$

Where β_0 and β_1 are the intercept and slope of the A_{max} vs. LMA relationships, respectively, and β_2 and β_3 are the intercept and slope of the A_{max} vs. N_{area} relationships, respectively. For big-leaf models (Equation 5), we used a height of 20.6 m that corresponded with the topmost unshaded leaves (Fig. 1; Kull & Jarvis, 1995; Raulier *et al.*, 1999). For multi-layer models, we estimated A_{max} per height interval. For models that did not account for seasonal changes in LMA and N_{area} , we used mid-summer values (July 22, 2013), herein referred to as ‘reference’ LMA or N_{area} . For models that accounted for seasonal changes in LMA and N_{area} , the variables allowed to vary over time were LMA_{min} , $N_{area.min}$, b_{LMA} , and b_N which were interpolated between sampling dates for

daily estimates. Leaf dark respiration (R_d) was predicted using relationships developed between A_{\max} and R_d , and I_c was predicted using relationships developed between R_d and I_c from data pooled across multiple studies (Table 4.1, Raulier *et al.*, 1999). The driving environmental variable of these models, irradiance at the leaf surface (I_a), was modeled from May 11th through October 19th using Equation 4. The extinction coefficient (k) of 0.5 was used for both models (Raulier *et al.* 1999).

5. Vertical gradients in *Acer saccharum* leaf cellular structure

display a trade-off between palisade layer thickness and mesophyll porosity

5.1. Abstract

Attributes of leaf cellular structure, such as leaf mesophyll porosity (proportion of air-space in leaf mesophyll) and palisade layer thickness, have been linked to mesophyll conductance of CO₂ and light capture, respectively. Our objectives were to investigate within-canopy gradients in both mesophyll porosity and palisade layer thickness in order to understand environmental limitations to leaf development at exposed and closed canopy positions in a sugar maple (*Acer saccharum*) forest. Variation in palisade layer thickness corresponded with light, whereas mesophyll porosity appeared to be linked with both height and light. Palisade layer thickness displayed stronger correlations with leaf mass per area (LMA), leaf density, and leaf thickness than did mesophyll porosity, indicating that light was strongly influencing gradients in leaf morphology in this broad-leaved deciduous tree species. For leaves growing in high light availability, large and tightly-packed palisade cells that comprised much of the leaf mesophyll likely contributed to greater leaf density and lower mesophyll porosity. Height appeared to influence epidermal cell width and mesophyll porosity, but the pattern of increasing epidermal cell width with height was unexpected and does not support the hypothesis that expanding epidermal cells and associated forces contributed to the creation of air-space in the mesophyll. While height appeared to limit the development of intercellular air-spaces in the mesophyll, this likely did not constrain photosynthesis for upper canopy leaves

given the commonly observed pattern of increasing photosynthetic capacity with increasing light and LMA in *A. saccharum*. Overall, decreasing mesophyll porosity with increasing palisade thickness indicates that there may be a trade-off between mesophyll conductance of CO₂ and light capture for *Acer saccharum* leaves.

5.2. Introduction

Within forest canopies, leaf cellular structure strongly influences leaf physiological processes, such as gas exchange and light absorption. The primary function of palisade cells is light capture via chlorophyll in chloroplast, and the structure of palisade cells facilitates light transmittance to greater depths in the spongy mesophyll to maximize overall absorption of light (Cui et al. 1991, Vogelmann and Martin 1993, Evans 1999). Increasing palisade layer thickness accompanied by increasing leaf thickness, leaf mass per area (LMA, g m⁻²), and photosynthetic capacity with greater light availability is a common pattern observed in greenhouse experiments (Chabot and Chabot 1977, Chabot et al. 1979, Oguchi et al. 2005, Tosens et al. 2012). Similarly, vertical gradients in both leaf thickness and LMA scales with natural light gradients in forest canopies (Hutchison et al. 1986, Hollinger 1989, Ellsworth and Reich 1993, Niinemets 1997, Niinemets et al. 1998, Bond et al. 1999, Niinemets et al. 1999, Niinemets et al. 2001, Coble and Cavaleri 2014), which are likely influenced by adjustments in palisade layer thickness. Thicker palisade layers in response to high light also corresponds with greater surface area of mesophyll palisade cells exposed to intercellular air-spaces, and this response has led to greater photosynthetic capacity and

mesophyll conductance of leaves (Nobel et al. 1975, Nobel et al. 1977, Hanba et al. 2002, Kenzo et al. 2004).

Likewise, leaf mesophyll porosity (proportion of air-space in leaf mesophyll) is an important component to cellular structure and leaf function. Reduced air-space can constrain mesophyll conductance, defined as diffusion of CO₂ from substomatal cavities to sites of carboxylation within chloroplasts (Parkhurst 1994, Syversten et al. 1995, Flexas et al. 2008, Marchi et al. 2008, Gu et al. 2010), which in turn, can be a major limitation to photosynthesis. Under drought conditions, increasing mesophyll conductance of CO₂ optimizes water-use efficiency (Hommel et al. 2014), and mesophyll conductance is equally as important as stomatal conductance in limiting forest carbon uptake (Keenan et al. 2010). In tall *Sequoia sempervirens* trees, reduced mesophyll porosity higher in the canopy due to hydraulic limitations on leaf development has resulted in declining mesophyll conductance with height, which was an important factor in limiting photosynthesis (Mullin et al. 2009, Oldham et al. 2010). Hydraulic constraints on leaf development in tall western conifer species may be caused by decreasing turgor pressure with height due to limited osmotic adjustments higher in the canopy (Woodruff et al. 2004). Since leaf turgor pressure is necessary for cell expansion and division and leaf expansion (Hsiao 1973), reduced turgor pressure can lead to the formation of small and densely packed cells and reductions in intercellular air space (Oldham et al. 2011). Thus, reduced mesophyll porosity higher in the canopy generally corresponds with smaller leaves with greater LMA and leaf density (Koch et al. 2004, Ishii et al. 2008). In contrast, limitations to photosynthesis higher in the canopy for two deciduous species (*Fagus sylvatica* and *Quercus petraea*) during drought conditions were primarily due to

constrained stomatal conductance, whereas mesophyll conductance was an important limitation lower in the canopy (Cano et al. 2013).

