

Evergreen broadleaf ecophysiology under rapid light fluctuations and transitional winter
conditions in the Southern Appalachian Mountains

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Abstract

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The southern Appalachian Mountains experience harsh winter conditions, including extremely cold air and soil temperatures, stochastic precipitation, often in the form of snow, high light, and strong winds. All of these contribute to the ecophysiological challenges that an evergreen plant faces during this season. Most wintertime research on evergreens has been done on conifers; we know less about how broadleaved angiosperms respond. Differences in leaf morphology between the two groups may influence mechanisms for coping with winter conditions. In addition, climate change is resulting in winters with stochastically dispersed warm days, and we do not know how broadleaved evergreens will respond to this. We are investigating the wintertime ecophysiology of the holly, *Ilex* x 'Nellie R. Stevens,' a broadleaved evergreen angiosperm commonly planted in this region. We are measuring diurnal gas exchange, water potential, maximum potential quantum efficiency (F_v/F_m), chlorophyll content, and leaf, soil, and air temperatures before, during, and after winter. We have found positive photosynthetic rates coupled with low stomatal conductances even at air temperatures slightly below or at 0°C , in part due to heating of the leaf above freezing when in full sun. Chlorophyll fluorescence measurements, however, indicate substantial photoinhibition for leaves exposed to high light at low temperatures, yet, they do not appear permanently injured and can achieve high rates on subsequent warmer days. Although rates are higher on warmer days in winter, cold soils may constrain the degree to which this species can take advantage of those days, due to the inability to transport water. Under sunny, winter conditions, low stomatal conductance may reduce latent heat loss, thus warming the broad leaves so they better cope with photoinhibition. This research can improve our understanding of how other broadleaf evergreen angiosperms may respond to natural as well as anthropogenically-influenced seasonal trends.

Introduction

Evergreen broad-leaved plants (EBLs) in the Appalachian Mountains of the southeastern U.S. must endure four distinct annual seasons, and to do so must produce leaves that can withstand the environmental variety within each season. In winter, evergreens face high light levels, cold temperatures, high wind speeds, frost stress, and snow and ice damage. In summer, evergreens tolerate high temperatures, high humidity, variable light intensity, sporadic precipitation, and drought. In spring, they must withstand sporadic freeze and thaw events that can cause injury to new leaves, while in fall, similar events can prove injurious before leaves are winter-hardened. Both deciduous and evergreens trees experience these seasonal variations in the Appalachian Mountains, however, deciduous trees have leaves that senesce in the fall, thereby avoiding the harsh environmental conditions of the winter season. An evergreen plant retains a cohort of leaves across the entire annual year, remaining “green” year-round.

There are two modes of evergreenness: in the first mode, plants produce two cohorts of leaves at different times of the year, keeping the plant effectively evergreen with leaves that live less than one full year. In the second mode, plants produce cohorts of leaves that last one or more years (Neufeld and Young 2014). In the latter group, individual leaves experience the annual variety of environmental conditions and therefore must be structurally tough and durable to withstand non-favorable conditions. The construction of these sclerophyllous (evergreen, small, thick, leathery) leaves requires large carbon investments, which may take longer than a single growing period or year to pay back (Chabot and Hicks 1982). Therefore, evergreen leaf longevities (LL) are generally extended so there is enough time for photosynthesis to achieve a carbon payback. If these evergreen plants grow in shaded habitats where incoming radiation amounts are reduced and photosynthesis is further constrained, payback times may become even

longer (Kikuzawa et al. 2013). This extended LL to pay back carbon investment requires evergreens to retain photosynthetically-capable leaves year-round. In fact, understory evergreen trees can reach their maximal photosynthetic capacity in winter, when the amount of overstory foliage is minimal and light levels are maximal (Katahata et al. 2014; Hamada et al. 2016). While it is beneficial for evergreen leaves with longer LLs to have more time to assimilate carbon, there come tradeoffs of overcoming annual environmental variability through higher construction costs and slower responses to rapid changes in environmental conditions (Smith et al. 2019). Regardless, a longer LL with stronger structural development leaf strategy ensures that an individual leaf can achieve a positive net carbon balance to contribute to total plant growth and ultimate survival in habitats with variable and stressful environmental parameters.

Two different types of evergreen trees exist: needle-leaved gymnosperms (conifers) and broad-leaved angiosperms (flowering plants). Though their evergreen leaf life strategies are similar, their different phylogenies and morphologies likely contribute to differing physiologies (Reich et al. 1995; Sanchez et al. 2016). Conifer needles are typically closely coupled to the environment (smaller leaves, less surface area, smaller boundary layer), meaning that energy exchange is fairly rapid when the surrounding environmental conditions change. Broad-leaved angiosperm laminae on the other hand may be less coupled to the environment (larger leaves, more surface area, larger boundary layer), which may contribute to slower rates in ecophysiological processes such as gas exchange or evapotranspirational cooling (Martin et al. 1999; De Kauwe et al. 2017). The physiologies between conifers and EBLs may potentially respond differently, even if both face similar environmental challenges year-round.

Both types of evergreen trees in the southern Appalachian Mountains experience highly variable light, temperature, and vapor-pressure deficit regimes due to intermittent cloud cover.

Such variable environmental conditions can homogenize intra-canopy differences in conifer evergreen response in gas exchange, water-use efficiency (WUE), and annual carbon gain (Hughes et al. 2015; Sanchez et al. 2016; Hernandez-Moreno et al. 2017). High light variability due to clouds is common in the southern Appalachian Mountains. These rapid light fluxes can disrupt the coordination of whole-plant ecophysiological responses (Smith and Hughes 2009) because various ecophysiological response times vary drastically, with light-dependent reactions occurring in pico-seconds to seconds, biochemical inductions in seconds to minutes, stomatal kinetics in minutes to hours, and growth in hours to weeks to months (Kaiser et al. 2015; McAusland et al. 2016). The asynchronicity or disconnect that can occur between carbon assimilation and stomatal conductance under dynamic light conditions will affect whole-plant carbon uptake, WUE, and has implications for long-term acclimation. It may also lead to inaccurate model predictions between field and controlled experiments (Yamori 2016; Matthews et al. 2017, 2018; Vialet-Chabrand et al. 2017a, 2017b; Schumann et al. 2017; Devireddy et al. 2020; Ehonen et al. 2020). These physiological disconnects can also lead to problems with dissipating radiative energy under sudden radiation increases, where a leaf could heat up to potentially lethal temperatures if its capacity to cool via transpiration is delayed by the rather slow response time of stomata (Leigh et al. 2012). Plants that are subject to sunflecks—short but intense changes in light due to intermittent cloud cover or self-shading—may be able to cope by relying on leaf water content to delay heat damage and turgor loss (Schymanski et al. 2013). However, EBLs may be more susceptible to these excessive radiation inputs since their broad leaves have a thicker boundary layer and cannot dissipate heat as efficiently as narrower needle-leaved conifers.

Dynamic environmental conditions also occur during the winter months, where EBLs deal with both high radiation loads and rapid light variations coupled with low temperatures. The seasonal combination of high light and cold temperatures can result in winter photoinhibition (WPI), in which the reduction of the temperature-dependent, enzymatically-driven Calvin Cycle causes a back-up of excess photon energy in the temperature-independent, light-driven light reactions. The accumulation of photon energy and reduction power can damage the photosynthetic electron transport chains, leading to chlorophyll bleaching and destruction, and eventual cell death (Öquist 1983). WPI effects and damage affect a diversity of plants subject to these conditions, including woody evergreens (Míguez et al. 2017). Plants, including evergreens, increase their use of sustained thermal energy dissipation to cope with WPI (Adams III et al. 1994; Verhoeven 2014) by upregulating their zeaxanthin cycle which can both absorb excess radiation and direct it through the electron transport chain and dissipate excess energy as heat, thereby preventing photoinhibition. As a result, evergreens are suggested as model species for studying photoprotective mechanisms in winter (Adams III et al. 2002; Demmig-Adams et al. 2012).

Although there has been plentiful research on needle-leaved conifer winter physiology (Verhoeven et al. 1999; Öquist and Huner 2003; Verhoeven 2013; Chang et al. 2015; Wang et al. 2019), less has been conducted on EBL winter physiology. After winter cold snaps, the alpine EBL, *Quercus guyavifolia*, substantially decreases both daytime electron flow and photosynthetic assimilation (Huang et al. 2016). Additionally, there is an overall acclimation response with strong spatio-temporal variability at the leaf level in some Mediterranean EBL species, which affects whole-plant gas exchange in the winter (Silva-Cancino et al. 2012).

