WATER SOURCES AND ECOPHYSIOLOGY OF SELECTED RIPARIAN SPECIES
OF THE SOUTHERN APPALACHIAN MOUNTAINS, USA

BY

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ABSTRACT

White, Joseph C.

WATER SOURCES AND ECOPHYSIOLOGY OF RIPARIAN SPECIES
OF THE SOUTHERN APPALACHIAN MOUNTAINS

Dissertation under the direction of William K. Smith, Ph. D.

Charles H. Babcock Chaired Professor of Botany

The following research examined water source utilization of two native riparian species along a perennial river in the southern Appalachian foothills, USA (Chapter II), including water source patterns over an entire year (III), and how these patterns changed according to differing hydrologic regimes (VI). Additionally, these source usage patterns were examined for dominant shrubs exhibiting strong spatial segregation along an elevational gradient (V). Finally, the hydraulic properties of these shrubs were examined in regards to adaptive leaf orientation during periods of light and water stress (VI).

Two native, broad-leafed riparian trees, *Acer negundo* and *Betula nigra*, relied strongly on deep ground water sources supplemented by shallow soil moisture. While deeper sources made greater contributions during the late growing season when plant demand was higher, neither species used substantial amounts of streamwater. In contrast, riparian *Liquidambar styraciflua* growing along differing surface water systems showed significant surface water usage, regardless of system. Trees growing along a perennial stream relied strongly on streamwater (up to 76%), while pondside trees were using both
pond water and soil water (up to 49%). Trees at neither site took significant contributions from deeper groundwater.

Water sources of three *Rhododendron* species adhering strongly to habitat preferences according to elevation were found to have little impact on distribution patterns. *Rhododendron catawbiense* and *R. minus*, found in considerably different habitats, both utilized high amounts of deep ground water sources (up to 74%), while riparian *R. maximum* relied predominantly on shallow soil layers (up to 91%) and used little stream or groundwater (up to 16%).

Aquaporins were found to have a limited role in leaf water transport in *R. catawbiense* and *R. maximum*; aquaporin inhibitor had little effect on leaf hydraulic resistance under cold or warm temperature trials. However, the expected relationship between water temperature and resistance may have been jeopardized by methodological errors.

In conclusion, water source utilization patterns of riparian vegetation in the southern Appalachians seemed to vary by species, but less so according to the hydrologic system. While some surface water use was demonstrated, it was not uncommon for streamside species to utilize groundwater and not streamwater.
CHAPTER I

WATER SOURCES AND ECOPHYSIOLOGY OF RIPARIAN SPECIES
OF THE SOUTHERN APPALACHIAN MOUNTAINS

The following dissertation details studies aimed at a better understanding of the water relations and ecophysiology of native riparian trees and shrubs in the southern Appalachian Mountain foothills (USA). I chose to pursue these projects because too little is known, in general, about the ecophysiology of these species, especially the influence of water sourcing, i.e. where these plants draw their water, the stream surface water or soil sources.

The southern Appalachian Mountains and surrounding foothills make up some of the most biologically rich and diverse temperate forests in the world. This biodiversity hotspot is home to hundreds of bird species and dozens of mammal, reptile, and amphibian species, many of which are listed as threatened or endangered (Pickering et al. 2002). The region is especially recognized for the diversity of aquatic species, vertebrate and invertebrate. The best known illustrations of this diversity are the spruce-fir tree islands, remnant forests found on seven isolated mountaintops in southeastern US (Oosting and Billings 1951).

The forests of the southern Appalachians are also threatened. While portions of this region are designated as national and state parks, areas not protected are imperiled by growing cities in the area, including Ashville, Knoxville, and Chattanooga. The implications of these urban areas extend beyond the loss of habitat to include increased air and water pollution, as well as increased water demand that may lead to damming and...
alteration of the natural hydrology. With the diversity of the riparian habitat and that of the southern Appalachians, anthropogenic pressures threaten a host of ecological services in the coming future. Thus, it is vital that we develop a better understanding of the biotic and abiotic interactions of riparian zones in this region.

While the ecology of watersheds of the southern Appalachians has received extensive study in the last century, especially at the Coweeta Hydrologic Laboratory (e.g. Monk and Day 1985, Webster et al. 1999), much less is understood about the ecophysiology of the plants that live in these watersheds. Additionally, much of our knowledge about the ecophysiology of North American riparian zones comes from the arid southwestern US, with only a few studies coming from the eastern half of the US. To fully understand the impacts of urbanization and other anthropogenic threats, including agricultural development and climate change, a better understanding of these ecosystems and their ecosystem services is an important necessity.

*Ecological importance of riparian zones*

Riparian zones are those vegetated buffers naturally lining surface bodies of fresh water, beginning at the water’s edge, through the floodplain, and to the edges of the surrounding upland habitats. These systems form an aquatic-terrestrial interface and, as such, are of fundamental importance in both aquatic and adjacent forested environments, with the ecology of these two ecosystems entwined on both biotic and abiotic levels.

Because of the location and associated environmental characteristics of riparian zones (e.g. increased water availability, high humidity, cooler temperatures, and more direct sunlight than the adjacent upland), they serve as habitat for a high diversity of
organisms endemic to these often disturbance-driven systems (Ward et al. 2002). Indeed, these areas are commonly more diverse than the surrounding uplands because of their heterogeneous environment (Catterall et al. 2001), providing habitat to a wide array of species. Especially divers are the plants, invertebrates, and birds (McIntyre 2000, Woinarski et al. 2000, Brown and Peet 2003). Riparia are also important for species that are not permanent residents in these habitats, including feeding habitats, migratory stopovers, and spatio-temporal refugia for species forced out of their native habitats (Naiman et al. 2005). A host of insectivores may rely on these environments for the aquatic insects that emerge from surface waters as adults to mate and reproduce (Jackson and Fisher 1986, Sanzone et al. 2003), other carnivores may benefit from the abundant amphibians and fish found in these systems (Hilderbrand et al. 1999). Riparian zones also serve as excellent migratory stopovers because of the numerous resources including shelter, food sources, and high water availability (Naiman et al. 2005). Finally, because of the unique physical characteristics of these systems, they may serve as short- or long-term refuges to species forced from their natural habitats due to changes in environmental conditions (Meave and Kellman 1994, Naiman et al. 2005).

Riparia provide many ecosystem services vital to the ecological integrity of the region, as well as for the survival of plants and animals dependent upon these habitats. In addition to the essential biotic interactions mentioned above, riparian zones perform a number of equally important abiotic services. These are dynamic systems, consistently being reshaped by disturbances from flooding, erosion and deposition of sediment, extended drought, and other natural perturbations (Naiman et al. 2005). One of the most important ecosystem services provided by riparian zones is the buffering against
widespread damage by flood waters, helping to slow streamflow and regulate volume downstream. Undisturbed, these systems accomplish this in two major ways. First, they intercept runoff from the surrounding uplands, slowing its flow, allowing the water to percolate through the soil profile and interception by vegetation, reducing overall water input to the stream (Tabacchi et al. 2000). Secondly, wide floodplains allow swollen rivers to expand beyond the channel into the riparian forests, slowing floodwaters and allowing temporary storage in backswamps and wetlands. Large erosion events, associated with high flows like flooding, where sediment is transported downstream, are typical in altered riparian zones. However, intact riparian buffers can significantly reduce or eliminate mass erosion during flood events, protecting upstream habitats and downstream water quality (Magette et al. 1989, Bromley et al. 1997, Poesen and Hooke 1997).

The quality of surface waters is often threatened by runoff contaminated with pollutants, including sediments, of natural or anthropogenic origins. In the same way that riparian vegetation aids in regulating high runoff volumes by slowing flow and allowing interception, polluted runoff also filters through soil, allowing uptake of nutrients by vegetation as well as biotic and abiotic processing of soil contaminants (Lowrance et al. 1984, Peterjohn and Correll 1984, Jacobs and Gilliam 1985, Sabater et al. 2003). While obviously important for organisms that may consume water directly, aquatic organisms are vulnerable to both direct and indirect effects of water pollution. Runoff contaminated with high nutrient concentrations, common from adjacent agricultural development, can lead to decaying algal blooms in surface waters that deplete oxygen, leading to significant mortality of aquatic life, including fish kills (Barica et al. 1980, Rabalais 2002). While
unaltered riparian forests can efficiently filter runoff before it enters surface water, damaged, removed, or bypassed buffers (i.e. effluent pipes draining directly into the surface water) prevent processing of runoff (Naiman et al. 2005).

Nutrient cycling in riparian zones tightly couples the adjacent terrestrial and aquatic systems in an important way. One of the best illustrations of this occurs in the hyporheic zones of stream banks, where surface and subsurface (e.g. groundwater) waters mix. These unique sites can act as nutrient sources or sinks depending on conditions, with the greatest nutrient retention occurring in lower order streams where greater proportions of surface water cycle through these areas (Findlay 1995, Hill et al. 1998). The oxygen conditions largely determine how nutrients are processed, with well-oxygenated flowpaths being sources of inorganic nitrogen and nitrate, while anoxic zones are dominated by denitrification (Carlyle and Hill 2001). With a similar level of integration, these aquatic and terrestrial systems provide reciprocal carbon and nutrient subsidies. Leaf litter and invertebrates that are swept from the uplands into the river, or fall in from overhanging branches, serve as energy and nutrient subsidies to the aquatic system (Webster and Meyer 1997, Nakano et al. 1999). Concomitantly, mature aquatic insects that leave the river to reproduce, fish carcasses, and detritus that washes ashore during flooding make significant contributions to the energy budget and nutrient cycle of riparian forests (Jackson and Fisher 1986, Hilderbrand et al. 1999, Sanzone et al. 2003).

For the reasons above, it is essential that we have a comprehensive understanding of the ecology and physiology of the plants that make up these important habitats. To determine the effects of anthropogenic and natural disturbances on riparian buffers, we must understand how these plants function in healthy, intact systems.
Possible water sources of riparian plants

Forstel and Hutzen (1982) were among the first researchers to demonstrate that the stable isotopes of water could be used to study water transport in plant tissues and that isotopic composition of the water was largely conserved during transport. This was soon followed by the first major riparian tree water sourcing study by White et al. (1985).

Field-based water sourcing studies such as that by White et al. (1985) rely strongly on differing isotopic composition of H and O in different compartments of the hydrologic cycle (Dansgaard 1954, Friedman et al. 1964, Allison et al. 1983, Gat 1996). These distinctions exist because of the mass difference between isotopes, which result in discrepancies in rates of phase change, with molecules containing lighter isotopes evaporating off more readily and condensing more slowly than those with heavy isotopes. Isotopic composition of precipitation in a given location depends on a number of factors, including temperature and humidity, as well as the type of precipitation (Dansgaard 1964, Gat 1980). After falling to earth, any changes in composition are largely dictated by the characteristics of the hydrologic compartment they join. Water that runs off land and drains into surface water bodies experiences high levels of evaporation, especially in large, exposed lentic bodies (e.g. lakes and ponds). This water will then become enriched in heavier isotopes as molecules with lighter isotopes evaporate off more quickly (Friedman et al. 1964). Water that is absorbed by shallow soil layers is similarly exposed to high levels of evaporation and will experience evaporative enrichment. Typically, the deeper in the soil profile, the lower the exposure to the environment and the less enrichment, often resulting in isotopically lighter water at deeper soil depths. Precipitation that percolates through the soil and to the water table joins a source that
experiences very little evaporative enrichment. Groundwater, however, may not reflect
the isotopic compositions of this precipitation. Aquifers are vast, often covering large
areas, with residence times of c. 20,000 years (Hornberger et al. 1998). As such, they are
buffered against relatively small seasonal inputs and instead reflect a history of inputs
and mixing with other water compartments (White et al. 1985). While differentiation
between compartments is often possible in arid to temperate habitats, it may be more
difficult in mesic locations because of potential mixing between sources (Smith et al.
1998).

These differences in isotopic composition of potential water sources to plants
allow for determination of water source use patterns by analyzing the mixture of sources
making up plant water. In almost all plants examined, isotopic composition of water
taken up by plant roots is conserved until it reaches the leaves, where evaporation due to
transpiration, causes fractionation of the ratio (Gonfiantini et al. 1965, Wershaw et al.
1966).

Since White et al. (1985) reported the surface water source use by *Taxodium
distichum* in an Arkansas swamp, the vast majority of plant water source studies have
involved more xeric locations, specifically the southwestern US and arid Australia. One
notable follow-up found little to no surface water use by riparian trees in an oak-maple
forest in northern Utah, even in individuals growing in streamwater (Dawson and
Ehleringer 1991). In the following years, a number of studies supported these findings,
showing limited usage of this highly available water source (Dawson and Ehleringer
1991, Busch et al. 1992, Thorburn and Walker 1993, Mensforth et al. 1994, Snyder and
Williams 2000, Singer et al. 2012, Chimner and Resh 2014). These results came from
different regions and in different plant types, but almost solely from arid locations. While not universal, even in dry habitats (Smith et al. 1991, Thorburn and Walker 1994, Drake and Franks 2003, Muttiah et al. 2005), the consistency of these findings led to hypotheses about why riparian plants, which were expected to use substantial amounts of surface water based on their distributions, would not use streamwater when it was so easily within reach. Two main theories emerged: (1) The ephemeral nature of many streams in these arid locations results in their not being dependable enough to merit investment in structures to absorb streamwater (Dawson and Ehleringer 1991, Ehleringer and Dawson 1992, Thorburn et al. 1992, Thorburn and Walker 1994, Lin et al. 1996). And, (2) because the water table generally follows surface topography, plants growing in riparian zones, which are typically at a lower elevation than the surrounding uplands, should have greater access to groundwater. Groundwater availability is typically high and less dynamic than streamwater, which may lead to a reliance on this source and render the use of surface water unnecessary (Dawson and Ehleringer 1991, Thorburn and Walker 1993, Mensforth et al. 1994, Thorburn and Walker 1994).

The first hypothesis, hereafter referred to as the “theory of unreliability,” states that because many streams in the southwest are snow-fed and dry up during the summer growth season, plants opt to allocate elsewhere, instead of this temporally variable source. This theory goes on to suggest that, regardless of ease of access (contrasting the 2nd hypothesis), if the plants can reach groundwater, it is more advantageous to invest resources into accessing the water table because of the greater annual consistency in water availability. The results of one follow-up study conducted along two streams, one ephemeral and the other perennial, found that trees along the perennial stream used some,
but very little surface water, while those adjacent to the ephemeral stream were not using streamwater (Kolb et al. 1997). Unfortunately, this does little to support or reject this hypothesis and leaves us with little additional insight.

Objectives

The initial goal of the research presented here was to answer the basic question “Which water sources are riparian trees in the southeastern US accessing?” We went on to ask more applied questions about source use over time, in co-occurring species, plants under different hydraulic regime, and the implications of species’ source use patterns in their ecology. We focused on the mesic riparian forests of the southern Appalachians and the surrounding foothills because of the ecological importance of these systems, as detailed above. However, we studied different forests and species in an attempt to gain a more comprehensive understanding of variability in source use patterns between species, systems, and environmental factors.

The first two studies (Chapters II and III) were conducted in the same riparian forest in the Appalachian foothills. The first study was performed over a single growing season and essentially served as a pilot project for the rest of the work presented in this dissertation. This earliest project sought to determine water source usage by riparian trees in an unaltered environment, establishing a baseline for the region, and allowing comparisons to findings from more arid regions and testing of the theory of unreliability. Chapter III continues this research over a 12-month period, designed to capture changes in plant phenology and seasonal changes in environmental variables and their effects on plant water use patterns.
Chapter IV applies what was learned from the two earlier projects, namely that riparian trees are not using surface water in the southeastern US, and asks if, in hydrologic conditions in which it would be highly advantageous to use surface water, the plants will deviate from these patterns. The study was conducted along a pond and adjacent stream, comparing a single species growing on the banks of bodies of water.

