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A Physiological Examination of the Age-related Decline in Photosynthesis in Picea rubens

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A PHYSIOLOGICAL EXAMINATION OF THE AGE-RELATED DECLINE IN PHOTOSYNTHESIS IN *PICEA RUBENS*

By

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A PHYSIOLOGICAL EXAMINATION OF THE AGE-RELATED DECLINE IN PHOTSYNTHESIS IN *PICEA RUBENS*

By Stephanie L. Adams

Thesis Advisors: Dr. Michael Greenwood
and Dr. Michael Day

An Abstract of the Thesis Presented
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Numerous conifer species undergo predictable age-related changes in productivity, photosynthesis and foliar morphology and anatomy. While these phenomena have been demonstrated for many species, the physiological mechanisms controlling them are not well understood. In order to better understand this issue, we examined four possible controls of the age-related decline in photosynthesis in red spruce: stomatal limitation, a decline in investment into photosynthetic capacity, nutrient limitations and a demand-side decline in sink: source relations.

We investigated these age-related trends in physiology for juvenile, mid-age and old (mean age ~13, ~54 and ~128 years old) red spruce trees in a multi-cohort stand in Maine. In order to examine stomatal limitations, we examined the diurnal trends in gas exchange parameters, whole tree conductivity and stable carbon isotopes. Photosynthetic capacity parameters (Vc_{max} and J_{max}) and the amount of chlorophyll were examined to evaluate investment in photosynthetic capacity. Amounts of biologically important foliar nutrients (N, P and K) were analyzed to rule out any age-related nutrient deficiencies. Finally, the amount of total non-structural carbohydrates (NSC) were examined in order

to better understand the supply and demand of photosynthetic end-product for each age class. All gas exchange parameters were measured *in situ*, on fully-expanded, current year foliage from the top 1/3 of the canopy using a LI-6400 gas exchange system.

The transition from juvenile (sub-canopy) to mid-age (emergent) trees is controlled by increased stomatal limitation and decreased photosynthetic capacity. This was demonstrated by decreases in diurnal trends of gas exchange parameters, photosynthetic capacity parameters (Vc_{max} and J_{max}) and chlorophyll between juvenile and older (mid-age and old) trees. Juvenile trees appear to be operating under a "go for broke strategy" to adapt to intraspecific and interspecific competition. The nutrient limitation hypothesis does not hold true, as there are no age-related difference in foliar nutrient quantities. The transition from mid-age to old trees is marked by a change from source (supply) control to sink (demand) control. Analysis of NSC demonstrates a build-up of photosynthate in old trees, indicating that older, established trees may be using a more conservative life-strategy. As a result, we conclude that the decrease in productivity in old *P. rubens* is not due to decreased photosynthesis, but to a decreased demand for new carbon compounds.

Chapter 2 attempts to distinguish between maturational influences and those due to environmental factors, factors external to the meristem by minimizing the confounding factors of tree size and complexity. Growth and gas exchange measurements were performed on reciprocal grafts in the summer of 2004 using field-grown mid-age and old rootstock and potted juvenile rootstock. Results indicate that factors external to the meristem appear to be the principle influence for age-related changes in photosynthesis and physiology in red spruce.

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Introduction

Many forest stands, including those with a large percentage of red spruce (*Picea rubens* Sarg.), show a decline in net primary productivity (biomass accumulation/ area/ year) with increasing tree age. Generally, forest growth is slow early on, peaks at mid-age and declines gradually thereafter (Ryan et al. 1997). The peak growth in mid-age stands coincides with maximum stand leaf area and canopy closure (Seymour and Kenefic 2002, Ryan et al. 1997). Stand-level behavior is in large part a function of individual trees following similar trends of an early peak and then a decline in growth. While this trend is well documented in many forest types and tree species, the cause of this decline remains under debate.

Conifers undergo many morphological and developmental changes as they age, including a decline in tree height and stem diameter growth. Branches may become thicker and more twisted, treetops become less conical and more flattened and branches exhibit less orthotrophism with increasing tree age (Bond 2000, Day et al. 2001, Greenwood and Hutchison 1993, Ryan and Yoder, 1997).

Changes in morphology and anatomy of needles have also been demonstrated for several conifers. Needle width, thickness (height) and cross sectional area have all been shown to increase with age in red spruce and eastern larch (*Larix laricina* (Du Roi)) (Day et al. 2001, Hutchison et al. 1990, Ward 2005), while needle length, roundness (ratio of shortest: longest cross-sectional axis) and cross sectional area increase in Douglas-fir (*Psuedotsuga menziesii* (Mirb.) Franco) (Apple et al. 2002). Leaf mass area (LMA = leaf

dry mass / total leaf area or specific leaf area (SLA) $^{-1}$), which is an indicator of needle massiveness, has been shown to increase in older red spruce trees and eastern larch (Day et al. 2001, Ward 2005, Hutchison et al. 1990). In red spruce these changes occur in both sun and shade leaves (Day et al. 2000). Ward (2005) also found an increase in the ratio of photosynthetically active mesophyll: non-photosynthetic tissue, tracheid diameter and in the ratio of xylem area to needle cross-sectional area and a decrease in internal air space with increasing red spruce age. Apple et al. (2002) found a decrease in the proportion of photosynthetic mesophyll in older Douglas-fir trees, a trend opposite that found in red spruce. It is important to note that the most dramatic changes in these parameters occur early in tree life; the differences between very young and older trees are greater than those between mid-age and old trees.

The decrease in growth rate is accompanied by a decrease in photosynthesis with increasing tree age in many conifer species, including lodgepole pine (*Pinus contorta*), ponderosa pine (*Pinus ponderosa*), red spruce, Douglas-fir, Norway spruce (*Picea abies*) and Scots pine (*Pinus silvestris*) (Bond 2000, Day et al. 2001, Ryan et al. 1997, Schulze et al. 1986). While the trend of decreasing photosynthetic rates with increasing tree age is demonstrated in many conifers, the physiological cause of this decrease is not well understood. One proposal, the hydraulic limitation theory, states that the decreases in photosynthetic rates are linked to an increasingly longer and more complex hydraulic pathway (from roots to needles) which in turn controls stomatal apertures (Ryan and Yoder 1997, Yoder et al. 1994).

Evidence for stomatal limitation to photosynthesis has been demonstrated in lodgepole pine, ponderosa pine and Douglas-fir (Hubbard et al. 1999, Yoder et al. 1994,

Woodruff et al. 2004). Yoder et al. (1994) found that instantaneous rates of photosynthesis of different aged trees for both lodgepole and ponderosa pines are similar in the early morning, indicating a similar photosynthetic capacity between the age classes. During the course of the day, photosynthetic rates of older trees diverged from those of younger trees, with an earlier and more severe mid-day depression of photosynthesis for older trees than for younger trees. In ponderosa pine, this divergence can occur as early as 10 A.M. In addition, stomatal conductance followed a similar trend, with a divergence between tree ages later in the day. This is supported with evidence from analysis of stable carbon isotope ratios, where older ponderosa pines had a larger amount of ^{13}C than younger trees (less negative $\delta^{13}C$) (Yoder et al. 1994). A greater proportion of ^{13}C can indicate that stomatal conductance was limiting at the time the carbon was fixed, resulting in less discrimination between ^{12}C and ^{13}C. While evidence for stomatal limitation to photosynthesis has been found for some large, Western tree species, no such evidence has yet been found for red spruce. In fact, Day et al. (2001) found that while older red spruce trees have significantly lower photosynthetic rates than younger trees, this could not be explained by differences in stomatal conductance or internal CO_2 concentration between age classes.

If hydraulic conductance decreases with increasing tree size or age, whole-tree conductance should be lower in older trees and carbon assimilation and hydraulic properties closely linked (Hubbard et al. 1999, Hubbard et al. 2001, Sellin 2001). Analogous to Ohm's Law and Darcy's Law, the flux of a fluid through a system, in this case roots, stems, branches, needles, etc., is the product of a driving force (or pressure differential) and a conductance (Fiscus 1986, Hubbard et al. 1999). We define tree

conductance as the ratio between transpiration and the water potential (Ψ) differential between leaf and soil; in other words,

Eq. 1.1 **conductance (k_{tree}) = transpiration (E) / $\Delta\Psi$**.

As previously mentioned, there is no direct evidence supporting a stomatal limitation to photosynthesis in red spruce. Two predictions can be tested to directly address this issue. If the age-related decrease in photosynthesis in red spruce is not due to increased stomatal resistance, then

1) Diurnal trends of gas exchange measurements between different age classes of *Picea rubens* will not diverge from each other and there will be no interactions between age and time of day, and

2) Picea rubens will not show age-related trends in tree conductance.

If photosynthesis in older red spruce trees is not limited by hydraulic constraints, we must look to other explanations. An alternative mechanism is that the decrease in photosynthesis in red spruce is related to a decline in the photosynthetic capacity with increasing tree age; that is to say, older trees have an inherently lower ability to photosynthesize than younger trees which is not a function of water stress.

The biochemical limitations to carbon assimilation vary based on light level, temperature and CO_2 levels (Farquhar and Sharkey 1982, von Caemmerer and Farquhar 1981, Wullschleger 1993). An analysis of these limitations gives us insight into the capacity for carbon assimilation. At low CO_2 concentrations, the CO_2 acceptor ribulose bisphosphate (RuBP) is saturating and carbon assimilation is limited by the activity of the enzyme RuBP carboxylase-oxygenase (Rubisco). The slope (rate) of the increase in carbon assimilation with increase in internal CO_2 at those concentrations is referred to as

the rate of carboxylation (Vc_{max}) and is a measure of how efficiently the Calvin cycle is operating. At higher CO_2 levels, carbon assimilation is limited by the rate of regeneration of RuBP via the electron transport chain (RuBp is now limiting) and is referred to as the electron transport rate (J_{max}). After utilization in the Calvin cycle, sufficient amounts of ATP and NADPH must be available to regenerate RuBP; the rates of ATP and NADPH production depend on the rate of electron transport. As these two components of the photosynthetic machinery, Vc_{max} and J_{max}, have evolved in balance, they are strongly correlated with each other and provide an estimate of the relative investment in or functionality of the photosynthetic machinery. Rates of Vc_{max} and J_{max} are obtained by plotting increasing internal CO_2 levels against carbon assimilation rates; under a given set of conditions, the slope of the initial portion of the graph represents Vc_{max} and the slope of the line after the inflection point represents J_{max}.

Chlorophyll is an energetically expensive compound to synthesize and maintain (Taiz and Zeiger 2002). In sun leaves, the level of carbon assimilation is tightly linked to chlorophyll content since all conversion of CO_2 into carbohydrates occurs in the chloroplast (Nobel 2005, Raven 1986). In fact, increased chlorophyll content was shown to be correlated with high levels of maximum photosynthesis at light saturation in larch (Hutchison et al. 1990). This study will use the amount of chlorophyll present in needles as an independent assessment of investment in the photosynthetic machinery. The following two predictions will be examined to determine if there are biochemical or phytochemical limitations to photosynthesis:

3) *Picea rubens* will show age-related differences in photosynthetic parameters; rates of carboxylation and electron transport will decrease with increasing tree age.

4) *Picea rubens* will demonstrate age-related trends in investment to the photosynthetic machinery; chlorophyll content will decrease with increasing tree age.

Relationships between sinks (photosynthate importing tissues, ie. actively growing tissues) and sources (photosynthate producing tissues) are currently being investigated as a source of control over photosynthetic rates. Photosynthesis is a highly regulated metabolic process; there must be a balance between the creation and maintenance of energetically expensive photochemicals and the need for carbon compounds (Paul and Foyer 2001). Although a specific mechanistic link between photosynthetic rates and amount of photosynthate present in foliage is not yet known, a growing amount of evidence suggests that through several possible feedback mechanisms, photosynthesis is tightly linked to the export and utilization of the photosynthetic product (sugars and starches) (Luxmoore 1991). High levels of non-structural carbohydrates (sugars and starches) in foliage can be an indication of end-product accumulation (source/production > sink/demand) and a potential cause of the down-regulation of photosynthesis (Körner 2003). If the age-related decline in photosynthesis in red spruce is a function of feedback limitation, the following prediction should be true:

5) *Picea rubens* will show differences in sink: source relations between the age classes; foliar sugar and starch concentrations will increase with increasing tree age.

Low nutrient (N, P, K, Mg, Ca, etc.) availability can potentially affect photosynthesis in several ways. Low nutrient availability may shift resources away from leaf area production and into fine root production or may lower concentrations of photosynthetic enzymes, thus decreasing photosynthesis (Ryan et al. 1997). The nutrient limitation to photosynthesis hypothesis states that fewer nutrients may be available to older trees, as they are bound up in living biomass and that older trees with reduced nutrient supply may have a lower photosynthetic capacity (Ryan and Yoder 1997). Nitrogen is especially important since it is often a limiting nutrient in many ecosystems and is a key component of chlorophyll and photosynthetic enzymes (Field and Mooney 1986). Although conifer growth may be linked to nitrogen supply (Ryan et al. 1997), recent work with ponderosa pine and lodgepole pine found no significant relationship between photosynthetic capacity and foliar nitrogen content (Hubbard et al. 1999, Yoder at al. 1994). This study also examines phosphorous, which is important to ATP synthesis, and magnesium, which is a building block of chlorophyll. If the nutrient limitation hypothesis does not explain the age-related decline in photosynthesis in *Picea rubens*, the following prediction should hold true:

6) *Picea rubens* will show no differences in foliar nutrient concentration between age classes.

Materials and Methods

Study Site

This study was conducted at the Penobscot Experimental Forest (PEF) in Bradley, Maine in July and August of 2005. Mid-age and old trees were located in a multi-species, multi-cohort stand managed by the USDA Forest Service under a 5-year selection silvicultural system (Seymour and Kenefic 2002). Juvenile trees were located in an adjacent (apx. 200 m away) stand that is managed under a shelterwood system. All trees were part of the same population. Close stand proximity minimized any potential site differences. Both stands have been described in greater detail in Day et al. (2001), Seymour and Kenefic (2002) and Ward (2005). Mid-age trees averaged 54 years and old trees averaged 128 years of age; both age classes produced strobili (Table 1.1). Juvenile trees averaged 13 years of age and produced no strobili. The upper crowns of all trees selected for this study were growing in full sunlight for a major portion of the day and foliage was selected from the upper third of the crown to avoid sun-shade interactions with the age-related comparisons. The upper canopy of the mid-age and old trees was reached using a 25 meter hydraulic lift (JLG Industries, Inc. Hagerstown, MD).

Table 1.1 Means ± standard deviation for age, diameter at breast height (DBH) for mid-age and old trees and diameter 10cm from the tree base for juvenile trees and height measurements for juvenile, mid-age and old trees.

Age class	**Age (years)**	**Diameter (cm)**	**Height (m)**
old	128 ± 26	40 ± 3	17 ± 2
mid-age	54 ± 8	19 ± 2	10 ± 1
juvenile	13 ± 1	1.7 ± 0.4	1 ± 0.1

Diurnal photosynthetic trends

Gas exchange measurements on fully expanded, current year foliage of ten trees of each age class were taken in July 2004 using a LI-6400 portable photosynthetic system (LI-COR Biosciences, Inc., Lincoln, Nebraska). Three branches per tree in the upper crown were flagged for subsequent measurements. Measurements were taken every two hours from 6am until 6pm. Shoots were exposed to a level of irradiance (2000 μmol m^{-2} s^{-1}) that approximates full-strength sunlight using a LI6400B red/blue LED light (LI-COR Biosciences, Inc., Lincoln, Nebraska). Within the cuvette, vapor pressure deficit was held below 2.0 kPa, leaf temperature was held between 22-26° C to avoid depression of photosynthesis (Day 2000) and ambient CO_2 levels (apx. 380 μmol mol^{-1}) were used. When conditions inside the 2 X 3 cm cuvette stabilized (total machine coefficient of variation <0.2 %), three measurements per shoot were taken approximately five seconds apart and averaged.