There has been some research on *Rhododendron maximum* and *R. catawbiense*, common EBLs in the southern Appalachian Mountains. These EBLs have a unique way of coping with WPI: when leaf temperatures drop below freezing, they curl their leaves to reduce the leaf area exposed to high light and change their leaf angle to a more vertical orientation, which also reduces the amount of incoming radiation, especially near midday (Liu et al. 2019; Nilsen 2019). The *Rhododendron* strategy of curling and changing leaf angles is rather unique, and many other EBLs do not have this capacity to re-orient their leaves, indicating other additional EBL mechanisms to cope with harsh winter conditions. One way to limit the amount of excess light penetrating into leaves is to produce anthocyanins. For example, *Lonicera japonica* and *Galax urceolata*, produce anthocyanins when growing under high light conditions in winter, with the red pigments serving as a photoprotective measure (Hughes 2011). However, not all EBLs produce anthocyanins under high light stress in winter, so there must be other mechanisms to cope with these conditions.

One prominent EBL genus in the southern Appalachian Mountains is *Ilex* (holly) from the Aquifoliaceae family. In a recent study, Wachendorf et al. (2019) determined that in the winter, the understory European holly (*Ilex aquifolium*), experiences reductions in carbon assimilation even when chlorophyll fluorescence measurements indicate that the photosystems have recovered, suggesting a decoupling of ecophysiological processes that can lead to WPI. Even without any visible anthocyanin production, or ability to re-orient leaves, some *Ilex* spp. do not show visible signs of WPI injury in the winter. The lack of WPI damage suggests there must be other winter protective mechanisms to deal with the stochastic and harsh winter conditions in the Southern Appalachians.

For this project, I focused on a common EBL that is extensively planted on the Appalachian State University campus: the Nellie R. Stevens (NRS) holly, *Ilex* x. 'Nellie R. Stevens'. This horticultural variety is derived from crosses of the English holly, *I. aquifolium*, and the Chinese holly, *I. cornuta* (Gilman and Watson 1993). Native hollies, such as the Yaupan holly, *I. vomitoria* or the American holly, *I. opaca*, are more commonly found as understory trees and are not exposed to such high light loads as the NRS holly variety (Virginia Cooperative Extension 2018). My objective in this study was to determine 1) how individual leaves of the holly respond to rapid changes in light and 2) how the ecophysiology of this plant transitions from fall into winter. To do this, I made measurements of gas exchange, water status, and photoinhibition during the winter months on three NRS hollies growing outside the Rankin Science Building.

Methods

Plant material and site

I studied three *Ilex* x 'Nellie R. Stevens' shrubs planted in front of Rankin Science South Hall at Appalachian State University in Boone, NC, USA (973 m; 36°12'50"N, 81°40'54"W). The shrubs were around 3 m in height and planted in a row, with the southeastern-facing canopies exposed to mostly full sun, while the northwestern-facing canopies were often self-shaded in the first half of the day and exposed to direct sunlight in the second half of the day. I restricted my sampling to the southeastern side of the canopy. The climate in Boone, NC ranges from mild to cold, with summer mean high temperatures of 16.3 °C and winter mean low temperatures of 4.0 °C. Annual precipitation of 133.8 cm is distributed evenly over the year, with more than half the total precipitation in the form of snow during the winter months (NC CRONOS Database).

Rapid gas exchange

Rapid gas exchange measurements provide leaf-level photosynthesis and evapotranspiration rates at second time scales. To complete the first objective, I measured rapid gas exchange responses using a LI-6800 Portable Photosynthesis System (LI-COR Biosciences, Lincoln, NE, USA) during January and February 2019 on two leaves of each of plant. I adjusted the instrument averaging time down from the standard 4 secs to just 2 secs. Such an adjustment will inevitably increase system noise, so I measured gas exchange over the course of 1 hr with an empty cuvette (data not shown) under ambient light, and I found negligible system noise fluctuations, where A varied by less than $0.1 \mu\text{mol m}^{-2} \text{s}^{-1}$ and g_s only by $0.005 \text{ mol m}^{-2} \text{s}^{-1}$. This

confirmed that the LI-6800 is capable of measuring accurate rapid gas exchange responses at 2 sec averaged intervals with minimal system noise.

I ran an auto-program to measure carbon assimilation (A) and stomatal conductance to water vapor (g_s) of individual leaves at 2 sec intervals for an average duration of 4-6 hr each measurement day. I set cuvette environmental conditions as follows: CO_2 to $410 \mu\text{mol mol}^{-1}$, relative humidity (RH) to 50%, and leaf temperature (T_{leaf}) to match that of the leaves every 30 min (see *Air and leaf temperatures*). I used the Clear-top Leaf Chamber (6800-12A, LI-COR Biosciences) to provide ambient light and I matched the LI-6800 infrared gas analyzers (IRGAs) about every 30 min.

Winter ecophysiology measurements

To complete the second objective, I completed three full days of transitional winter ecophysiology measurements in 2019: Oct 29, Nov 5, and Nov 14. I collected measurements during four time periods: 7-8 am (early morning, AM), 10-11 am (early afternoon, EA), 1-2 pm (late afternoon, LA), and 4-5 pm (before sunset, PM). The hour-long time periods allowed me to take into consideration EDT changing to EST on Nov 3, 2019. On each day, I measured diurnal gas exchange, leaf and air temperatures at all four time periods, chlorophyll fluorescence and stem water potential in the AM and LA, soil temperatures in the AM and PM, and soil moisture in the AM. I was unable to record PM measurements for Nov 14, due to inclement weather. During each time period, I staggered individual measurements across the three plants to avoid confounding rates with light intensity or time.

Diurnal gas exchange:

I measured winter diurnal gas exchange (A , g_s , and C_i/C_a , the internal to ambient CO₂ ratio) using the LI-6800 system at all four time periods. I sampled 2-3 leaves per plant, allowing the leaves to stabilize for at least 2 min in the cuvette before taking a 4 sec average reading. I set cuvette conditions CO₂ to 410 $\mu\text{mol mol}^{-1}$, relative humidity (RH) to 50%, leaf temperature (T_{leaf}) to match that of the leaves at the beginning of the time periods (see below), and I used the Clear-top Leaf Chamber to provide ambient light. I matched the IRGAs before each time period.

Chlorophyll fluorescence:

Chlorophyll fluorescence can provide an indication of the potential photosynthetic efficiency stress and degree of WPI. Fully functional leaves can have a maximum value of ~ 0.85 . Values significantly less than this indicate injury primarily to photosystem II. I measured potential quantum efficiency of photosystem II (F_v/F_m) using a Handy PEA+ fluorescence meter (Hansatech Instruments, King's Lynn, NOR, England) on three leaves per plant at two time periods, AM and LA, when photoinhibition would be minimal and maximal, respectively. I dark-acclimated the leaves for 30 min using leaf clips before exposing them to a 1 sec saturating flash of actinic light set at 3500 $\mu\text{mol m}^{-2} \text{s}^{-1}$.

Stem water potential:

Stem water potential provides an indication of plant water status and low values (more negative) indicate greater water stress. I measured water potential (Ψ) using a Scholander Pressure Chamber Model 600 (PMS Instrument Company, Albany, OR, USA). I measured two twigs per plant at two time periods: AM and LA, when water stress would be minimal and

maximal, respectively. I cut the samples with gardening shears at 20 cm from the apical meristem shoot, then immediately placed the twigs into the pressure chamber and recorded the balancing pressure where water appears on the cut end of the stem protruding from the chamber. If the twig replicates deviated substantially from each other (which happened only once on 11/5), I measured a third twig and discarded the aberrant reading.

Air and leaf temperatures:

Plant ecophysiological processes are dependent on temperature, so I recorded leaf (T_{leaf}) temperatures ($^{\circ}\text{C}$) using an OMEGASCOPE Handheld Infrared Thermometer (Omega Engineering, Inc., Norwalk, CT, USA) on three leaves per plant at each of the four time periods. I used the average temperature of each time period to set T_{leaf} in the LI-6800 cuvette prior to each rapid and diurnal gas exchange time period. I used the LI-6800 to obtain air temperature (T_{air}) at each of the four time periods.

Soil temperature and moisture:

I measured soil temperatures (T_{soil}) at two depths (15 and 30 cm) using two Reotemp Stainless Steel Bi-Metal Thermometers (Reotemp Instruments, San Diego, CA, USA). I used three locations around the shrubs: under full canopy, edge of canopy, and outside of canopy at two time periods, AM and PM, to capture any changes that may have taken place during a measurement day. I randomly placed both thermometers in tandem within each zone and allowed them to acclimate for at least 2 min before taking readings. I measured soil moisture as volumetric water content (VWC%) at a depth of 20 cm using a HydroSense II Handheld Soil

Moisture Sensor (Campbell Scientific, Inc., Logan, UT, USA). VWC was measured once a day in the early morning in each of the three root system zones described above.