The fourth study (Chapter V) was conducted along an elevational gradient in the southern Appalachian Mountains and was aimed at improving our understanding of mechanisms causing the apparent habitat preferences of three *Rhododendron* species, some of which dominate riparian forest understories, common to the region. Water source use strategies have gone unstudied in *Rhododendron*; the sources these species use could have direct and/or indirect impacts on species’ distributions.

Chapter VI is unique among the work presented here in that it is the only study that does not include a water sourcing aspect. This project investigated the hydraulic properties of two *Rhododendron* species investigated in the Chapter V study, one of which was riparian. The primary focus was to determine the effects of temperature change on leaf water transport and the role of aquaporins in that process.

Overall, this dissertation aims to increase our understanding of plant-water relations in the riparian habitats of the southern Appalachians. The studies presented here were designed to build a foundation for future, more applied questions about riparian plants, perhaps examining the impacts of water pollution, damming, or other alterations of the these forests.
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Freshwater Biology 47:517-539.


CHAPTER II

PRELIMINARY FINDINGS ON THE WATER SOURCES IN RIPARIAN TREE SPECIES OF THE SOUTHERN APPALACHIAN FOOTHILLS, USA

The following manuscript has been published in *Riparian Ecology and Conservation* (2013, 1:46-52). Stylistic variations are due to the requirements of the journal.
Abstract

The aim of this study was to determine the sources of water uptake for two common riparian tree species found in the southeastern United States, *Acer negundo* and *Betula nigra*. The study site was located within a riparian zone typical of this region and those of the temperate USA. Water sources were determined by analyzing signatures of stable isotopes found naturally in water, $^2$H and $^{18}$O. Samples from surface water, groundwater, and soil, plus woody tissue from mature individuals of each species, were taken once each month during the 2011 summer growth season. Both species relied strongly on an unidentified ground water source, although *A. negundo* also showed a strong correlation with deep soil moisture ($r^2= 0.972$). Sampling limitations did not permit an accurate determination of the fractional contribution of each source to plant water, limiting the strength of the results. The evidence collected leads to conclusions comparable to those of studies that have been conducted in arid parts of North America, corroborating that streamside species may rely heavily on groundwater sources, not surface streamwater. Further studies are needed in this region to confirm the evidence reported here to establish a baseline for vegetation in these systems.

Key Words: Deuterium, Groundwater, Stable Isotopes, Streamwater, Soil Water, Boxelder, River Birch
1. Introduction

Use of the stable isotopes of hydrogen and oxygen as a non-radioactive tracer, has enabled the determination of plant water sources in a large array of environments. Because root presence is not necessarily an indicator of water uptake, the ability to use stable isotopes as tracers fulfills a need for analysis of water uptake and storage in plant tissue [1, 2]. More specifically, this determination is made by analyzing differences in isotopic compositions between water extracted from woody tissue, soil, or other potential water sources created by the fractionation of ratios produced by phase changes [3, 4]. Dawson and Ehleringer [3] evaluated the basic premise that riparian species growing on stream banks, or in the stream itself, are benefiting from increased water availability. However, this research revealed that mature individuals of multiple species in the southwestern US utilized little or no streamwater, even for individuals growing in the stream. Several subsequent studies reported similar findings where, in contrast to juveniles, mature trees in the Southwest were relying predominately on sources other than surface water [3-6]. The question arises-- with large amounts of available water, why are streamside species not utilizing surface water? Several studies have hypothesized that because many streams in arid regions experience severely decreased flows in mid-summer, streamwater is too unreliable for individuals to invest resources into developing roots that can absorb surface water [5, 7]. One of the few studies that tested this “unreliability” hypothesis for riparian systems, where streamflow is usually perennial, found that while some streamwater was utilized, it did not contribute an important amount to plant water [10].
Objectives of the present study included determining the sources of water uptake, and differences therein, for two species common to the riparian zone of the foothills of North Carolina, USA—Acer negundo L., a tree common to most of North America that has been studied previously in the southwestern United States [10, 11] and Betula nigra L., a more regional riparian species, in a system with reliably high streamwater. Four potential sources for plant water uptake were tested: streamwater, shallow and deep soil moisture, and groundwater.

The study was conducted in the foothills of the southern Appalachian Mountains in western North Carolina (USA) and aimed to determine the source of water uptake for Acer negundo (boxelder) and Betula nigra (river birch), two species representative of riparian vegetation in the region. Each possible source was sampled along with woody tissue of both species for comparison of isotopic signatures during each month of the 2011 summer season. Additionally, plant water status and environmental variables were measured to detect relationships between these and possible changes in water source over a growth season. It was predicted that, with the reliably high water levels and quality of the stream in this study, mature individuals will utilize a substantial amount of streamwater.

2. Methods

2.1 Study Site

The study site was located on a 40.5 ha parcel located at 35.6299° W, -81.3101° N in Newton, North Carolina. The land is preserved as a city park (Jacob Fork Park) and is bordered by rivers on two sides—the Jacob Fork along its northern-most border, and the Henry Fork on the eastern side. Both the Jacob Fork and the Henry Fork have their
headwaters in South Mountains State Park. Jacob Fork Park also contains the municipal water supply uptake for the City of Newton, which features a small dam on the Jacob Fork before it joins the Henry Fork in the northeast corner of the parcel. Individual trees were selected from an approximate 240 m x 10 m stretch of riparian zone running along the Jacob Fork River which borders the site. Vegetation in this area is representative of mixed deciduous forest riparian zones of the region, and elsewhere in the temperate zone of North America, including many common species besides *A. negundo* and *B. nigra*, such as American sycamore (*Platanus occidentalis*) and Christmas fern (*Polystichum acrostichoides*). As is typical for this region, several common invasive species, aided by disturbance due to land-clearing and development, including briar (*Smilax* spp.), Chinese privet (*Ligustrum sinense*), and Japanese honeysuckle (*Lonicera japonica*), also exist in these areas [12].

The Jacob Fork River has been designated “Outstanding Resource Water” by the North Carolina Department of Environment and Natural Resources for its high water quality (NC Water Quality Classifications 2012). The river has a sediment-covered bottom of sand or clay and is ca. 1-3 m deep on average. The banks of the study site along the Jacob Fork have been sharply eroded and rise 1-2 m above the stream, while those on the opposite side slope more gradually toward the stream. The stream is ca. 8-10 m wide during average rainfall conditions and is probably artificially wider and deeper at the study location due to the damming near its juncture with the Henry Fork. The high water quality and consistently high water level of the river makes the site an ideal place to test the “unreliability” hypothesis.

### 2.2 Isotope Sampling
To complete the plant water isotopic analysis, groundwater, streamwater, and soil water samples at various depths (30 cm and 1 m) were taken along with plant tissue samples. Groundwater was sampled via a small self-installed well located about 6 m from the stream edge and with a depth of approximately 3 m (at which the soil was saturated). The well was pumped free of standing water upon arrival on the day of data collection and would typically refill by noon. For these reasons, it was expected that samples would be representative of the groundwater resource available to the trees in the area.

The installed well was created using a Hoffco Inc. PH 980 motorized post-hole drill with a 2” earth bit (EB-2) and bit extensions. Once the saturated zone had been reached, a 2” PVC pipe, with numerous ¾” holes drilled into the lowest meter, was inserted into the well. The pipe was fitted with a threaded coupler and a screw-on cap to prevent the entrance of precipitation. Soil removed from the hole during drilling was used to pack around the pipe to minimize surface runoff down the pipe, potentially contaminating the groundwater isotopic ratio. Groundwater samples were taken from the well using a syphon to draw water into the collection vial. The well was syphoned of standing water and allowed to refill before groundwater samples were collected.

Streamwater samples were taken from a well-mixed location in the middle of the stream about 0.5 m below the stream surface. Soil samples were taken at two locations within the riparian zone of the study site, one at each end of the corridor (ca. 100 m apart), near individuals selected for water source analysis. Samples (n=4) were taken at depths of approximately 30 cm and 1 m, and were collected using the same post-hole drill and bits used in drilling the groundwater well. Along with groundwater sampling, collection
of soil at these depths corresponds with what is known about the rooting architecture and depths of these species. Cost and logistics limited additional sampling. Because previous findings have shown that there is a significant delay in the uptake of rainfall, during which evaporative enrichment likely occurs [16, 17], shallow soil samples should be representative of rainfall available for plant uptake. Thus, precipitation analyses were not conducted.

Plant tissue collected from selected individuals (n=3, for both A. negundo and B. nigra) consisting of 15-20 centimeters of stem material, approximately 0.5-1.25 cm in diameter, was taken from randomized locations on each individual. Samples were stripped of any loose bark and cut into lengths short enough to be placed in the collection jar. Individual plants were systematically selected based on their health, age, and location, i.e. mature, healthy trees, some growing on the banks of the river, others, farther from the river (up to 10 m), while still being located in the riparian zone. Plant tissue samples were collected in the late morning hours (10am-12pm) of each collection day. Similar to other comparable studies, three individuals of each species were sampled for isotopic analysis, with 3 replicates per individual [18-20].

All water, soil, and woody tissue samples were stored in screw-top glass vials and sealed with Parafilm M to prevent evaporation. Samples were kept chilled in the field and frozen vertically upon return to the lab until shipped. Collections took place on a single day in the latter half each month of the summer season (June, July, August, and September 2011).

2.3 Isotopic Analysis
Samples were shipped to the Stable Isotope Ratio Facility for Environmental Research (SIRFER) at the University of Utah for isotopic analysis. Water was extracted from woody tissues and soils via cryogenic vacuum distillation using the methodology of West et al. [21]. Analysis of hydrogen and oxygen isotopic ratios was completed using a Delta Plus XL isotope ratio mass spectrometer (ThermoFinnigan) coupled to a temperature conversion/elemental analyzer into which microliter quantities of the sample water were directly injected. Values are reported relative to V-SMOW. Precision of analysis was ±2.1‰ for $\delta^2$H and ±0.3‰ for $\delta^{18}$O.

**Plant Water Status**

In addition to isotopic analyses, species’ xylem water potentials ($\Psi$) were monitored throughout the growing season on individuals (n=6) selected for plant isotopic water sampling, enabling direct comparison. Water potentials were measured for apical leaves sampled from the lower canopy twice on each day of isotope sampling (once per month). Measurements were taken early morning, but not predawn, and again in the late afternoon, using a Scholander-type pressure chamber (Model 1000, PMS Instrument Comp., Corvallis, OR) to evaluate how these species deal with water stress over the course of a day. Theoretically, those species utilizing a more reliable, consistent source (such as groundwater) should experience less water stress throughout the day. For that reason, the change between the water potential measurements was of primary interest, and predawn water potentials were not necessary. One replicate for each of 6 individuals per species was taken for both morning and afternoon water potential measurements.

**Riparian Microclimate**
Air temperature and ambient humidity were collected on-site using a HOBO Pro V2 data logging temperature and humidity sensor (U23-001) which was shielded from direct sunlight and nighttime sky exposure and attached to a tree at breast height. Rainfall was measured in an adjacent open location using an Onset tipping-bucket rain gauge (RG1) and HOBO data logger (H07-002-04). Depth to and height of groundwater was measured within the installed well.

2.4 Statistics

After ensuring the normality of the data set, One-Way Analyses of Variance (ANOVA) were used to examine differences in isotopic values between months for each species, accompanied by Tukey’s test for intraspecific pairwise comparison. T-tests, or Mann–Whitney U tests for non-parametric data, with a Bonferroni correction, were used to compare interspecific values between collection periods. Linear regressions were used to analyze relationships with isotopic values of woody tissue, potential water sources, and environmental variables. All analyses and figures were created using SigmaPlot v. 11.

3. Results

Isotopic compositions of waters from woody tissue were consistently more negative than those of waters from all four collected sources (Figure 1). Streamwater and groundwater tended to be most enriched in deuterium throughout the season followed by shallow and deep soil moisture, while shallow soil water was consistently the most enriched in $^{18}$O. The lines of best fit for each set of samples exhibits a slope similar to that of the global meteoric water line (GMWL). However, stream and groundwater are shown to have undergone enrichment in $\delta^3$H, likely due to evaporation (Figure 1).
Possible relationships between isotopic values of woody tissue and potential sources were evaluated by comparing changes in $\delta^2$H over the duration of the study (Figure 2). A strong, significant relationship existed between the values of *Acer negundo* and deep soil water ($r^2=0.972$, $P=0.028$); a similar, but non-significant, trend was found for *Betula nigra* (Figure 3). These findings were supported by $\delta^{18}$O results ($r^2=0.915$, $P=0.043$ for *A. negundo*). There was no other significant relationship between woody tissue values and water sources analyzed ($P>0.11$). It was also apparent that at least one water source utilized by these species was not collected (Figure 1).

Intraspecific differences in isotopic signatures between collection dates showed similar trends for both species; however, the means of July and September values were significantly different in *B. nigra* but not *A. negundo* (Figure 4). Multiple pairwise tests, with a Bonferroni correction, were used to compare interspecific values for each month and showed that there was no difference in isotopic values of plant water between these species over the duration of the study ($P>0.029$).

*Influence of precipitation and plant water status*

Neither the current or previous month’s rainfall were related to the isotopic values of water from the woody tissue of either species ($P>0.17$, Fig. 5). Similarly, there was no relationship between the ambient variables monitored, air temperature and humidity, and the isotopic signature of either species over the duration of the study ($P>0.23$; data not shown). In addition, the distance from the soil surface to well water and the height of standing water in the well, both before and after draining for sample collection, were not associated with woody tissue isotopic values of either species ($P>0.051$; data not shown).
Finally, there was no relationship between the deuterium values of the water extracted from woody tissue of either species and that species’ morning or afternoon $\Psi$ measurements ($P>0.12$). Similarly, there was no correlation between isotopic values and average $\Delta\Psi$ between the daily measurements ($P>0.454$; Fig. 6).

4. Discussion

Most riparian tree species must withstand flooding, severe erosion and sediment deposition, pollution of water from upstream, sediment abrasion of roots extending into the stream, and many other factors other plants do not encounter. Establishing the source of uptake under favorable conditions is the first step in examining how these plants thrive in such an environment, especially as riparian degradation and water pollution continue to be a factor [22, 23].

Because root presence is not necessarily an indicator of water uptake, the ability to use stable isotopes as tracers fulfills a need for analysis of water uptake and storage in plant tissue and determination of water sources [3, 8]. More specifically, this determination is made by analyzing differences in isotopic signatures [1, 2].

4.1 Identification of Water Source

The isotopic compositions of the water extracted from the woody tissue of both species were more depleted in deuterium than those of either shallow or deep soil water or streamwater, indicating consistent reliance on a source highly protected from evaporative enrichment (Figure 1). Also indicated is a potentially important methodological error—soil and woody tissue values are shown to be isotopically lighter than water collected from the installed well (“groundwater”). Because groundwater experiences a very low rate of evaporation, it should be one of the most depleted sources available [24].
Therefore, the water collected from the installed well was likely not representative of the groundwater of this area and the unsampled source is some groundwater source available to the trees at this site. While evidence suggests that the missing source is groundwater, we cannot be certain of the depth of that source (deep unsaturated zone, the capillary fringe, or the saturated zone). Because of this uncertainty, the term “deep groundwater source” will be used hereafter to refer to the unsampled source.