One tree of each age class was measured in succession in order to avoid weather-related confounding factors between age classes. The order in which age classes were measured was changed every day to avoid smaller time scale issues; for example, differences in photosynthesis between a 6 A.M. start time vs. a 7 A.M. start time. A maximum of two trees per age class were measured per day and a minimum of one tree of each age class was measured per day. After the final reading was taken each day, the shoots were collected and transported to the laboratory in a cooler in airtight bags with a moist paper towel to avoid desiccation. Needles were then removed from the branch and total projected area measurements were estimated using a high-resolution scanner with WinSeedle™ software (Regent Instruments, Quebec, QC, Canada). Gas exchange

measurements were recalculated using the estimated needle areas. The needles were then dried at 60°C for three days and dry needle mass was recorded using an analytical balance.

Assimilation versus intercellular CO_2 concentration (A v. C_i)

Six trees of each age class were examined for their response to changing levels of CO_2 using the LI-6400 system. Needles were exposed to a saturating irradiance (1200 μmol m^{-2} s^{-1}) (Day et al. 2001) and temperature inside the cuvette was held to 22-24°C. Carbon dioxide levels were varied between 100-1200 μmol mol^{-1} in the following order: 400, 350, 300, 250, 200, 100, 400, 400, 600, 800, 1200 μmol mol^{-1}. Varying the CO_2 levels in this fashion (mid to low to high) enables the stomata to adequately adjust to the changing levels (LI-COR Biosciences 1998). Foliage samples were collected and treated as mentioned in the "Diurnal Trends" section.

Vc_{max} and J_{max} were estimated from A-C_i curves modeled with the Nelder-Mead simplex method using the Photosynthesis Assistant software package (Dundee Scientific, Scotland, UK) with equations based on those of Farquhar et al. (1980).

Conductivity (k)

Tree-level conductance (k_{tree}) was estimated six trees of each age class. Transpiration rates (E) were measured using the LI-6400 system and water potential (Ψ) measurements were made pre-dawn and at noon using a pressure bomb (PMS Instrument Co., Corvallis, OR). Pre-dawn foliar water potential was used as a proxy for soil water potential. Tree level conductance was calculated using

Eq. 1.2 $$\boldsymbol{k_{tree} = t_r / \Delta\Psi},$$

where t_r = transpiration (μmol H_2O $m^{-2}s^{-1}$) and ΔΨ = noon ΔΨ - pre-dawn ΔΨ (MPa), corrected for gravity (0.1 MPa for every 10m tree height).

Chlorophyll content

Foliar samples from ten trees of each age class were collected, the needles were stripped from the branch and then air dried. Seven ml of dimethyl sulfoxide (DMSO) was added to approximately 0.1g of needles and incubated overnight at 65°C (Hiscox and Israelstam 1979, Wellburn 1994). Needles were then washed with an additional 3 ml of DMSO, bringing the total volume to 10 ml. Absorbance was read at 435, 415, 666, 648 and 740 nm on a Spectronic 1201 spectrophotometer (Milton Roy Company) using a tungsten light source. An additional sample from three trees per age class were weighed, dried for 48 hours and weighed again to calculate average percent dry mass for each age class (dry weight / fresh weight).

Amounts of chlorophyll a, chlorophyll b, ratio chlorophyll a:chlorophyll b, total chlorophylls and total carotenoids were calculated using the following equations:

Eq. 1.3 **chlorophyll a (mg chl / g dry weight) = (Ca * 0.01) / needle dry weight**

Eq. 1.4 **chlorophyll b (mg chl / g dry weight) = (Cb * 0.01) / needle dry weight**

Eq. 1.5 **chlorophyll a: chlorophyll b = chl a / chl b**

Eq. 1.6 **total chlorophylls (mg chl / g dry weight) = chl a + chl b**

Eq. 1.7 **total carotenoids (mg chl / g dry weight) = (car * 0.01) / needle dry weight**

where Ca (μg / ml of chlorophyll a) = (17.04*absorbance @ 666nm) – (6.96*absorbance @ 648nm), Cb (μg / ml of chlorophyll b) = (38.8*absorbance @ 648nm) –

(11.41*absorbance @ 666nm) and car (μg / ml of carotenoids) = ((1000*absorbance @ 470nm) – (3.27*Ca) – (104*Cb)) / 229 (Arnon 1949, Wellburn 1994).

Total non-structural carbohydrates (NSC)

Current-year foliar samples were collected in August 2004 from 10 trees of each age class to analyze sugar and starch levels. Due to a low amount of current-year foliar mass on individual branches, samples were collected from several branches per tree and combined. Samples were wrapped in foil and placed in liquid nitrogen to be transported back to the laboratory. Samples were immediately weighed and dried at 60°C until no further weight change could be determined, at which point they were ground with a mortar and pestle. Samples were weighed when dry and a dry weight: fresh weight ratio was calculated. Approximately 50 mg of dried, ground foliar sample per tree was then treated with 5 ml of 80% ethanol as described in Rose et al. 1991 (Appendix: Enzyme Method 3) in order to separate simple sugars (supernatant) from starches (residue).

Sugar analysis was performed via the phenol-sulfuric acid method (Buysse and Merckx 1993). A 28% concentration of phenol was used as sucrose, glucose and fructose all have the same absorbance in 80% ethanol when reacted with 28% phenol (Buysse and Merckx 1993). Absorbance was measured at 490 nm on a Spectronic 1201 spectrophotometer using a tungsten light source. A standard curve was created using known concentrations of sucrose (Sigma-Aldrich, Inc. CAS 57-50-1). A regression equation to calculate percent sugar was created using the SAS system general linear model (glm) (version 9.0, SAS institute, Inc. Cary, NC).

Eq. 1.8 **% sugar = 100*((absorbance – 0.0356) / 669.2)**, r^2= 0.99.

Starch content was analyzed using the enzymatic digestion method discussed in Rose et al. (1991) and Haissig and Dickson (1979). Enzymatic digestion converts mobile starch to glucose using, in this case, amyloglucosidase and α-amylase (Sigma-Aldrich 2005). While this method may overestimate starch content (Chow and Landhäusser 2004), any bias would be uniform across age classes in this comparative study. Starch content was calculated using:

Eq. 1.9 $\mathbf{\% \text{ starch} = 10 * ((\Delta A_{TEST} * 900) / (\Delta A_{STD} * \text{sample weight}))}$

where ΔA_{TEST} = (absorbance of foliar sample – absorbance of reagent blank) and ΔA_{STD} = (absorbance of standard – absorbance of standard blank) when measured spectrophotometrically at 540 nm (Sigma-Aldrich 2005).

Stable Carbon Isotope Analysis ($\delta^{13}C$)

Current year foliage from ten trees of each age class was analyzed to determine relative amounts of ^{13}C and ^{12}C. Tissue was collected in the same manner as described above for NSC analysis. Cellulose was extracted by the Jayme-Wise method. Carbon isotope ratios were determined using a Finnigan/MAT 251 isotope ratio mass spectrometer (Thermo Electron Corporation, Waltham, MA) at the University of Idaho Stable Isotopes Laboratory (Moscow, ID). Ratios reported here are in standard delta notation relative to the conventional standard PDB (Pee Dee Belemnite) ($\delta^{13}C$). This process has been described in detail in Yoder et al. (1994).

Nutrient analysis

Foliar samples from ten trees of each age class were collected for nutrient analysis. Samples were transported to the lab in cool, moist, air-tight conditions, dried for 3 days at 60°C and ground in a Wiley mill with a 20 mesh screen. Samples were

analyzed at the Maine Agricultural and Forest Experimental Station's Analytical Lab (Orono, ME). Total nitrogen was analyzed using the combustion (or Dumas) method in a LECO CN-2000 (LECO Corporation, St. Joseph, Michigan). All other nutrients were analyzed using the dry ash method (Kalra and Maynard 1991).

Statistical Analysis

All statistical analyses were performed using the SAS System for Windows (version 9.0, SAS Institute, Inc., Cary, NC). Diurnal trends were analyzed using an analysis of variance (ANOVA) for a repeated measures design with a 0.05 α-level to test the effects of age class, time of day and the interaction between age and time of day. Means were separated using the Tukey's studentized range test and a Levene's test was performed to assess homogeneity of variances. In order to meet the assumptions of the ANOVA, a natural log transformation was performed on the diurnal stomatal conductance data.

A single factor ANOVA was performed on all A-C_i, conductivity, chlorophyll, NSC and nutrient data to test age class differences. Means were separated using t tests, the Levene's test was used to check homogeneity of variances and an α-level of 0.05 was used. Natural log transformations were made on percent starch, chlorophyll a and percent phosphorous data and a square root transformation was made on carotenoid data in order to meet the assumptions of the ANOVA.

Results

Diurnal trends

On both an area basis and a mass basis, juvenile photosynthetic rates differed significantly from mid-age and old photosynthetic levels (Table 1.2). On an area basis, photosynthetic rates of mid-age trees ranged from 16% to 38% lower than those of juvenile trees and rates of old trees ranged from 11% to 46% lower than juvenile trees. There were no significant differences between the photosynthetic rates of mid-age and old trees. There were significant differences in photosynthetic rates as a function of time of day while the interaction between age and time of day was not significant (Figure 1.1). This trend was similar but more pronounced on a mass basis, with significant differences between juvenile and the two older age classes and no significant differences between mid-age and old trees (Table 1.2). Results from mid-age trees were between 31% and 63% lower than those of juvenile trees and results from old trees were between 30% and 53% lower than those of juvenile trees. There were highly significant time of day differences in photosynthesis on a mass basis and no significant interactions between age and time of day (Figure 1.2). On a chlorophyll basis (photosynthesis mg chl^{-1}), there were significant differences between all age classes, with mid-age trees having the lowest rates of photosynthesis per unit chlorophyll (Table 1.2). Photosynthetic rates of juveniles varied between 30-136% higher than those of mid-age trees and the rates for old trees were between 14-59% higher than those of mid-age trees. There were significant time of day differences and no significant interactions between age and time of day (Figure 1.3).

Table 1.2 P values for ANOVA factors and interaction for diurnal trends in photosynthesis on an area, mass and chlorophyll basis and for intercellular CO_2 concentration and stomatal conductance.

Measurement	age	time of day	age * time of day
Photosynthesis/ area (μmol CO_2 m^{-2} s^{-1})	0.0001	0.0001	0.7464
Photosynthesis / mass (μmol CO_2 kg^{-1} dry weight s^{-1})	0.0001	0.0001	0.9984
Photosynthesis / chlorophyll (μmol CO_2 mg^{-1} chlorophyll s^{-1})	0.0001	0.0001	0.9185
Intercellular CO_2 concentration (μmol mol^{-1})	0.0001	0.0001	0.5536
Stomatal conductance (μmol CO_2 m^{-2} s^{-1})	0.0001	0.0001	0.9985

Figure 1.1 Diurnal photosynthetic trends on a leaf area basis by tree age category. Means ± SE are graphed. Age classes not followed by the same letter are significantly different at $p< 0.05$.

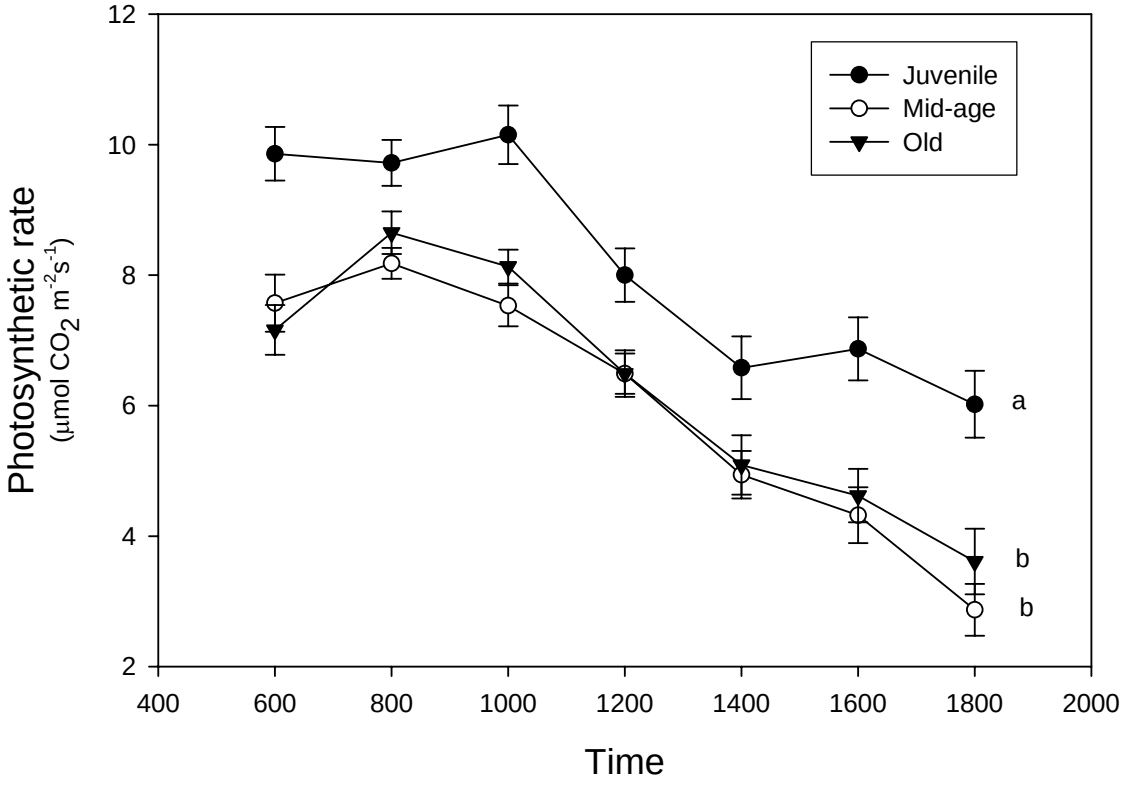


Figure 1.2 Diurnal photosynthetic trends on a foliar dry mass basis by tree age category. Mean values ± SE are graphed. Means not followed by the same letter are significantly different at $p < 0.05$.