Results

Rapid gas exchange

I examined leaf A and g_s responses to ambient changes in photosynthetically active radiation (PAR) at 2-sec intervals across a 6-hr block from 9:45-15:45 EST. Across the first 2 hours, PAR had an average high light intensity of $1558 \mu\text{mol m}^{-2} \text{s}^{-1}$ but low variation with a coefficient of variation (CV) of 12.4%. For the remaining 4 hours, PAR decreased in mean light intensity by more than 50% and increased in variation to a CV of 92.7%. During the 2-hr period of low variability in PAR, both A and g_s also showed low variation and had similar CVs. While A was maintained at a substantial mean rate for winter of $6.3 \mu\text{mol m}^{-2} \text{s}^{-1}$, g_s had a low mean flux of $0.066 \text{ mol m}^{-2} \text{s}^{-1}$. During the 4-hr period of high variability in PAR, both A and g_s had roughly 40% decreases in mean rates while variation increased by almost 7x and 2.5x, respectively (Fig. 1).

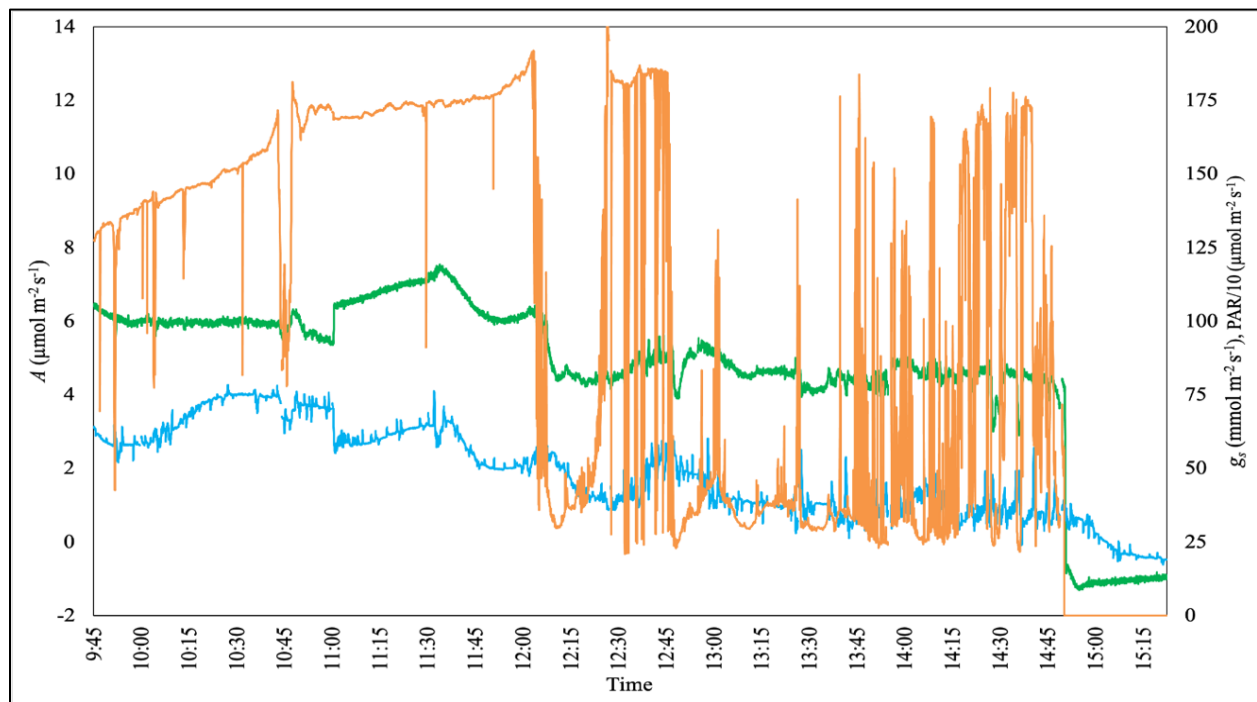


Figure 1. Full day of gas exchange measurements at 2 s intervals: photosynthesis (A , green) and stomatal conductance (g_s , blue) responding to ambient light (PAR/10, orange) over time on 2/7/19.

I examined both A and g_s under two 20 min periods during either relatively stable or variable PAR (Fig. 2). Under relatively stable PAR, both A and g_s steadily increased, although the absolute increases were relatively minor. Their coefficients of variation at this time were 3.7% and 3.7%, respectively (Fig. 2a and 2b). During the period of variable PAR, A had a CV of 7.7%, more than 2x the variation when under stable PAR (Fig. 2c), and g_s had a CV of 12.1%, more than 3x the variation when under stable PAR (Fig. 2d).

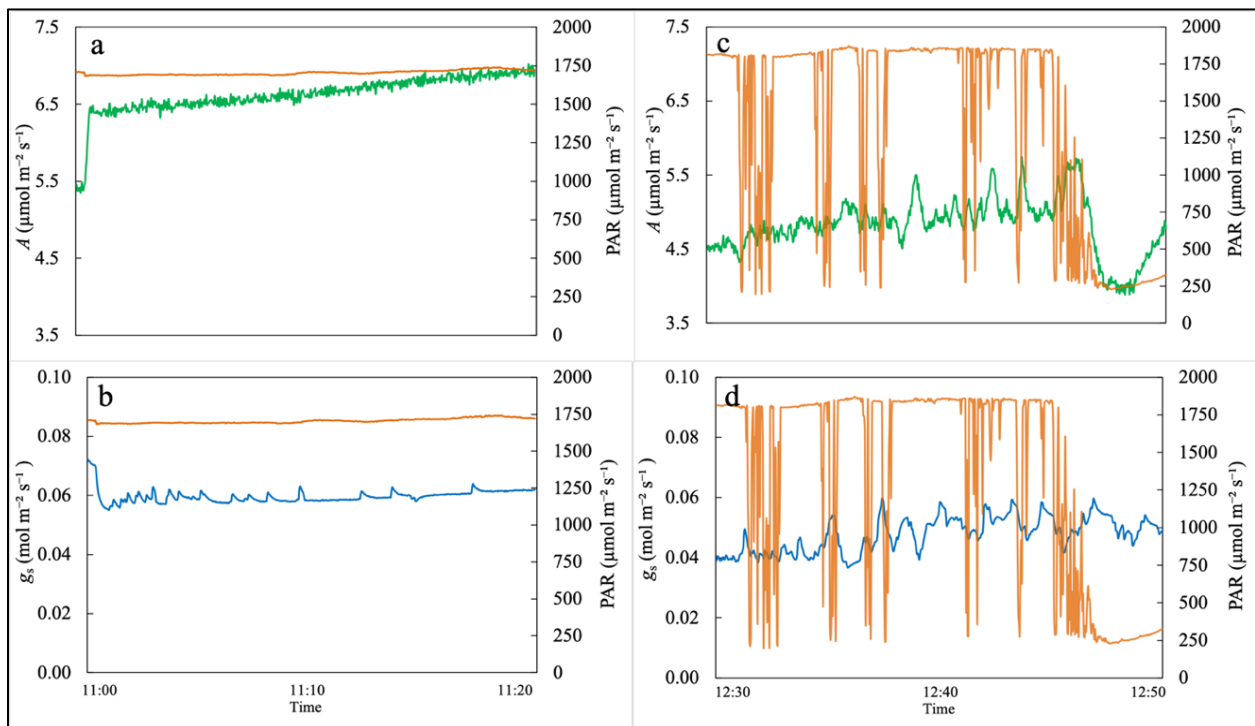


Figure 2. Zoomed-in perspective of the full day of 2/7/19 comparing steady (a and b) vs. variable (c and d) light conditions. Carbon assimilation rate (green) and stomatal conductance rates (blue) in response to changes in PAR (orange).

Winter abiotic characteristics:

In the AM of 10/29, PAR was less than 1% full sunlight, and T_{air} and T_{leaf} were 12.3 and 12.6 °C, respectively. By the LA, PAR increased to 67% full sunlight, and T_{air} and T_{leaf} increased to 17.7 and 18.7 °C. In the PM, PAR decreased back to less than 10% full sunlight, and both T_{air}

and T_{leaf} coalesced around 17.2 °C (Fig. 3, green). Over the course of the day, shallow T_{soil} increased by 15% while deep T_{soil} increased by 2% to a final temperature of 14.0 and 14.4 °C, respectively (Fig. 4, green). Soil VWC indicated that soil was adequately moist across all 3 measurement days, where mean VWC was 26.6% (Fig. 4).