As mentioned above, missing samples representative of groundwater are an obvious error when interpreting the findings of this study. Because of the inability to install a drilled well nearer to the study site, and the seemingly shallow depth to which the self-installed well reached, proper sampling and characterization of the groundwater source was a concern. However, isotopic values of water taken from a nearby drinking well (approximately 4 km), reaching a depth more than 50 m, were in agreement with those taken from the well at the study site, thus groundwater samples taken from the study site were thought to be reliable. Because the isotopic values were so similar, and because of the relative nearness to the stream, it is likely that water collected from the well was from a hyporheic flowpath fed by streamwater. While no actual values for the missing source were measured, the data suggest that it is isotopically lighter than the values for *A. negundo* and *B. nigra*, and significantly lower than the lines of best fit for all other sources on the plot, suggesting that it must be highly preserved—another indication that the unsampled source is groundwater.

In an ideal situation, where all sources were sampled, isotopic mixing models could be used to determine fractional contribution of each source to plant water [25]. However, the failure to accurately sample all sources forces the use of less powerful
forms of analysis (due to low statistical power) [6, 9]. Regression analyses indicated a strong positive relationship between the isotopic signatures of A. negundo woody tissue and deep soil water over the duration of the study (Figure 3). While other sources cannot be ruled out, it appears that the unsampled deep ground water source and deep soil moisture are making important contributions to this species, as the high r²-value (0.972) indicates that the large majority of the variation in woody tissue values was due to variation in the isotopic values of the deep soil water samples. This would provide further evidence that the missing source was groundwater, as any other source contributing to woody stem water should have a relatively constant isotopic composition, a characteristic expected of groundwater. A similar, but non-significant trend occurred in B. nigra (P=0.116).

Overall, it appears that both species relied on a similar composition and utilization of water sources. With the lack of correlation between B. nigra and deep soil moisture, it is possible that the species utilizes deep soil water, but that it makes up a smaller portion of plant water than in A. negundo, and therefore does not exhibit the same trend. Additionally, neither species seemed to have a notable shift in source through the duration of the study.

4.2 Rooting Habits

While the use of root presence to identify the source of water uptake has been both cautioned and encouraged [1, 2, 24], the water utilization pattern found here is not surprising based on the rooting habits of these species [13-15]. Both species have relatively shallow roots (up to 2 m) allowing soil water uptake, but may also form a deeper tap root. Depth to groundwater in riparian zones of the region is often 5-10 m,
permitting uptake from the capillary fringe and, often, direct access to groundwater for species with deeper root systems.

4.3 Comparison with Previous Findings

The findings reported here are in contrast with the few studies conducted in the southeastern US which have reported surface water as the primary source of plant uptake [17, 25]. However, these previous studies have not been conducted streamside, and the variable results could be due to differences in the water conveyance system or species. These results seem to support studies performed in the southwestern US for *A. negundo* and similar species in the riparian zones of that region. As noted in multiple studies, plant water of many species of mature riparian trees in the arid Southwest, and Australia, is taken almost solely from soil and groundwater resources [3-6, 10].

The reliance on a deep ground water source of the species in this study suggests that broadleaved riparian trees may not be growing in these zones in an attempt to capture the abundant surface water. One theory as to why these species are only found naturally in riparian zones is that higher groundwater levels often exist in these areas allowing access to increased soil moisture and, possibly, groundwater resources [27, 28].

4.4 Unreliability Hypothesis

The data reported here contrast with the idea that riparian species do not utilize streamwater because streams are often too unreliable for uptake during the critical late summer period [1, 2, 9, 27]. The characteristics of the stream at this site (i.e. high water quality, reliably high water levels with an increased width and low flow rate due to damming) and selection of healthy, mature individuals should create an ideal scenario for streamwater uptake if it were going to occur. However, evidence collected at this site
suggests source utilization similar to those of Kolb et al. [10], and thus, the unreliability hypothesis does not appear to be an appropriate explanation for a lack of streamwater uptake at this site.

While it is possible that the roots of these species are not plastic or do not exhibit hydrotropism, and are unable to take advantage of stream resources when available, there is currently no good explanation as to why trees at this site would not use more substantial amounts of streamwater

4.5 Environmental Influence

None of the environmental variables measured in this study seemed to have an impact on which water source trees were using, i.e. temperature and humidity fluctuations were not associated significantly with changes in isotopic values. Although plant gas exchange measurements were not taken, increased temperatures and lower humidity would likely increase transpiration and lower xylem water potentials ($\Psi$), requiring access to a reliable water source to maintain photosynthetic gas exchange.

Monthly rainfall amounts also seemed to have little effect on water source utilization, with statistical analyses revealing no significant correlation. However, these analyses are hindered by the inability to examine the relative contribution from each source. It is possible that selected trees take up more soil moisture when rainfall increases, but not to a degree that would lead to a substantial change in isotopic signature, while mixing model data may differ enough to be statistically significant. It is likely that, as rainfall increases soil moisture, a higher amount of that moisture would contribute to plant water.

4.6 Plant Water Status
Comparison of isotopic analyses and Ψ failed to demonstrate any relationship between the apparent sources used and xylem water potential measurements in either species. While individuals examined seem to be consistently drawing from the combination of groundwater and/or deep soil moisture, Ψ and ΔΨ between morning and afternoon measurements varied throughout the study season (Fig. 6). It was expected that there would be consistency in measurements within each species, which would be linked to heavy reliance on a particular source. With both species relying heavily on groundwater, it would be expected that both exhibit a consistently high Ψ with little change between morning and afternoon measurements, as species with easy access to available water should become less water stressed. The lack of relationship between isotopic values and Ψ indicates that Ψ measurements cannot be used in place of isotopic analyses for determining water sources in A. negundo or B. nigra, similar to the findings of Snyder and Williams [6].

4.5 Conclusions
The inability to calculate fractional contribution of each source to plant water prevents the determination of conclusive utilization data. However, evidence from Figure 1 and a strong relationship with deep soil water indicate the missing source is highly conserved, suggesting a deep ground water source. This study also provides a preliminary agreement with studies conducted in arid systems, where groundwater makes up an important portion of plant water and little or no surface water is utilized.

Additional studies in the southeastern US, where all sources can be accurately sampled are needed to confirm the evidence collected here. As these projects begin to define the baseline of source utilization in systems with high water quality and
consistently reliable stream flows, this methodology could be used to monitor the impacts of environmental variations, in precipitation and temperature, and of anthropogenic manipulation, such as stream pollution from industrial and agricultural sources.

**Acknowledgement**

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Figure Captions

Figure II-1. Mean $\delta^{2}$H values v. mean $\delta^{18}$O values of water from woody tissue of A. *negundo* (filled circle) and *B. nigra* (empty circle) and water collected from 4 possible sources, with lines of best fit for each set of points. Data show that woody tissue values are isotopically lighter than any of the sources, and that shallow soil water (filled triangle) and deep soil water (empty triangle) are tightly coupled and enriched in $\delta^{2}$H compared to woody tissue values. Groundwater (filled square) and streamwater (empty square) are isotopically the heaviest (the most enriched) and are also tightly coupled. Error bars show the greatest cumulative variation at ±1 s.d. for each curve. The dashed line shows the Global Meteoric Water Line.

Figure II-2. Mean $\delta^{2}$H over time for water from woody tissue of A. *negundo* (filled circle) and *B. nigra* (empty circle) with possible sources examined. Woody tissue of both species is isotopically lighter than any of the sources throughout the study with the exception of a peak in September measurements where *A. negundo* values are more enriched, than those of deep soil water (empty triangle). Here, deep soil water values appear to be isotopically lighter than any other sources collected, while shallow soil water (filled triangle), groundwater (filled square), and streamwater (empty square) are tightly coupled and are the most enriched in $\delta^{2}$H.

Figure II-3. Relationship between $\delta^{2}$H values of *A. negundo* (filled circle) and *B. nigra* (empty circle) with deep soil water. A significant relationship exists between *A. negundo* woody tissue ($y=103.59 + 4.28x$; $r^2 = 0.972$, P=0.028); the same relationship was not found with *B. nigra* (P=0.12).
Figure II-4. $\delta^2$H values of *A. negundo* (filled circle) and *B. nigra* (empty circle) over time. The isotopic values of both species show similar trends, decreasing from the previous month in August and October measurements, while increasing in September. There was no difference in isotopic signatures between species during this period (P>0.052). Error bars show ± 1SD.

Fig. II-5. Regressions of $\delta^2$H values of *Acer negundo* (filled circle) and *Betula nigra* (empty circle) with a) current monthly rainfall and b) the previous month’s rainfall. There was no relationship found between rainfall at either time period and woody tissue values (P>0.171).

Fig. II-6 Regressions of $\delta^2$H values of *Acer negundo* (filled circle) and *Betula nigra* (empty circle) with a) morning Ψ measurements, b) afternoon Ψ measurements, and c) the change in Ψ between morning and afternoon measurements. Trends between morning Ψ and woody tissue values conflict between species, with *A. negundo* exhibiting a positive trend and *B. nigra* displaying a negative one. In contrast, both species exhibit positive trends with afternoon Ψ. No relationship between woody tissue values and Ψ were significant (P>0.128). Both species exhibit a non-significant, but positive trend with change in Ψ between the two daytime measurements (P>0.454).
Figure II-1

Figure II-2
Figure II-5
Figure II-6
CHAPTER III

SEASONAL VARIATION IN WATER SOURCES OF THE RIPARIAN TREE SPECIES *ACER NEGUNDO* AND *BETULA NIGRA*, SOUTHERN APPALACHIAN FOOTHILLS, USA

The following manuscript is in press in *Botany* (2015), and reprinted with permission from NRC Research Press. Stylistic variations are due to the requirements of the journal.
Abstract

Determining which water sources a plant accesses throughout a year is an important step in understanding how natural or anthropogenic changes in source characteristics affect utilization by plants. Sources of *Acer negundo* and *Betula nigra* of the foothills of the southern Appalachians Mountains were examined during the phenological stages of leaf bolt, flowering, and leaf senescence/abscission. Source utilization was monitored by comparing the isotopic composition of water samples taken from woody tissue to those of possible water sources at the site. Species used a combination of deep ground and shallow soil water sources, with a greater reliance on deeper sources during the late growing season when plant water demand was likely high. Water extracted from *B. nigra* was typically more depleted in $\delta^{2}$H than all sources measured, while values from *A. negundo* were more variable throughout the study period. Intraspecifically, isotopic values did not vary monthly or seasonally for either species (P>0.56), while interspecific values were different for December, January, and July samplings (P<0.02). Positive relationships occurred between air temperature and isotopic values of both species (P<0.04), and may reflect increased evaporation from the upper soil layers at warmer temperatures, which both species appeared to use most of the year.

**Keywords:** Deuterium, Groundwater, Streamwater, Phenology, Stable Isotopes
Introduction

Fluctuations and changes in water sources over time in riparian vegetation have been documented in numerous studies. These changes have been tied to plant phenological stage (Phillips and Ehleringer 1995; Snyder and Williams 2007), seasonal meteorological variation (Burgess et al. 2000; Dawson and Pate 1996; Ehleringer et al. 1991; Nippert et al. 2010; Snyder and Williams 2000; Zencich et al. 2002), and biotic and abiotic alteration of natural hydrological events such as streamflow diversion and regulation (Busch et al. 1992; Smith et al. 1991). These seasonal changes in water sources may be linked to reduced reliability of supply (e.g. decreased soil moisture availability) and/or changes in plant water demand. Determining when these fluctuations occur, and their association with plant physiological changes, should enable a better understanding of the mechanistic interaction between the plant and soil in terms of water uptake on a seasonal basis.

Determination of water source utilization is made possible through the analysis of the isotopic composition of all water sources and xylem water extracted from the woody tissue of the species of interest. The second most abundant isotopes of water’s atoms (\(^2\)H and \(^18\)O) evaporate at differing rates compared to their more common counter parts (\(^1\)H and \(^16\)O). This differential evaporation results in sources (i.e. surface water, soil moisture, or groundwater) with isotopic compositions distinct from one another in many ecosystems (Allison et al. 1983; Allison and Hughes 1983; Gat 1996). Xylem water of the examined plants should be representative, isotopically, of the source or combination of sources on which an individual is relying (Brunel et al. 1991; Dawson and Ehleringer 1991; Thorburn et al. 1993; Wershaw et al. 1966; White et al. 1985). Thus, monitoring
changes in isotopic values over longer periods enables determination of fluctuation or changes in the utilization of each source at different phenological stages.

While there have been a small number of studies that have determined water source utilization in the southeastern US over relatively short time periods (e.g. on a single sampling date or over a single growing season; White and Smith 2013; White 1989; White et al. 1985), there have been few to monitor riparian vegetation for extended periods. Thus, little is known about how changes in plant phenology, micrometeorology, or source availability affect water source usage in this region. The purpose of the current study was to identify and track the water sources utilized by two representative riparian tree species (Acer negundo L. and Betula nigra L.; Radford et al. 1968; Weakley 2012) during a 12-month period (October 2011-September 2012) in the Appalachian foothills of western North Carolina, USA. One of the few source utilization studies conducted in the Southeast region, suggested that these species rely significantly on deep ground water sources, supplemented with shallower soil water uptake during the late growing season (June-September) (White and Smith 2013).

The water sources of Acer negundo, which is widely distributed across North America, has been examined in a number of studies, providing a background with which current findings can be compared (Dawson and Ehleringer 1991, 1993; Kolb et al. 1997; Thorburn and Ehleringer 1995; White and Smith 2013). In contrast, Betula nigra, most common in the southeastern US, has received much less attention (White and Smith 2013). Both species leaf out during early spring (mid-March-early April), flower just afterwards, and begin to fruit in the early summer (late May-early June); however, A. negundo may not drop its fruit until late fall while B. nigra sheds its fruit during the
summer season. Leaf senescence occurs as cooler temperatures become frequent and more pronounced in early to mid-Fall in both species.

Based on the life history of these species, with leaf-out and flowering occurring during late spring when soil moisture is near its peak, it was predicted that both species would utilize a combination of soil moisture and groundwater throughout the year, using a greater amount of soil water when it is available (typically in the spring and fall) and relying more strongly on groundwater when soil moisture is unable to meet the plant’s water demands. It is expected that this inability to meet plant demands will occur due to increased water requirements during flowering and fruiting, or because of reduced soil moisture availability caused by increased evapotranspiration or competition from shallow-rooted annuals (Dawson and Pate 1996; Ehleringer et al. 1991; Liu et al. 2010; White and Smith 2013; Zencich et al. 2002).

Methods

The possible water sources identified in this system were streamwater, soil water, and groundwater. Water extracted from woody tissue and soil samples, along with water collections, underwent isotopic analyses. Microclimate and hydrological factors, including temperature and rainfall, were also monitored in an attempt to document any relationships between fluctuations in these variables and variations in water source. Collections of woody tissue from each species, soil, and water were made once each month during the study period (October 2011-September 2012).

Study Site and Species

The current study was conducted in an intact and largely undisturbed riparian corridor along the Jacob Fork River in Newton, NC. This zone is part of a 40.5 ha parcel
located at 35.6299° N, 81.3101° W which has been preserved as a city park. The site has undergone minor development, and features an intake for the municipal water supply, which includes a small dam downstream from the study site, increasing stream depth and width along the site. Adjacent to the corridor under study, the stream is approximately 3 m deep and up to 12 m wide with a sediment-covered bottom. The stream banks along the study site are sharply eroded and rise 0.3-2 m above the stream while those on the adjacent side slope gradually to the water. Nearly the entire park area falls into the 100-year floodplain (North Carolina Floodplain Mapping Program, floodmaps.nc.gov 2011).