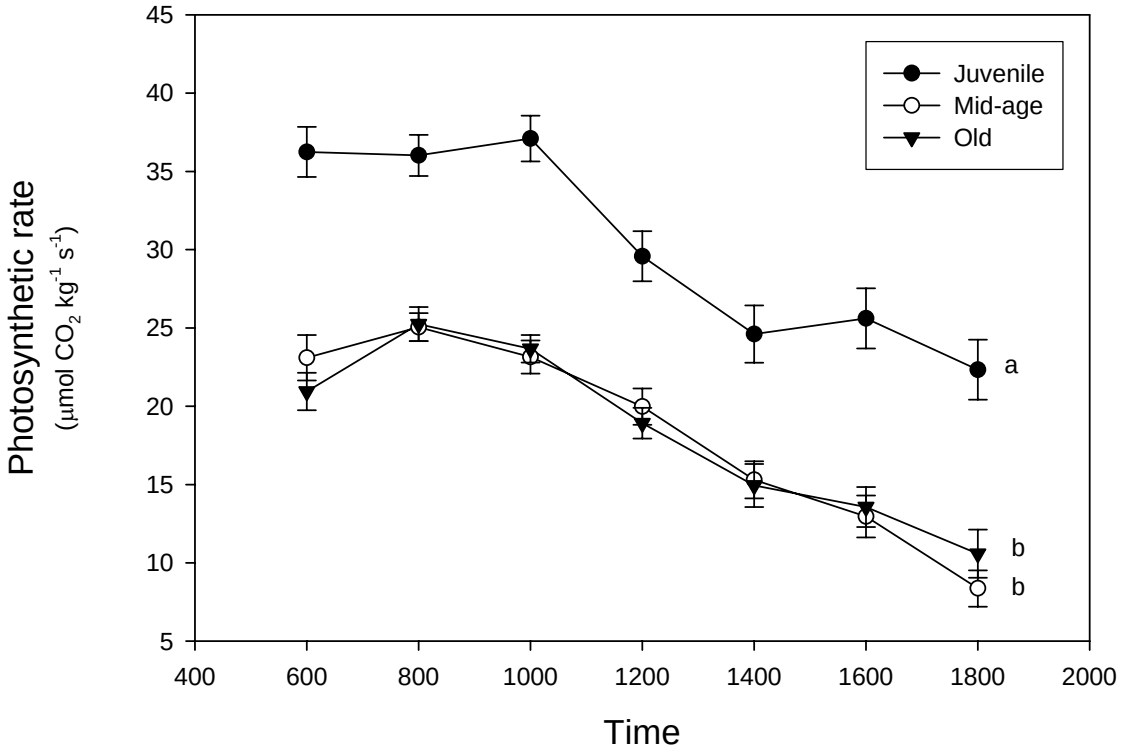
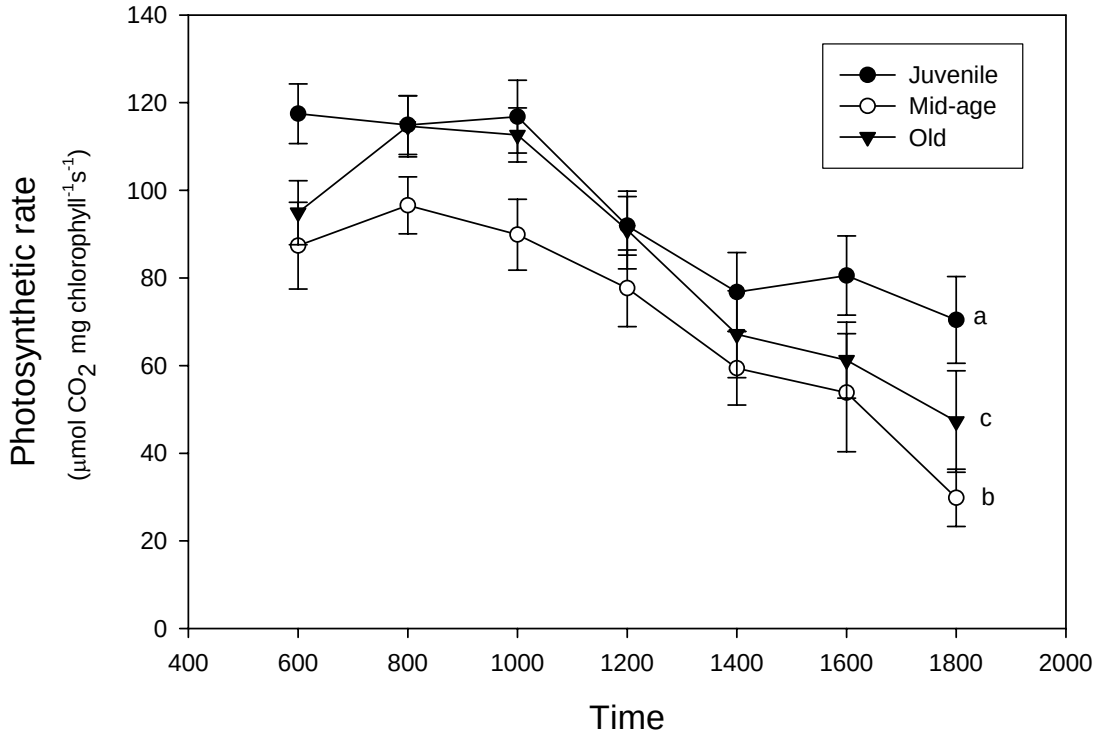


Figure 1.3 Diurnal photosynthetic trends on a unit chlorophyll basis by tree age category. Mean values ± SE are represented. Age classes not followed by the same letter are significantly different from each other at $p < 0.05$.



Diurnal trends for both stomatal conductance (g_s) and intercellular CO_2 concentration (C_i) mirrored those of photosynthesis on an area and a mass basis, with levels for juvenile trees significantly greater than those of either the mid-age or old trees (Table 1.2). C_i was 11-19% lower in mid-age trees than in juvenile and 8-19% lower in old trees than in juvenile trees (Figure 1.4). Rates of g_s were 41-73% lower in mid-age than juvenile trees and 51-68% lower in old trees than in juvenile trees (Figure 1.5). While C_i was lower in old trees than in mid-age trees, there were no significant differences in either C_i or g_s between mid-age and old trees. For both parameters there were significant time of day factors and no significant interactions between age and time of day (Table 1.2).

Figure 1.4 Diurnal trends of intercellular CO_2 concentration by tree age. Means ± SE are shown. Ages not followed by the same letter are significantly different at $p < 0.05$.

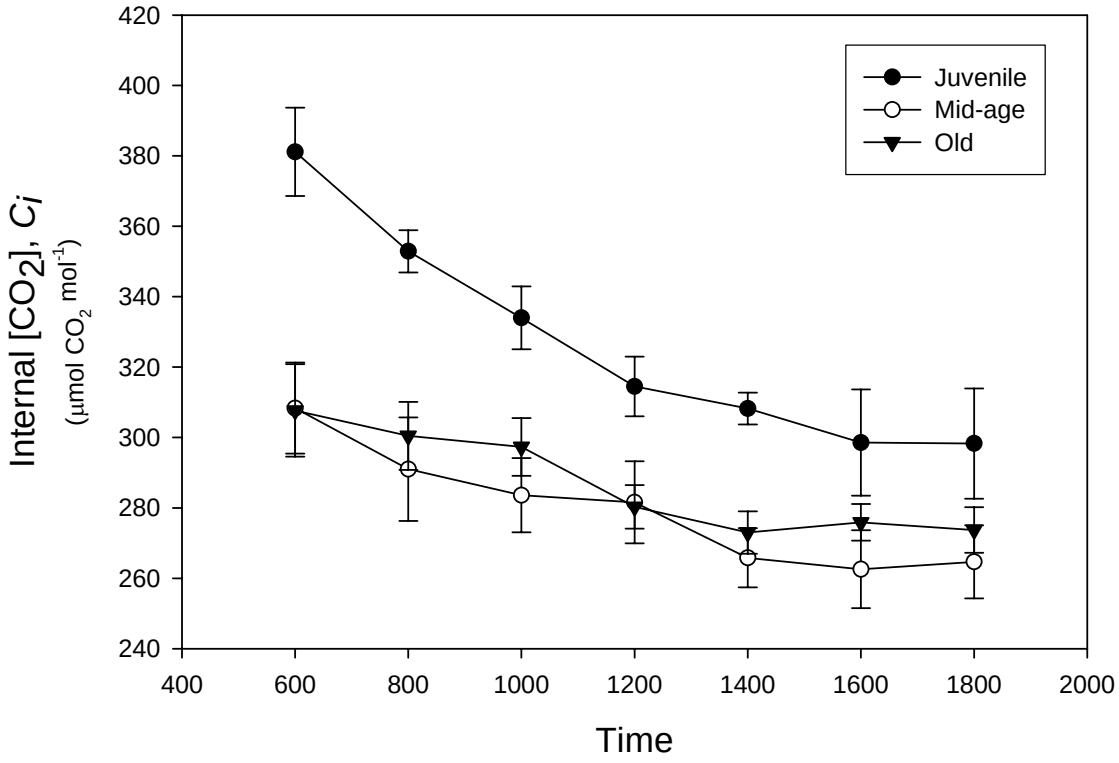
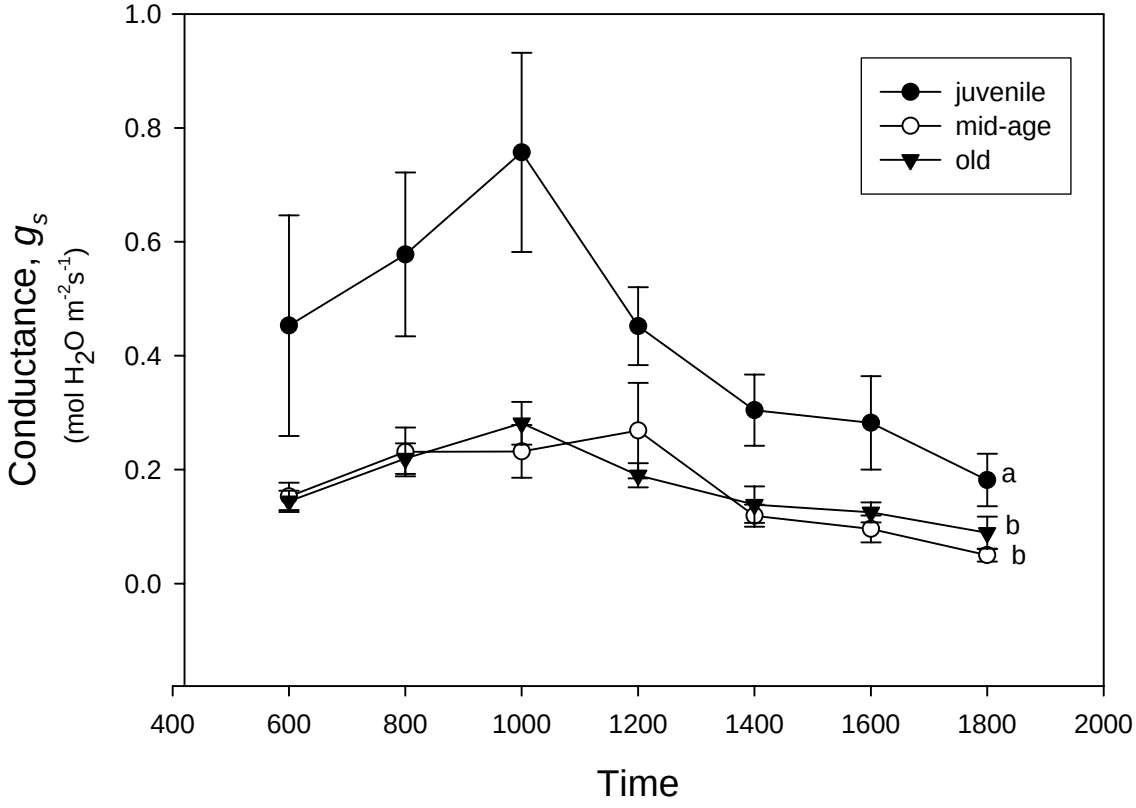


Figure 1.5 Diurnal trends in stomatal conductance by tree age. Mean values ± SE are shown. Age classes not followed by the same letter are significantly different from each other at $p < 0.05$.



A-C_i data

Vc_{max} and J_{max} declined with increasing tree age (Table 1.3). Differences between the juvenile and the two older age classes were significant for both parameters. Mid-age and old trees had 28% and 32% lower Vc_{max} rates, respectively, than juvenile trees and 31% and 35% lower rates for J_{max}.

Table 1.3 Means ± SE for Vc_{max} and J_{max} for different age classes. For each parameter, means not followed by the same letter are significantly different at $p<0.05$. N=6

Measurement	juvenile	mid-age	old	p
Vc_{max} ($\mu mol\ m^{-2}\ s^{-1}$)	33.67 ± 2.06 a	24.24 ± 1.55 b	22.87 ± 2.28 b	0.0037
J_{max} ($\mu mol\ m^{-2}\ s^{-1}$)	93.02 ± 5.53 a	64.40 ± 4.72 b	59.88 ± 5.77 b	0.0012

Conductivity

There were no significant age-related differences between any age classes for rates of transpiration (Table 1.4). Tree level conductance (k_{tree}) was significantly different between juvenile trees and the older trees. There was no significant difference in k_{tree} between mid-age and old trees. Whole tree conductance was 49% lower in mid-age trees than in juveniles and 42% lower in old trees than in juvenile trees. For both parameters, mid-age trees had the lowest values.

Table 1.4 Means ± SE for mid-day transpiration and tree level conductance for different age classes. For each parameter, means not followed by the same letter indicate significant differences ($p<0.05$). N= 5 for juvenile trees, N= 6 for mid-age and old trees.

Measurement	juvenile	mid-age	old	p
Mid-day transpiration ($\mu mol\ H_2O\ m^{-2}\ s^{-1}$)	2.72 ± 0.50 a	2.05 ± 0.30 a	2.26 ± 0.34 a	0.4800
Tree level conductance, k_{tree} ($\mu mol\ H_2O\ m^{-2}\ s^{-1}\ MPa^{-1}$)	3.90 ± 0.48 a	1.57 ± 0.23 b	1.78 ± 0.32 b	0.0186

Chlorophyll content

Amounts of chlorophyll a, total chlorophylls, total carotenoids and chl a: chl b were significantly different between all age classes (Table 1.5) and all parameters decreased with increasing tree age. Chlorophyll b levels were not significantly different for any age classes. It is important to note that the difference in chlorophyll a between the age classes drives the significant differences of total chlorophylls and chl a: chl b. Chlorophyll a was 28% higher in juvenile trees than in mid-age and 71% higher in juvenile trees than old trees. Total carotenoids showed approximately the same increases; 24% between juveniles and mid-age and 76% between juvenile and old trees.

Table 1.5 Means ± SE for chlorophylls a and b, total chlorophylls, total carotenoids and chl a: chl b for trees of different age classes. For each parameter, means not followed by the same letter are significantly different at $p<0.05$. N= 10.

Measurement	**juvenile**	**mid-age**	**old**	**p**
Chlorophyll a (mg chl / g dry weight)	2.39 ± 0.12 a	1.86 ± 0.07 b	1.39 ± 0.07 c	0.0001
Chlorophyll b (mg chl / g dry weight)	0.82 ± 0.04 a	0.77 ± 0.04 a	0.73 ± 0.05 a	0.3293
Total chlorophylls (mg chl/ g dry weight)	3.21 ± 0.16 a	2.63 ± 0.11 b	2.12 ± 0.10 c	0.0001
Total carotenoids (mg car / g dry weight)	0.31 ± 0.01 a	0.25 + 0.01 b	0.18 ± 0.02 c	0.0001
Ratio chlorophyll a:b	2.91 ± 0.08 a	2.46 ± 0.06 b	1.96 ± 0.10 c	0.0001

Total non-structural carbohydrates

Sugar content on a dry matter basis was significantly higher in old trees than in juvenile or mid-age trees (Table 1.6). Differences between juvenile and mid-age trees were not significant. Sugar levels were 33% and 71% higher in old trees than in mid-age and juvenile trees, respectively. Percent starch on a dry matter basis was significantly

different between all age classes. Old trees contained 136% and 340% more starch than mid-age and juvenile trees, respectively, and juvenile trees contained 87% more starch than mid-age trees.

Table 1.6 Means ± SE for sugar content and starch content for different age classes. Means not followed by the same letter are significantly different ($p<0.05$). For sugar content N= 10, for starch content N= 6.