Leaf epidermal cells may play a role in leaf expansion and subsequent formation of mesophyll cells. Prior to leaf expansion, leaf primordia contain little to no air-space, and mesophyll cells are tightly packed without any differentiation (Eschrich et al. 1989). During leaf expansion, cells differentiate into epidermal, palisade, and spongy mesophyll cells (Eschrich et al. 1989, Tosens et al. 2012) that increase in size during the development of intercellular air-spaces (Dale 1988, Knight and Roberts 1994, Marchi et al. 2008, Zhang et al. 2011b, Tosens et al. 2012). Given this observed pattern, we would expect that a driving force would be required to separate spongy mesophyll cells. Avery (1933) considered intercellular air-space to be formed by expanding epidermal cells that separated spongy mesophyll cells, while Jeffree et al. (1986) provides evidence that this view is oversimplified because initial signs of air-space and cell separation at the junction of cells is formed by the breakdown of cell walls. Both Jeffree et al. (1986) and Jarvis et al. (2003) suggested that the primary driving force of subsequent formation of larger intercellular air space is cell turgor pressure or mechanical forces in rapidly expanding leaves; however, the specific cells involved in creating air-space are unclear.

The main objectives of this study were to investigate environmental drivers of leaf cellular structure, palisade layer thickness and mesophyll porosity, and to assess inter-related anatomical and morphological traits to identify potential mechanisms behind gradients in leaf morphological traits in a broad-leaved deciduous forest (*Acer saccharum*). Within the same stand, Coble and Cavaleri (2014) concluded that light was the primary driver of vertical gradients in LMA, leaf density, and thickness. While there

was evidence of height-related constraints on leaf morphology early in the growing season, morphological acclimation to light appeared to optimize leaf functional traits (N_{area} and LMA) over time (Coble and Cavaleri 2015). Considering these changes in leaf morphology as observed throughout the growing season, our aim was to reduce any bias associated with seasonal changes in *Acer saccharum* leaf anatomy by estimating time-integrated light availability that occurred during the life-span of each leaf following leaf expansion and prior to leaf collection. In this study we will test the following hypotheses: 1) Light primarily drives palisade layer thickness, which corresponds with variation in LMA; and 2) Height constrains the formation of intercellular air-space, leading to reduced mesophyll porosity and denser leaves, as a result of limitation on expansion of epidermal cells.

5.3. Methods

5.3.1. Study site

The study was conducted in an *Acer saccharum* forest at the Michigan Technological University Ford Center and Forest near Alberta, MI, USA (46.65°N, 88.48°W). The mean annual temperature and precipitation at the Ford Forestry Center are 4.8 °C and 810 mm, respectively (NOAA, WS ID 15608). The *A. saccharum* stand was the ‘uncut control’ stand of a study initiated in 1956, which consisted of nine silvicultural experimental trials (Erickson et al. 1990). This stand also included *Betula alleghaniensis*, *Ostrya virginiana*, *Tilia americana*, and *Ulmus americana*. The tree density of *Acer saccharum* was 259 trees ha⁻¹, which was 97% of the tree density of the stand (267 tree ha⁻¹). The mean height of the stand was 23.0 m and the basal area was 33

$\text{m}^2 \text{ha}^{-1}$. In a closed canopy portion of this stand, three cable zip-lines provided canopy access from 0 to 15 m in height along three two-dimensional planes. Arborist climbing techniques were used to access the canopy above the zip-lines (>15 m). A 19-m aluminum walk-up tower was constructed 55 meters from the zip-lines in the same stand in August 2012 in order to access portions of tree crowns that were exposed to brighter light conditions at lower heights due to a canopy opening. At both sites, we used a telescoping pole-pruner to collect leaves at the tops of trees (up to 30 m). The tower and zip-line sites will be herein referred to as the ‘exposed canopy’ and the ‘closed canopy’, respectively. More information about the site history and methodology can be found in Coble and Cavaleri (2014).

5.3.2. Height and Light Measurements

Height above the ground at each sampling location was measured using a tape measure. Light conditions or ‘canopy openness’ at the exposed and closed canopies were measured as diffuse non-interceptance (DIFN), the fraction of radiation that is transmitted through the canopy (Norman and Welles 1983), using two plant canopy analyzers (LAI-2000 and LAI-2200, LI-COR, Inc., Lincoln, NE, USA). Light measurements were made during overcast conditions or following sunrise until 1 hour following sunrise. Open sky measurements (proxy for ‘above-canopy’ light conditions) were collected in a nearby open field (~ 400 m from the site) using the LAI-2000 mounted on a tripod at 30 second intervals, and below-canopy measurements were collected using the LAI-2200. Prior to and following below-canopy measurements, open sky measurements were collected with the LAI-2200 next to the LAI-2000 in order to calibrate open measurements collected by

the LAI-2000. At each sampling point, we collected two light measurements and used a 180° view cap to prevent climbing ropes and the tower from obstructing the view. The average of both light measurements at each sampling point was used for further analysis. We used FV2200 software (LI-COR, Inc., Lincoln, NE, USA) to adjust open sky measurements and to estimate DIFN by matching open sky and below-canopy readings closest in time. For leaves collected at the top of trees (pole-pruner collection), we assumed a DIFN of 1 (i.e., 100% canopy openness) because light measurements using the LAI-2200 were not possible for these leaves. From May 10 to October 19, we measured open sky photosynthetic photon flux density ($PPFD_{above}$, $\mu\text{mol m}^{-2} \text{s}^{-1}$) at 10-min intervals using a light sensor (S-LIA-M003, Onset Computer Corporation, Bourne, MA) in a nearby open field ~400 m from the site.

We estimated integrated photosynthetic photon flux density ($PPFD_{INT}$ mol m^{-2}) over the life-span of leaves at each sampling location in order to account for seasonal changes in leaf morphology due to light acclimation (Coble and Cavaleri 2015) with the following equation:

$$PPFD_{INT} = \sum_{\text{Harvest}}^{50\% \text{ Expansion}} DIFN * PPFD_{above} \quad \text{Eq. 1}$$

We assumed that DIFN was equal to the fraction of PPFD transmitted through the canopy, which has been shown in a previous study (Machado and Reich 1999).

5.3.3. Leaf morphology

We collected one leaf per sampling location following leaf expansion until the end of August at the closed-canopy and at the end of August at the exposed canopy. Leaf sampling occurred later at the exposed canopy because the tower was constructed in

August. Immediately following collection, leaves were placed in sealable, plastic bags with a moist paper towel and temporarily stored in an ice chest prior to bringing leaf samples to the lab. Leaves were then stored at 2 °C until leaf morphology measurements were made. Leaf area was measured by scanning leaves with a bench-top leaf area meter (LI-3100, LI-COR, Inc.). Leaf volume was derived by immersing fresh leaves in a beaker of water placed on a balance and using Archimede's principle (Coble and Cavaleri 2014). Leaves were dried at 65 °C for 48 hours and weighed to the nearest 0.1 mg. LMA was calculated as the leaf dry mass (g) divided by leaf area (m²), and density was calculated as the leaf dry mass (g) divided by leaf volume (cm³).