Soils are classified as Ronda loamy sand, a well-drained, deep soil found in floodplains and riparian zones of the foothills of North Carolina and Virginia. These soils feature relatively low total plant available water at ca. 10.75 cm/m (UC Davis Soil Resource Laboratory, casoilresource.lawr.ucdavis.edu 2014). Vegetation at the site is typical of riparian zones of the region, including A. negundo, B. nigra, Quercus spp., Platanus occidentalis, and Smilax spp. among others. There is moderate undergrowth in this system, made up of both native and invasive species (Radford et al. 1968; Weakley 2012).

The year was divided into 3 major study seasons, based on plant phenology. The dormancy period ranged from October-February, from the time of leaf drop to leaf bolt. The early growing season lasted from March-May and included leaf-out, flowering, and up to fruiting, while the late growing season was from June-September, during fruiting and up to leaf abscission. The phenological stage of each species was documented qualitatively during each field visit. The foothills of the southern Appalachians experience moderately cold, dry winters (December-early March); moist springs with
warmer temperatures (March-May); hot, wet summers (though periods of drought are characteristic) (June-early September); and mild, moist falls (September-November).

**Site Microclimate**

Environmental variables were measured onsite throughout the study period. Air temperature and relative humidity were monitored using a HOBO Pro V2 sensor (model U23-001; Onset, Bourne, MA, USA) shielded from direct sunlight and nighttime sky exposure. Onset’s tipping-bucket rain gauge (RG1) and data logger (H07-022-04; Onset, Bourne, MA, USA) were used to monitor rainfall at the site; however, due to consistent insect infestation that prevented accurate collection of precipitation, rainfall data were taken from those reported from a regional airport (KHKY) less than 15 km from the site. Reliable rainfall measurements from the study site were similar to those reported at the airport.

**Sample Collection**

To determine water source utilization, water samples from each potential source were collected. Streamwater samples were taken from an undisturbed, but well mixed location at an approximate depth of 20 cm. Groundwater samples were syphoned from a self-installed well located about 5 m from the stream. The well was created using a Hoffco Inc. PH 980 post-hole drill with a 2” earth bit to drill to a depth of ca. 3m (where soil was saturated), and was fitted with a 2” PVC pipe with numerous holes drilled into the lower meter. The pipe was capped and soil taken from the drilling was used to backfill around the well, a measure to prevent precipitation and runoff from leaking around the pipe and potentially contaminating the isotopic composition of groundwater. The well was bailed upon arrival on each collection date, and would typically refill by
noon when samples were collected. Precipitation was not sampled for isotopic analysis at the site, as previous studies have shown that the time gap between the precipitation event and plant uptake likely allows for evaporative enrichment (Allison et al. 1983; White et al. 1985). For this reason, shallow soil samples were assumed to be representative of the precipitation available for plant uptake. Data from previous projects (unpublished data) demonstrate that the isotopic ratios of precipitation of the region fall closely along the global meteoric water line (GMWL; shown in Fig. 2).

Soil samples were taken at each end of the site within 2 m of trees selected for isotopic analyses. Collections were taken at 2 depths: 30 cm (shallow soil) and 1 m (deep soil). Sampling at these depths, along with groundwater, should have provided collection of water sources available to both species based on their dimorphic rooting habits, with shallow lateral roots running parallel to the soil surface in the upper 30 cm and, in deeper moist soils, tap roots at least capable of reaching the capillary fringe (Green 1934; Maeglin and Ohmann 1973).

Three healthy, mature individuals of both *A. negundo* and *B. nigra* of similar age and size, which were within reach of the stream (up to 5 m), were selected for woody tissue sampling. Consistent with several previous studies (Lambs et al. 2003; Mensforth et al. 1994; Thorburn and Walker 1994), three stem segments, 0.5-1 cm in diameter and up to 0.5 m in length, were taken randomly from each individual. Stems were cut from the tree, leaves and loose bark were removed, and segments were cut into lengths short enough to be placed in a collection vial.

All samples were placed in screw-top glass vials and sealed with Parafilm M to prevent any evaporation and potential fractionation of isotopic compositions. Samples
were kept in a chilled cooler until returned to the lab where they were frozen until being shipped for isotopic analyses.

*Isotopic Analysis*

Samples were shipped frozen to the Stable Isotope Ratio Facility for Environmental Research (SIRFER) at the University of Utah for isotopic analyses. Water was extracted from woody tissue and soil via cryogenic vacuum distillation which, when completed, preserves the isotopic ratios of the water sample (West et al. 2006). Four replicates for each sample were injected into a wavelength-scanned cavity ring-down spectrometer water analyzer (model L1102-I; Picarro, Sunnyvale, CA, USA). Hydrogen and Oxygen isotopic ratios reported were the mean of the 3rd and 4th injection and were reported as $\delta$-values (in per mill) relative to V-SMOW. Precision of analysis was $\pm 3.8\%\text{oo}$ for $\delta^2$H and $\pm 0.4\%\text{oo}$ for $\delta^{18}$O. It is worth noting that this method of analysis of isotopic composition can be affected by contamination by secondary compounds in plant tissue (Schmidt et al. 2012; West et al. 2010). However, because all samples were analyzed in this way and because previous studies have found only small deviations between results from isotope ratio mass spectrometry and isotope ratio infrared spectroscopy in related species of both *Acer* and *Betula* (Schmidt et al. 2012; Schultz et al. 2011; West et al. 2010; West et al. 2011), it was assumed that any errors due to contamination were likely to be minor and similar between species.

*Plant Water Status*

Xylem water potentials ($\Psi$) were taken at midday on each collection day (once per month). Water potentials were measured from multiple individuals of both species ($n=6$ per species), including those selected for isotopic analyses. Samples of terminal
stems were removed from random locations of similar height (>5-8m), sealed in a zip-top bag, and kept in a cool, dark bag until measurements were taken using a Scholander-type pressure chamber (Model 1000, PMS Instrument Co., Corvallis, OR, USA). Water potentials were measured during the afternoon because this period often reflects highest daily water stress, especially for species accessing shallower water sources.

**Statistics**

Normality of each dataset was analyzed using Shapiro-Wilk tests. One-Way ANOVAs and Kruskal-Wallis One-Way ANOVA on Ranks, for non-parametric data, were used to compare differences between months for each species. Tukey’s tests were then performed to identify significant differences in the dataset. T-tests, and Mann-Whitney U-tests when data were non-normal, were used to compare interspecific differences for each month. Relationships between woody tissue values and environmental variables were analyzed using linear regressions.

**Results**

**Site Microclimate**

Average daily temperature was lowest in the winter months (the dormant period) and highest in the late growing season; more specifically, January 2012 had the coldest daily temperatures at 5.02 °C and July 2012 had the hottest temperatures at 24.65 °C (Fig. 1). Mean daily highs and lows followed similar patterns. Alternatively, humidity was highest during the summer and early fall, and lowest during the late fall and spring (data not shown). Rainfall during the study was slightly lower than average (~1200 mm) at 1048 mm (Fig. 1). The late growing season (June-September) experienced alternating months of high and low rainfall, but still had the greatest cumulative total rainfall of any
period (453.90 mm), with the highest rainfall in July 2012 (168.15 mm). The dormant period saw the lowest rainfall totals per month, with February being the driest month at 40.13 mm of precipitation.

**Identification of Water Sources**

Isotopic compositions of plant water from both species were often more depleted in deuterium ($^2$H) than all possible sources during the study period (October 2011-September 2012; Fig. 2). While water extracted from woody tissue and sources were similarly depleted in oxygen-18 ($^{18}$O) over the same period, there were clear differences in $\delta^2$H between sources and plant water. The collected groundwater and streamwater were consistently more enriched in $\delta^2$H than other sources. Shallow and deep soil water values fell between those of ground and streamwater and those of plant water (Fig. 2). During each season, soil moisture lay nearest the Global Meteoric Water Line (GMWL), while ground and streamwater were more enriched in $\delta^2$H, indicating evaporative enrichment. Source $\delta^2$H and $\delta^{18}$O were similar over the study period, with no differences in any source, between seasons (P>0.37).

While water extracted from *B. nigra* was typically more depleted in $\delta^2$H than all collected sources, values from *A. negundo* were more variable throughout the study period (Fig. 3 and 4a). Excepting 3 sampling periods, *B. nigra* values were isotopically lighter than any possible sources analyzed. Only the relationships between *A. negundo* with shallow soil water (P=0.04, $r^2=0.82$) and streamwater (P=0.04, $r^2=0.80$) during the dormancy period were significant. There were no other significant relationships between isotopic values of water extracted from woody tissue of either species and analyzed sources on an annual or seasonal scale (P>0.05). An important note: Fig. 2 also shows
that a source, more depleted in $\delta^2$H than all others, was not collected at any point in the year.

Isotopic values did not vary seasonally for either species (P>0.56), and similarly, woody tissue values were consistent on a monthly scale. Interspecific values also showed consistency, with differences only at December, January, and July samplings (P<0.02; Fig. 4a). While there were fluctuations in the values for both species throughout the study, they were most consistently alike during the June-September period.

**Plant Water Status**

No relationships existed between midday water potentials with $\delta^2$H of water extracted from woody tissue of either species on a monthly scale (P>0.06, Fig. 4b). There were no notable intraspecific differences in $\Psi$ for either species during the study, but the two species did differ for the October, February, August, and September (P<0.02) measurements, with *A. negundo* being more water stressed in the dormant period and *B. nigra* more stressed during the late growing season.

**Environmental Influence**

There were few notable relationships between woody tissue isotopic composition and environmental variables. However, there were significant relationships between average daily air temperature and both species during the late growing season (*A. negundo* - P=0.01, $r^2=0.98$; *B. nigra* - P=0.04, $r^2=0.92$) and with *A. negundo* during the dormant period (P=0.001, $r^2=0.93$).

**Discussion**

Decreased water availability, due to lower groundwater levels, increased evaporation from soils, or meteorological drought, can lead to increased plant water stress
and shifts in water sources (Burgess et al. 2000; Dawson and Pate 1996; Ehleringer et al. 1991; Nippert et al. 2010; Singer et al. 2012; Snyder and Williams 2000; Zencich et al. 2002). Alternatively, changes in plant phenology, such as leaf-out, flowering, and fruit production may cause increased water demand (Phillips and Ehleringer 1995; Snyder and Williams 2007) and increased water stress when additional water resources are not available. These factors likely result in a reliance on dependable water sources, where they exist, that have consistent and ample availability, even during mild to moderate drought episodes. Added to these potential stressors are exogenous factors such as competition for sources by other vegetation and anthropomorphic alteration of the natural hydrology of a habitat, e.g. channelization of a stream, draining of groundwater resources for consumption, and alterations of flooding regimes by damming (Busch et al. 1992; Dawson 1993b; Smith et al. 1991). These complications can have significant implications for a species' water uptake, and could cause localized extinction of species that cannot tolerate these conditions.

The use of the stable isotopes of water has been shown to be a reliable means of tracing water sources, both in *Acer* (Dawson 1993a; Dawson 1996; Dawson and Ehleringer 1991; Kolb et al. 1997; Phillips and Ehleringer 1995; Thorburn and Ehleringer 1995; Ward et al. 2002; White and Smith 2013) and *Betula* (Dawson and Ehleringer 1993; Roden and Ehleringer 1999a, b, 2000; Smith et al. 1991; White and Smith 2013; White 1989). Previous riverine studies have shown that riparian tree species are often relying strongly on ground water resources rather than streamwater throughout the growing season in arid (Dawson and Ehleringer 1991; Mensforth et al. 1994; Thorburn and Walker 1993) and temperate (White and Smith 2013) locations. There have been,
however, several non-riverine studies that show surface water utilization (Lin and Sternberg 1994; White et al. 1985) suggesting water conveyance system may impact water uptake strategies.

**Identification of Water Sources**

Water sampled from the installed well and shallow soil water were consistently the most enriched in deuterium, followed closely by streamwater and deep soil moisture (Figure 2). The regression lines of $\delta^2$H for water extracted from the woody tissue of both species run lower than those of all analyzed sources—an important complication. While similar findings have been reported previously (Brunel 2009; Horton et al. 2003; Jackson et al. 1995; Snyder and Williams 2000; Snyder and Williams 2007; Walker and Richardson 1991), there is no good explanation for this finding. After carefully examining the collection methodology and isotopic reports, the best explanation is that there was a failure to accurately collect all water sources available to vegetation at this site. Because xylem water, on an annual scale, is more depleted in deuterium than the collected water sources, and because the collected groundwater was among the most enriched sources, it seems likely that the error occurred in the collection of groundwater sources. While groundwater supplies can be affected by local inputs (e.g. streamwater), in most cases, it is the isotopically lightest water source because it is highly conserved and experiences the least evaporation (Allison et al. 1983; Allison and Hughes 1983; Gat 1996). Due to the proximity to the stream and the similarity in isotopic values, it appears that the installed well accessed a hyporheic flowpath where there was mixing of ground and streamwater, or possibly the capillary fringe associated with the stream, instead of
solely groundwater. Additionally, because of the depletion in $\delta^2$H of water sampled from woody tissues, it is likely that the unsampled source is of some ground water origin.

Because of the above complications, it can only be said that some ‘deep ground water’ source consistently contributed to plant water throughout the year. Both species began the dormant period depleted in deuterium relative to sources collected, but as the last leaves fell in November, isotopic values of the species diverged, with values of *A. negundo* increasing throughout this period (Fig. 3a). These interspecific differences in mean $\delta^2$H are likely due to differing periods of inactivity, with *A. negundo* entering its dormant period a few weeks after *B. nigra*, and utilizing enriched shallow soil moisture in the interim, as indicated by the similarity in $\delta^2$H values in this span. These differences are unlikely due to a change in source utilization, as it is unusual for a tree to cycle through significant amounts of water during this period (Phillips and Ehleringer 1995).

As suggested by the stability in isotopic values, it appears that *B. nigra* entered dormancy between the October and November measurements, while *A. negundo* did not appear to go into the dormancy phase until the following month, December. Each species’ dormant period, however, lasted about 3 months, with the apparent flush of new water in February for *B. nigra* and March for *A. negundo*.

During the early growing season, as the trees bolted and entered the leaf growth phase, $\delta^2$H values for *B. nigra* remained similar to those at the end of the dormant period (February), while those of *A. negundo* trended toward depletion. This notable decline in *A. negundo* values shown in Fig. 3b may be due to a change in source or cycling of fresh water from deeper soil moisture as leaves flushed. Isotopic values for both species continued to become more depleted throughout the early growing season and into the
summer. This evidence suggests that the trees moved to a greater reliance on a deep ground water source, with a smaller amount of surface soil water making contributions to these plants. While rainfall was greater during the late growing season (Fig. 1), the increased water demands from transpiration of a fully-leaved tree, maintenance of remaining fruit, and increased evaporation from upper soil layers due to an increased vapor pressure deficit, may have overcome rainfall inputs, forcing a shift to a stronger reliance on groundwater. The isotopic values of both species were similar in the late growing season, but the more variable *A. negundo* δ²H values had minor fluctuations during this period and δ²H of both species peak in July, both likely responses to rainfall events (Chimner and Cooper 2004; Ehleringer et al. 1991). As the growing season ended, δ²H for both species declined to near those of the October 2011 measurements, suggesting an annual pattern of a decline in δ²H values at the onset of dormancy.