Measurement	juvenile	mid-age	old	p
Sugar content (% dry mass)	6.67 ± 0.46 a	8.55 ± 0.70 a	11.44 ± 0.74 b	0.0001
Starch content (% dry mass)	6.62 ± 0.65 a	3.54 ± 1.25 b	15.63 ± 3.04 c	0.0011

Carbon isotope analysis

$\delta^{13}C$ was significantly different for all age classes (Table 1.7). Mean $\delta^{13}C$ for mid-age trees was 8% less negative than that of juvenile trees and 12% less negative for old trees than for juvenile trees.

Table 1.7 Mean ± SE for $\delta^{13}C$ for different age classes of *Picea rubens*. Means not followed by the same letter are significantly different ($p<0.05$). N = 10.

Measurement	juvenile	mid-age	old	p
$\delta^{13}C$	-27.741 ± 0.2957 a	-25.609 ± 0.2695 b	-24.522 ± 0.2134 c	0.0001

Nutrient analysis

There were no significant age-related trends for amounts of phosphorous (P) or for magnesium (Mg) (Table 1.8). Percent nitrogen (N) showed a downward trend with increasing tree age, with juvenile trees having significantly different nitrogen than old trees. There were no significant differences in nitrogen levels in adjacent age classes. Juvenile trees contained 12% more nitrogen than old trees. There were no significant differences between age classes when comparing the N: P ratio.

Table 1.8 Means ± SE for % nitrogen, % phosphorous, % magnesium and N: P for different age classes. Means not followed by the same letter are significantly different at $p<0.05$. N= 10.

Measurement	juvenile	mid-age	old	p
Nitrogen content (% dry mass)	1.016 ± 0.031 a	0.944 ± 0.033 ab	0.900 ± 0.040 b	0.0636
Phosphorous content (% dry mass)	0.141 ± 0.002 a	0.139 ± .006 a	0.127 ± 0.005 a	0.1093
Magnesium content (% dry mass)	0.105 ± 0.006 a	0.119 ± 0.005 a	0.115 ± 0.008 a	0.2837
Ratio N:P	7.24 ± 0.30 a	6.84 ± 0.25 a	7.11 ± 0.29 a	0.6028

Discussion

Trees are faced with different environments and stresses as they grow. When trees are very young and just becoming established in a stand, the ability to collect light, convert that energy into height growth and ascend to canopy level is critical to survival. Once canopy ascension is achieved, trees are exposed to a varying set of external stressors, such as intense wind and sun and in many cases, increased exposure to pathogens (Kimmins 1997). In addition, the internal environment, including hormonal changes (Greenwood 1984), changes in carbohydrates (Myers et al. 1999) or changes in water or nutrient availability (Ryan et al. 1997) of a tree varies during this time. All of these factors and others, either alone or in combination, will act to place differing limitations on photosynthesis and growth. Different life strategies are utilized to deal with these varying conditions. As a result, there will be different controls over photosynthesis between 1) juvenile (sub-canopy) trees and mid-age and old (overstory level) trees and 2) between mid-age (high productivity) and older (low productivity) trees.

Do tree hydraulics limit photosynthesis in older red spruce?

Ryan and Yoder (1997) give three general predictions for the hydraulic limitation of photosynthesis; 1) transpiration and photosynthesis must vary with differing hydraulic resistances, 2) hydraulic resistance must increase with tree size and 3) therefore, photosynthesis must be lower in older trees. The third prediction is the easiest to examine and has been confirmed in many conifer species including red spruce (Day et al. 2001, Ward 2005), Norway spruce (Niinemets 2002), ponderosa pine (Hubbard et al. 1999 and Yoder et al. 1994), lodgepole pine (Yoder et al. 1994), *Pinus sylvestris* (Scots

pine) (Kull and Koppel 1987, Niinemets 2002), Douglas-fir (McDowell et al. 2002) and *Sequoia sempervirens* (redwood) (Koch et al. 2004).

If the first prediction of the hydraulic limitation hypothesis, that age-related changes in transpiration and photosynthesis vary with changes in hydraulic resistance, holds true, any decrease in foliar water availability, such as occurs during the course of a day, should decrease stomatal conductance and thus decrease photosynthesis. The mid-day depression in photosynthesis is due to increased internal water stress as the tree transpires the locally available water and closes its stomata to avoid cavitation. If this trend is age-related (or size-related), older (larger) and younger (smaller) trees should follow different patterns of carbon assimilation (A) and g_s throughout the day (Yoder et al. 1994).

Both lodgepole and ponderosa pines displayed similar age related trends in diurnal gas exchange (Yoder et al. 1994). In ponderosa pine, the mid-day depression of photosynthesis of older trees began approximately 2 hours before that of young trees. In addition, the decrease in photosynthesis from the daily maximum was much more drastic in older ponderosa pines (from 5 μmol m^{-2} s^{-1} to 2 μmol m^{-2} s^{-1}) than in young ponderosa pines (>5 μmol m^{-2} s^{-1} to 3 μmol m^{-2} s^{-1}). In red spruce, the mid-day depression of photosynthesis began at the same time of day, regardless of tree age (Figures 1.1, 1.2 and 1.3). While the decrease in photosynthesis in ponderosa pine was rapid, with a large drop off early, and then a slight recovery and a plateau later (Yoder et al. 1994), the depression in red spruce was gradual and photosynthesis did not rebound or plateau later in the day. In both lodgepole pine and ponderosa pine, the maximum daily rate of photosynthesis was similar in both young and old trees, while in red spruce, the maximum rate of

photosynthesis for juvenile trees was significantly higher than that of the two older age classes. There were no interactions between age and time of day when examining photosynthetic levels in red spruce, which indicates that any hydraulic limitation operating on older trees is operating in the same manner in younger trees.

While these analyses hold true for photosynthesis on both an area and a mass basis, the differences in photosynthesis between young and older trees were greater on a mass basis than on an area basis (Figs. 1.1 and 1.2 and Day et al. 2001). This is due to the decrease in SLA in *Picea rubens* with increasing tree age (increase in LMA). While leaf area increases with increasing tree age, leaf mass increases to a greater degree and unit area per unit mass decreases with increasing age (unit mass per unit area increases with increasing tree age). This change has several benefits and disadvantages. The more massive ("heavy duty") needles of the older trees are able to withstand the more stressful environment at the top of the canopy, but may sacrifice some ability to photosynthesize due to internal shading or increased resistance to CO_2 diffusion. Dijkstra and Lambers (1989) found that fast growing *Plantago major* had a greater photosynthetic rate than a slow growing variety, which they attributed to greater internal shading and less light absorption due to a higher SLA in the fast growing trees. Norway spruce and Scots pine showed a decrease in net assimilation on a mass basis with increasing tree age, but not on an area basis (Niinemets 2002). The author hypothesizes that a change in foliar morphology, the increase in needle dry mass per area (LMA) with increasing age (or decrease in SLA), is a mechanism to compensate for water stress in older trees and that this compensation is incomplete (not enough to return photosynthetic rates to that of young trees) for Norway spruce and Scots pine. In red spruce, Ward (2005) found that

older needles had larger vascular bundles and showed an increase in the ratio of xylem area to leaf perimeter. This could be viewed as a mechanism of hydraulic compensation if this change is accompanied by a concomitant increase in the vascular tissue of the stem and branch. Ward (2005) also found decreased mesophyll airspace with increasing tree age, which may increase resistance to CO_2 diffusion through the gas phase and increase liquid phase resistance to CO_2 absorption by the mesophyll cells.

In both ponderosa and lodgepole pines, photosynthetic rates first thing in the morning for old trees were similar to those of the young trees (comparable to mid-age trees in this study), which indicate that these trees all have the same photosynthetic capacity (Yoder et al. 1994). In fact, in the early morning, older ponderosa pines were actually operating at a higher photosynthetic level than younger pines. First thing in the morning, older red spruces trees were already photosynthesizing at a much lower rate than the younger ones (about 20% lower on an area basis and about 40% lower on a mass basis) (Figures 1.1 and 1.2). Early morning plant Ψ are high and non-limiting and show only minor differences across age classes related to gravitational effects; this indicates that younger red spruce trees may have a greater photosynthetic capacity than older ones.

If stomatal resistance limits carbon assimilation, we should see corresponding diurnal trends between stomatal conductance, g_s (resistance $^{-1}$) and the internal concentration of CO_2, C_i. Stomatal conductance in the older age classes of red spruce did not vary greatly during the day and internal CO_2 concentration dropped very gradually over the course of the entire day (310 μmol mol^{-1} to 270μmol mol^{-1}) with no peaks. However, the trends for the juvenile trees were not as tightly linked. Juvenile trees showed a peak in stomatal conductance at 10 A.M. (the time of maximum

photosynthesis) and then a more drastic decline for the rest of the day than the older trees do. However, internal CO_2 concentration in juvenile trees did not show a corresponding peak and the change over the course of the day was more pronounced than in older trees (380 μmol mol^{-1} to 300 μmol mol^{-1}). In addition, results for the $\delta^{13}C$ analysis indicated that at some time during the life span of the foliage there was some stomatal limitation with increasing tree age (Table 1.7). The $\delta^{13}C$ for old trees was less negative than that of the two younger age classes and the difference between mid-age and juvenile trees was great. A less negative number for $\delta^{13}C$ tells us that there is less discrimination between ^{13}C and ^{12}C in the older trees, which can result from stomatal limitation (Yoder et al. 1994). This analysis shows that we may have some stomatal restriction between mid-age and old trees, but this was not reflected in the photosynthetic data.

These results reflect a more conservative water use strategy, greater WUE (water use efficiency), by older trees. Since juvenile trees will not survive long in a sub-canopy position, water stress is less limiting in juvenile trees. Juvenile trees also have a much smaller root system and if they conserve water resources, competitors with better developed root systems are likely to utilize any "conserved" water. In older trees, survival against water stress (high WUE) may increase long-term reproductive success. This study has not found evidence that g_s limits photosynthesis to a greater degree in older trees than in younger *Picea rubens*.

While the results for $\delta^{13}C$ for red spruce follow the same trend as those for ponderosa pine (Yoder et al. 1994), the diurnal trends for both g_s and C_i did not and the trends for g_s and C_i did not match each other. There are several possible explanations for this. First, both g_s and C_i, as discussed in this experiment, are instantaneous

measurements, while $\delta^{13}C$ is integrated over the life of the needle. It is very possible that over the course of the year, trees of different age classes responded differently to dry periods, especially if these occurred during the growing season, as demonstrated by McDowell et al (2005). Foliage analyzed for $\delta^{13}C$ in this study was one year old, but there may have been seasonal water stress differences that were not recorded during the instantaneous measurements taken in July and any carbon incorporated during bud-set was potentially several years old. The results for $\delta^{13}C$ were also consistent with Ward's (2005) findings that older trees may have more mesophyll resistance to CO_2 diffusion, indicating that increased resistance to gas diffusion in older trees is within the cell and not related to stomatal resistance. Another possible explanation for these apparently inconsistent trends may be due to how the gas exchange measurements were taken. In this study, we measured potential (saturating light) and not actual (ambient light) photosynthetic rates. Photosynthesis responds quite rapidly to changes in light, while stomatal adjustment can take up to 30 minutes. Older trees are exposed to full sunlight earlier in the day than juvenile trees. As soon as they are hit with bright sunlight, they begin photosynthesizing at high levels, depleting internal CO_2, and there is lag time before the stomata open to replenish internal CO_2. The apparent inconsistencies in g_s and C_i may be a result of this component. Early in the day, juvenile trees may not be "realizing" their full potential for photosynthesis. Taken as a whole, these trends indicate that while there may be some stomatal limitation to photosynthesis in red spruce, it does not explain the decrease in photosynthesis with increasing tree age.

The final portion of the hydraulic limitation theory is that hydraulic limitation increases with tree size (age), which limits photosynthesis, and this should be evident in

whole tree conductances (Hubbard et al. 1999, McDowell et al. 2002, Ryan and Yoder 1997). Changes in tree level conductance could be due to a change in resistance in the soil-to-air pathway, changes in sapwood permeability or to the difference in the gravitational potential caused by increased height (Ryan et al. 2000). Young red spruce trees had a greater tree level conductance than older trees do, but the increased resistance to water flow was not necessarily the cause of the decline in photosynthesis. In addition, these fluxes did not correspond well to photosynthetic rates in red spruce; mid-age trees had the lowest conductances, while old trees had the same photosynthetic rates. Differences in hydraulic conductivity may explain the differences in photosynthetic levels between juvenile and canopy level trees, but not those between canopy level trees. Despite any differences in k_{tree}, the lack of differences in photosynthetic performance between mid-age and old trees indicates that something other than stomatal resistance is controlling photosynthetic rates between these age classes. Although photosynthetic performance was similar in mid-age and old trees, there was a drop in foliar efficiency of about 50% between mid-age and old *P. rubens* (Day et al. 2001, Seymour and Kenefic 2002). Leaf level hydraulic conductance in Douglas-fir decreased up to 50% with increasing tree height although this does not correspond to a decrease in photosynthesis; the authors indicate that the expected trend in conductance and photosynthesis may be seasonal in nature (McDowell et al. 2002, McDowell et al. 2005). The fact that red spruce does not display an age-related decrease in hydraulic conductance is not likely due to methodology. Ryan and Yoder (1997) indicate that when leaf water potential is measured with a pressure bomb and resistance expressed on a leaf area basis, not a mass basis, as in this study, hydraulic resistances were generally greater in old trees.

To summarize, while stomatal limitation has not been completely disregarded as an explanation for the decline in photosynthesis with increasing tree age in red spruce, age-related hydraulic limitations do not adequately account for this decline, especially between mid-age and old trees. Diurnal photosynthetic measurements did not support the idea that trees of different ages behave differently over the course of the day and in fact, provided some evidence that older (mid-age and old) red spruces had a lower photosynthetic capacity than younger trees. Older trees had a greater resistance to water movement than juvenile trees do. It does not seem likely that the same factors limiting photosynthesis in tall, Western species are operating in red spruce. The fact that there may be different limitations to photosynthesis in trees growing in very different ecosystems makes biological sense. In fact, King (1990) made a strong argument for region-specific co-evolution of tree heights between commonly occurring species. If tree height is influenced, at least to some degree, by the behaviors of other species, it is conceivable that traits other than height may have co-evolved.

Biochemical limitations to photosynthesis

If photosynthesis is not limited by hydraulic conductance in red spruce, another possible explanation is that it may be limited in one of the initial biochemical steps of carbon assimilation. Analysis of the efficiency of the Calvin cycle (Vc_{max}) and of the rate of electron transport (J_{max}) gives us insight into the potential to photosynthesize at a given CO_2 concentration that is independent of stomatal limitations (Farquhar et al. 1980, von Caemmerer and Farquhar 1981, Wullschleger 1993). For instance, highly productive herbaceous species were reported to have consistently higher rates of both Vc_{max} and J_{max} than slower growing woody species (Wullschleger 1993).

Red spruce showed a significant decline in both Vc_{max} and J_{max} with increasing tree age, with a drop of approximately 30% for both parameters between young and older trees. Since trees tend to be limited by Vc_{max} at high light levels and by J_{max} at low light levels, the fact that both of these parameters decline in a similar manner indicates that in young and old trees electron transport and carboxylation capacity are up- or down-regulated in parallel. While these parameters were significantly different only between the juvenile and the two older age classes, the trend of declining rates continued from mid-age to old trees. This indicates that older red spruce trees have a lower capacity for photosynthesis than younger trees.