Leaf density can be estimated by dividing LMA by leaf thickness. To compare this estimation with our measured leaf density, we plotted leaf density values as derived from laboratory measurements (measured density) against leaf density values as derived from LMA and thickness (estimated density; Figure 5.1). There was a strong correlation between both leaf density values from the same leaves ($r^2 = 0.65$); however, the measured leaf density values appeared to be greater than estimated values, particularly at low leaf density (Figure 5.1). We used both values of leaf density for subsequent analysis because measured values may be biased due to small air-bubbles that form along the leaf cuticle (Coble and Cavaleri 2014) and estimated values may be biased because leaf veins in *Acer saccharum* are thicker than photosynthetic tissue and were not included in leaf thickness measurements.

5.3.4. Leaf anatomy

Prior to leaf morphology measurements, a small section of leaf (~16 x 8 mm) was cut from the right lobe of each leaf and temporarily stored in formaldehyde - acetic acid – ethanol - water solution (10:5:50:35, by vol.; Reinoso et al. 2002). Leaf sections were further divided into two to three sections using a scalpel and embedded in paraffin to create a block. Leaf specimens in each block were cut at 5 μm , perpendicular to the leaf adaxial surface, using a microtome (Finesse 325, Thermo Shandon, Pittsburgh, PA, USA). This was repeated two times for each block to produce six to nine leaf transverse sections. Between each leaf section, 200 μm of the block was sliced off. Leaf transverse sections were mounted on slides, stained with hematoxylin and eosin in an automatic stainer (Model Linistain GLX, Thermo Shandon), and covered with a coverslip. Ten images at 20x magnification were collected from three leaf transverse sections per leaf (i.e., one transverse section per microtome cut) using a microscope (Eclipse E400, Nikon, Inc., Melville, NY, USA) with a camera (Leica DFC295, Leica Microsystems, Buffalo Grove, IL, USA) mounted above the objective lenses. We randomly selected one image from each leaf transverse section used in the analysis.

ImageJ software (Schneider et al. 2012) was used for all image analysis. For each image, we created five evenly spaced vertical lines that were randomly offset. Palisade layer thickness (μm) and horizontal width of epidermal cells (μm) were measured (Figure 5.2a) at each of the vertical lines and averaged to obtain one palisade layer thickness and epidermal cell width measurements per leaf. Mesophyll porosity, defined as the area occupied by intercellular air-space divided by the total area occupied by the leaf cross section (Oldham et al. 2012), was estimated from the same set of images. To estimate

mesophyll porosity, images were first converted to a 32-bit gray-scale image, and the image threshold was adjusted to the point just prior to the rapid increase in the color histogram in order to convert to a binary image (Oldham et al. 2012). All cells were filled in with black using the ‘brush tool’ and the intercellular air-space was kept white (Figure 5.2b). The image was again converted to a black and white image, and the area of the cells (black area) was measured. The image was inverted so that the black area became white, and we removed the black area that was not part of the leaf, so that only the air-space was black (Figure 5.2c). The area of the air-space was measured, and the area of the air-space and the cells were added to estimate the total leaf cross-sectional area.

5.3.5. Data analysis

Statistical analyses were conducted using R statistical software (R Development Core Team 2013). Relationships between palisade layer thickness (μm), mesophyll porosity, epidermal cell width (μm), height (m), and light (PPFD_{INT} , mol m^{-2}) were examined using regression analysis. Comparisons of these relationships between the two canopy types (closed and exposed canopy) were made using ANCOVA. We assessed the contribution of light and height to the full model for predicting palisade layer thickness, mesophyll porosity, and epidermal cell width using partial R^2 analysis. Leaf anatomical parameters and light were natural log-transformed (\ln) to satisfy regression assumptions of linearity and homoscedasticity and to develop linear models for ANCOVA and partial R^2 analysis. We also assessed the contribution of palisade layer thickness and mesophyll porosity to the full model for predicting LMA and leaf density (estimated and measured)

using partial R^2 analysis. We compared a subsample of palisade thickness values collected only in August at the closed ($n = 6$) and exposed ($n = 7$) at 17-21 m in height using one-way ANOVA to assess the timing of sample collection as a possible source of differences between canopy types.

5.4. Results

5.4.1. Leaf anatomy relationships with height and light

To assess the influence of environmental factors on leaf anatomy, we compared relationships among light, height, palisade layer thickness, epidermal cell width, and mesophyll porosity between the exposed and closed canopy positions. $PPFD_{INT}$ increased more rapidly with height at the exposed canopy compared with the closed canopy (Figure 5.3). Similarly, palisade layer thickness exponentially increased with height at the exposed and closed-canopies (Figure 5.4, 5.5a), and the slope of the height and palisade layer thickness relationship was steeper at the exposed canopy (Table 5.1). Palisade layer thickness increased non-linearly (log-linear) with light for both canopy types (Figure 5.5b). The slopes and intercepts of the light and palisade layer thickness relationship were significantly different (Table 5.1). The partial R^2 analysis showed that for palisade layer thickness, light contributed more to the full model than height (Table 5.2). In our post hoc analysis of palisade layer thickness at the exposed and closed canopies at 17-21 m for August, we found that palisade layer thickness at the exposed canopy was significantly greater ($P < 0.01$) than at the closed canopy.

Mesophyll porosity exponentially decreased with height at both canopy types (Figure 5.5c), and the slopes of this relationship did not differ between canopy types.

Mesophyll porosity displayed a negative log-linear decrease with light (Figure 5.5d), and the slopes did not differ between canopy types (Table 5.1). The partial R^2 analysis showed that for mesophyll porosity, light and height equally contributed to the full model (Table 5.2). Epidermal cell width exponentially increased with height at both canopy types (Figure 5.5e), and the slopes of these relationships did not differ among canopy types (Table 5.1). Epidermal cell width non-linearly (log-linear) increased with light at both canopy types (Figure 5.5f), the slopes of these relationships did not differ among canopy types, and the intercepts of these relationships were different among canopy types (Table 5.1). The partial R^2 analysis showed that for epidermal cell width, height contributed more to the full model than light (Table 5.2).