Overall, both species appear to have similar source utilization strategies by season, with the few interspecific differences in δ²H possibly due to use of the same sources in slightly different proportions. Based on the relationships shown in Fig. 2 and Fig. 3, it appears that throughout the year, xylem water of both species was a combination of water from deep ground water sources, shallower soil layers, and possibly streamwater, with a greater reliance on deeper sources during the late growing season. These patterns may be due to increased water demand after leaf flush and during flowering and fruiting when shallow soil moisture cannot keep up with higher transpiration levels (Hultine et al. 2007; Phillips and Ehleringer 1995; Schilling and Jacobson 2009), but are unlikely to be caused by desiccation of upper soil layers as seen in previous studies (Dawson and Pate 1996; Ehleringer et al. 1991), as indicated by increased rainfall in this period.
**Environmental Influence**

The strong positive relationships between air temperature and isotopic values of both species (*A. negundo* in the dormant period and both species during the late growing season) revealed an increase in $\delta^2$H with increases in air temperature likely due to increased evaporation of moisture, associated with higher temperatures, from the upper soil layers. This relationship indicates that this source was used to some degree by both species most of the year.

**Plant Water Status**

Xylem water potentials between species were significantly different at seemingly random points during the year. Interestingly, three of the differences (at October, August, and September measurements) occur at periods where $\delta^2$H of the species are among the closest measured during the study (Fig. 4). This implies differing water demands at a given water availability, and may explain the apparently slight difference in water source use strategies. There was no statistical relationship between $\Psi$ for either species and rainfall as seen in several previous water utilization studies (Burgess et al. 2000; Chimner and Cooper 2004; Ehleringer et al. 1991; Snyder and Williams 2000; White et al. 1985; Williams and Ehleringer 2000).

**Summary and Conclusions**

Although apparent failure to sample all water sources available at this site (likely due to possible mixing between compartments sampled within the installed well) limits the conclusiveness of this study, there is ample evidence that the interpretation is accurate. The cumulative results including isotopic data (Fig. 2 and Fig. 3) and the strong positive relationships between $\delta^2$H of both species and temperature (Fig. 1 and Fig. 4) indicate
that both *A. negundo* and *B. nigra* are using a deep ground water source and shallow soil water (fed by rainfall) throughout the year, though likely in differing amounts. The greatest differences in water source utilization occurred during the dormant period, with very similar source usage throughout both segments of the growing season. The shift towards greater deep water reliance in the late growing season occurred at a time when rainfall was the highest, indicating that increased water demand due to biotic and abiotic processes (i.e. transpiration, fruiting, and increased evaporation from upper soil layers) may have overcome the additional precipitation inputs.

**Acknowledgements**

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Literature Cited


List of Captions

Figure III-1. Mean air temperature (line) and precipitation (bars) during the 12-month study. The dark gray section highlights the dormant period, while the light gray section indicates the early growing season, and the white section shows the late growing season.

Figure III-2. Mean $\delta^2$H v. $\delta^{18}$O of woody tissue of Acer negundo (solid circles) and Betula nigra (solid triangles) with all possible sources (open symbols), plus the regression lines for each set of points for the full 12-month study. Dashed line is the Global Meteoric Water Line (GMWL).

Figure III-3. Mean $\delta^2$H for woody tissue of Acer negundo (solid circles) and Betula nigra (solid triangles) and all possible sources (open symbols) during (a) the dormant period, (b) the early growing season, and (c) the late growing season.

Figure III-4. (a) Mean $\delta^2$H for woody tissue and (b) xylem water potentials of Acer negundo (solid circles) and Betula nigra (open circles) over the duration of the study (dark gray section = dormant period, light gray section = early growing season, and white section = late growing season). * show significant interspecific differences (P< 0.02). Error bars show ± 1sd.
Figure III-1

Figure III-2
Figure III-3

The diagrams illustrate the variation of δ²H (% in parts per thousand) over time for different water sources and species. The graphs show the seasonal changes in isotopic composition from October to September, with data points for four distinct months: October (Oct), November (Nov), December (Dec), January (Jan), February (Feb), March (Mar), April (Apr), May, June (Jun), July (Jul), August (Aug), and September (Sep). The data is represented by various symbols and line types, indicating different water sources and species.
Figure III-4
CHAPTER IV

SURFACE FLOW REGIME AND WATER SOURCE UTILIZATION IN THE RIPARIAN SPECIES LIQUIDAMBAR STYRACIFLUA, SOUTHERN APPALACHIAN FOOTHILLS, USA

The following manuscript is in review for publication in *Ecohydrology* (2014). Stylistic variations are due to the requirements of the journal.
Abstract

A number of studies have revealed that mature riparian trees of the southern Appalachian foothills utilize little streamwater, relying heavily on soil moisture and groundwater instead. In contrast, previously studied riparian plants growing in swamp systems have been shown to use greater proportions of surface water. The purpose of this study was to determine if surface flow regime is linked to these differences in water source utilization. Water sources of Liquidambar styraciflua growing along Whetstone Creek in Stoneville, NC were compared to those growing along adjacent ponds by analyzing signatures of stable isotopes found naturally in water. Potential sources included surface water, soil water, and groundwater. Plant water status and environmental variables were also monitored. It was expected that, due to the lower flow rate and increased availability, pondside individuals would utilize a greater amount of surface water, while streamside trees would rely more strongly on groundwater. Results showed streamside trees were taking the majority of plant water from the stream (up to 76%), while pondside trees relied about evenly on soil water and pond water. Generally, groundwater contributed only a minor proportion to trees at both sites. There were no meaningful relationships between source and environmental factors, and source use did not have a notable impact on the water status of trees at either site. This study is the first to show consistent use of surface water by riparian trees in the southeastern United States.

Keywords: Stable Isotopes, Deuterium, Groundwater, IsoSource, Mixing Models, Riparian, Southern Appalachians
Introduction

Because riparian species are generally defined by growing along surface water, it is commonly assumed that their distribution is linked to the abundance of surface water in these habitats. However, with only a few exceptions (Smith et al., 1991; Thorburn and Walker, 1994; Drake and Franks, 2003; Muttiah et al., 2005), mature riparian trees have been shown to rely strongly on soil or groundwater and use little surface water, if any (Dawson and Ehleringer, 1991; Busch et al., 1992; Thorburn and Walker, 1993; Mensforth et al., 1994; Snyder and Williams, 2000; Singer et al., 2012; White and Smith, 2013; Chimner and Resh, 2014; White and Smith, 2014). One report of mature riparian trees utilizing surface water (although defined as groundwater in the paper), is the study by White et al. (1985), which reported abundant use of surface water in bald cypress trees growing in a swamp system, a rare example of a source utilization study conducted in a lentic system.

In comparing differences between streamside sites where trees have been found to rely primarily on groundwater, and swamps where White et al.’s study indicated increased surface water use, the most obvious contrast is the flow regime (e.g. lotic vs lentic) and the associated hydrologic differences. Indeed, their findings suggest that uptake of surface water may, in some way, be linked to surface water flow regimes (i.e. lentic vs. lotic). Other studies have examined the effects of changing flow and flooding regimes on riparian vegetation (most commonly related to damming and flood regulation) and have shown that alteration of hydrologic regimes can have major impacts on seed germination and stand dominance relations (Smith et al., 1991; Busch et al., 1992;
suggesting that these changes can have critical implications for plant-water relations.

The primary aim of the current study was to evaluate plant source usage under such differing flow regimes. A secondary goal was to collect additional information on source utilization of inland forests of the southeastern US, a region that has received much less attention (White et al., 1985; White, 1989; Retzlaff et al., 2001; White and Smith, 2013; Berry et al., 2014; White and Smith, 2014) than other areas of the country, i.e. the southwestern US (Dawson and Ehleringer, 1991; Busch et al., 1992; Phillips and Ehleringer, 1995; Kolb et al., 1997; Snyder and Williams, 2000; Williams and Ehleringer, 2000; Brunel, 2009; Warren, 2011) and coastal Florida (Sternberg and Swart, 1987; Lin and Sternberg, 1994; Ewe et al., 1999; Ewe et al., 2007).

The present study compared differences in water sources between individuals of a single species, *Liquidambar styraciflua* Linnaeus, found growing in both a lotic (stream) and lentic (pond) habitat. Stable isotopes of water were utilized as non-radioactive tracers to determine the contribution each water source made to plant water. Isotopic compositions of plant water taken from woody tissue samples were measured, plus all possible water sources (surface water, soil water, and groundwater). Based on previous findings, the hypothesis for this study was that individuals growing pondside would utilize greater amounts of surface water than streamside individuals, which were expected to rely primarily on groundwater.

**Methods**

*Study Site*
The study was conducted within riparian zones bordering Whetstone Creek and two nearby ponds in Stoneville, North Carolina, USA (36.4392 N, -79.8465 W), with data collected once each month June-September 2013. Whetstone Creek is a moderately-sized, spring-fed, perennial stream, typical of streams of the region. The creek varied in morphology along the study reach, ranging from about 1 m wide and 30 cm deep at its most narrow and shallow to ca. 4 m wide and 75 cm deep at its widest and deepest locations. The stream bed, although sand covered through much of the reach, was scoured to a claypan in downstream portions. The two stream-adjacent ponds, along which study trees were distributed, have spring-fed sources, are decades-old, and have been used in the aquaculture industry. The ponds were relatively large (>2 ha each) and were sequential, with the upstream pond emptying into the downstream pond, which then drained into Whetstone Creek below the study reach.

The riparian vegetation along the stream and pond sites described above was representative of the area and includes *Platanus occidentalis*, *Betula nigra*, species of *Acer* and *Quercus*, and others along with *Liquidambar styraciflua*. There was also low to moderate undergrowth in these zones, which was composed of both native and invasive species (Radford *et al.*, 1968; Weakley, 2012).

**Site Microclimate**

Temperature (±1.5 °C) and humidity were measured on-site using an Onset HOBO Pro V2 sensor (model U23-001; Onset, Bourne, MA, USA) shaded from midday sun and nighttime sky exposure. Rain gauges were placed in several locations throughout the area to monitor rainfall during the study period, with mineral oil (~2mm) placed in the gauges to prevent evaporation of collected precipitation between visits to the site.
Due to complications with accurately collecting rainfall during the first half of the study, rainfall measurements were taken from reports by an adjacent airport (ca. 0.6 km from site). Reliable on-site rainfall measurements later in the study (data not shown) were in close agreement with those from the airport, which were therefore considered to be representative of those at the site.

Sample Collection

Water from each source available to plants at these sites (surface water, soil water, and groundwater), along with woody tissue from which water was extracted, were sampled at each collection date to determine each source’s contribution to plant water. Groundwater was collected from a residential drinking well, located 1.1 km from the center of the study site, via the outdoor spigot nearest the well. The spigot was allowed to run for about 1 min before replicate samples were taken. Isotopic similarity between water from this source and streamwater from the site, as well as with more surficial groundwater values reported for the region (unpublished data), indicate the water sampled from the well is a suitable proxy for groundwater directly accessible to the trees. Due to past issues with accurately collecting more surficial groundwater with piezometers in these habitats in the region, collection via this method was deemed unfeasible. While sampling from springs at the site would have been informative, they had been excavated to create ponds, and were therefore unavailable for sampling.

Surface water sources were sampled at multiple (n=3) locations in the stream and each pond. Streamwater was collected near the surface from several locations in the study reach of Whetstone Creek. Multiple samples were taken from each pond using an extension pole fitted with a cup, allowing sampling away from the bank and at a depth of
ca. 20 cm from the surface. Soil was collected near the base of each tree selected and was sampled at depths of 30 cm (shallow soil) and 1 m (deep soil) at each location. Soil samples were taken using a 2.75 inch, one-piece auger (model 400.48, AMS Inc., American Falls, ID). By sampling at these 2 soil depths and groundwater, course sampling of sources available to trees should have been achieved based on rooting habit of *L. styraciflua*, which often features a dimorphic rooting system with numerous lateral roots and, regularly, a taproot on well-drained soils (Sargent, 1922; Kormanik, 1990). Soils were regularly saturated at ca. 30 cm; even so, no free water was observed during soil sampling and any mixing of water between soil layers due to sampling was expected to be minimal.

Woody tissue samples were taken from healthy appearing, mature individuals at both sites (n=4 per site), each of which were growing within ca. 3 m of the respective surface water. Multiple twigs were taken from random locations within the crown of each individual using pole pruners. Similar to several previous studies, three replicates were taken from each selected individual on each collection date (Lambs et al. 2003; Mensforth et al. 1994; Thorburn and Walker, 1994). Woody material 30-50 cm in length and ca. 10 mm in diameter was taken for each sample, removing all leaves and loose bark (potential sites of evaporative enrichment) before storage.

All samples (water, soil, or woody tissue) were sealed in screw-top vials, which were wrapped in Parafilm to ensure an air-tight seal. After collection, samples were stored in a chilled cooler and frozen upon return to the lab—additional measures to prevent any evaporation.

*Isotopic Analysis*
At the completion of sample collection, frozen samples were sent to the Stable Isotope Ratio Facility for Environmental Research (SIRFER) at the University of Utah for water extraction and analysis of isotopic ratios. Extraction was performed via cryogenic vacuum distillation which, at completion, preserves isotopic composition of the water samples (West et al., 2006). Isotopic analysis was conducted using a wavelength-scanned cavity ring-down spectrometer water analyzer (model L1102-I; Picarro, Sunnyvale, CA, USA), into which 4 replicate samples were injected; mean isotopic concentrations of hydrogen and oxygen were measured using the 3\textsuperscript{rd} and 4\textsuperscript{th} injection. Samples were analyzed using 3 laboratory standards calibrated with Vienna Standard Mean Osmotic Water (VSMOW) values. Results were reported relative to VSMOW with precision of analysis of ±1.6‰ for $\delta^2$H and ±0.2‰ for $\delta^{18}$O. Within-tree variance (n=3) for each individual was ±5%. This method of isotopic analysis can be affected by contamination due to secondary compounds in plant tissue of some species, causing discrepancies between isotope ratio mass spectrometry and isotope ratio infrared spectroscopy results (West et al., 2010; Schmidt et al., 2012). However, similar species have shown only minor differences (West et al., 2010; Schultz et al., 2011; West et al., 2011; Schmidt et al., 2012).

**Plant Water Status**

As an indicator of plant water status, xylem water potentials ($\Psi$) were measured twice per collection day—once in the morning (ca. 0800) and once in the afternoon (ca. 1500) using a Scholander-type pressure chamber (model 1000; PMS Instrument Co., Corvallis, OR). Multiple terminal leaves were taken from random locations within the crown of the same individuals sampled for source determination. Sample leaves were
sealed in zip-top plastic bags and stored in a cool, dark bag until measurements were taken.

**Mixing Models**

Isotopic ratio data from each individual and each possible water source were used to determine the mean and range of possible source contributions to plant water using the IsoSource program (v. 1.3.1; Phillip and Gregg, 2003; www.epa.gov/wed/pages/models/stableIsotopes/isosource/isosource.htm). Linear mixing models (two ended) can only determine percent contribution of $n+1$ sources, where $n$ is the number of isotopes used in source identification. IsoSource overcomes this complication by constructing mixing polygons (examples given in Fig. 1), and calculating feasible contributions from each source that could influence plant water isotopic values (Phillips and Gregg, 2003). Isotopic values from each replicate were input into IsoSource and output data were averaged for each individual and site. The IsoSource software was utilized with the increments of 1% and the tolerance value set to 0.05‰ or to the lowest possible value enabling an analysis up to 1.75‰.