Once inside the sub-stomatal cavity, CO_2 must diffuse from the intercellular air spaces to the site of carboxylation in the chloroplast. This involves diffusion between the intercellular air space and diffusion through the cell wall, cell membrane and the liquid phase into the chloroplasts (Evans and von Caemmerer 1996). Increased resistance to CO_2 diffusion could contribute to the decrease in Vc_{max}, although this potential increase in resistance is not enough to adequately account for the differences in photosynthesis that we see between age classes. Ward (2005) found proportionally less internal air space in the needles from older trees, which may increase the resistance to diffusion of CO_2 (Mediavilla et al. 2001). Since CO_2 diffuses more slowly in liquid than in air (10,000 times more slowly), the length of the diffusive liquid pathway must be short in order to ensure an ample supply of CO_2 to the chloroplast (Evans and von Caemmerer 1996). When adjusted for internal air space, Ward (2005) found a slight increase in the proportion of mesophyll in the oldest trees. This could be a potential limitation to photosynthesis in old trees, but does not account for the more drastic drop-off in

photosynthesis between juvenile and mid-age trees. Additionally, air space decreased between mid-age and old trees with no decrease in Vc_{max} and J_{max} should not be affected by CO_2 diffusion. With that said, an age-related increase in mesophyll resistance could contribute to the less negative $\delta^{13}C$ in older trees by decreasing effective C_i at the chloroplasts. When examining just the $\delta^{13}C$, we can't say if the decrease in isotope discrimination with increasing tree age is due to CO_2 resistance or photosynthetic capacity (less discrimination = greater CO_2 resistance or lower photosynthetic capacity), although it appears that any increase in resistance to CO_2 diffusion is intracellular and not stomatal. Since our photosynthetic parameters, Vc_{max} and J_{max}, both decreased with increasing tree age, it is likely that the noted differences in $\delta^{13}C$ were due to increased WUE or seasonal water stress limitations and not to an overall resistance to CO_2 diffusion.

Differential photochemical potentials have also been shown in Norway spruce and Scots pine (Niinemets 2002). While age-related changes were not examined, Vc_{max} declined in these species on a height basis (height as a proxy for age), which the author attributed to increased resistance between needle intercellular air space and the site of carboxylation. As previously discussed, Ward (2005) found that younger trees have a higher proportion of needle internal air space, suggesting an increased air phase resistance in older trees. Ponderosa pine, a species with substantial evidence supporting hydraulic limitation to photosynthesis, showed no difference in the slopes of the A-C_i curves of different aged trees (Hubbard et al. 1999). The decline in the photosynthetic capacity parameters in red spruce supports the hypothesis that older trees simply have a lower ability to photosynthesize than younger trees do.

Is photosynthesis constrained by nutrient limitations?

Ryan and Yoder (1997) suggest two possible nutrient-related explanations for the decline in photosynthetic capacity. The first is that hydraulic resistance limits the flow of nutrients to the foliage and the second is that chronic water deficiencies are accompanied by reduced nitrogen allocation to foliage under water stress. In many species, photosynthetic capacity (A_{max}) is highly correlated to foliar nitrogen concentration when compared on both an area and a mass basis (Field and Mooney 1986). Specifically, the nitrogen in Rubisco, chlorophyll and other soluble leaf proteins represents the majority of nitrogen investment in needles, although increases in nitrogen do not always translate into increases in growth (Bauer et al. 2001, Evans 1989). Niinemets (2002) suggested that a low nitrogen concentration may limit the formation of "high capacity photosynthetic apparatus" in older/taller trees.

Work with lodgepole and ponderosa pine indicated that photosynthetic capacity is not nitrogen-limited in older trees because nitrogen levels did not display age-related trends (Hubbard et al. 1999, Ryan and Yoder 1997, Yoder et al. 1994) and nitrogen concentration was also independent of tree age or height in Norway spruce and Scots pine (Niinemets 2002). Red spruce showed significant differences in nitrogen concentration only between juveniles and the oldest trees and there was only a 10% reduction in the amount of nitrogen in old trees. It is unclear what effect these slight nitrogen differences in red spruce may have; nitrogen concentration is not correlated with photosynthetic capacity and long term nitrogen additions do not cause a response in photosynthesis for this species (Elvir et al. 2006, Schaberg et al. 1997). It is speculated that red spruce, a slow-growing, stress-tolerating conifer, may practice luxury consumption (absorbing

resources in excess of immediate growth needs). This study argues only that photosynthesis in red spruce is not limited by nitrogen and not that the similarities in foliar nitrogen concentration indicate a similar photosynthetic capacity. A possible avenue for further investigation into A_{max}-nitrogen relations in red spruce is the potential differences in nitrogen partitioning (Rubisco vs. chlorophyll vs. proteins) between the age classes (Bauer et al. 2001).

As mentioned above, nitrogen is a key component of chlorophyll and chlorophyll should be tightly correlated with photosynthesis. While it may seem that an increase in chlorophyll would increase photosynthesis, the opposite has been shown in red spruce grafts (Rebbeck et al. 1993). Photosynthesis was shown to decline with age and chlorophyll content in mature scions was greater than or equal to that in juvenile scions. However, in eastern larch, chlorophyll content increased with tree age, which paralleled the trend for photosynthesis, although the ratio between chlorophyll a and b did not change (Greenwood et al. 1989, Hutchison et al. 1990). In our population of non-grafted red spruce, chlorophyll content decreased significantly across all age classes, with the exception of chlorophyll b (Table 1.5). Chlorophyll a is associated with Photosystems I and II and is more indicative of the investment in reaction centers while chlorophyll b is associated with the light harvesting antennae (Nobel 2005). These results potentially represent a decrease in the investment in the photosynthetic reaction centers with increasing age, although chlorophyll is not as tightly linked to photosynthetic rates as Vc_{max} or J_{max}. This would indicate that either older trees have more efficient reaction centers or that older trees absorb excess PAR, even though there may be more mutual shading in old foliage. Another possibility is that since only 40 % of chlorophyll a goes

to the reaction centers and the rest into light harvesting (Nobel 2005), the reduction in chlorophyll a may represent a redistribution of the ratios of a:b within the chloroplast. This would appear to give trees a greater ability to adapt to future conditions, since chlorophyll b only plays a role in light gathering, while chlorophyll a serves both roles.

Differences in foliar nitrogen content within the range of this study have been shown to not influence photosynthesis in *Picea rubens* (Elvir et al. 2006). Elvir et al. (2006) indicated that the lack of response in red spruce to nitrogen additions may be due to an imbalance with other nutrients, such as Mg and Ca. Reductions in magnesium were associated with stomatally induced reductions in photosynthesis and reductions in the electron transport rate (J_{max}) in *Pinus radiata* (Monterey pine) (Laing et al. 2000). There were no age-related differences in Mg or other nutrients in red spruce. Therefore age-related trends in photosynthesis in red spruce do not appear to be related to a nutrient deficiency.

Sink: source relationships

The relationships between sink and source strengths have been linked to changes in photosynthetic rates (Lavigne et al. 2001, Luxmoore 1991, Myers et al. 1999, Schaberg et al. 2000). A basic assumption behind this relationship is that organs will meet their own carbon, water, nutrient, etc. needs and then export any excess to neighboring tissues. If sink strength in the whole tree is high, photosynthates will continue to be produced and exported, thus the source remains active. When sinks are low, exportation of carbohydrates will slow or cease and this will be a feedback mechanism limiting photosynthesis. Experimental manipulations in *Pinus taeda* (loblolly pine) demonstrated that lowered carbohydrate sink strength decreased photosynthesis (Myers et al. 1999). In

the long-term, reduced sink strength can trigger the down-regulation of genes that code for components of the photosynthetic system and result in reduced photosynthetic capacity (Paul and Foyer 2001).

According to Luxmoore's model (1991) simple sugars, such as sucrose, can be considered to be short-term storage molecules and the more complex starch molecules longer-term storage molecules. Recent work with *Metasequoia*, *Taxodium*, and *Larix* demonstrated that starch was more highly correlated with the down-regulation of photosynthesis in conifers (Equiza et al. 2006). Foliar sugar content increased with age in red spruce, indicating that younger trees were stronger sinks than older trees. Foliar starch concentrations were drastically higher in old trees, which indicated that starch may be operating as a feedback mechanism in older red spruces. While the age-related trend of sugar concentration paralleled that of photosynthesis, this did not hold true for starch levels. Mid-age trees had lower amounts of starch than juvenile trees, but higher photosynthetic rates. Mid-age trees were the fastest growing age class and were putting on more biomass per year than the other age groups (growth will be discussed in Chapter 2), so it stands to reason that their foliage would be exporting more photosynthate to meet this high sink demand than that of trees in the other age groups. Day et al. (2001) suggested that the reduced photosynthetic capacity exhibited by older *Picea rubens* may result from reduced carbohydrate demand associated with inherently lower growth potential.

This issue can get quite complicated as the mechanism linking carbohydrate build-up and photosynthesis has not yet been determined. This raises the issue of which parameter (photosynthesis or carbohydrate concentration) is the cause and which is the

effect (Luxmoore 1991). Does a reduction in photosynthesis decrease the export of carbohydrates because there is less excess photosynthate or does a build up of carbohydrates cause the decrease in photosynthesis through a "black box" feedback mechanism? To complicate matters even more, there may be some compensation for increased export loads. Ward (2005) found that phloem: mesophyll ratios were greater in 3 year old trees than in 12 year old trees and speculated that the phloem in young trees may be larger due to increased export, although no causal evidence was offered. Regardless, non-structural carbohydrate levels did, generally, increase with increasing tree age in red spruce, which is expected if the decline in photosynthesis is due to decreased sink strength.

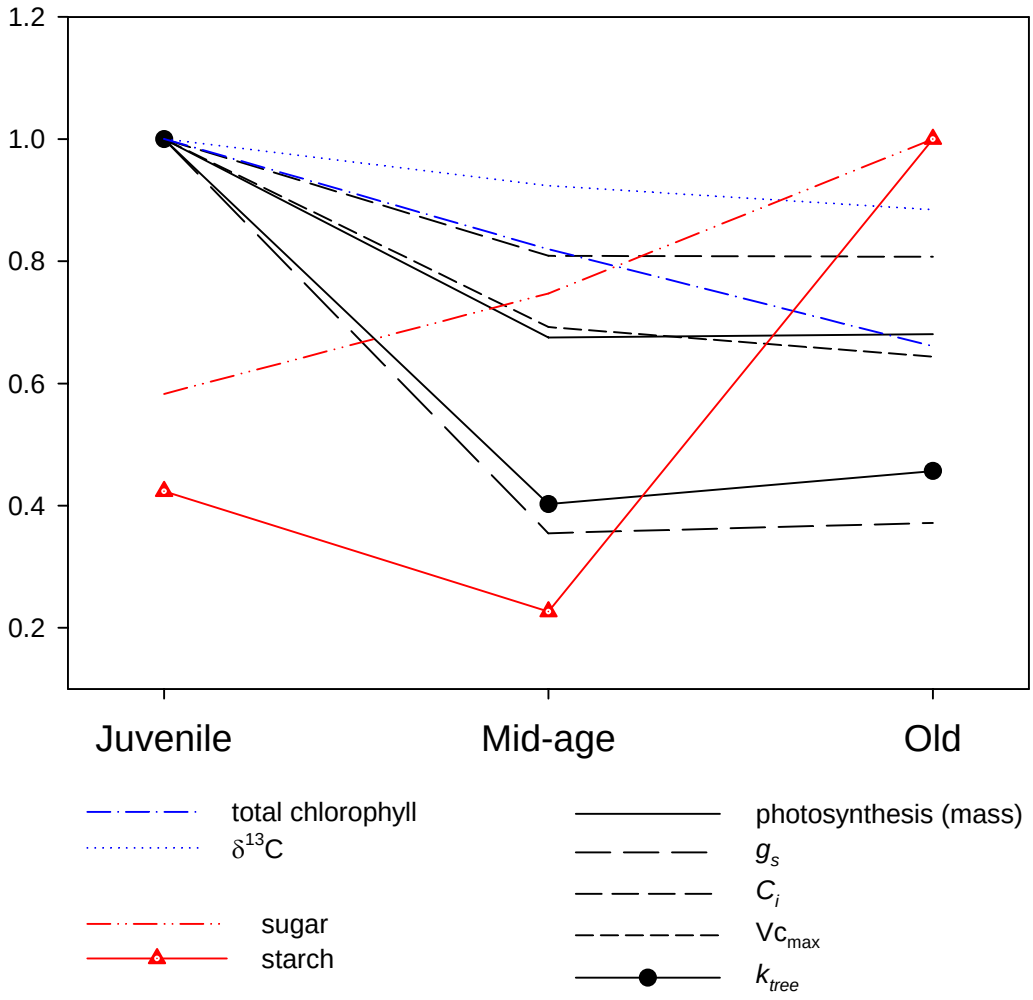
In conclusion, while stomatal limitation may play a small role in the decline in photosynthesis with increasing tree age in red spruce, there appears to be an inherently lower ability to photosynthesize in older trees. Photosynthetic parameters and chlorophyll analyses indicated that older trees invested less in photosynthetic machinery than younger trees. Exploration of non-structural carbohydrates lead us to speculate that decreased sink strength may be a negative feedback mechanism limiting photosynthesis in old trees. Much like the change in SLA, the most drastic differences in photosynthesis, Vc_{max}, J_{max} and sugar content occurred early in the lifespan of a tree (juvenile vs. mid-age). This corresponds well to the early peak and then plateau/ gradual decline in productivity on a stand level.

It appears that there are two segments of changes that occur throughout the lifespan of *Picea rubens*, first with the change from juvenile to mature and then from

mature to old. Photosynthetic capacity did decrease between juvenile and mid-age trees, as evidenced from photosynthetic rates, chlorophyll content and Vc_{max} (Figure 1.6). Juvenile trees maximize their investment in potential productivity and are able to optimize their growth with the available foliage, but a decrease in potential photosynthesis does not explain the decreased productivity between mid-age and old trees. It is possible that the decrease in productivity between mid-age and old trees is the result of varying photosynthetic capacity during dry seasons or during the non-growing season. C_i and g_s both showed large decreases between juvenile and old trees, but not between mid-age and old (Figure 1.6), so the decrease in photosynthesis between juvenile and older trees may be stomatally controlled, to some degree. Juvenile trees may be at a greater risk for embolism (they have a smaller root system), but if they are not able to attain canopy status, they won't survive anyway. The trend in $\delta^{13}C$ between juvenile and mid-age/old trees demonstrates greater water use efficiency in older trees and supports the idea that older trees operate under less risk of embolism.

As trees gain canopy status and are no longer limited by light, they no longer need to operate under the "go for broke" strategy and would actually be putting decades worth of investment in biomass at risk if they continued to operate this way. Mid-age trees had the lowest amount of foliar starch (Figure 1.6), which coincides with the period of maximum growth. After this period, sugar and starch increased, which indicates sink limitations may be operating in the transition from mid-age to old trees. As shown in Figure 1.6, all measured attributes decreased between juvenile and older trees and remain level between mid-age and old trees, except for sugar and starch content.

Figure 1.6 Percent relative to maximum for any age class of 9 physiological parameters. Values were calculated by dividing the value for that age by the maximum value for that parameter for any age. For photosynthesis, C_i, and stomatal conductance, maximum values used were the maximum for the entire day.



Biologically, it makes sense that decreases in photosynthesis and reduced biomass accumulation have some adaptive advantages in older age trees. Older trees are already established and competition from neighbors is less intense than it is for younger trees. Any additional increase in height may make older trees more susceptible to pathogen or wind damage and when height growth slows additional increases in basal area are not

necessary for structural support. A recent analysis of carbon limitation in several ecotypes indicates that while C_3 plants are not CO_2 saturated at current atmospheric levels, mature trees are not carbon limited (Körner 2003). It has been estimated that deciduous trees contain enough carbon stores to replace their canopy four times over (Hoch et al. 2003). If carbon limitation is not an issue in older trees, why spend energy producing and maintaining high levels of expensive photosynthetic machinery? This study leads to the possibility that carbon accumulation and growth in older red spruce is unlikely to increase significantly with increased nitrogen deposition or increases in atmospheric levels of CO_2. As the above measurements were made only during the peak growing season, there is still the possibility that younger and older red spruces may begin and end active carbon accumulation at different times of the growing season. An analysis of these physiological parameters over the course of an entire year would offer a greater insight into this issue.