5.4.2. Inter-related morphological and anatomical traits

We investigated relationships between anatomical and morphological traits to identify potential mechanisms behind gradients in LMA and leaf density, as well as mechanisms associated with the creation of intercellular air-space (mesophyll porosity). Mesophyll porosity decreased with increasing epidermal cell length at both canopy types (Figure 5.6a). Mesophyll porosity index also decreased with increasing palisade layer thickness at both canopy types (Figure 5.6b). We also assessed inter-related morphological and anatomical traits to identify association of anatomical structure (palisade layer thickness and mesophyll porosity) with LMA and density using partial R^2 analysis (Table 5.3). Palisade layer thickness was positively correlated with LMA, density, and thickness, and mesophyll porosity was negatively correlated with LMA,

density, and thickness (Table 5.3). Palisade layer thickness contributed more to the full model compared with mesophyll porosity for LMA, density, and thickness (Table 5.3).

5.5. Discussion

5.5.1. Patterns and mechanisms associated with palisade layer thickness

Our results support our first hypothesis that light primarily drives palisade layer thickness, which corresponded with variation in LMA. Cumulative light availability increased more rapidly with height at the exposed canopy above 10 m in height compared with the closed canopy. Similarly, we observed a greater increase in palisade layer thickness above 10 m at the exposed canopy compared with the closed canopy, suggesting that light is driving changes in palisade layer thickness. LMA, density, and thickness were more strongly correlated with palisade layer thickness compared to mesophyll porosity, highlighting the strong role of light in determining leaf morphology. Our results are consistent with other studies that have found that light availability strongly influences within-canopy variation in leaf morphology in *A. saccharum* (Ellsworth and Reich 1993, Sack et al. 2006, Jones and Thomas 2007, Coble and Cavaleri 2014, Coble and Cavaleri 2015). While there is evidence that thickness and density may respond independently to different environmental factors (Witkowski et al. 1991, Niinemets 2001), there are instances where both respond to similar environmental factors such as light availability (Witkowski et al. 1991, Niinemets et al. 1999, Coble and Cavaleri 2014), which may be due to more densely packed palisade cells in leaves growing in higher light availability (Niinemets et al. 1999). We speculate that more

densely packed palisade cells for leaves growing in high light availability, as observed in this study (Figure 5.2), resulted in greater leaf density.

In the same stand, Coble and Cavaleri (2015) found that LMA and density increased throughout the growing season, suggesting that palisade thickness may also increase following leaf expansion. Vertical elongation of palisade cells following full leaf expansion has been reported in numerous species indicating that leaves continue to thicken after achieving a final area (Miyazawa and Terashima 2001, Miyazawa et al. 2003, Yano and Terashima 2004). Our previous conclusions regarding the strong influence of light on LMA and leaf thickness were likely not biased by seasonal changes in LMA because we specifically tested for this and found that LMA was greater at higher light availability compared with lower light for leaves collected at similar heights and during the same week (Coble and Cavaleri 2014). In this study, we accounted for seasonal increases in leaf anatomy by using time-integrated irradiance ($PPFD_{INT}$), which showed strong correlations with palisade thickness.. This suggests that $PPFD_{INT}$ may serve as a useful parameter that incorporates light availability and seasonal changes in leaf morphology and function.

5.5.2. Patterns and mechanisms associated with mesophyll porosity

Our results provide partial support for our second hypothesis that height constrains the development of intercellular air-space, but do not support the hypothesis that reduced mesophyll porosity was due to height limitations on expansion of epidermal cells. Mesophyll porosity decreased with height within both canopy types and this relationship did not differ among canopy types that differed in light availability. Our

results were consistent with the findings of Oldham et al. (2010) who observed a strong linear decrease in mesophyll porosity with height in extremely tall (100+ m) *Sequoia sempervirens* trees despite sampling leaves from the inner (shaded) and outer (exposed) crowns that differed in light availability. There is evidence that light only influences LMA lower in the canopy where light is limiting, whereas height appears to influence LMA higher in the canopy in tropical forests, western conifer trees, and in *Eucalyptus* plantations (Ishii et al. 2008, Cavaleri et al. 2010, Coble et al. 2014). Height is often used as a proxy for gradients in leaf water potential because leaf water potential, in the absence of transpiration, declines linearly height with due to gravity (Scholander et al. 1965, Hellkvist et al. 1974, Bauerle et al. 1999). In tall western conifer trees such as *S. sempervirens* and *Pseudotsuga menziesii*, leaf water potential gradients have been implicated as a key limitation to leaf development, leaf morphology, and gas exchange (Koch et al. 2004, Woodruff et al. 2004, Ishii et al. 2008, Mullin et al. 2009). In *A. saccharum*, Coble and Cavaleri (2015) found that height-related constraints on leaf morphology were prevalent earlier in the growing season, but these constraints were overcome by morphological acclimation to light during leaf maturation.

Our results suggest that larger epidermal cells that experienced greater increases in expansion did not correspond with the creation of mesophyll air-space (Figure 5.5a). In this study, thicker leaves also appeared to have more area occupied by vascular tissue and surrounding collenchyma tissue (Figure 5.2: Exposed canopy, 22-23 m), which may also contribute to reduced mesophyll porosity. Lower mesophyll porosity higher in the canopy also corresponded with greater palisade layer thickness, which comprised much of the total cross-sectional leaf area, particularly at high light. This may partially explain

why height and light were equally as important in determining mesophyll porosity (Table 5.2). Similarly, Tosen et al. (2012) found that light and leaf water-stress were important in influencing mesophyll porosity in *Populus tremula*.

Our results point to an alternative hypothesis that thicker palisade layers in high light occupy more space and reduce mesophyll porosity. Reduced air-space in leaves and thicker cell walls have been found to be important limitations to mesophyll conductance (Loreto et al. 1992, Parkhurst 1994, Syvertsen et al. 1995, Niinemets 1999, Flexas et al. 2008, Gu et al. 2010). Reduced mesophyll porosity in leaves higher in the canopy has been considered one of the primary reasons for decreasing mesophyll conductance with height in tall *Sequoia sempervirens* trees (Mullin et al. 2009, Oldham et al. 2010). Flexas et al. (2008) found that LMA set an upper limit to mesophyll conductance, where leaves with greater LMA constrained mesophyll conductance. Consistent with these findings, our study shows that mesophyll porosity tended to be lower in leaves with a thicker palisade layer and LMA appeared to be strongly associated with palisade layer thickness (Table 5.3). Thus, there may be a trade-off between light capture and mesophyll conductance, if we assume that greater air-space leads to greater CO₂ conductance in the mesophyll.