**Statistics**

Shapiro-Wilk tests were used to test the normality of each dataset. One-Way ANOVAs, followed by Tukey’s or Dunn’s tests, were used to determine any isotopic differences between sources on a monthly basis. Kruskal-Wallis One-Way ANOVA on Ranks were used to test these differences where data were non-normal. T-tests, and Mann-Whitney Rank Sums when data were non-parametric, were used to determine differences in inter-site water potentials. Linear regressions were used to analyze relationships between isotopic data, environmental variables, and plant water potentials.
Results

Site Microclimate

During the four-month study period (June-September 2013), the hottest month was July, with a mean temperature of 23.5 °C (Fig. 2). June had the greatest mean daily high temperature (28.1 °C), while September had the widest range between the mean high and low temperatures. Relative humidity was consistent throughout the study period ranging from 90 to 93%. June was the wettest month during the study, with 179 mm of rainfall, while September was the driest (33 mm). Overall, the season was slightly drier (393 mm) than the average of 421 mm over the study period (Fig. 2).

Plant and Source Isotopic Variation

Water sources available to both streamside and pondside L. styraciflua were consistent isotopically during the course of the season, with only pond water values varying significantly during the study (P<0.001; Fig. 3). At the stream site, on a whole-season scale, there was no significant difference between soil at different depths (P>0.585), while both were significantly different from streamwater in δ²H values (P<0.001) but not in δ¹⁸O (P>0.09). Shallow soil water at the creek site was different from groundwater in both δ²H and δ¹⁸O (P<0.01), but deep soil water differed only in δ²H (P=0.01). Groundwater and streamwater also differed from each other (P<0.001).

At the pond site, isotopic values of soil water at the 2 sampled depths were statistically similar (P>0.63). Soil water at neither depth was different from groundwater in δ²H (P>0.06), but both were in δ¹⁸O (P<0.015) values. Both soil depths and groundwater were significantly different from pond water (P<0.001).
While isotopic compositions of plant water were similar between the two sites, there was notable variation in plants at both sites along an evaporation line during the season, most pronounced in $\delta^{18}$O values (Fig. 3). Groundwater and streamwater were highly consistent in isotopic signature throughout the study period, and pond water ranged tightly along an evaporation line. Soil water showed much greater variation at both sites, on both monthly and whole-season scales, with the greatest spread in pond site values. Groundwater was more enriched in deuterium ($\delta^{2}$H) than the global meteoric water line (GMWL), while all other sources were more depleted in $\delta^{2}$H at a given $\delta^{18}$O.

**Water Source Utilization**

Isotopic and mixing model data rejected the hypothesis that pondside trees would be using more surface water than streamside trees. At each sampling period, streamside *L. styraciflua* used more surface water than those growing pondside (Table 1, Fig. 4a). Surface water typically contributed about 50% of plant water to these trees and as much as 76% in June, while contributing about 30% on average to pondside trees. The maximum contribution from streamwater to streamside trees likely occurred in June, declined in July, and remained stable for the remainder of the study. Pond water made a much more variable contribution to pondside trees, from as little as 11% in September to as much as 49% in July.

Pondside plants used more groundwater than those growing along the stream, with the exception of the August sampling (Table 1, Fig. 4b). Trees at neither site relied predominantly on groundwater—it typically made up less than 5% of plant water in streamside trees (except the August contribution of up to 15%), while contributing a maximum of 34% to pondside trees. While streamwater made up the greatest portion of
plant water in streamside trees, trees along the pond utilized similar portions of soil water at different depths and pond water. With the exception of the July sampling where surface water contributed up to 49% to plant water, soil water contributions made up more than 50% of plant water, peaking at a time when pond and groundwater contributions were at their lowest (Table 1).

Soil water from shallow and deep depths contributed a much more variable amount to plants at both sites. Overall, soil water seemed to make up a moderate amount of plant water, falling between the high contributions from surface water and the minimal contributions from groundwater, and consistently contributing more than 10%, but rarely more than 50%. In June and July, there was considerable overlap in contribution from shallow soil layers to trees growing streamside and pondside, while there were greater similarities in deep soil layer contributions later in the study period (Table 1). Increased variation in percent contribution data was likely due to the increased variation in isotopic data for soil water (Fig. 3).

Plant Water Status

Trees at both sites appeared to experience greater morning water stress later in the season, while afternoon xylem water potentials (Ψ) were relatively constant throughout the study period (Fig. 5). Only morning measurements at the stream site showed intra-site differences (August v. September, P=0.004) and there were no apparent patterns in water stress in the morning measurements. Mean afternoon Ψ values for streamside individuals were consistently, though not statistically, more negative than those of pondside trees July-September (Fig. 5). Differences in the decline in Ψ between morning
and afternoon were greater in the streamside *L. styraciflua*, while pondside trees showed recovery and greater values in the afternoon for August and September measurements.

**Discussion**

Dawson and Ehleringer (1991) reported limited surface water use in riparian trees, one of the first studies to demonstrate that the distribution of plant species may not be as intrinsically tied to apparent water source abundance, as often thought. Many water sourcing studies have since been conducted, focusing primarily on plants in drier habitats (i.e. Busch *et al.*, 1992; Donovan and Ehleringer, 1994; Phillips and Ehleringer, 1995; Burgess *et al.*, 2000; Snyder and Williams, 2003; Cook and O'Grady, 2006; Costelloe *et al.*, 2008). A number of these studies have supported Dawson and Ehleringer’s (1991) conclusions, indicating little or no use of surface (stream) water by riparian vegetation in both xeric (Busch *et al.*, 1992; Thorburn and Walker, 1993; Mensforth *et al.*, 1994; Snyder and Williams, 2000; Costelloe *et al.*, 2008) and mesic regions (Singer *et al.*, 2012; White and Smith, 2013; White and Smith, 2014). Several other studies have found limited uptake of surface water (Smith *et al.*, 1991; Thorburn and Walker, 1994; Drake and Franks, 2003; Muttiah *et al.*, 2005). A popular hypothesis for the concentrated distribution of riparian plants around bodies of water has been that they required surface water—a theory challenged by many of these isotopic water source studies. Thus, two questions arise: (1) why do riparian species often not utilize surface water and (2) if they do not, why are their distribution patterns so tightly linked to the riparian habitat?

In arid locations, one hypothesis for this water source use pattern has been that streams are often ephemeral and, thus, too unreliable for plants to invest in root access to such resources (Dawson and Ehleringer, 1991; Ehleringer and Dawson, 1992; Thorburn...
et al., 1992; Thorburn and Walker, 1994; Lin et al., 1996). While this theory seems plausible, similar source usage has been found in more temperate, mesic locations where streams are perennial and provide a more reliable source of water year-round (Singer et al., 2012; White and Smith, 2013; White and Smith, 2014). Another possible explanation is that riparian species grow in these locations because of the increased access to groundwater, not the more ephemeral surface water (Dawson and Ehleringer, 1991; Thorburn and Walker, 1993; Mensforth et al., 1994; Thorburn and Walker, 1994). Because of the lower elevation of stream channels and floodplains, the depth to the water table is often much less compared to the adjacent uplands. This would explain why riparian species might use large amounts of groundwater, but fails to answer why they would not utilize more surface water, especially when it is dependable (perennial).

One of the few studies showing significant use of surface water in riparian species was that by White et al. (1985), which demonstrated plant use of swamp water (though referred to as groundwater by the authors). Because of these findings, the present study sought to determine if surface water flow regime has an impact on plant water source utilization. While studies involving flow and flood regime modification demonstrate that ecosystem hydrology can have an impact on plant water status, White et al.’s study (1985) suggests that flow regime of the surface water could have similar effects on water source use.

**Water Source Utilization**

In contrast to the hypothesis, streamside individuals of *L. styraciflua* relied strongly on surface water (using up to 76%) compared to pondside trees (which used as little as 11%). In broader terms, this is the first study in the southeastern US to
quantitatively demonstrate usage of streamwater by riparian plants (White and Smith, 2013; White and Smith, 2014), and one of the few in the literature to show such strong reliance by riparian species on surface water. Though previous studies in the southeastern US have shown reliance on groundwater similar to those in the drier southwestern US (Dawson and Ehleringer, 1991; Ehleringer and Dawson, 1992; Thorburn et al., 1992; Thorburn and Walker, 1994; Lin et al., 1996), the high surface water use of trees in the current study is entirely logical. While there was no study of root morphology conducted at the current sites, previous studies have indicated that the rooting habit of *L. styraciflua* is a dimorphic system consisting of shallow lateral roots and deeper tap roots, especially in well-drained soils (Sargent, 1922; Kormanik, 1990). At most sampling dates, the soil was near saturation under ca. 30 cm at many locations. With the reliability of surface water and apparently high availability of soil water, it seems unlikely that these trees would need to invest in deep roots for groundwater access and, instead, maintain a shallow rooting system. However, even a tree at the stream site growing on banks several meters higher than the stream and in notably drier soil took more than 54% of plant water from the stream (data not shown).

While the ranges of feasible contributions were generally constrained for both riparian sites, there were some wide ranges. These wider ranges, of up to 38%, were consistent for soil and groundwater use in pondside plants throughout the study period. The range of contributions from shallow soil layers to streamside trees was also generally greater than those from surface or groundwater sources at that site. These wider ranges in soil water contributions at both sites, but most pronounced in pondside samples, is likely due to the greater variation in isotopic signatures for the soils compared to surface and
ground waters (Fig. 3). The isotopic variation itself could be due to mixing of subsurface waters or differing exposure to evaporative demand caused by the individual microclimates experienced in the location of each sample. Additionally, the August samplings showed much greater ranges in possible contributions from each source than what was seen on average.

The high water content of soils, the shallow water table (indicated by the springs feeding the ponds at the site), and the consistently wide ranges of possible contributions at the pond site suggest some amount of soil, ground, and possibly surface water mixing. At the site of sample collection, shallow and deep soil layers were not significantly different from each other, and neither was different from groundwater (based on $\delta^2$H values), while both were different from pond water. These differences suggest the most likely site of mixing was between soil water and groundwater at that site. However, there is no convincing explanation for the increased breadth of ranges from the August sampling, and it is possible that the higher rainfall in June and July resulted in a shallower water table and a greater mixing of sources, even though precipitation was considerably lower during August itself.

Another possible explanation for the higher variation in soils at the pond site is contamination by compounds that could confound the laser-based isotopic analyses. However, soils at both stream and pond sites are classified as Clover sandy loam or Clover sandy clay loam (Web Soil Survey, websoilsurvey.nrcs.usda.gov 2014) and were consistent in color, texture, and soil moisture at most sampling times, while variation in stream soil was much more constrained (Table 1, Fig. 3a).
**Inter-site comparisons.** Contributions from each source were similar between sites throughout the study period, suggesting that source-use strategies were similar, and that the differences in site hydrology (i.e. stream v. pond) had little impact on which sources were utilized during the growing season. There were, however, greater differences between sites for the June sampling, with streamside trees using much more surface water and less groundwater. It is unclear why this difference existed, especially when the percent contribution is consistent throughout the remainder of the season. One possibility is that the increased rainfall in June raised streamwater levels enough to increase the availability of the water to the streamside trees, while having smaller impacts on the pondside trees’ access to pond water.

**Intra-site comparisons.** Intra-site differences in source usage were similar between months of the early study period (June and July) and those later in the study (August and September) at both sites. Plant phenology was relatively consistent throughout the study, leaving changes in microclimate as the most notable difference the trees experienced between these periods. There is no clear reason why trees shifted from usage of deep soil layers to shallow ones in the drier August and September period (Table 1), as the opposite trend of increasing use of deep resources during periods of lower rainfall would be expected. This trend is suggested by lower shallow soil water use during periods of increased temperature in pondside trees. The weeks before the June and July samplings had more rainfall than those before the August and September samplings, suggesting that the confounding trend is not the result of short-term rainfall history.

*Plant Water Status*
Xylem water potentials (Ψ) at both sites were consistently high throughout the study. In mesic systems such as this, with higher water availability and humidity, it is not surprising that water stress remained low throughout the day and season. An interesting finding from the Ψ measurements was the trend of slightly increasing afternoon values compared to morning values, where Ψ of some individuals (most often pondside) was consistently higher in the afternoon. In general, an improvement in water potentials later in the day is unusual for most plant species and difficult to explain (Larcher, 2003). One possible explanation is that stomata in these plants closed at midday due to excessive transpiration, and their water status improved as tissue water was replenished. It also appears that source utilization pattern had limited effects on plant water status at this site, likely because water from each source was highly available.

Summary and Conclusions

The present study in the southeastern US is the first to quantitatively demonstrate reliance of riparian trees on surface water (White and Smith, 2013; White and Smith, 2014), and adds to the rare literature showing similar results in other regions (Smith et al. 1991; Thorburn and Walker 1994; Drake and Franks 2003; Muttiah et al. 2005). There is no obvious difference in this particular riparian tree species that explains why it is using so much more surface water than reported for other species, but this finding does support the idea that riparian species are commonly coupled to riparian zones due to the availability of surface water. The findings also lend support to theories about reliability and source utilization developed in more arid systems (Ehleringer and Dawson 1992; Thorburn and Walker 1994; Dawson and Ehleringer 1991; Lin et al. 1996; Thorburn et al. 1992). Environmental variables had no apparent effect on the sources L. styraciflua.
accessed at this site, as no meaningful relationships were detected during the study.

Finally, there was no obvious relationship between plant water status and isotopic data, but water status was consistently high throughout a given day during the entire study period, suggesting that the abundance of surface water enabled maintenance of a favorable water status.

Acknowledgements

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Literature Cited


White JC, Smith WK. 2014. Seasonal variation in water sources of the riparian tree species *Acer negundo* and *Betula nigra* in the southern Appalachian foothills, USA. *Plant Ecology* in review


Table and Figure Captions

Table IV-1. Mean monthly percent contribution from each possible source to plant water during the study period (ranges are shown in parentheses). Values were generated by the IsoSource software.

Figure IV-1. Example mixing polygons for a) streamside and b) pondside *L. styraciflua* (solid circles) as utilized by IsoSource to determine percent contribution ranges of each source (open symbols) to plant water.

Figure IV-2. Rainfall and mean monthly temperature at the site during the study period. Bars show rainfall (mm) and lines show temperature (°C) with error bars (± s.d.).

Figure IV-3. $\delta^2$H v. $\delta^{18}$O values of woody tissue of a) streamside and b) pondside *L. styraciflua* (solid circles) and each possible source (open symbols) with regression lines for each set of points. Dashed lines show the Global Meteoric Water Line (GMWL).

Figure IV-4. Mean contributions of surface water (thick lines) and groundwater (thin lines) to streamside (solid symbols) and pondside (open symbols) *L. styraciflua* during the study period. Values were generated by the IsoSource software.

Figure IV-5. Mean a) morning and b) afternoon xylem water potentials for streamside (solid circles) and pondside (open circles) *L. styraciflua* during the study period. There were no inter-site differences at either daily measurement, however, there was a difference between morning values in streamside trees between August and October (P=0.00).
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Figure IV-2
Figure IV-3
Figure IV-4

Figure IV-5
CHAPTER V

WATER SOURCE PARTITIONING IN THREE RHODODENDRON SPECIES ALONG AN ELEVATIONAL GRADIENT, SOUTHERN APPALACHIAN MOUNTAINS, USA

The following manuscript is in review for publication in *Plant and Soil* (2015), and reprinted with permission from NRC Research Press. Stylistic variations are due to the requirements of the journal.
Abstract

Background and Aims The dominant rhododendron species of the southern Appalachian Mountains (USA) are commonly limited to preferred habitats, with little overlap in small-scale distribution patterns. A number of environmental factors and physiological constraints appear to influence these habitat preferences. Differences in distribution may result from each species’ ability to cope with these stresses, which may include decreased water availability. Thus, the water sources of *Rhododendron catawbiense* Michx., *R. minus* Michx., and *R. maximum* L., growing along an elevational gradient, were determined using the stable isotopes of water.