Chapter Two: Physiology and growth of foliage from reciprocal grafts on juvenile, mid-age and old *Picea rubens*

Introduction

The previous chapter discussed some of the physiological controls of the age-related decline in photosynthesis in red spruce, but the developmental mechanisms ultimately controlling these factors were not examined. A potential problem when examining age-related changes in trees is that as trees grow older, they also increase in size and complexity and the meristems may be subjected to radically different environments between juvenility and old age. Maturation is a developmental process that occurs when an apical meristem exhibits stable changes in its growth habits, including morphological, physiological and biochemical changes (Hackett 1985, Greenwood 1995). While age-related changes are often correlated with changes in size and complexity, these maturation and size effects may be distinct from one another (Greenwood 1984, Greenwood and Hutchison 1993).

As trees age, they undergo changes in both maturational state and in size and complexity. To truly examine age-related changes, these compounding factors must be separated from each other. The environment external to the apical meristem will be different in trees of different sizes, regardless of age (Day et al. 2002). An excellent way to avoid this confusion is to remove one of these factors. By grafting different aged scions onto common rootstock or by using vegetative propagation, we are able to remove the influence of size and can examine age-related changes without the compounding factor of size.

Age-related changes in conifers may be driven by factors external to the meristem. Foliage in older, taller trees can be exposed to increased levels of sun and wind and competition for resources and the pressure of pests and pathogens can change in complex ways as trees age (Day et al. 2002). In addition, a longer and more complex hydraulic pathway may change the dynamics of water, hormone, photosynthate and nutrient transport throughout the tree (Greenwood 1984, Ryan and Yoder 1997, Day et al. 2002). Changes in the sensitivity to hormones, ratios of certain hormones to each other (e.g. abscisic acid (ABA), gibberellins and cytokinins) and the role hormones play in assimilate partitioning also affect maturation (Hackett 1985).

If factors controlling age-related changes are external to the meristem, juvenile habits should return when conditions are restored to those experienced by younger trees. Decreased vigor is noted for some species with increasing age which is retained when old foliage is rooted or grafted onto younger rootstock (Hackett 1985, Poethig 1990, Greenwood et al. 1989, Huang et al. 1992). Rooting ability, a defining characteristic of juvenile plants which decreases with increasing age (Greenwood and Hutchison 1993), has also been shown to increase only when scions from older donors are serially grafted onto juvenile rootstock (Huang et al. 1992). Huang et al. (1992) repeated (4 times) grafting of adult foliage onto rooted juvenile cuttings causing gradual reversion in rooting ability, number of roots per shoot, stem elongation and number of lateral branches per shoot to levels equal to or greater than that of juvenile *Sequoia sempervirens*. These changes were accompanied by the appearance and disappearance of distinctive leaf proteins, although the precise role of these proteins is unknown. The change in leaf proteins indicates a change in meristematic gene expression, but these changes are

obviously not permanent and appear to be controlled by the environment external to the meristem.

Matsuzaki et al. (2005) found no differences between net photosynthesis, stomatal conductance, photosynthetic capacity (per unit nitrogen), carboxylation efficiency, internal to ambient CO_2 concentration ratio or carbon isotope discrimination between young scions grafted onto older *Cryptomeria japonica* and the ungrafted foliage of the old tree. *Picea rubens* rootstock age and size affected foliar characteristics such as needle width, height, cross-sectional area, SLA, net photosynthesis and stomatal conductance, to varying degrees (Ward 2005). These results also suggest that factors extrinsic to the meristem limit photosynthesis in older trees and that the age-related decline in photosynthesis is not due to irreversible, intrinsic changes in the meristem. However, intrinsic and extrinsic factors may both play a role in *P. rubens* since, as discussed later, scion age also played a significant role in determining foliar characteristics (Ward 2005).

There is a good deal of evidence supporting an intrinsic control for some aspects of maturation (Hackett 1985, Poethig 1990, Huang et al. 1992, Greenwood and Hutchison 1993, Matsuzaki et al. 2005). Differences in needle and branch morphology and reproductive capacity remained for two years in *Pinus taeda* after grafting onto young rootstock, which indicated a persistent change in the meristem (Greenwood 1984). Decreases in shoot diameter, number of branches, branch length and the number of flushes per year with increasing scion age have been reported for *Psuedotsuga menziesii* (Ritchie and Keeley 1994), while increases in lateral branching, leaf mass area (LMA, SLA^{-1}), needle thickness and leaf cross-sectional area have all been shown to increase

with increasing scion age in *Larix laricina* (Greenwood et al. 1989, Hutchison et al. 1990). In addition to these morphological changes, persistent physiological differences between scions of different ages have also been reported. Both chlorophyll content and net photosynthesis was shown to increase with increasing scion age in *L. laricina* (Greenwood et al. 1989, Hutchison et al. 1990).

In *Picea rubens*, mature characteristics persisted for at least three years after grafting onto juvenile rootstock (Rebbeck et al. 1993, Day et al. 2001). Rebbeck et al. (1993) found that mature scions of *P. rubens* had a greater needle mass, length, thickness and surface area and more chlorophyll than juvenile scions. There were also increases in needle width, height, cross-sectional area, perimeter and vascular bundle area with increasing scion age (Ward 2005). Photosynthesis and stomatal conductance have been shown to decrease in red spruce with increasing scion age (Rebbeck et al. 1993, Day et al. 2001, Ward 2005). It is important to note that while Rebbeck et al. (1993) and Ward (2005) found that the most dramatic changes occurred between juvenile and mature scions, which agrees with trends found in *Picea sitchensis* (Steele et al. 1989), Day et al. (2001) found that significant changes occurred between young mature (mid-age) and old-growth scions, but not between juvenile and mature. Since both scion age and rootstock age were significant factors in the behavior of grafted *Picea rubens*, this species shows evidence of both intrinsic and extrinsic control over the meristem (Ward 2005).

While the above studies indicate a change in gene expression, the exact mechanism that causes this change is unclear. Phase change occurs from progressive events and is not an abrupt shift in habits (Hackett 1985). This process is autonomous in the apical meristem and may not be controlled by the amount of previously differentiated

tissue (Robinson and Wareing 1969). Within tree maturational gradients (the terminal shoot is more mature than lateral branches closer to the base of the tree) support a cellular basis for maturation (Greenwood and Hutchison 1993) and indicate that maturation may be a function of the number of cell divisions that the meristem has undergone (Hackett 1985, Greenwood and Hutchison 1993). A possible marker for changes in gene expression is DNA methylation since increased levels of methylation are associated with decreased levels of transcription-level gene expression (Greenwood and Hutchison 1993). Work with *Larix laracina* and *Acacia mangium* did not find corresponding changes in methylation to age-related changes in foliar traits, although this may have been due to methodology (Greenwood et al. 1989, Baurens et al. 2004).

Despite years of research, it is still unclear whether age-related changes are pre-programmed genetic changes in the meristem, plastic responses to varying environments or some combination (Greenwood 1995, Day et al. 2002). Day et al. (2002) propose four models to describe potential pathways of control of these changes. The extrinsic model states that the meristem remains inherently unchanged, but can respond plastically during organ development to external variation. A meristem following the stimulus-response pathway will also remain inherently unchanged, but in this case a developing organ will respond to external stimuli. In both cases, the factor controlling the physiological and morphological changes is external to the meristem. The intrinsic pathway proposes that the meristem itself "ages" and goes through genetic changes and then produces organs that are inherently different, regardless of the external environment. The final pathway, the intrinsic-extrinsic pathway, hypothesizes that the meristem undergoes fundamental changes in gene expression that are in turn controlled by factors external to the meristem.

In this study, the confounding factors of size and complexity were removed from the equation by grafting different aged foliage onto rootstock of common ages. This approach minimizes the effects of factors external to both the tree and the meristem (Greenwood and Hutchison 1993, Day et al. 2002). Age-related trends in the physiology and growth of the different graft combinations were examined. If factors extrinsic to the meristem are controlling the age-related changes, scions of any age should begin to exhibit characteristics associated with the age of the rootstock. If factors intrinsic to the meristem control these changes, scions should retain characteristics associated with their donor age, regardless of the age of the rootstock. Support for a combination of intrinsic and extrinsic factors would be demonstrated by an interaction between the scion-donor age and the age of the rootstock.

Methods and Materials

Grafting

During April of 2002, a series of reciprocal grafts were done using different aged *Picea rubens* scions and rootstock. This process was described in detail in Ward (2005). Grafts from juvenile, mid-age and old trees were placed on the top third of the crown in mid-aged and old trees of the same population discussed in Chapter 1. Grafts from juvenile, mid-age and old trees were also placed on the top third of the crown in potted three year-old trees. The three year-old trees were kept in a shadehouse (30% interception) at the University of Maine in Orono. This population has been described in greater detail in Ward (2005). This juvenile population was kept well watered and the summer in which measurements were made (2005) had plenty of rainfall; as a result there should be little or no differences in water availability for the two populations.

Gas exchange measurements

During mid-August of 2004 (August 17th for mid-age and old rootstock and August 18th for juvenile rootstock), gas exchange measurements were made using a LI6400 gas exchange system (LI-COR Biosciences, Inc., Lincoln, Nebraska) on fully expanded grafted foliage. Measurements were made using a saturating irradiance of 1200 μmol m^{-2} s^{-1}, ambient CO_2 levels and leaf temperature was held between 22-24° C. Measurements were made on five mid-age and old trees; on each tree, one graft of each age class (juvenile, mid-age and old) was used and measurements were also made on adjacent ungrafted foliage of that tree. Measurements were made on 15 juvenile rootstocks; five with juvenile grafts, five with mid-age and five with old grafts. Leaf area

was determined using the method described in Chapter 1 and gas exchange parameters were recalculated using those values. These measurements were made on foliage that had flushed three times since the grafting process; the first flush after grafting contained foliage that was pre-formed on the donor tree.

Growth measurements

Total scion length and current year shoot length was collected from all grafted material in August of 2004. The number of rootstock-scion combinations that were measured are shown in Table 2.1.

Table 2.1 Number of grafts utilized for growth measurements of *P. rubens* by rootstock age and scion donor age. These values are the N values for each rootstock-scion combination.

	juvenile scion	**mid-age scion**	**old scion**
juvenile rootstock	13	24	25
mid-age rootstock	17	27	11
old rootstock	11	15	12

Statistics

All statistical analyses were performed using the SAS System for Windows (version 9.0, SAS Institute, Inc., Cary, NC). Gas exchange measurements and growth measurements were analyzed using an analysis of variance (ANOVA) with a 0.05 α-level to test the effects of rootstock age, scion age and the interaction between rootstock and scion age. Means were separated using t-tests and the Levene's test was used to check for homogeneity of variances. In order to meet the assumptions of the ANOVA, a natural log transformation was performed on current year shoot length.

Results

Gas exchange measurements

Rootstock age was a significant factor for net photosynthesis, on both a mass and an area basis (Table 2.2), although no continuous trend is observed, as photosynthetic rates were highest for mid-age rootstock (Table 2.3). On a mass basis, there was a 12% increase in net photosynthesis of grafted material from juvenile to mid-age rootstock and a decrease of 21% and 30% from juvenile to old and mid-age to old, respectively (Table 2.3). On an area basis, there was an 8% increase in net photosynthesis from juvenile to mid-age rootstock and a decrease of 17% from juvenile to old rootstock and 23% from mid-age to old, although the difference between mid-age and old was not significant (Table 2.3). All above age-related changes in photosynthesis were analyzed with scion ages pooled. Rootstock age was not a significant factor for specific leaf area (SLA), stomatal conductance (g_s), internal CO_2 concentration (C_i) or the ratio between the ambient and internal CO_2 concentrations (C_i: C_a) (Tables 2.2 and 2.3). Scion age and the interaction between rootstock age and scion age were not significant factors for any parameter, although the scion X rootstock interaction for mass based photosynthesis was almost significant (p= 0.0644) (Tables 2.2 and 2.4).

Table 2.2 P values for ANOVA factors and interaction for specific leaf area and gas exchange measurements of grafted material from *P. rubens*, sorted by scion-rootstock combinations. Values followed by * are significant at an α-level of 0.05. N= 5 for all rootstock-scion combinations.

Measurement	scion	rootstock	scion X rootstock
Specific leaf area (SLA) ($cm^2\ g^{-1}$)	0.2963	0.1014	0.4517
Net photosynthesis ($\mu mol\ CO_2\ kg^{-1}\ s^{-1}$)	0.1752	**0.0004***	0.0644
Net photosynthesis ($\mu mol\ CO_2\ m^{-2}\ s^{-1}$)	0.3774	**0.0017***	0.1102
Stomatal conductance ($\mu mol\ H_2O\ m^{-2}\ s^{-1}$)	0.0671	0.0571	0.5811
Internal CO_2 concentration ($\mu mol\ mol^{-1}$)	0.1538	0.2649	0.3345
Ratio internal: ambient CO_2 concentration	0.5561	0.5819	0.3858

Table 2.3 Means ± SE for specific leaf area and gas exchange measurements of grafted foliage of *P. rubens* by rootstock age with scions of all age classes pooled. For each parameter, means not followed by the same letter are significantly different at $p< 0.05$. For age J and O, N=15. For age M, N=15 for net photosynthesis, internal CO_2 concentration, internal: ambient CO_2 and N=13 for conductance.

Measurement	juvenile	mid-age	old	p
Specific leaf area (SLA) ($cm^2\ g^{-1}$)	38.16 ± 1.04 a	39.42 ± 0.81 a	36.47 ± 0.99 a	0.1008
Net photosynthesis ($\mu mol\ CO_2\ kg^{-1}\ s^{-1}$)	30.24 ± 1.56 a	33.79 ± 2.15 a	23.81 ± 1.48 b	0.0009
Net photosynthesis ($\mu mol\ CO_2\ m^{-2}\ s^{-1}$)	7.89 ± 0.29 a	8.52 ± 0.45 a	6.55 ± 0.39 b	0.0024
Stomatal conductance ($\mu mol\ H_2O\ m^{-2}\ s^{-1}$)	0.511 ± 0.062 a	0.571 ± 0.074 a	0.355 ± 0.061 a	0.0656
Internal CO_2 concentration ($\mu mol\ mol^{-1}$)	316.0 ± 2.4 a	316.8 ± 3.7 a	310.0 ± 3.5 a	0.2843
Ratio internal: ambient CO_2 concentration	0.880 ± 0.005 a	0.885 ± 0.010 a	0.872 ± 0.009 a	0.5772

Table 2.4 Means ± SE for specific leaf area and gas exchange measurements of grafted red spruce foliage by scion donor age with rootstock ages pooled. Means not followed by the same letter are significantly different from each other at $p < 0.05$. N=15 for all parameters except stomatal conductance in juvenile and mid-age scions, where N=14.