However, mesophyll conductance is not always constrained by reduced by air-space, in fact, leaves with reduced mesophyll porosity may have greater mesophyll conductance. In leaves with lower mesophyll porosity, development of thicker palisade layers in high light also correspond greater surface area of mesophyll cells exposed to intercellular air-spaces, which has been found to increase mesophyll conductance in numerous species (Hanba et al. 1999, Hanba et al. 2002, Tosens et al. 2012). Thus,

thicker palisade layers and greater surface area of mesophyll cells exposed to air-space corresponded with greater photosynthetic capacity in leaves of three *Acer* species and *Populus tremula* growing in high light (Hanba et al. 2002, Tosens et al. 2012). Given that LMA is strongly correlated with photosynthetic capacity (Ellsworth and Reich 1993, Jones and Thomas 2007) and palisade layer thickness (this study) in *A. saccharum*, the decline in mesophyll porosity at higher heights likely does not constrain photosynthesis via reduced mesophyll conductance.

5.5.3. Conclusions and Implications

Our study highlights the strong influence of light availability on palisade layer thickness, and consequent effects on LMA, leaf thickness, and leaf density. Results from this study and other studies in *A. saccharum* suggest that increasing palisade thickness with light may be the primary structural adjustment that increases light capture and photosynthetic capacity along natural light gradients. Leaf mesophyll porosity appeared to be constrained by both height and high light availability. Contrary to our expectations, epidermal cell expansion did not appear to be constrained by height, and therefore did not explain decreasing mesophyll porosity with height. Rather, we show that as palisade cells increase in size and number, they occupy more leaf volume and decrease air space inside the leaves, suggesting that there is a trade-off between palisade layer thickness and mesophyll porosity in *A. saccharum*. Reduced air-space may limit mesophyll conductance of CO₂, but this may be counter-balanced by increasing surface area of palisade mesophyll cells, which has been found to increase mesophyll conductance. Overall, the results from this study shows that light strongly influences leaf

morphological and anatomical structure and supports previous research that has investigated environmental factors on leaf morphology and function in *A. saccharum* canopies. Also, our results suggest that integrated PPFD is a useful parameter for predicting leaf traits because it incorporates both light-availability within the canopy and seasonal effects of light acclimation.

5.6. References

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5.7. Tables and Figures

Table 5.1. Summary of ANCOVA results for test of height, canopy type (CT), and height x CT effects on palisade layer thickness, epidermal cell length, and mesophyll porosity; and (ln)light (PPFD_{INT}; mol m⁻²), CT, and light x CT effects on palisade layer thickness, epidermal cell length, and mesophyll porosity. Degrees of freedom (df), mean square, F-ratio, and level of significance are listed for main, interaction, and error terms. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Source	(ln)Palisade Layer Thickness (μm)			(ln) Epidermal Cell Length (μm)			(ln) Mesophyll Porosity		
	df	Mean square	F-ratio	df	Mean square	F-ratio	df	Mean square	F-ratio
Height	1	4.84	259.4***	1	3.57	178.2***	1	3.60	51.4***
CT	1	0.75	40.1***	1	0.01	0.3	1	0.40	5.7*
Height x CT	1	0.76	41.0***	1	0.06	2.8	1	0.06	0.8
Error	83	0.02		83	0.02		83	0.07	
(ln)Light	1	5.56	248.7***	1	1.89	56.6***	1	3.61	50.4***
CT	1	0.22	5.23**	1	0.63	18.9***	1	0.22	3.0
(ln)Light x CT	1	0.15	3.21*	1	0.00	0.0	1	0.08	1.1
Error	83	0.02		83	0.03		83	0.07	

Table 5.2. Partial R^2 analysis for relationships among palisade layer thickness (μm), epidermal cell width (μm), mesophyll porosity (\ln) light (PPFD_{INT} , mol m^{-2}), and height (m). Sample size, regression coefficients, and R^2 values are displayed for the log-linear regressions.

Response variable	n	(ln)Light Only			Height Only			Light and Height			Partial R^2 for adding:	
		β_0	β_1	R^2	β_0	β_1	R^2	β_0	β_1	β_2	Light	Height
(ln)Palisade Layer Thickness	86	2.45***	0.21***	0.70	3.26***	0.03***	0.61	2.64***	0.14***	0.01***	0.16	0.07
(ln)Mesophyll Porosity	86	-1.15***	-0.17***	0.37	-1.78***	-0.03***	0.36	-1.34***	-0.10**	-0.02**	0.06	0.06
(ln)Epidermal Cell Width	86	2.27***	0.12***	0.36	2.62***	0.03***	0.67	2.61***	0.00	0.03***	0.00	0.32

Table 5.3. Partial R² analysis for relationships among palisade layer thickness (μm), mesophyll porosity, LMA (g m⁻²), estimated and measured density (g cm⁻³), and thickness (μm). Sample size, regression coefficients, and R² values are displayed for the log-linear regressions.

Response variable	n	Palisade Layer Thickness Only			Mesophyll Porosity Only			Palisade Thickness and Mesophyll Porosity			Partial R ² for adding:	
		β_0	β_1	R ²	β_0	β_1	R ²	β_0	β_1	β_2	Palisade Thickness	Mesophyll Porosity
(ln)LMA (g m ⁻²)	86	2.79***	0.03***	0.90	4.53***	-5.47***	0.42	2.94***	0.02***	-0.74*	0.49	0.00
(ln)Density (g cm ⁻³) Measured	86	-2.12***	0.02***	0.60	-0.51***	-5.42***	0.36	-1.77***	0.02***	-1.68*	0.27	0.02
(ln)Density (g cm ⁻³) Estimated	86	-1.04***	0.01***	0.42	-0.51***	-1.91***	0.32	-0.86***	0.01***	-0.88*	0.14	0.04
Thickness (μm)	86	17.36***	2.05***	0.89	151.09***	-385.12***	0.32	10.88	2.12***	31.45	0.58	0.00

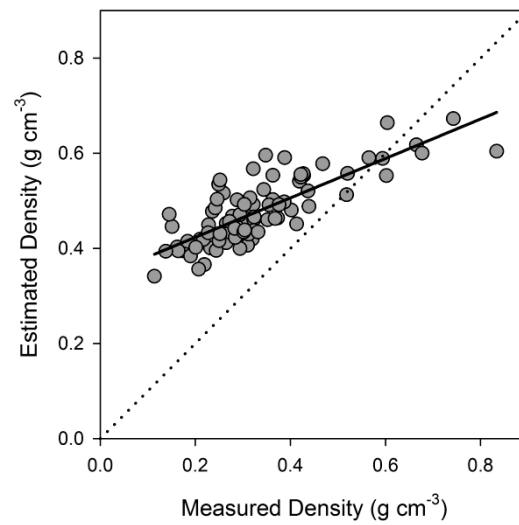


Figure 5.1. Relationship between estimated density and measured density with the 1:1 line (dashed line). Estimated density was calculated as $\text{Density} = \text{LMA}/\text{Thickness}$, and measured density was derived from leaf volume and dry mass measurements.