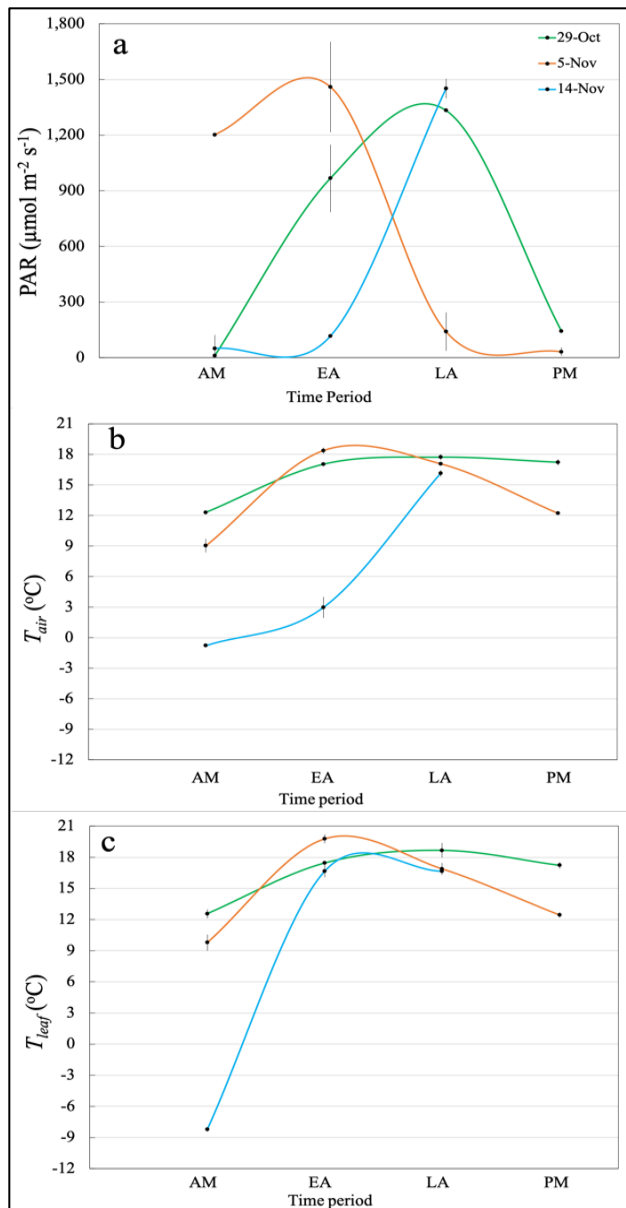


Figure 3. Mean light and temperature measurements \pm se of (a) photosynthetic active radiation levels (PAR, $\mu\text{mol m}^{-2} \text{s}^{-1}$), (b) air temperature (T_{air} , °C), and (c) leaf temperature (T_{leaf} , °C) on 3 days at 4 time periods: AM, EA (early afternoon), LA (late afternoon), and PM. Sample sizes are $n=6$ for a and b; $n=9$ for c. I was unable to complete measurements in the PM of 14-Nov.

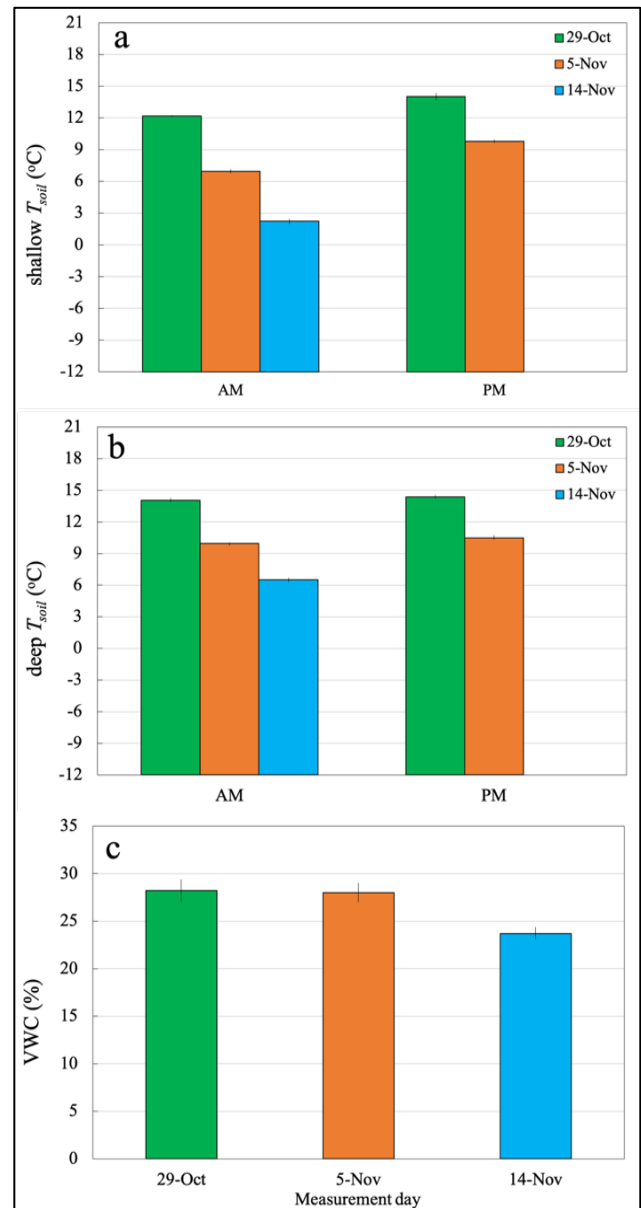


Figure 4. Mean temperature (°C) \pm se of (a) shallow soil at 15 cm and (b) deep soil at 30 cm on 3 days at 2 time periods: AM and PM. I was unable to complete measurements in the PM of 14-Nov. Mean soil volumetric water content (VWC, %; c) for the 3 days. Sample sizes are $n=9$. I was unable to complete measurements in the PM of 14-Nov.

In the AM of 11/5, PAR began at 60% full sunlight. T_{air} and T_{leaf} were a bit cool at 9.0 and 9.8 °C, respectively. By the EA, PAR increased to 73% full sunlight, and T_{air} and T_{leaf} increased to 18.4 and 19.8 °C. In the PM, PAR decreased back to less than 2% full sunlight, and both T_{air} and T_{leaf} coalesced around 12.3 °C (Fig. 3, orange). Over the course of the day, shallow T_{soil} increased by 41% while deep T_{soil} increased by 6% to a final temperature of 9.8 and 10.5 °C, respectively. (Fig. 4, orange).

In the AM of 11/14, PAR began at 2% full sunlight, and T_{air} and T_{leaf} began slightly below 0 and -8 °C, respectively. By the EA, PAR had only increased to 8% full sunlight, T_{air} increased to 3.0, yet T_{leaf} significantly increased to 16.7 °C. In the LA, PAR increased to 73% full sunlight, and T_{air} and T_{leaf} coalesced around 16.4 °C (Fig. 3, blue). Shallow and deep T_{soil} began at 2.2 and 6.5°C, respectively (Fig. 4, blue).

In general, T_{leaf} was slightly warmer (less than 1 °C) than T_{air} under high light. Under low light, T_{leaf} was still slightly warmer than T_{air} , excluding two outliers, both on 11/14. In the AM, T_{leaf} at -8.2 °C was much cooler than T_{air} at -0.8 °C, and in the EA, T_{leaf} at 16.7 °C was much warmer than T_{air} at 3.0 °C (Fig. 5).

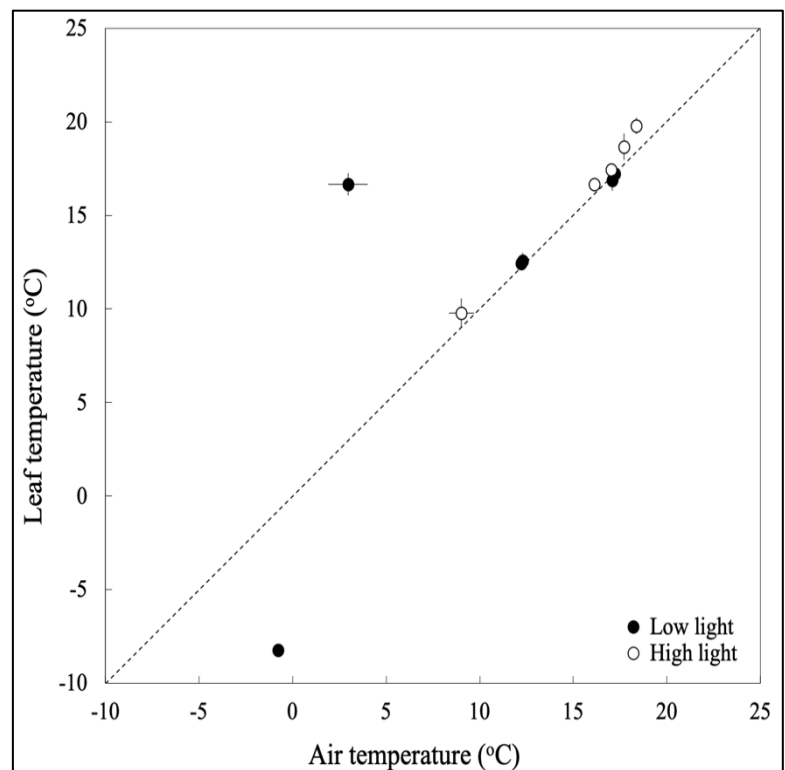


Figure 5. Relationship between mean air temperature and mean leaf temperature, distinguished by low light ($< 300 \mu\text{mol m}^{-2} \text{s}^{-1}$) and high light ($\geq 900 \mu\text{mol m}^{-2} \text{s}^{-1}$) intensities. Dashed line is the 1:1 relationship. Sample sizes are $n=9$.