Measurement	juvenile	mid-age	old	p
Specific leaf area (SLA) ($cm^2\ g^{-1}$)	39.15 ± 1.13 a	37.84 ± 1.02 a	37.05 ± 0.74 a	0.3161
Net photosynthesis ($\mu mol\ CO_2\ kg^{-1}\ s^{-1}$)	31.79 ± 2.33 a	28.31 ± 2.21 a	27.74 ± 1.38 a	0.3178
Net photosynthesis ($\mu mol\ CO_2\ m^{-2}\ s^{-1}$)	8.07 ± 0.49 a	7.44 ± 0.48 a	7.45 ± 0.29 a	0.4987
Stomatal conductance ($\mu mol\ H_2O\ m^{-2}\ s^{-1}$)	0.594 ± 0.092 a	0.383 ± 0.044 a	0.448 ± 0.052 a	0.0821
Internal CO_2 concentration ($\mu mol\ mol^{-1}$)	319.4 ± 3.9 a	311.7 ± 2.9 a	311.7 ± 2.8 a	0.1614
Ratio internal: ambient CO_2 concentration	0.886 ± 0.010 a	0.874 ± 0.008 a	0.877 ± 0.007 a	0.5505

As previously reported (Day et al. 2002), net photosynthesis was not limited by hydraulic resistance at the graft union. In this study, no differences were found between ungrafted foliage and autografts (scions grafted onto rootstock of the same tree) for any gas exchange parameter, with C_i as an exception. While scion age was a significant factor for C_i when non-grafted material was included in the analysis ($p=0.0426$), the only significant differences were between juvenile grafts and the non-grafted juvenile growing stock. However, this apparent graft-related effect did not limit photosynthesis since rates were approximately 2% higher in the grafted foliage than in the ungrafted foliage.

Table 2.5 Means ± SE for SLA and gas exchange parameters for all rootstock: scion age combinations. Means in bold are significantly different from values for other rootstock age at $p < 0.05$. Scion age is not a significant factor for any parameter. N=5, except N=4 for g_s for juvenile and mid-age scions on mid-age rootstock.

Scion age	Measurement	juvenile rootstock	mid-age rootstock	old rootstock
juvenile	Specific leaf area (SLA) ($cm^2\ g^{-1}$)	38.31 ± 1.8	41.41 ± 1.62	37.74 ± 2.38
juvenile	**Net photosynthesis ($\mu mol\ CO_2\ kg^{-1}\ s^{-1}$)**	**29.77 ± 2.60**	**40.24 ± 3.88**	**25.37 ± 2.49**
juvenile	**Net photosynthesis ($\mu mol\ CO_2\ m^{-2}\ s^{-1}$)**	**7.78 ± 0.63**	**9.70 ± 0.83**	**6.74 ± 0.60**
juvenile	Stomatal conductance ($\mu mol\ H_2O\ m^{-2}\ s^{-1}$)	0.565 ± 0.147	0.818 ± 0.170	0.445 ± 0.151
juvenile	Internal CO_2 concentration ($\mu mol\ mol^{-1}$)	324.2 ± 5.7	326.2 ± 5.5	307.8 ± 6.7
juvenile	Ratio internal: ambient CO_2 concentration	0.882 ± 0.016	0.909 ± 0.012	0.867 ± 0.019
mid-age	Specific leaf area (SLA) ($cm^2\ g^{-1}$)	39.87 ± 2.12	37.47 ± 1.12	36.17 ± 1.85
mid-age	**Net photosynthesis ($\mu mol\ CO_2\ kg^{-1}\ s^{-1}$)**	**33.96 ± 29.83**	**30.71 ± 3.87**	**20.27 ± 1.36**
mid-age	**Net photosynthesis ($\mu mol\ CO_2\ m^{-2}\ s^{-1}$)**	**8.50 ± 0.49**	**8.13 ± 0.85**	**5.70 ± 0.55**
mid-age	Stomatal conductance ($\mu mol\ H_2O\ m^{-2}\ s^{-1}$)	0.421 ± 0.099	0.406 ± 0.034	0.327 ± 0.075
mid-age	Internal CO_2 concentration ($\mu mol\ mol^{-1}$)	312.4 ± 1.2	309.6 ± 8.0	313.0 ± 4.5
mid-age	Ratio internal: ambient CO_2 concentration	0.873 ± 0.003	0.867 ± 0.023	0.881 ± 0.012
old	Specific leaf area (SLA) ($cm^2\ g^{-1}$)	36.29 ± 1.41	39.38 ± 1.04	35.49 ± 0.75
old	**Net photosynthesis ($\mu mol\ CO_2\ kg^{-1}\ s^{-1}$)**	**26.98 ± 1.97**	**30.42 ± 1.72**	**25.80 ± 3.20**
old	**Net photosynthesis ($\mu mol\ CO_2\ m^{-2}\ s^{-1}$)**	**7.40 ± 0.30**	**7.72 ± 0.37**	**7.22 ± 0.79**
old	Stomatal conductance ($\mu mol\ H_2O\ m^{-2}\ s^{-1}$)	0.547 ± 0.082	0.506 ± 0.074	0.292 ± 0.084
old	Internal CO_2 concentration ($\mu mol\ mol^{-1}$)	311.4 ± 1.7	314.6 ± 3.7	309.2 ± 7.8
old	Ratio internal: ambient CO_2 concentration	0.884 ± 0.006	0.878 ± 0.008	0.870 ± 0.018

Figure 2.1 Means ± SE for photosynthesis on an area basis of grafted *P. rubens* foliage for all scion: rootstock age combinations. For each age combination, N=5.

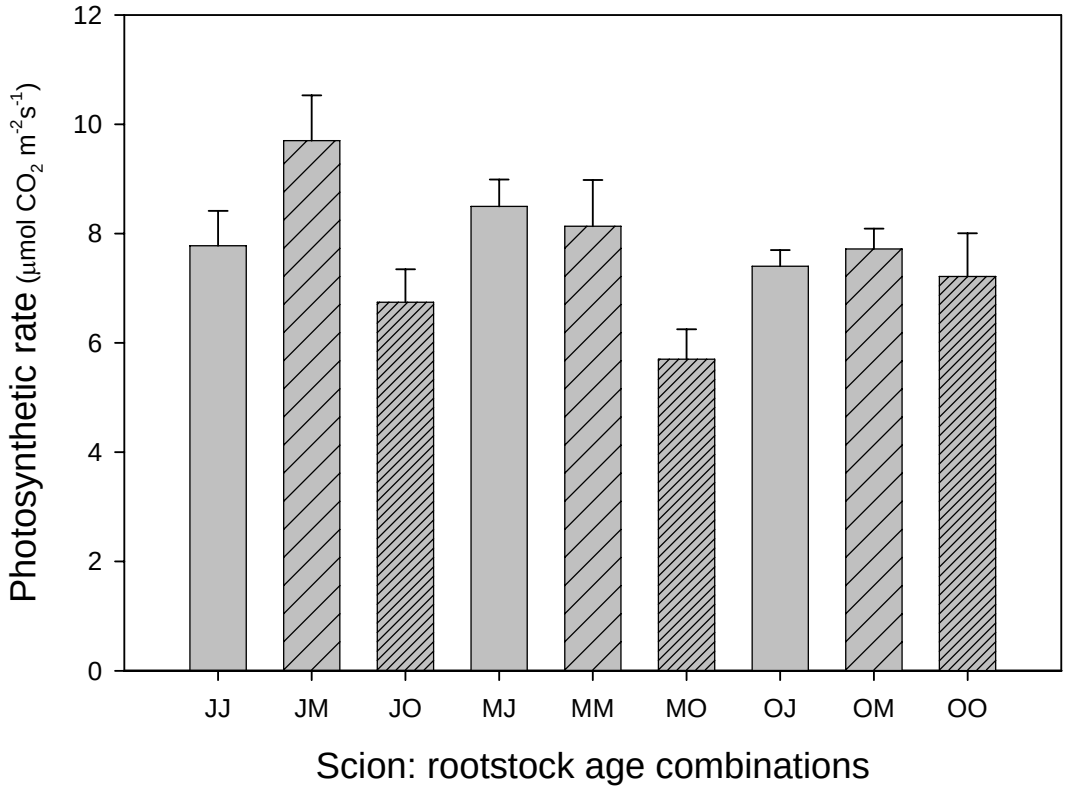
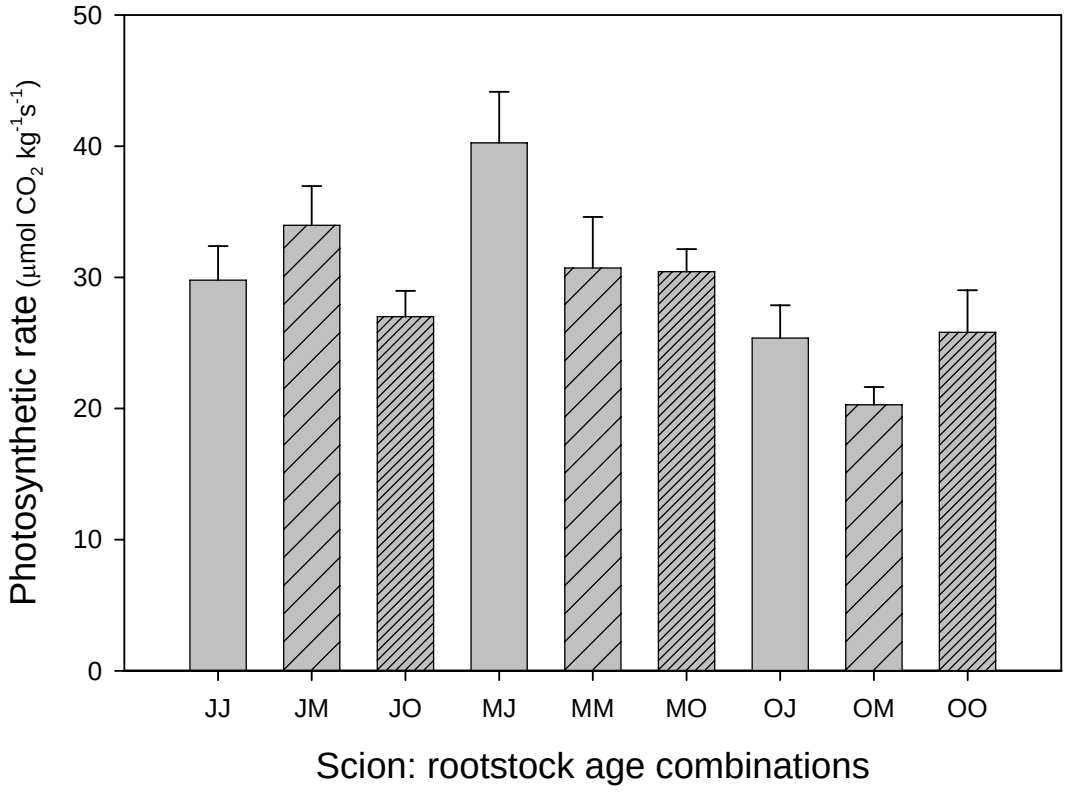


Figure 2.2 Means ± SE for photosynthesis on a mass basis of grafted *P. rubens* foliage for all scion: rootstock age combinations. For each age combination, N=5.



Growth measurements

Rootstock age, scion age and the interaction between rootstock and scion age were all significant factors for total scion length (Table 2.6). Rootstock age and the interaction between rootstock and scion age were significant factors for current growth (Table 2.6). For both parameters, all rootstock age comparisons were significantly different from each other, with both parameters decreasing significantly with increasing rootstock age (Table 2.7). Total growth of grafted material decreased 46% from juvenile rootstock to mid-age rootstock, 31% from mid-age to old and 63% from juvenile to old.

Current year growth decreased 61% from juvenile rootstock to mid-age rootstock, 50% from mid-age to old and 78% from juvenile to old.

Table 2.6 P values for ANOVA factors and interaction for growth measurements of grafted material from *P. rubens*, sorted by scion-rootstock combinations. Values followed by * are significant at an α-level of 0.05. N values for each scion-rootstock combination are shown in Table 2.1.

Measurement	scion	rootstock age	scion X rootstock
Total scion length (cm)	0.0181*	0.0001*	0.0145*
Current growth (cm)	0.5238	0.0001*	0.0138*

Table 2.7 Means ± SE for growth measurements of grafted *P. rubens* material by rootstock age with scion ages pooled. Means not followed by the same letter are significantly different from each other at $p<0.05$. For age J, N=62, for age M, N=55 and for age O, N=38.

Measurement	juvenile	mid-age	old	p
Total scion length (cm)	46.23 ± 2.14 a	24.68 ± 1.67 b	17.09 ± 0.80 c	0.0001
Current growth (cm)	17.69 ± 1.04 a	7.08 ± 0.60 b	3.57 ± 0.22 c	0.0001

Although scion age, rootstock age and the interaction between the two were significant for total scion length for the whole model (Table 2.6) this was not the biologically important parameter for our growth analysis. Initial scion length at the time of grafting was not factored into these measurements so total scion length did not accurately demonstrate post-grafting behaviors. Scion age was not a significant factor for the differences seen in current year growth, although there was a trend of increasing growth with increasing age (Table 2.8).

Table 2.8 Means ± SE for growth measurements of grafted foliage from red spruce by scion age with pooled rootstock ages. Means not followed by the same letter are significantly different from each other at $p<0.05$. For age J, N=41, for age M, N=66 and for age O, N=48.

Measurement	juvenile	mid-age	old	p
Total scion length (cm)	30.74 ± 3.00 a	33.07 ± 2.27 a	29.79 ± 2.42 a	0.6101
Current growth (cm)	8.44 ± 0.98 a	10.54 ± 1.05 a	12.07 ± 1.38 a	0.3746

Figure 2.3 Means ± SE for length of current year growth of grafted *P. rubens* for all scion: rootstock age combinations. N values for each age combination are shown in Table 2.1

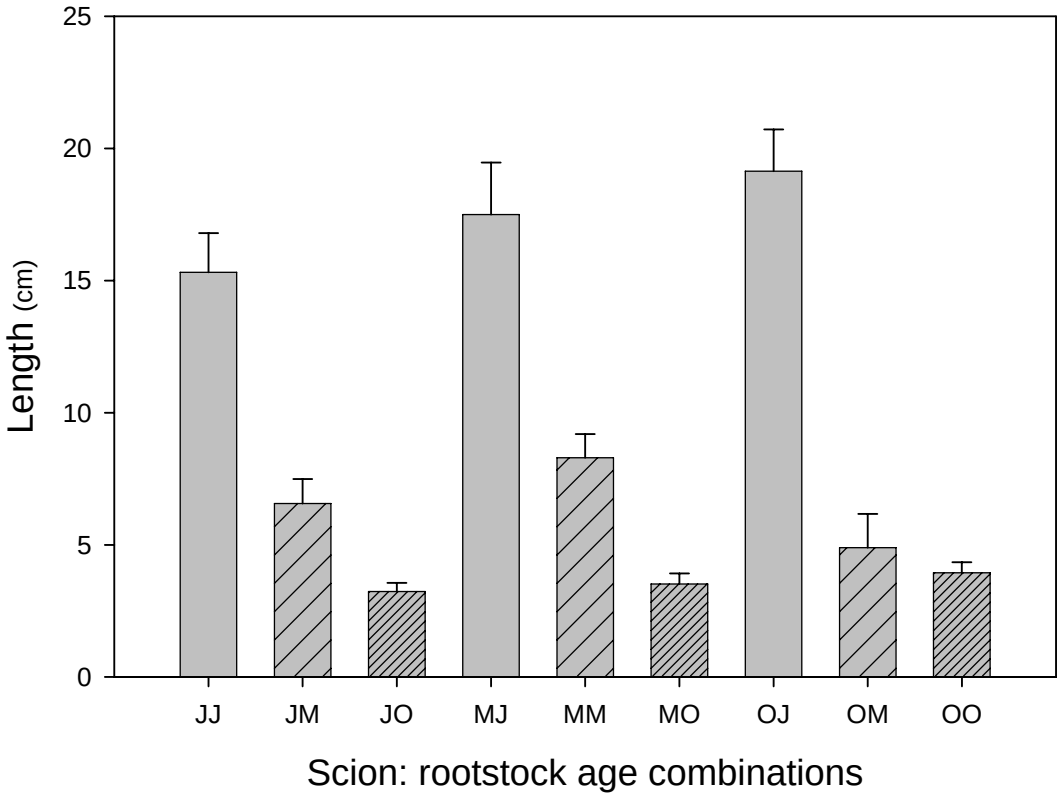


Table 2.9 Means ± SE for current year growth (cm) of grafted *P. rubens* foliage for rootstock age: scion age combinations. N values for each age combination is shown in Table 2.1.

	juvenile rootstock	**mid-age rootstock**	**old rootstock**
juvenile scion	15.31 ± 1.49	6.56 ± 0.93	3.23 ± 0.33
mid-age scion	17.46 ± 1.97	8.30 ± 0.89	3.52 ± 0.40
old scion	19.14 ± 1.58	4.89 ± 1.28	3.94 ± 0.40

As mentioned above, the interaction between rootstock age and scion age was significant for current year growth, although there was no over-riding trend for all age combinations. When on juvenile and old rootstock, growth increased with increasing

scion age (Figure 2.3 and Table 2.9). Grafts on mid-age rootstock showed no clear trend, with mid-age scions having the highest growth and old scions having the lowest growth.