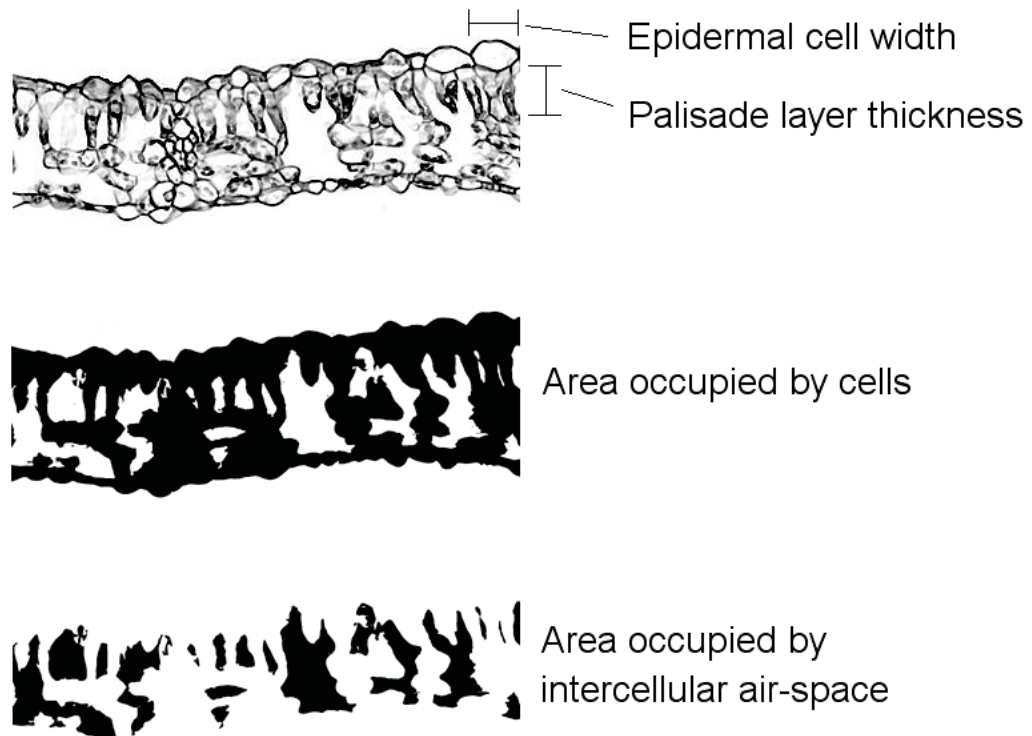


Figure 5.2. Image showing measurements of epidermal cell width and palisade layer thickness (a). All cellular area was filled-in and converted to a black-and-white, binary image (b). The image was then inverted so that the area occupied by intercellular air-space was black for measurements of total intercellular air-space (c). Mesophyll porosity was estimated by dividing the area occupied by intercellular air-space by the total area of the leaf cross section (cellular area plus area of air-space).

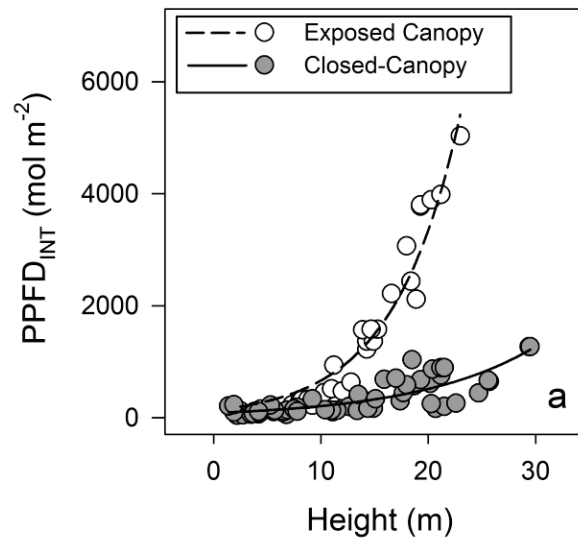


Figure 5.3. Relationship between PPFD_{INT} (mol m⁻²) and height (m) at the exposed and closed canopy for sampled leaves. PPFD_{INT} represents the time integrated light conditions over the life-span of sampled leaves beginning at 50% leaf expansion and ending at leaf collection.

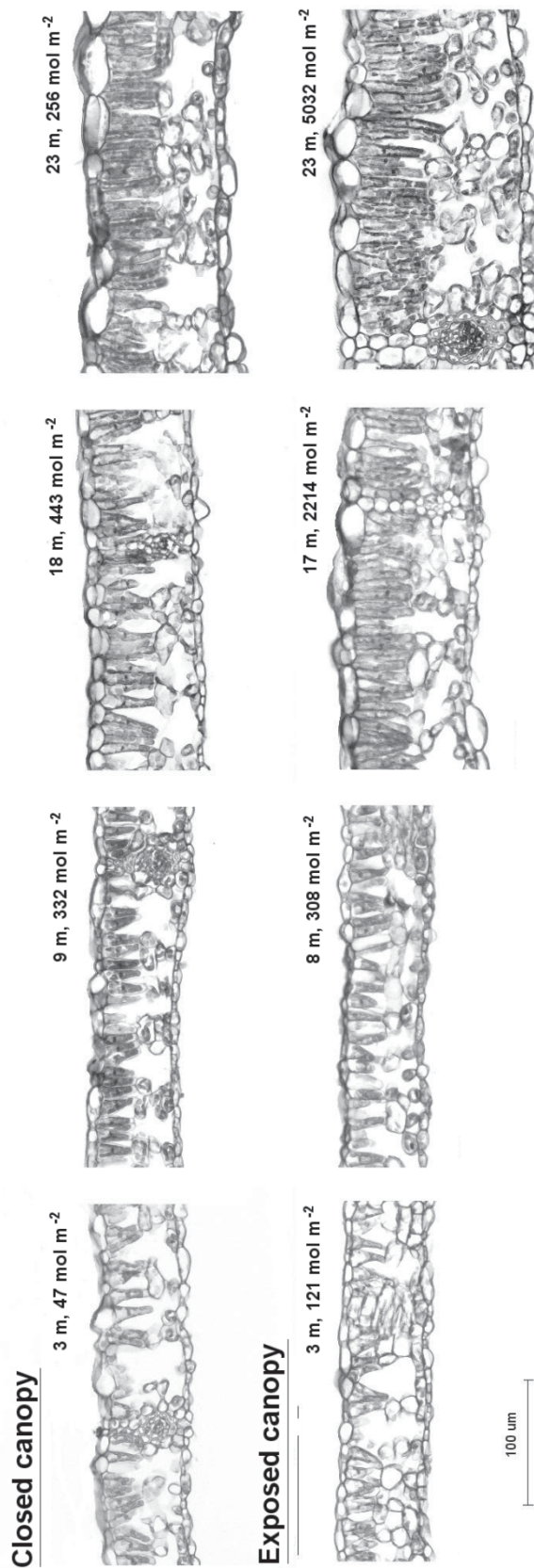


Figure 5.4. Leaf anatomical images for leaves at the closed- and exposed canopy at four heights (2-3, 8-9, 17-18, 22-23 m).