Winter gas exchange:

On 10/29, A began at $0.0 \mu\text{mol m}^{-2} \text{s}^{-1}$ in the AM, indicating cellular respiration outweighed carbon assimilation. This correlated strongly to low PAR at about 1% full sunlight despite warm temperatures around 12°C .

Rates of A peaked in the LA to $12.0 \mu\text{mol m}^{-2} \text{s}^{-1}$, in conjunction with LA peaks in PAR ($1334 \mu\text{mol m}^{-2} \text{s}^{-1}$), T_{air} , and T_{leaf} . Rates of g_s started out low in the morning at $0.074 \text{ mol m}^{-2} \text{s}^{-1}$ and reached an earlier maximum in the EA at $0.206 \text{ mol m}^{-2} \text{s}^{-1}$, which decoupled from the LA peaks in A , PAR, T_{air} , and T_{leaf} .

The C_i/C_a ratio began near 1 in the AM, then reached a minimum in the LA at 0.69. This minimum dip coincided with the LA maximum peak in A when internal carbon would be lower due to high fixation rates inside the leaf. Overall, the trend of C_i/C_a showed an opposite trend to A (Fig. 6, green).

On 11/5, A began at $9.0 \mu\text{mol m}^{-2} \text{s}^{-1}$ in the AM, in correlation with very high PAR yet reasonably cool temperatures. Rates of A peaked in the EA to $11.2 \mu\text{mol m}^{-2} \text{s}^{-1}$, in conjunction with EA peaks in PAR, T_{air} , and

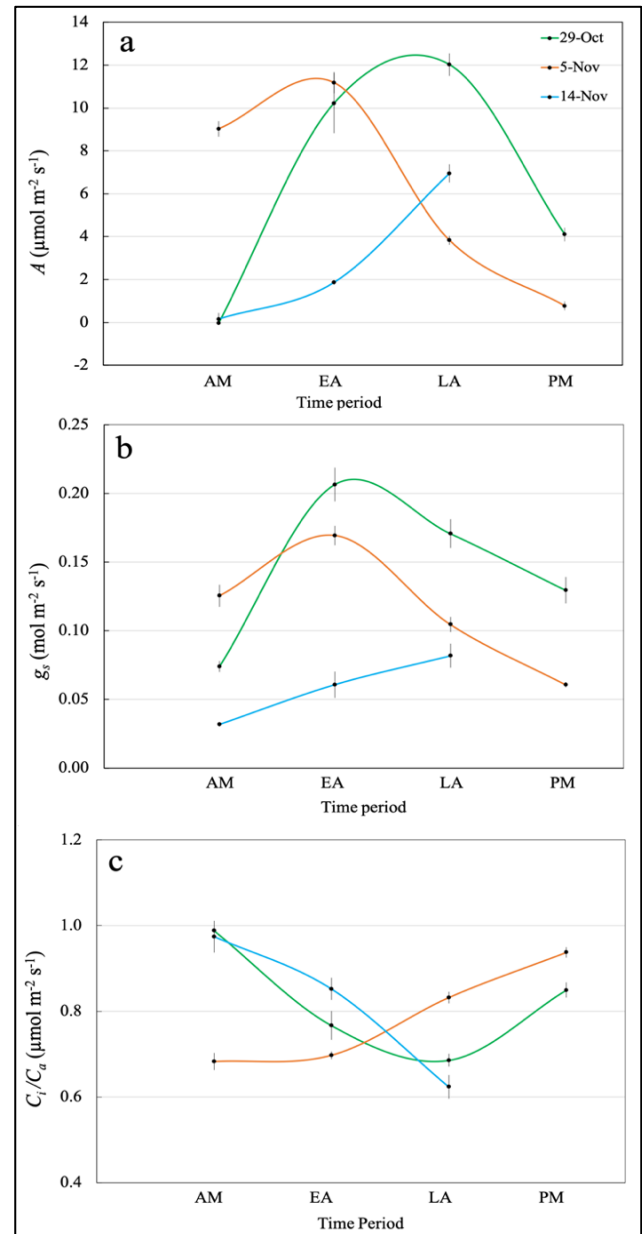


Figure 6. Mean diurnal gas exchange measurements \pm se across the 3 days at 4 time periods: AM, EA, LA, and PM. Measurements include (a) carbon assimilation (A , $\mu\text{mol m}^{-2} \text{s}^{-1}$), (b) stomatal conductance (g_s , $\text{mol m}^{-2} \text{s}^{-1}$), and (c) ration of internal to ambient carbon dioxide (C_i/C_a). Sample sizes are $n=6$. I was unable to complete measurements in the PM of 14-Nov.

T_{leaf} . Rates of g_s started at $0.125 \text{ mol m}^{-2} \text{ s}^{-1}$ and reached an early maximum in the EA at $0.169 \text{ mol m}^{-2} \text{ s}^{-1}$, which coupled with EA peaks in A , PAR, T_{air} , and T_{leaf} . Both peaks in A and g_s were slightly lower than 10/29 maximums. The C_i/C_a ratio began near 0.69 in the AM and EA, then steadily increased over the day to 0.94, where the overall trend of C_i/C_a similarly showed an opposite trend to A (Fig. 6, orange).

On 11/14, A began at $0.2 \text{ } \mu\text{mol m}^{-2} \text{ s}^{-1}$ in the AM, in correlation with very low PAR and freezing temperatures below 0. Rates of A slightly increased in the EA, potentially due to warmer leaf temperatures, though PAR and air temperatures were still relatively low. Finally, rates of A peaked in the LA to $7.0 \text{ } \mu\text{mol m}^{-2} \text{ s}^{-1}$ coupled with LA peaks in PAR, T_{air} , and T_{leaf} . Rates of g_s started at $0.032 \text{ mol m}^{-2} \text{ s}^{-1}$ and reached a maximum in the LA at $0.082 \text{ mol m}^{-2} \text{ s}^{-1}$, coupled with LA peaks in A , PAR, T_{air} , and T_{leaf} . Both peaks in A and g_s were considerably lower than 10/29 and 11/5 maximums. The C_i/C_a ratio began near 0.97 in the AM, then steadily decreased over the day to 0.62, where the overall trend of C_i/C_a also showed an opposite trend to A (Fig. 6, blue).

Mean rates of A and g_s from all three measurement days indicated a plateau across the afternoon

in rates of A averaging around $7.7 \text{ } \mu\text{mol m}^{-2} \text{ s}^{-1}$, while g_s peaked in the EA around $0.146 \text{ mol m}^{-2} \text{ s}^{-1}$ (Fig. 7).

Across the 3 measurement days, reduced shallow and deep T_{soil} depths coincided with reduced rates in A and g_s (Fig. 3 and 6).

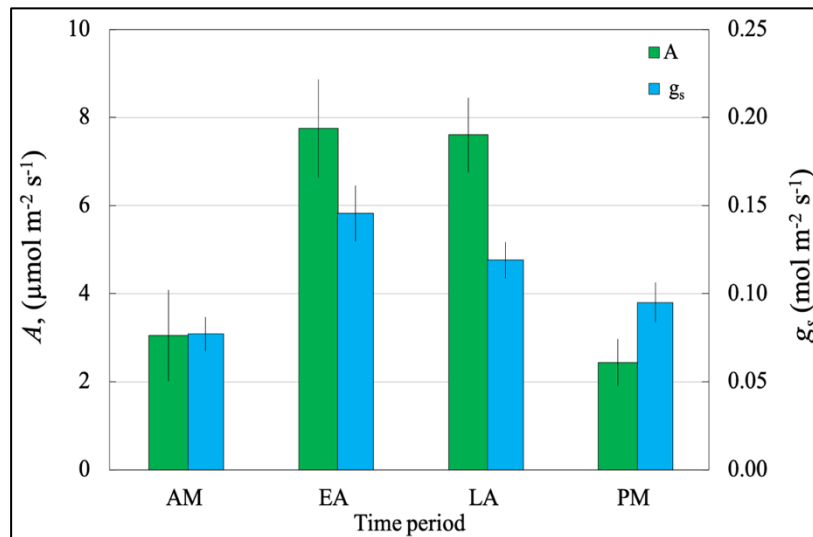


Figure 7. Overall mean gas exchange rates \pm se across the 3 days at 4 time periods: AM, EA, LA, and PM. Measurements include carbon assimilation (A , $\mu\text{mol m}^{-2} \text{ s}^{-1}$) and stomatal conductance (g_s , $\text{mol m}^{-2} \text{ s}^{-1}$). Sample sizes are $n=18$ for AM, EA, and LA, and $n=12$ for PM.