Methods Isotopic compositions from possible water sources were compared to plant water collected from each species growing in its respective habitats.

Results Results show *R. catawbiense* and *R. minus* accessed deep soil and groundwater sources predominantly (up to 93.6%), while *R. maximum*, which had increased access to streamwater, drew water almost exclusively from the upper 30 cm of the soil profile (>75%).

Conclusions Although differences in source use patterns appeared to exist, they seem unlikely to be a dominant limitation determining habitat preferences in these species. Instead, they are likely only one factor in a suite of site characteristics, including solar input and water content within the entire soil profile, influencing these distributions.

Keywords: *Rhododendron catawbiense*, *Rhododendron minus*, *Rhododendron maximum*, Stable Isotopes, Mixing Models, Elevational Gradient
Introduction

*Rhododendron catawbiense, R. minus, and R. maximum* are the dominate rhododendron species of the southern Appalachians and, although widely distributed, each species appears to be tightly constrained to a preferred habitat. *Rhododendron maximum* is most often limited to the riparian zone and surrounding mesic low-lands (Cordero and Nilsen 2002; Lipp and Nilsen 1997; Monk and Day Jr 1985; Monk et al. 1985; Peattie 1948; Radford et al. 1968; Weakley 2012). In contrast, *R. minus*, while also found in mesic woodlands, typically occurs in the floodplain and upland forests beyond the riparian buffers that surround streams. *Rhododendron catawbiense* occurs in drier, more open sites and at higher elevations. These differences in preferred habitat have been associated specifically with such factors as slope, aspect, and overall distance from valley or ridge (Dobbs 1995; Dobbs and Parker 2004; Monk and Day Jr 1985).

The micrometeorological implications of these topographic factors (i.e. incident solar radiation, evaporative demand, and precipitation input) could have important impacts on plant water status, carbon uptake, and growth. In addition, these shrubs are evergreen and, thus, must cope with environmental stress year-round, including drought- and freeze-thaw induced cavitation as well as excessive solar radiation after deciduous canopies shed their leaves (Cordero and Nilsen 2002; Lipp and Nilsen 1997; Nilsen et al. 2014; Powles 1984; Russell et al. 2009). Probable differences in abiotic stress factors, plus the dominance of *R. maximum* thickets in the riparian zone may act to exclude the other two species from establishing in its preferred habitat. Yet, *R. maximum* may only be able to grow in these mesic understory locations due to hydraulic limitations and increased risk of drought-induced cavitation in more sun-exposed microsites (Horton et al.
2009; Lipp and Nilsen 1997). Similar interactions among abiotic and biotic factors have been noted in other riparian species (Dawson and Ehleringer 1993; Hultine et al. 2007).

One potentially important characteristic that may increase understanding of observed differences in small-scale distribution patterns of these species is their water source use. Therefore, the principal aim of the present study was to identify water sources used by three *Rhododendron* species common to the southern Appalachian Mountains and investigate possible implications for their small-scale distribution patterns. Three populations, one each of *R. catawbiense*, *R. minus*, and *R. maximum*, growing along an elevational and moisture gradient were studied in the Appalachian Mountains of North Carolina, USA. The hypotheses were that *R. catawbiense*, growing in the most exposed habitat, would rely strongly on deeper soil moisture and groundwater, and that *R. minus*, found in mesic closed canopy forests, would use soil moisture from more shallow depths. Finally, it was predicted that, given its riparian habitat, *R. maximum* would take up significant amounts of streamwater, possibly complemented by shallow soil water.

**Methods**

Woody tissue samples from several individuals of *Rhododendron catawbiense* Michx., *R. minus* Michx., and *R. maximum* L. along with samples from each possible source, including soil samples from a range of depths and water samples from groundwater and a bordering stream, were collected for isotopic analyses. These data were then used to determine contribution of each source to plant water. Water status of each species was measured multiple times each sampling day to determine any relationships with plant water source. Environmental variables were also monitored throughout the study period, in hopes of discovering any connection between changes in
site conditions and water source use patterns. Measurements were taken once each month from June-August 2014.

Study Site

This study was conducted throughout a summer growth period in South Mountains State Park (Connelly Springs, NC; 35.5964 N, 81.6005 W), within three rhododendron populations found along a 4.3 km transect running NW-SE. Temperate deciduous forest, including *Quercus* spp., *Acer* spp., and *Platanus occidentalis*, covers much of these park lands, though evergreens (mostly *Pinus* spp.) are common throughout. Each population grew under a canopy composed of multiple species, while low to moderate undergrowth of native and invasive species was also present at each site (Radford et al. 1968; Weakley 2012). While the soil types varied somewhat between sites, each is well-drained with depth to the water table of >2 m and generally low water storage capacity (USDA, Natural Resources Conservation Service, Web Soil Survey, websoilsurvey.sc.egov.usda.gov).

The northwest-most and highest elevation site was a *R. catawbiense* population covering ca. 330 m$^2$, while a ca. 235 m$^2$ population of *R. minus* was the southeastern-most population studied (at mid-elevation along the transect), and the population at the lowest elevation was composed of *R. maximum*. This latter population was in the riparian zone adjacent to the Jacob Fork River, a major stream (ca. 15 m width) along the entire reach of this lower study site (ca. 90 m).

Site Microclimate

Air temperatures during the study were measured within each population using HOBO Pro V2 (model U23-001; Onset, Bourne, MA, USA) and HOBO Pro RH/Temp
(model H08-032-08; Onset, Bourne, MA, USA) sensors, mounted near the center of each population and shielded from midday sun and nighttime sky exposure. Rainfall was collected via standard rain gauges installed at each site, each containing a thin layer of mineral oil to prevent evaporation of precipitation between collections. Rainfall amounts were consistent between sites and, thus, rainfall data are reported as means of all three study populations for each month of the study.

Sample Collection

To determine the patterns of water source use in each species, samples of woody tissue and each possible water source were taken at each site. Groundwater was collected from 3 locations via well-fed spigots on the park grounds. Spigots were allowed to run for about 1 min to prevent sampling water that may have experienced fractionation in the pipes. While sampling groundwater within each population would have been ideal, these wells were the only access at the site. The wells surrounded the *R. minus* and *R. maximum* populations, but were up to ca. 5 km from the *R. catawbiense* site. Multiple water samples from the Jacob Fork River (a possible water source for *R. maximum*) were taken from well-mixed parts of the river at about 20 cm depths using a cup attached to a telescoping pole, allowing collection away from the banks.

Soils were sampled at 3 locations in each population, at 4 depths each: 15 cm, 30 cm, 45 cm, and 60 cm. Attempts were made to take collections at greater depths, but due to the shallow rocky soils, sampling below 60 cm was rarely successful. These attempts often resulted in the collection of ground rock created during the sampling process. Soils were collected using a 2.75 in, one-piece auger (model 400.48, AMS Inc., American Falls, ID). Sampling the full soil profile (nearly always <100 cm) as well as groundwater, and
streamwater for *R. maximum* populations, should have included all possible sources available to these shallow-rooted species (Monk and Day Jr 1985), and allowed for determining the water source utilization of each.

Woody tissue was sampled from multiple individuals (n=4) of each species. Individuals were systematically selected for sampling based on several criteria: they were mature, appeared healthy, and were large enough that repeated sampling over the duration of the project would not affect their survival. Several twigs (n=3), ca. 30 cm in length and up to 1 cm in diameter, were taken from each individual. All leaves and loose bark, potential sites of fractionation of the plant water isotopic composition, were removed and stems were cut into lengths short enough to be placed in sample vials. All samples were stored in screw-top glass vials and sealed with Parafilm, stored in a chilled cooler in the field, and frozen upside down upon returning to the lab. Sealing with Parafilm and freezing samples were precautions taken to prevent any fractionation of isotopic composition of the samples.

*Plant Water Status*

Xylem water potentials (Ψ) for each species were measured twice, at 0900 and 1500, each day of sample collection (once monthly June-August 2014) with multiple terminal leaves from each individual selected for water sourcing measurements. Healthy, mature leaves were sampled from random locations on each plant, sealed in zip-top sandwich bags, and stored in a cool dark container until measurements were made using a Scholander-type pressure chamber (model 1000; PMS Instruments Co., Corvallis, OR).

*Isotopic Analysis*
Frozen samples were shipped to the Earth Systems Center for Stable Isotopic Studies (ESCSIS) at Yale University for isotopic analyses at the completion of sample collection. Water extractions from soil and woody tissue samples were conducted by cryogenic vacuum distillation, a process that preserves the isotopic composition of the sample upon completion (West et al. 2006). Isotopic analyses were completed using a Thermo-Finnigan DeltaPlus XP isotope ratio mass spectrometer (Berlin, Germany) with a high-temperature conversion elemental analyzer (TC/EA). Replicate samples were injected into the spectrometer and mean isotopic compositions of hydrogen and oxygen were taken from the final 2 replicates. Analyses were conducted using 3 or more standards and results were reported relative to Vienna Standard Mean Osmotic Water (V-SMOW). The precision of analysis was ±3.3‰ for δ²H and ±0.3‰ for δ¹⁸O.

**Mixing Models**

To determine the relative contribution of each potential water source to plant water, isotopic data were input into the mixing model software IsoSource (v. 1.3.1; Phillip and Gregg 2003; www.epa.gov/wed/pages/models/stableIsotopes/isosource/isosource.htm). Linear mixing models are limited to determining contribution from n+1 sources, where n is the number of isotopes analyzed. Because there were two isotopes used in this study, ²H and ¹⁸O, but 4-5 potential sources, a more robust model was required. IsoSource overcomes this issue by constructing mixing polygons (see examples in Fig. 1) using source isotopic compositions to generate estimates of feasible contributions from each possible source. Isotopic results from each woody tissue sample were entered into the program, along with
mean isotopic values from possible sources available to that plant, and output data were averaged for each plant and each population.

Statistics

Shapiro-Wilk tests were used to ensure the normality of each dataset. One-Way ANOVAs, followed by Tukey’s or Dunn’s post hoc tests, were used to analyze inter-population differences between isotopic values of woody tissue and sources and Ψ, as well as intra-population variation in isotopic compositions and Ψ over time. Kruskal-Wallis One-Way ANOVA on Ranks were used where data were non-parametric. Linear regressions were used to determine relationships between Ψ and environmental variables.

Results

Site microclimate

At all sites, air temperatures and precipitation were nearly identical and no consistent pattern was apparent between the highest or lowest temperature or rainfall according to site (Fig. 2). At each site, July had the highest average daily temperature, with the site of the *R. maximum* population being the warmest at 22.3 C. The coolest month at all sites was August and the coolest site was the *R. maximum* site (mean maximum, 20.4 C). The greatest mean daily maximum air temperature was in June for each site, with the warmest (27.5 C) occurring in the *R. catawbiense* population; the coldest mean daily minimum was also in June, with the lowest temperature in the *R. minus* population at 17.0 C.

Precipitation was greatest in August (101.6 mm) and lowest in July (66.9 mm). Total precipitation at the site was 263.7 mm during the study period; precipitation amounts at the nearest meteorological monitoring station (<9.5 km away) were
considerably lower than the average 334.5 mm during the same period (June-August), suggesting a drier than normal season (NOAA, National Weather Service Forecast Office, NOWData, weather.gov/climate).

**Isotopic compositions**

In the *R. catawbiense* population, soil at 60 cm depths was consistently more depleted in $\delta^{18}$O than other soil sources, while 15 cm and 30 cm soil isotopic values overlapped considerably and were more enriched in $\delta^2$H and $\delta^{18}$O (Fig. 3a). In both *R. minus* and *R. maximum* populations, all sampled soil sources were isotopically similar within each site. Streamwater and groundwater were also similar and overlapped significantly, suggesting significant mixing between these two possible water sources along the *R. maximum* population.

Although there was some variation between isotopic compositions of plant water between species and over time, there were no differences, at any sampling date, between species (p>0.06). Intraspecifically, plant water from *R. maximum* was significantly heavier in June than August (p<0.01), while June samples from *R. catawbiense* were more enriched in $\delta^2$H and $\delta^{18}$O than both July and August samples (p<0.01).

It should be noted that in all three populations, isotopic values of some plant water samples were more negative than those of any source collected (Fig. 1). Additionally, due to the frequent occurrence of large boulders in the soil, sampling much deeper than 60 cm was often impossible. Thus, isotopic values of the few 100 cm soil samples that were collected are included in Fig. 3, but were not included in calculations.

**Mixing models**
Rhododendron catawbiense relied strongly on deep sources, particularly groundwater, throughout the study period (Table 1 and Fig. 4a). Groundwater alone contributed up to 74.5% to plant water in June; soil at 60 cm depths and groundwater together contributed 55.3-95.3% to these individuals throughout the study. Upper soil layers (15 and 30 cm depths) contributed less to R. catawbiense at this site (as little as 37%), but these contributions were considerably variable and appeared greater in July.

Rhododendron minus took significant contributions from 60 cm soil and groundwater sources in June—as high as 93.6% (Table 2, Fig. 3b). However, plant water of this species was more equally composed of water deep from and shallow sources in July and August. Additionally, in August there was a notable shift away from the high amounts of groundwater utilized in June and July and increased use of soil water from 60 cm.

Rhododendron maximum used significant portions of shallow soil water (15 and 30 cm) at each sampling date (Table 1, Fig. 4c). With these upper soil layers contributing a feasible 90%+ throughout the study period (15 cm soil water alone contributed up to 91% alone in August), other sources (60 cm soil water, groundwater, and streamwater) contributed less than 17%, individually, at any given sampling date. Streamwater, the water source unique to this site, contributed no more than 16.1% to R. maximum plant water during the study period.

The most obvious differences in source utilization between the three species were in deep source (60 cm soil water and groundwater) use, where R. catawbiense and R. minus relied significantly more on groundwater than R. maximum. There were also a number of notable patterns in source utilization over time in each species. Rhododendron
catawbiense and R. minus took majority contributions from groundwater in June but less than 25% by the end of the study. Rhododendron maximum showed significant variation in water use from 15 cm and 30 cm, with 55%+ of plant water taken from 15 cm soil in June and as little as 4.5% in August, and 30 cm soil water contributions declining from a feasible 69.3% in July to 8.0% at most in August.

Because a number of values for woody tissue isotopic compositions fell outside of those of the collected sources (Fig. 1), some plant water compositions were unable to be analyzed via mixing model methods. As a result, some of the values reported in Table 1 and Fig. 4, particularly those of R. minus and R. maximum for August, are based on a reduced number of replicates.

Xylem Water Potentials (Ψ)

Morning (0900) Ψ were similar between species in June and July (p>0.191), with the only interspecific difference coming in August when R. catawbiense was more water stressed than R. minus and R. maximum (p=0.003; Fig. 5a). All species appeared least water stressed in June and the decline in R. catawbiense Ψ, to -1.0 MPa, was significantly lower than June and July measurements (p=0.005). Rhododendron maximum had significantly lower Ψ at 0900 h in August (p=0.041) compared to June.