PPFD_{INT} is also listed next to the height of each leaf.

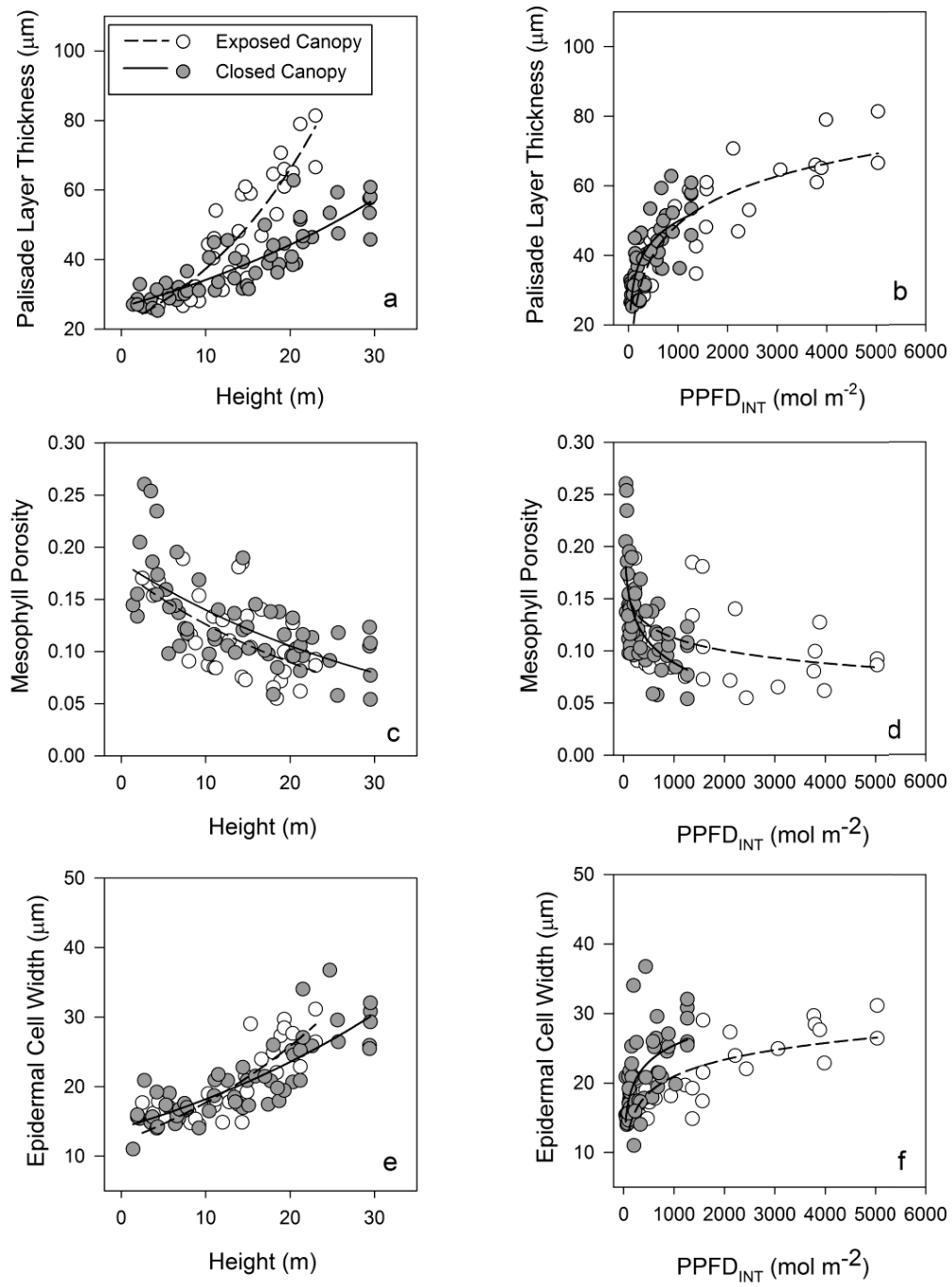


Figure 5.5. Relationships between height, PPFD_{INT} , palisade layer thickness (a, b), mesophyll porosity (c, d), and epidermal cell width (e, f) at the exposed and closed canopy.

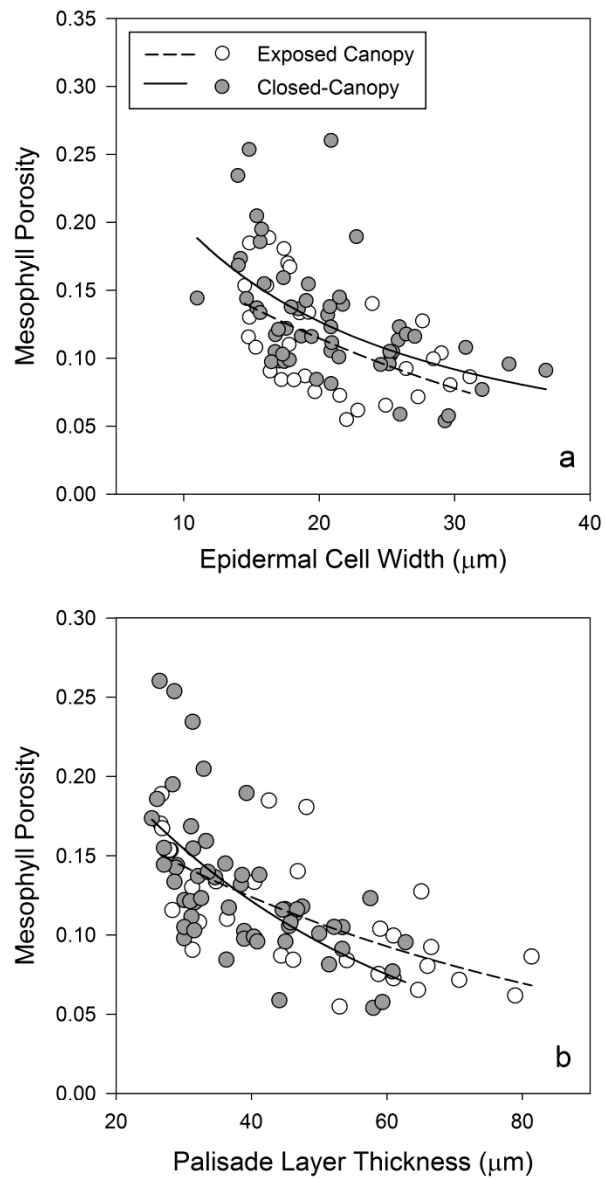


Figure 5.6. Relationships between mesophyll porosity, epidermal cell width (a), and palisade layer thickness (b) for the exposed and closed canopy.