Rates of photosynthetic assimilation had a strong dependency on light intensity. A was relatively low at $0-4 \mu\text{mol m}^{-2} \text{s}^{-1}$ under low PAR ($\leq 300 \mu\text{mol m}^{-2} \text{s}^{-1}$) and relatively high at $6.5-12 \mu\text{mol m}^{-2} \text{s}^{-1}$ under high PAR ($\geq 900 \mu\text{mol m}^{-2} \text{s}^{-1}$). Rates of g_s did not differ between low light or high light, however there was a positive linear relationship between both gas exchange rates and PAR that tended to decrease across the measurement days (Fig. 8a and 8e).

Additionally, there was a positive linear relationship between the two gas exchange rates and both T_{air} (Fig. 8b and 8f), and less of a relationship with T_{leaf} (Fig. 8c and 8g). In general, the relationship between A and g_s did not vary in relation to the difference between leaf and air temperatures, $T_{leaf} - T_{air}$. Only on 11/14 did two outliers stand out: on the AM when T_{leaf} was much cooler than T_{air} , A and g_s had low rates of $0.2 \mu\text{mol m}^{-2} \text{s}^{-1}$ and $0.032 \text{ mol m}^{-2} \text{s}^{-1}$, respectively. That following EA when T_{leaf} was much warmer than T_{air} , A and g_s had increased slightly to $1.86 \mu\text{mol m}^{-2} \text{s}^{-1}$ and $0.061 \text{ mol m}^{-2} \text{s}^{-1}$, even though PAR doubled between that time period. If I eliminate these two outliers, there is no significant relationship between either of the gas exchange responses and $T_{leaf} - T_{air}$ (Fig. 8d and 8h). Gas exchange responses are less dependent on differences in leaf and air temperature, and more so on light and air temperature.

Winter chlorophyll fluorescence:

Overall, F_v/F_m decreased across the 3 measurement days. F_v/F_m decreased during the day on 10/29 and 11/5. Only on the coldest day, 11/14, did increase over the course of the day (Fig. 9). There was a strong positive linear relationship between AM F_v/F_m and AM T_{air} , where both values decreased as the days transitioned further in late fall (Fig. 10).

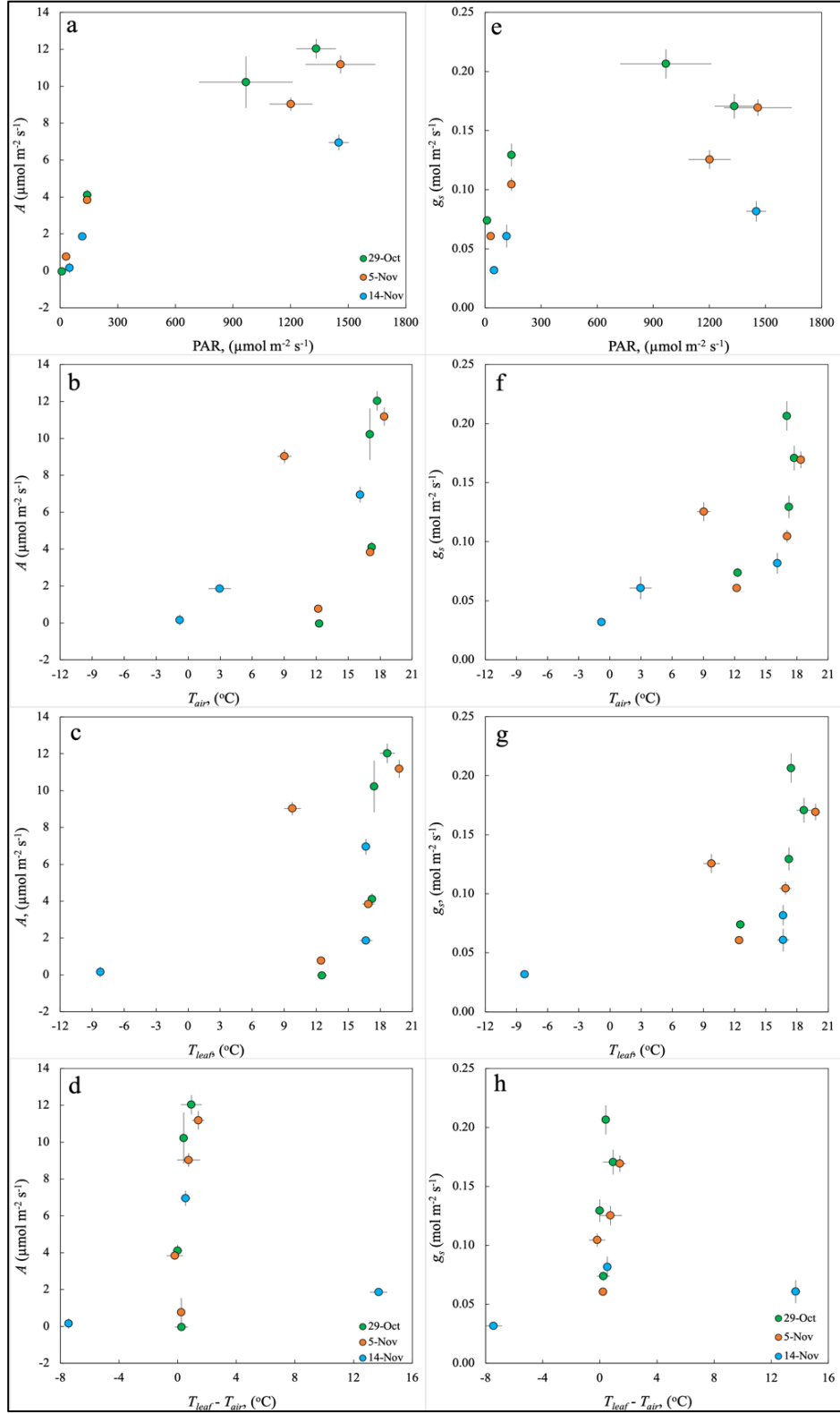


Figure 8. Relationship between mean carbon assimilation (A , $\mu\text{mol m}^{-2} \text{s}^{-1}$) and mean stomatal conductance (g_s , $\text{mol m}^{-2} \text{s}^{-1}$) and (a, e) mean photosynthetically active radiation (PAR, $\mu\text{mol m}^{-2} \text{s}^{-1}$), (b, f) mean air temperature (T_{air} , $^{\circ}\text{C}$), (c, g) mean leaf temperature (T_{leaf} , $^{\circ}\text{C}$), and (d, h) the difference between leaf temperature and air temperature ($T_{leaf} - T_{air}$, $^{\circ}\text{C}$).

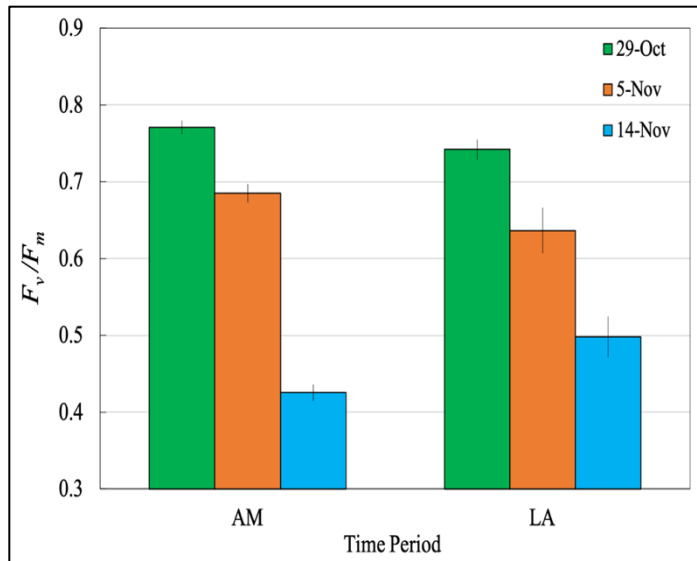


Figure 9. Mean maximum potential quantum efficiency \pm se (F_v/F_m) on 3 days at 2 time periods: AM and LA. Sample sizes are $n=9$.