Discussion

As discussed in the previous chapter, many photosynthetic parameters, including photosynthesis, stomatal conductance (g_s), intercellular CO_2 concentration (C_i) and measures of carbon assimilation capacity (Vc_{max} and J_{max}), decrease with increasing tree age. But will these trends apply to grafted material that differ in age from the rootstock? The most drastic changes in these parameters occur between juvenile and mid-age trees. If the extrinsic hypothesis is correct, scion behavior will reflect that of differently aged rootstock. If the intrinsic hypothesis is correct, scion behavior will reflect scion donor, not rootstock age. Changes reflecting a combination of both scion and rootstock age would suggest a combination of both intrinsic and extrinsic controls. Changes in gas exchange parameters may also occur in concert with the changes in leaf morphology that have been previously described (Rebbeck et al. 1993, Day et al. 2001, Ward 2005).

Physiological trends in scion behavior found in this study did not agree with previously reported findings for *Picea rubens* (Rebbeck et al. 1993, Day et al. 2001, Ward 2005). This study found no influence of scion donor age for any gas exchange parameters after 3 flushes of graft growth (Tables 2.2 and 2.4), which suggests that control over these parameters is now largely external to the apical meristem. This is in contradiction to previous work with grafted scions of red spruce, which did find differences between scion ages (Rebbeck et al. 1993, Day et al. 2001, Ward 2005). The studies done by Ward (2005) and Rebbeck et al. (1993) represented behavior on foliage two flushes after grafting and did show differences in photosynthesis based on scion age, while measurements for this study were done after 3 flushes had accrued and did not show differences based on scion age. This indicates that initial control over scion

behavior may be internal to the apical meristem, but that this trend may diminish with time due to reinvigoration. Results from Day et al. (2001) did not support this analysis, as they showed a significant scion influence 4 flushes after grafting. A potential reason for this apparent discrepancy could be due to the initial size of the rootstock. Juvenile rootstock in this study was one year old at time of grafting, while Day et al (2001) grafted scions onto rootstock that was three years of age and was already quite large. Older rootstock may be able to exert enough dominance that any potential scion age influence is suppressed. In addition, in this study, mid-age rootstock showed the highest levels of photosynthesis (Table 2.3), while Day et al. (2001), Rebbeck et al. (1993), Ward (2005) and results from ungrafted foliage in this study (Chapter 1) all showed that juvenile rootstock had the highest photosynthetic rates.

Table 2.10 Comparisons of results from juvenile and mature scions on juvenile rootstock from *Picea rubens* grafting studies. Approximate photosynthetic rates are shown on a mass basis (μmol CO_2 $kg^{-1}s^{-1}$).

	Initial rootstock age	# flushes after grafting	Measurement date	N	Juvenile photosynthesis	Mature photosynthesis
Rebbeck et al. 1993	2	2	July 9	80	22	22
Rebbeck et al. 1993	2	2	August 8	80	48	29
Day et al. 2001	3	4	June-August	4	42	20
Ward 2005	1	2	July-August	15	73	54
This study	1	3	August 18	15	30	34

This study found significant changes in net photosynthesis (on both a mass and area basis) that were a function of rootstock age and not scion age (Tables 2.2 and 2.3). However, work on the same population during the previous growing season found differences not only in net photosynthesis, but also in SLA, g_s and C_i, based on scion donor and rootstock age (Ward 2005), while this study, conducted a year later on the same material, found significant differences only in net photosynthesis. In addition, no significant age-related differences were found in SLA of grafted material, although these differences were found in ungrafted material (Chapter 1). Even more surprising was the fact that the only significant difference in net photosynthesis was between the two younger age rootstocks (juvenile and mid-age) and the old rootstock (Table 2.3), although previous work (Chapter 1; Ward 2005) had shown that most change occurs between the juvenile and the older two age classes.

Although Ward (2005) found a decrease in needle massiveness and photosynthesis (on a mass basis) with increasing scion and rootstock age, these changes were more strongly associated with rootstock age as opposed to scion age. Ward (2005) also found that there were significant rootstock differences in photosynthesis between the juvenile rootstock and the two older age classes, while this study found differences between the two younger age classes and the old rootstock. A possible explanation for this difference could be meteorological. The summer of 2003 was much drier than the summer of 2004. In 2003, the field grown rootstock (mid-age and old) could have been under water stress. The container grown juvenile rootstock were consistently watered, although the irrigation was uneven throughout the shade-cloth house. In combination, these two studies on the same populations point to an increasing influence of factors

external to the meristem with increased time from initial grafting. This increasing lack of internal control over the habits of the apical meristem indicate that once a graft has acclimated to its new environment, any previous age-related differences in the photosynthetic habits of the meristems are not permanent. Evidence for extrinsic control of the decrease in photosynthesis with increasing tree age/height, specifically by the mechanism of stomatal closure, had also been demonstrated in *Crytomeria japonica*, although the authors compared photosynthesis on a height and not strictly an age basis (Matsuzaki et al. 2005). This study found no significant decreases in g_s on either a scion or a rootstock basis (Tables 2.2 and 2.3).

These results suggest several additional important points. First, care must be taken to ensure a sufficient time after the grafting process before age-related changes can be examined. Since most conifers have pre-formed organs within the meristem from the previous year, the first flush after grafting does not represent the influences of the new environment, since the organ was formed in the donor environment. To truly examine the effects of the new environment, observations should be made from at least the second flush after grafting (which was done by Day et al. (2001) and Rebbeck et al. (1993)), as these new organs will have developed under water, hormonal, nutrient, etc. regimes influenced by the new rootstock. When looking at scions grafted onto juvenile rootstock, trends for photosynthesis in this study (Table 2.5 and Figures 2.1 and 2.2) were similar to results from Day et al. (2001), with a decrease in photosynthesis between juvenile and old scions, although these differences were not significant in this study. However, mid-age scions in this study showed the highest photosynthetic rates (not significantly different), while in Day et al. (2001) photosynthetic rates of mid-age scions were intermediate

between those of juvenile and old scions. Second, one year of examination may not be sufficient to fully explore whether age-related changes are controlled by intrinsic or extrinsic factors. It likely takes several flushes for grafted foliage to fully acclimatize to their new growing environment; any change in scion behavior may be missed if the timing of measurement does not account for this acclimatization period. It is also worth mentioning that the time of year that measurements are taken may affect these results. Rebbeck et al. (1993) found much greater differences in photosynthetic rates between juvenile and mid-age scions in August and September than in July; measurements in this study were taken in August, when differences should have been at their greatest.

Results for growth trends were not as clear-cut as were those for gas exchange measurements. Both total scion length and current year growth were significant for rootstock age with significant differences between all age classes (Tables 2.5 and 2.6). Total scion length, although reported in the Results section, will not be discussed in depth. Current growth showed a significant decrease with increasing rootstock age (Table 2.7). Unlike the results for gas exchange measurements with the more drastic changes between mid-age and old foliage (Table 2.3), juvenile and older foliage (Ward 2005) or between younger and old growth foliage (Day et al. 2001), changes in growth in this study occurred relatively equally throughout the age continuum, with large drops between each age category. Current growth showed a 60% drop between juvenile and mid-age rootstock and another 50% decrease between mid-age and old trees (Table 2.7).

Growth did not appear to be as tightly linked to rootstock age as carbon assimilation, as there was a significant interaction between rootstock and scion age for current growth. This interaction was significant because scions on mid-age rootstock act

differently than scions on juvenile and old rootstock. Day et al. (2001) found decreasing growth with increasing scion age when grafted onto juvenile rootstock, while in this study, growth increased with increasing scion age when grafted onto juvenile rootstock, although scion age was not significant (Figure 2.3). Growth decreased with increasing rootstock age, but increased (non-significantly) with scion age on juvenile and old rootstock. The difference in the behavior of scions grafted onto mid-age rootstock appears to come from old scions (Figure 2.3 and Table 2.9). Old scions, if they had followed the trend shown in scions grafted onto juvenile and old rootstock, would have had more growth than juvenile and mid-age scions, which was not the case. However, it is difficult to draw any biological conclusion from this behavior since old scions grafted on juvenile rootstock did not demonstrate reduced vigor. This may indicate that both intrinsic and extrinsic factors may be controlling growth behaviors, but rootstock (extrinsic factors) appears to play the largest role. A possible source of variation in this analysis comes from the fact that grafts grown on juvenile rootstock may have faced less competition due to pruning. In addition to the previously mentioned difference in initial juvenile rootstock age between this study and Day et al. (2001), during the summer of 2003 and 2004, non-grafted foliage on juvenile trees was pruned back so that no ungrafted leader was greater than the scion leader. It is possible that while mid-age and old meristems cannot control the juvenile rootstock, with less competition from rapidly growing juvenile foliage, they may be able to respond to it. A better understanding of this could be gained by following the fate of grafts on both pruned and unpruned rootstock for several continuous years, although pruning mid-age and old rootstock is impractical.

In conclusion, rootstock age was the factor that controls the behaviors of grafted material in this study. This tells us that all parameters examined in this study were controlled by factors external to the apical meristem, which supports either the extrinsic or the stimulus-response developmental pathway. Examination of the developing organs, not just post-development as done in this study, could help bring this issue into sharper resolution. It would also be helpful to track these changes not only through the entire growing season (and beyond), but also across several years as relationships may not be the same from year to year.

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Appendix: Means ± standard errors for diurnal trends for various gas exchange parameters

Table A.1 Means ± SE for photosynthesis on a leaf area basis (μmol CO_2 $m^{-2}s^{-1}$) by age category and time of day. This table corresponds to Figure 1.1.

Time of day	juvenile	mid-age	old
600	9.856 ± 0.527	8.206 ± 0.730	7.154 ± 0.462
800	9.721 ± 0.417	8.180 ± 0.333	8.649 ± 0.400
1000	10.494 ± 0.724	7.536 ± 0.453	8.133 ± 0.359
1200	8.449 ± 0.507	6.490 ± 0.583	6.488 ± 0.496
1400	7.183 ± 0.595	4.939 ± 0.598	5.090 ± 0.773
1600	7.582 ± 0.391	4.663 ± 0.909	4.618 ± 0.648
1800	6.723 ± 0.577	2.822 ± 0.603	3.613 ± 0.875

Table A.2 Means ± SE for photosynthesis on a foliar dry mass basis (μmol CO_2 $kg^{-1}s^{-1}$) by age category and time of day. This table corresponds to Figure 1.2.

Time of day	juvenile	mid-age	old
600	36.246 ± 2.192	23.114 ± 2.535	20.793 ± 1.456
800	36.062 ± 1.725	25.030 ± 1.332	25.144 ± 1.370
1000	36.022 ± 2.349	23.112 ± 1.595	23.598 ± 1.239
1200	30.500 ± 3.038	19.943 ± 1.880	18.843 ± 1.566
1400	25.999 ± 3.086	15.258 ± 1.943	14.900 ± 2.368
1600	25.674 ± 3.274	14.041 ± 2.972	13.509 ± 2.029
1800	22.342 ± 3.217	8.188 ± 1.688	10.539 ± 2.676

Table A.3 Means ± SE for photosynthesis on a unit chlorophyll basis (μmol CO_2 mg chlorophyll$^{-1}s^{-1}$) by age category and time of day. This table corresponds to Figure 1.3.

Time of day	juvenile	mid-age	old
600	117.467 ± 6.807	87.353 ± 9.890	94.892 ± 7.304
800	114.896 ± 6.702	96.551 ± 6.490	114.610 ± 6.975
1000	116.781 ± 8.299	89.858 ± 8.093	112.618 ± 6.149
1200	91.899 ± 6.692	77.644 ± 8.749	90.939 ± 8.869
1400	76.775 ± 9.014	59.380 ± 8.406	67.142 ± 9.929
1600	80.538 ± 9.051	53.813 ± 13.459	61.224 ± 8.659
1800	70.409 ± 9.882	29.819 ± 6.515	47.251 ± 11.587

Table A.4 Means ± SE for intercellular CO_2 concentration, C_i (μmol CO_2 mol^{-1}) by age category and time of day. This table corresponds to Figure 1.4.

Time of day	juvenile	mid-age	old
600	381.13 ± 12.55	308.33 ± 12.92	307.67 ± 13.13
800	352.89 ± 5.98	291.00 ± 14.71	300.44 ± 9.68
1000	334.00 ± 8.95	283.60 ± 10.55	297.30 ± 8.19
1200	314.50 ± 8.49	281.60 ± 11.67	280.30 ± 6.15
1400	308.22 ± 4.51	265.80 ± 8.42	273.00 ± 6.01
1600	298.57 ± 15.10	262.57 ± 11.06	275.89 ± 5.21
1800	298.29 ± 15.66	264.67 ± 10.38	273.71 ± 6.50

Table A.5 Means ± SE for stomatal conductance, g_s (mol H_2O m^{-2} s^{-1}) by age category and time of day. This table corresponds to Figure 1.5.

Time of day	juvenile	mid-age	old
600	0.4529 ± 0.1937	0.1530 ± 0.0240	0.1444 ± 0.0187
800	0.5779 ± 0.1438	0.2312 ± 0.0428	0.2192 ± 0.0267
1000	0.7570 ± 0.1748	0.2319 ± 0.0463	0.2814 ± 0.0375
1200	0.4519 ± 0.0685	0.2659 ± 0.0838	0.1900 ± 0.0211
1400	0.3044 ± 0.0625	0.1189 ± 0.0194	0.1384 ± 0.0322
1600	0.2821 ± 0.0820	0.0959 ± 0.0236	0.1249 ± 0.0174
1800	0.1817 ± 0.0460	0.0497 ± 0.0120	0.0893 ± 0.0282

BIOGRAPHY OF THE AUTHOR

Stephanie L. Adams was born in Toms River, New Jersey. She graduated from Mainland Regional High School in Linwood, NJ in 1994. She continued her education at the University of Maryland, College Park, MD and graduated *Magna cum laude* from the Richard Stockton College of New Jersey in 2003 with a B.S. in Environmental Sciences. After receiving her B.S., she enrolled in the graduate program in the department of Forest Ecosystem Sciences at the University of Maine. Stephanie is a candidate for a Master of Science degree in Forestry from the University of Maine in May, 2006.