6. Conclusions

From landscape to global scales, leaf functional traits (LMA and leaf nitrogen) are strongly correlated with leaf photosynthesis (Reich et al. 1999, Wright et al. 2004). Thus, simple leaf traits that are easy to measure can be used to predict more difficult traits, which can facilitate scaling from leaf to ecosystem-level processes and modeling landscape to global estimates of gross primary productivity [(GPP); Wright et al. 2004, Thornton and Zimmerman 2007, Ollinger et al. 2008, Hollinger et al. 2010, Ryu et al. 2011]. Within forest canopies, the commonly observed pattern of increasing LMA and area-based leaf nitrogen (N_{area}) with height has traditionally been viewed as a consequence of natural light gradients, where leaf carbon and nitrogen are optimally distributed to maximize canopy photosynthesis (Hollinger 1989, Ellsworth and Reich 1993, Bond et al. 1999, Niinemets et al. 1999, Sack et al. 2006). However, a recent challenge to this assumption has implicated leaf water potential gradients as the primary driver in determining gradients in LMA and leaf nitrogen, which may lead to sub-optimal distributions in leaf traits (Koch et al. 2004, Burgess and Dawson 2007, Ishii et al. 2008, Cavaleri et al. 2010).

Consistent with the traditional assumptions behind vertical gradients in leaf morphology, our study in an *Acer saccharum* stand provides strong evidence that light availability drives gradients in leaf functional traits, LMA, leaf thickness, leaf density, and N_{area} . Greater LMA, leaf thickness, and leaf density at high light availability were associated with thicker palisade layers and more densely packed palisade cells in response to increasing light availability. N_{area} corresponded to changes in LMA due to the strong association of N_{area} with LMA ($N_{\text{area}} = \text{LMA} * N_{\text{mass}}$). This study corroborates

studies that have attributed vertical gradients in leaf morphology and chemistry to natural light gradients (Hollinger 1989, Ellsworth and Reich 1993, Bond et al. 1999, Niinemets et al. 1999). The range of tree heights at our study site was shorter (25-34 m) compared with *Sequoia sempervirens* trees (85-113 m) in other studies (Koch et al. 2004, Ishii et al. 2008), which may explain why water potential was not as limiting in our study as compared with *S. sempervirens*. Previous studies in *A. saccharum* showed that LMA and N_{area} were strongly correlated with photosynthetic capacity (Jurik 1986, Reich et al. 1991, Ellsworth and Reich 1992, Ellsworth and Reich 1993, Raulier et al. 1999, Liu et al. 1997, Jones and Thomas 2007). Thus, photosynthetic capacity appears to be regulated by light availability and associated changes in cellular structure and leaf chemistry.

There was evidence of height-related constraints early in the growing season during and soon after leaf expansion. Constraints on leaf expansion were apparent for leaves growing higher in the canopy, which was likely due to cell wall properties that limited cell extensibility and turgor pressure threshold required to expand cells. Cell wall extensibility has been found to decrease in response to reduced leaf water potential (Nonami and Boyer 1990a, 1990b, Zhang et al. 2011), which may explain limitations to leaf development for upper canopy leaves as observed in this study. Following leaf expansion, midday leaf water potential declined and $\delta^{13}C$ increased with height, suggesting that the supply of CO_2 was constrained by stomatal behavior. However, morphological acclimation to light over time appeared to optimize leaf functional traits.

Increasing leaf carbon and LMA and reduced stomatal sensitivity to VPD or midday leaf water potential over time corresponded with acclimation to light and functional optimization over time. The theoretical optimal pattern in leaf photosynthetic

capacity with respect to light is an important assumption in big-leaf models that integrate canopy structure and leaf function parameters over the entire canopy (Field 1983, Hirose and Werger 1987, Sellers et al. 1992, Amthor 1994). Our model simulations revealed that models that do not account for seasonal changes in N_{area} or LMA overestimate daily canopy net CO₂ assimilation in the early season during acclimation; however, biases associated with models that do not account for canopy profiles in leaf structure and function are much greater throughout the majority of the growing season. Overall, our results indicate that vertical gradients in leaf morphology and chemistry and morphological acclimation to light are important factors that influence canopy photosynthesis in *Acer saccharum* and should be incorporated in canopy function modeling.

Results from this study have important implications for modeling carbon exchange. First, big-leaf models that estimate canopy photosynthesis using one layer of leaves that are functionally similar do not account for detailed structural and physiological parameters along canopy the profile. For example, we show that for exposed tree crowns, leaf morphology and function can be considerably different than portions of crowns at the same height but at different light conditions. Canopy function models that account for the three-dimensional structure of forest canopies, such as MAESTRA, capture detailed light regimes within non-homogeneous forest canopies (Bauerle et al. 2004, Medlyn et al. 2004). Second, seasonal changes in leaf traits and function can potentially bias estimates of forest productivity. Recent incorporation of seasonal changes in photosynthetic capacity in Earth System Models led to a >3% reduction in global net primary productivity (Bauerle et al. 2012). Our results provide

further support that canopy function models can be further refined by accounting for seasonal changes in leaf function.

Finally, recognizing the distribution of leaf area within stands and the contribution of canopy layers to total canopy photosynthesis is important in understanding the potential consequences of vertical and seasonal constraints on stand-level CO₂ carbon exchange. In this *A. saccharum* stand, a majority of the leaf area of the canopy is within the center of the canopy. However, the upper quarter of leaf area may contribute to over 50% of total canopy photosynthesis, which emphasizes the importance of height-related constraints on leaf morphology, chemistry, and function on upper canopy leaves. As trees grow taller, height-constraints are likely to gain greater importance to overall canopy photosynthesis. While there is evidence that light acclimation may overcome these constraints over the course of the growing season under current climate conditions, whether or not these constraints can be overcome in trees under increasing temperatures, as predicted with future climate change, requires further investigation.

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