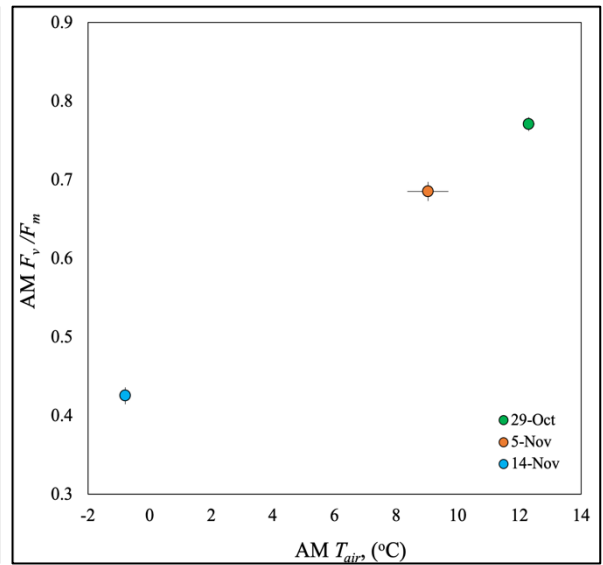


Figure 10. Relationship between mean air temperature (T_{air} , $^{\circ}\text{C}$) and mean maximum potential quantum efficiency (F_v/F_m) in the AM. Sample sizes are $n=6$ for T_{air} and $n=9$ for F_v/F_m .

Winter water potential:

Pre-dawn ψ increased over the course measurement days, whereas mid-day ψ varied.

Overall, ψ measurements indicated that water stress was not a major limitation for the plants on these days (Fig. 11).

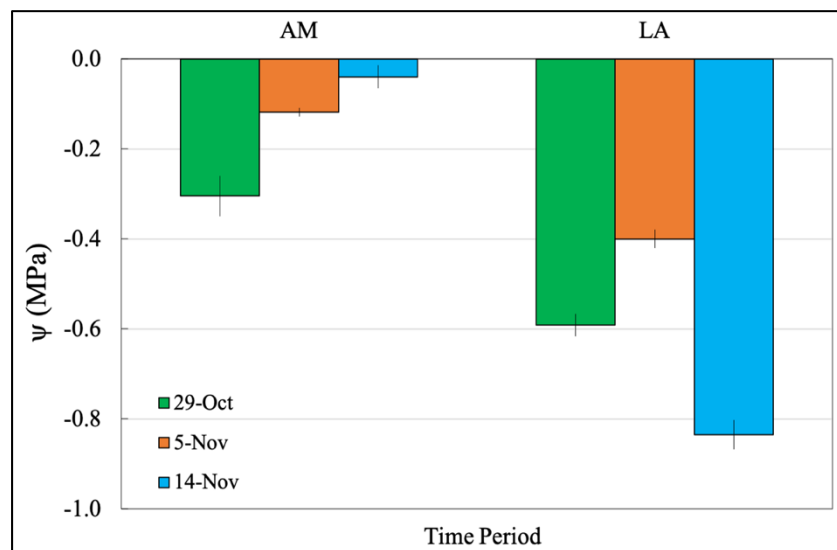


Figure 11. Mean stem water potential \pm se (ψ , MPa) on 3 days at 2 time periods: AM and LA. Sample sizes are $n=6$.

Discussion

In this study, I focused on elucidating the ecophysiological capabilities and limitations of the EBL, *Ilex x.* ‘Nellie R. Stevens’, under two time-scales: rapid light fluctuations and the long-term transition from fall into winter. I first examined ambient, rapid light fluctuation effects on leaf-level gas exchange at 2-second intervals across a 6-hour period. I then analyzed ecophysiological responses across the transition into winter using leaf-level diurnal gas exchange, chlorophyll fluorescence, and stem water potential measurements.

Advancements in scientific instrumentation now allow researchers to investigate gas exchange responses at shorter time scales than previously studied. In earlier studies, diurnal gas exchange measurements consisted of spot measurements conducted minutes or hours apart and averaged across an extended period of time. Newer gas exchange instruments now have the capability of measuring such responses at minute and even second time scales. In this experiment, I was testing a proof of concept to determine if the LI-6800 is capable of measuring gas exchange responses under ambient light at fine time scales on the order of just a few seconds. One could further study the overall difference in values under different second time intervals, i.e. 2, 5, and 10 sec, however with negligible system noise at 2 sec, this can offer key insights at a very short time frame.

My rapid gas exchange results highlight an important point: diurnal gas exchange measurements conducted at hourly or longer intervals may not capture accurate estimates of daily carbon assimilation, especially when leaves are subject to rapid fluctuations in PAR. Under steady light, carbon assimilation achieved considerable rates with low variation, while under variable light, average rates were halved, and variation increased significantly. The same response pattern occurred for stomatal conductance to water vapor (Fig. 1 and Fig. 2). Stomatal

conductance to water vapor and carbon assimilation had a tendency to become decoupled, or unsynchronized, when under an extended period of variable light. The decoupling of gas exchange mechanisms results from different response times among biochemical and physiological processes when leaves are under sudden environmental changes, and where correlations between instantaneous light and instantaneous gas exchange may break down. These asynchronous changes in gas exchange responses under variable light confirm that leaf-level gas exchange rates can display complex patterns with high variance under rapid light fluctuations compared to steady light periods (Matthews et al. 2018). Both this temporal heterogeneity in stomatal behavior and spatial heterogeneity in stomatal size, speed, and responsiveness influence carbon assimilation and transpiration, ultimately impacting WUE and net plant productivity (Lawson and Blatt 2014; Lawson and Vialet-Chabrand 2019; Ehonen et al. 2020)

Rapid light fluctuation impacts on gas exchange not only depend on the overall rate and pattern of variation, but also on the absolute magnitude of this variation. For example, if PAR fluctuates rapidly without minimum values dropping below the light saturation point (LSP) as determined from light response curves (Young 2018, unpublished study), then there may be minimal changes in A or g_s , especially if this does not cause changes in T_{leaf} , as that would alter the leaf to air vapor pressure deficit and possibly affect transpiration. However, if the minimum PAR values in stochastic light regimes drop below the LSP, this could result in down-regulation of RUBISCO activase, a light-activated enzyme that activates carboxylation sites on RUBISCO, and that would initiate a down-regulation of the carboxylation rates. This in turn would result in a rise in C_i , which can then exert feedback on stomata and initiate a stomatal closure response (Vialet-Chabrand et al. 2017b). Each of these processes occur on vastly different timescales: deactivation of RUBISCO-activase can occur rapidly within seconds, whereas stomatal closure

operates on timescales of minutes (Kaiser et al. 2015; Ehonen et al. 2020), which may result in the decoupling of leaf gas exchange responses from instantaneous light intensities at any particular moment.

My rapid gas exchange results highlight that diurnal gas exchange measurements made at hourly intervals may not capture accurate estimates of interacting gas exchange responses, especially when leaves are in environments with extreme light variation, highlighting the need for studies to consider entire plant responses (Monsi and Saeki 2005; Smith and Hughes 2009; Schymanski et al. 2013; Company et al. 2016; Ehonen et al. 2020). This has larger implications for annual carbon assimilation models that depend upon widely spaced measurements, as they may be misleading if they do not take into account these rapid light fluctuations that are caused by clouds, sunflecks, and intra-canopy self-shading.

Additionally, rapidly varying light can exert effects on leaf energy budgets (Violet-Chabrand and Lawson 2019, 2020) and impact rapid acclimation over longer time frames (Morales and Kaiser 2020; Walker et al. 2020). Under a scenario of sudden and large radiation increases, stomata could be slow to respond, and the leaf would not be able to effectively dissipate excess energy through transpiration as the latent heat of evaporation (Lee and Gates 1964). One way to deal with this sudden increase in energy input would be to shunt some of it through the xanthophyll cycle, where photonic energy can be released as heat (Demmig-Adams and Adams III 1992, 1996; Demmig-Adams et al. 1995, 1996, 2012; Violet-Chabrand and Lawson 2019, 2020). However, the xanthophyll cycle may not be capable of dissipating all of the incoming radiation and the only recourse is for the leaf to heat up. Such increases in temperature could have differing consequences for gas exchange dependent on the season. In the summer, if the leaf is near its thermal maximum, these sudden inputs could lift leaf temperatures past their

thermal maxima, causing heat shock to the leaf (Leigh et al. 2012; Schymanski et al. 2013). Holly leaves are sclerophyllic, and likely have a moderate to low water content compared to other non-sclerophyllic broad-leaved plants (Gilman and Watson 1993; Potter and Kimmerer 1988). Leaves are also moderately sized at five to ten cm in length and less than one mm in thickness and are smaller than most native or ornamental Rhododendrons. Research on an ornamental Rhododendron growing next to these hollies showed that their leaves could become up to 5 °C warmer than air temperatures, when at the same time the holly leaves I was working with were near or maybe at air temperature (Houston and Buzard 2019, unpublished study). This suggests that solar heating may not subject holly leaves to lethal temperatures in summer, especially in the mountains, where maximum air temperatures do not get too high.

On the other hand, if solar heating raises leaf temperatures in the winter, this might reduce their susceptibility to WPI. The pre-winter ecophysiology results indicated that gas exchange responses were correlated to light and leaf temperature. A combination of high leaf temperatures and high light contributed to considerable carbon assimilation rates, whereas colder temperatures decreased carbon assimilation potential in conjunction with low F_v/F_m values. This suggests that there could be a negative impact on carbon assimilation under cold leaf temperatures when coupled to high light intensity, as is often the case under WPI. However, if the leaf warmed up due to overall increases in the radiation load, then carbon assimilation rates could recover. In February 2019, my gas exchange data illustrate that photosynthesis rates could get as high as $7.4 \mu\text{mol m}^{-2} \text{s}^{-1}$ and stomatal conductance as high as $0.076 \text{ mol m}^{-2} \text{s}^{-1}$, which suggests that this species can assimilate carbon even in the dead of winter provided they have sufficient light and moderate temperatures. They also suggest there may be some robust ability of the NRS holly to recover from WPI when under favorable high light and warm temperatures.

This finding supports the research done by Wachendorf et al. (2019), in which potential PSII efficiency (F_v/F_m) in *I. aquifolium* maintained high plasticity and potential to recover in the winter, dependent on which stage of the frost-hardening phase leaves were in. The NRS holly, an ornamental cross from *I. aquifolium*, may also experience this frost-hardening phase in order to tolerate extreme winter conditions, dictated by cold temperatures and low light. Thus, in the winter, high light may favor carbon assimilation of an NRS holly by increasing leaf temperatures, reducing WPI, and assimilating carbon at a time when most deciduous plants are dormant.

In this experiment, I wanted to investigate how the NRS holly made the transition into winter, but I did not investigate any potential mechanisms to cope with WPI. Additional speculative mechanisms include: (1) an efficient repair of photosynthetic electron pathways, (2) a highly upregulated xanthophyll cycle to dissipate excess photonic energy as heat, (3) variable phloem loading, or (4) a responsive antioxidant system to detoxify any reactive oxygen species that are created as a result of WPI (Adams III et al. 1994, 2002; Logan et al. 1998; Demmig-Adams and Adams III 2006). My data suggest that potential photoinhibition damage may be reduced if the broad leaves of the NRS holly can raise internal leaf temperatures, preventing photochemical damage. Both this study and the study of Wachendorf et al. (2019) confirm that *Ilex* species can maintain evergreen leaves with longer LLs and can endure frost damage and WPI, at the potential expense of reduced carbon gains (Savitch et al. 2002; Ensminger et al. 2006). Thus, holly species fall under a classification of leaves that live longer, yet “stronger”, by producing expensive, yet durable leaves to endure harsh winter conditions.

Another concern of EBL plants in winter is the coupling of above- and belowground ecophysiological processes, which are often in different temporal environments, as changes in

soil temperature can lag behind air temperature (Soong et al. 2020). Frozen soils could impact water transport as cold soils are known to signal stomatal closure in *Picea rubens* (Schaberg et al. 2000). Thus, the potential response of this holly species could have been constrained to some degree by cold soils. This suggests that short high temperature events in winter may have a limited, but still significant impact, on carbon assimilation, but if individual events lengthen in duration, and become more frequent, then these hollies may be capable of achieving even higher rates of gas exchange. A major question is whether this will have positive or negative effects on the carbon balance of these plants in the winter. While higher air and soil temperatures may allow greater leaf gas exchange, it is unknown whether this will increase or decrease the water use efficiency (ratio of assimilation to stomatal conductance). If it lowers this parameter, hollies might suffer greater water stress in the winter. The other aspect to consider is respiration; Krebs cycle and photorespiration in leaves increase with temperature, as will root respiration with higher soil temperatures. If the combined increases in respiratory carbon losses exceed those gained by leaf and stem photosynthesis, then winter warming could be harmful to this species. If assimilation exceeds respiratory increases, then warming in winter could benefit these plants. Further research is warranted as studies indicate that winter warming is becoming more common (Kunkel et al. 2020).

A general challenge in the winter can be water transport, either due to lack of adequate precipitation or due to frozen stems and soils. Trees must be able to retain high frost resistance in order to survive in their habitats (Charrier et al. 2013, 2015; Verhoeven et al. 2018) The plants I worked with were well-watered, as indicated by their relatively high values of water potential. However, there can be times when these trees undergo severe water stress. For example, I had previously measured the water potentials of these same bushes in September 2019, when

Watauga County experienced a long-term drought. The water potentials of these same plants dropped as low as -2.0 MPa for pre-dawn and -2.5 MPa for midday water potential, indicating severe water stress. Yet these plants were not visibly altered by this high degree of water stress, although stomatal conductances were greatly reduced, indicating that it did induce stomatal closure. That would also limit carbon uptake because CO₂ would not be able to diffuse into the leaf when the stomata aperture is shut. This suggests that this variety of holly is capable of tolerating considerable water stress, at least during the summer and early fall, but how this interacts with the transition to winter is not well understood and deserving of more research.

In this experiment, I did not consider controlling for self-shading, intra-canopy variation, or sun flecks, which all strongly influence whole-plant function, ecophysiology, and growth (Smith and Hughes 2009; McAusland and Murchie 2020). Ornamental species such as the one I studied here also pose their own horticulturally selected advantages when it comes to high light, water stress, and other abiotic and biotic stressors (Gilman and Watson 1993). If I had the opportunity to do this study again, I would first design an experiment to examine the effects of intra-canopy variation and self-shading. I would collect rapid gas exchange measurements on second intervals in addition to more widely spaced diurnal gas exchange measurements all the way from pre-winter to post-winter. This would enable me to obtain a more complete, detailed dataset on winter responses of this evergreen broadleaved plant. Finally, I would expand my study to include native American hollies, e.g., *Ilex opaca*, in order to determine whether there are any physiological differences among native and ornamental species.

This study highlights future research directions. First, intra-canopy variation and whole-plant rapid responses should be examined under both stable and variable light regimes. Second, one could investigate how holly leaves respond to highly variable and rapid changes in light

intensities in both summer and winter. Third, evergreen broadleaf coordination between shoot and root temperatures needs to be elucidated under warming winter temperatures, and attempts should be made to separate warm period length, magnitude of temperature change, and frequency of warming periods to determine how plant responses will change in the face of climate change. Lastly, I would like to better understand the physiological mechanisms holly leaves use to cope with winter stress since they cannot move their broad leaves like rhododendrons do, yet they do not seem to experience noticeable winter photoinhibition damage.

Conclusions

It is important to understand EBL carbon assimilation, water use, and nutrient cycling patterns in winter, and under warming conditions, to better predict the potential impact of global warming on EBL plants, and how these responses may affect their biogeographical distribution (Monk 1966; Church et al. 2016; Ge and Xie 2017; Conedera et al. 2018). The Nellie R. Stevens Holly, a woody ornamental temperate EBL, is capable of rapidly responding to variable light and temperatures that are common during southeastern U.S. winters. Climate model projections indicate an increase in winter warm spells (Kunkel et al. 2020), which may impact above- and belowground plant temperatures and cause disconnects in whole-plant function. Our study has three main take-home messages: (1) variable light can cause extreme variability in gas exchange responses of evergreen broadleaved plants, potentially uncoupling intertwined biochemical and physiological processes and affecting whole-plant ecophysiological responses to environmental variation; (2) considerable carbon assimilation can occur during the winter months, possibly contributing to a greater annual carbon budget, and (3) winter assimilation could potentially be essential for evergreen broadleaved trees, particularly in understory habitats, since it is in the winter months when the overstory is leafless that light levels are at their highest. On the flip side, it is still not known whether high respiration rates of leaves and non-photosynthetic organs (i.e. roots) would overwhelm any increases in carbon assimilation during short periods of winter warming. Shoot and root response during the winter may become decoupled, especially in light of projected warm periods in winters (Bowling et al. 2018; Soong et al. 2020). There is much to learn about how individual leaves respond to warming winters and extrapolating these responses to the whole plant will provide data necessary to determine how global climate change will ultimately impact the survival and distribution of woody evergreens on a global basis.

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