There was generally more variability in afternoon Ψ measurements, with no interspecific differences during the study period (p>0.061; Fig. 5b). Rhododendron catawbiense had the lowest afternoon Ψ in June and August, as low as -1.0 MPa, while R. minus and R. maximum were the most similar throughout the study. Again, all 3 species appeared to be the least water stressed during June measurements. Intraspecifically R.
minus Ψ values were significantly lower in July (p<0.01) and R. maximum Ψ were significantly lower in July and August (p<0.01) All species saw the greatest declines in Ψ from 0900 to 1500 in July, with R. minus experiencing the greatest change of -0.6 MPa, although there were no interspecific differences between measurements during the study (p>0.12). Interestingly, R. catawbiense experienced a recovery of 0.05 MPa in Ψ for the August measurements, while R. minus and R. maximum Ψ declined, as expected. The only meaningful association between Ψ and environmental conditions found was the positive relationship between the change in R. catawbiense water status (morning and afternoon measurements) and average monthly temperature (p=0.02; r²=0.99).

Discussion

Ericaceous shrubs, including Rhododendron spp. and Kalmia latifolia, are dominant in the understory of the Appalachian Mountains (USA), especially in the southern extent where they cover nearly 31,000 km² (Dobbs 1995; Dobbs and Parker 2004). These species can be found in many habitats and across a wide range of elevations, with the most abundant R. maximum growing in dense thickets in valleys, along streams (Cordero and Nilsen 2002; Monk and Day Jr 1985; Monk et al. 1985; Peattie 1948; Radford et al. 1968; Weakley 2012). Rhododendron spp., as a group, are the most abundant understory species in the southern Appalachians, yet, the biotic/abiotic factors responsible for the observed small-scale distribution patterns are unknown. With such a high abundance in the region, these species have considerable ecological influence, including a major role in forest regeneration and species composition after disturbance (Dobbs and Parker 2004), especially around R. maximum thickets (Horton et al. 2009;
Monk and Day Jr 1985; Monk et al. 1985). This group of shrubs may also provide a number of ecosystem services, including regulation of mineral and nutrient cycles, protection of water quality, and providing habitat for a host of animal species (see review by Chastain and Townsend 2004).

Identifying Water Sources

While there were shifts between soil water use from the 60 cm layer and groundwater use in *R. catawbiense*, these deeper sources dominated the contribution to plant water. This source-use strategy makes sense in these more sun-exposed habitats where the upper soil layers are likely driest, and suggests that the greatest plant-available water was in the deeper soil layers.

Similar to the source use patterns of *R. catawbiense*, *R. minus* was also found to rely heavily on water from deeper soil layers. However, there are clear differences between the habitats of these two species—one of the most obvious is the more mesic nature of the *R. minus* habitat. While, it may be advantageous for *R. catawbiense* to utilize greater amounts of deeper soil moisture in its more open and presumably drier habitat, it is difficult to make the same argument for *R. minus*. The dense, closed canopy and thick detritus layer on the soil surface at the *R. minus* habitat should contribute to greater moisture in the upper soil layers which, when combined with the shallow rooting habit of rhododendron species, would suggest the use of water from this source would be optimal. Indeed, these factors may account for the spike in shallow soil water usage in July.

There are two other factors that may help explain this source-use strategy for *R. catawbiense* and *R. minus*. First, the slope of the *R. minus* site was significantly greater
than at the other two sites, and could act to reduce water availability due to greater run-off and drainage (Lipscomb and Nilsen 1990). While this would not seem to be an issue in the *R. minus* habitat during the study period, as upper soil layers were moist, it is possible that the soils do drain historically and hold less water, leading the plants to invest in roots accessing deeper soil layers. Secondly, and perhaps most likely, is that rhododendron at this site were able to access a deeper soil layer with much greater water content, e.g. the capillary fringe or possibly the saturated zone. Because water tables tend to follow surface topography, and occur at shallower depths at lower elevations (Haitjema and Mitchell-Bruker 2005; Naiman et al. 2005), plants at this site may have been able to access this source more easily.

An interesting finding for *R. maximum* water source utilization was the limited use of streamwater by these plants (less than 16.1% on any sampling date; Table 1, Fig. 4c). Because each individual sampled was growing on the stream banks, it was expected that this abundant and perennial water source would make much larger contributions to these plants. This water source strategy, however, is similar to that found for numerous riparian tree species of the southeastern US (White and Smith 2013; White and Smith 2015) and elsewhere (Busch et al. 1992; Chimner and Resh 2014; Dawson and Ehleringer 1991; Mensforth et al. 1994; Singer et al. 2012; Snyder and Williams 2000; Thorburn and Walker 1993). A common explanation for why streamside plants use little streamwater is that they often have access to the shallow water table and, as mentioned above, it may be more advantageous to invest heavily into accessing that source instead of the adjacent streamwater, which in some cases may be inconsistent or intermittent. That theory makes sense when the plants are using large amounts of groundwater. But here, *R. maximum*
was using less groundwater than streamwater—no more than 12% during the study. Instead, they were relying strongly on shallow soil layers (15 and 30 cm) throughout the season. These upper layers were moist and may have been able to serve as the sole source for plant water. If this were the case, it may be beneficial to forgo the costs of developing fewer deep roots and invest in more shallow roots to further exploit these moist layers. It would still seem advantageous, however, to access at least one of the two highly available sources within reach. It is possible that streamwater and/or groundwater fed the soil water *R. maximum* was utilizing from the shallow soil layers. This indirect use could have gone undetected because of isotopic fractionation that may occur before the water is absorbed by plants (Allison et al. 1983; White et al. 1985).

Finally, the limited use of stream or groundwater by *R. maximum* may be due to a lack of adaptation to regular submersion and the accompanying anoxic conditions (Blom and Voesenek 1996; Blom et al. 1994). If using water directly from these sources requires coping with low oxygen levels, costs of attempting to maintain roots capable of taking water up from these sources may outweigh the benefits.

It should be noted that throughout the study, some woody tissue isotopic compositions were unable to be analyzed by IsoSource because they fell out of the relatively narrow mixing polygons (Fig. 1). These samples typically fell out of the left most bounds of the polygons; they were isotopically more negative than the sources collected, often in both isotopes. While these issues occurred in all species, they were most common in *R. minus* and *R. maximum* samples. This resulted in reduced replicates for these species, especially for the August sampling. Therefore, while mixing model results for August do seem to be in line with those from earlier in the study, it should be
stressed that these values were generated with a small replicate size. The fact that these data fell outside the IsoSource mixing polygons indicate a possible missing water source. It seems likely that the missing source(s) were those soil layers below the rocky layer encountered between 60 and 100 cm. The fact that these data are a much smaller subset of the entire study’s sample set seems to support this explanation, with the knowledge that the vast majority of rhododendron roots are found in the upper 60 cm (Monk and Day Jr 1985).

Implications for Rhododendron Distribution Patterns

While there are a few notable differences in source use, especially between *R. minus* and *R. maximum*, source usage between species does not appear different enough for water sources alone to explain the observed habitat preferences. *Rhododendron maximum*’s limited use of streamwater and *R. minus*’s similarity to *R. catawbiense*’s source usage patterns removes many of the distinguishing patterns that were hypothesized. While previous research has suggested that *R. maximum* distribution is tied strongly to water availability (Horton et al. 2009; Lipp and Nilsen 1997), there must be additional factors that influence the small-scale distribution patterns of these three species.

Prior work indicates that *R. maximum* distribution is strongly influenced by the aspect of slope, with south and west facing slopes being less preferred due the increased sunlight and, therefore, drier environment which may increase water stress and the likelihood of hydraulic failure (Lipp and Nilsen 1997; Lipscomb and Nilsen 1990). Photodamage may also be common in this species when growing on these slopes, especially during the winter if the canopy had shed its leaves (Nilsen 1985; 1987; 1991;
Wang et al. 2009). These factors seem to result in *R. maximum* being restricted to lower slopes with north and east aspects in more mesic habitats like those found in riparian zones (Lipp and Nilsen 1997; Lipscomb and Nilsen 1990).

*Rhododendron minus*, and *R. maximum* for that matter, may be restricted to the understory under dense canopies due to their vulnerability to drought-induced cavitation (Lipp and Nilsen 1997). The decreased sunlight, which should reduce transpiration, and increased moisture typical of these habitats, should lead to lower water stress. While *R. catawbiense* has been found to be more vulnerable to hydraulic failure than *R. maximum* (Cordero and Nilsen 2002), it has a number of modifications that may allow it to thrive in more exposed, stressful habitats and maintain favorable water status. These adaptations include waxy deposits around the stomata and lower stomatal density, likely reducing water stress, and additional layers of palisade cells that may help protect against photodamage (Wang et al. 2008). Being able to tolerate these drier and brighter habitats might also have some advantages, including greater opportunities for photosynthesis and may explain why *R. catawbiense* thrives there.

More ecophysiological measurements of photosynthetic rates, stomatal conductance, and fluorescence are needed for all three species and would offer insight into efficiency of each species in its habitat.

*Plant water status*

While there were some obvious differences in source use strategy during the study, each species appeared to avoid water stress in their respective habitats. All three species maintained favorable Ψ, similar to those reported for rhododendron previously (Kadioglu and Terzi 2007; Lipp and Nilsen 1997), and did not approach ranges shown to result in a
reduction of photosynthesis or loss of xylem conductivity due to embolism (Lipscomb and Nilsen 1990).

Throughout the study, *R. minus* and *R. maximum* had the most similar water status, possibly reflecting their similar habitats and corresponding water availability, even though they appear to rely on different sources. Though consistent at 0900 h measurements and early in the study, *R. catawbiense* was significantly more stressed in August compared to the other species and its own water status earlier in season. With temperature and rainfall remaining consistent, there is no obvious explanation for these differences.

**Summary and Conclusions**

The three rhododendron species, evaluated here, which dominate the understory of the southern Appalachians, are often distributed along an elevational gradient. There were several notable differences between anticipated water source usage patterns and the sources actually utilized by these species, namely the reliance on deep sources in *R. minus* and the limited use of stream and groundwater by *R. maximum*. While there were interspecific differences in source use, there were no clear indications that these factors alone were driving the observed differences in habitat preferences. Most likely, water source utilization strategy is one component of a suite of species characteristics, including drought and high-light tolerance, that result in these patterns. Further ecophysiological measurements are needed to more fully understand the factors driving habitat selection and segregation.

**Acknowledgements**
We thank the North Carolina State Parks system and the staff at South Mountains State Park, especially Park Superintendent Jonathan Griffith and Ranger Allen Rogers, for their permission to conduct the study at the park and their time and effort in helping locate suitable sites. This project was funded in part by the Vecellio Award from Wake Forest University. We thank Mary Jane Carmichael, Brenton Smith, Sean Taylor, and Jennifer White for their invaluable assistance in the field and in manuscript preparation.
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Figure Captions

Table V-1. Mean monthly source contributions to individuals of each species during the study period. Feasible ranges are reported in parentheses. Values were generated via the IsoSource software.

Figure V-1. Example mixing polygons for a) *R. catawbiense*, b) *R. minus*, and c) *R. maximum* (shaded symbols) as utilized by IsoSource to determine percent contribution ranges for each source (open symbols) to plant water.

Figure V-2. Mean temperature (line) and precipitation (bar) during the study period. Temperatures presented are those recorded in the *R. catawbiense* population, however, temperatures were similar across populations and there were no significant differences (p=0.844). Error bars show ± 1 sd.

Figure V-3. $\delta^2$H v. $\delta^{18}$O values of woody tissue from a) *R. catawbiense*, b) *R. minus*, and c) *R. maximum* (shaded symbols) and possible sources at each site (empty symbols) with regression lines for each set of points. The dashed line in each panel shows the local meteoric water line (LMWL).

Figure V-4. Mean contributions from a) 15 cm soil water, b) 30 cm soil water, c) 60 cm soil water, and d) groundwater to plant water of *R. catawbiense* (empty circles, solid line), *R. minus* (shaded squares, dashed line), and *R. maximum* (solid triangles, dotted line) during the study period. Points show mean contribution from each source, while error bars show ranges of feasible contributions. Values were generated via the IsoSource software.
Figure V-5. Mean a) morning (0900) and b) afternoon (1500) water potentials for *R. catawbiense* (empty circles, solid line), *R. minus* (shaded squares, dashed line), and *R. maximum* (solid triangles, dotted line) during the study period. Error bars show ± 1 sd.
<table>
<thead>
<tr>
<th>Table V-1</th>
<th>( R. \text{ catawbiense} )</th>
<th>( R. \text{ minus} )</th>
<th>( R. \text{ maximum} )</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Soil water</strong></td>
<td>( R. \text{ catawbiense} )</td>
<td>( R. \text{ minus} )</td>
<td>( R. \text{ maximum} )</td>
</tr>
<tr>
<td>( 15 \text{ cm} )</td>
<td>June: 6.9 (0.2-23.5)</td>
<td>July: 17.3 (0.6-40.4)</td>
<td>August: 7.0 (0.4-16.9)</td>
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<td></td>
<td>June: 13.2 (7.2-20.8)</td>
<td>July: 43.1 (39.2-47.7)</td>
<td>August: 53.1 (52.5-54.0)</td>
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<tr>
<td><strong>Soil water</strong></td>
<td>( R. \text{ minus} )</td>
<td>( R. \text{ maximum} )</td>
<td></td>
</tr>
<tr>
<td>( 15 \text{ cm} )</td>
<td>June: 7.8 (3.6-12.2)</td>
<td>July: 22.6 (15.7-30.5)</td>
<td>August: 24.8 (22.7-27.7)</td>
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<tr>
<td></td>
<td>June: 32.9 (30.8-36.2)</td>
<td>July: 8.9 (0-21.7)</td>
<td>August: 61.6 (58.7-64.7)</td>
</tr>
<tr>
<td><strong>Groundwater</strong></td>
<td>June: 56.3 (55.2-57.4)</td>
<td>July: 38.1 (36.0-39.7)</td>
<td>August: 10.5 (9.0-12.3)</td>
</tr>
<tr>
<td><strong>Soil water</strong></td>
<td>( R. \text{ maximum} )</td>
<td></td>
<td></td>
</tr>
<tr>
<td>( 15 \text{ cm} )</td>
<td>June: 59.7 (55.4-62.7)</td>
<td>July: 14.0 (4.5-22.4)</td>
<td>August: 88.8 (86.0-91.0)</td>
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<tr>
<td></td>
<td>June: 32.2 (23.0-38.1)</td>
<td>July: 65.2 (60.9-69.3)</td>
<td>August: 1.8 (0-8.0)</td>
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<tr>
<td><strong>Groundwater</strong></td>
<td>June: 2.5 (0.4-6.1)</td>
<td>July: 4.2 (0.2-10.6)</td>
<td>August: 8.3 (5.00-12.0)</td>
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<tr>
<td><strong>Streamwater</strong></td>
<td>June: 2.3 (0.3-5.4)</td>
<td>July: 10.3 (5.7-16.1)</td>
<td>August: 0.7 (0-4.0)</td>
</tr>
</tbody>
</table>
Figure V-1
Figure V-2

[Bar chart showing mean monthly rainfall and temperature for June, July, and August.]
Figure V-3
Figure V-4

Figure V-5
CHAPTER VI

IMPORTANCE OF AQUAPORIN ACTIVITY TO RAPID WATER TRANSPORT IN
THE LEAVES OF TWO COLD-TOLERANT RHODODENDRON
SPECIES, SOUTHERN APPALACHIAN MOUNTAINS, USA
Abstract

The curling and drooping of some Rhododendron leaves is well documented in the literature, with species exhibiting reorientations at near freezing temperatures, in a matter of minutes. While these movements appear to be functionally significant, the mechanism allowing the transport of such the large volumes of water required for these movements is unknown. Previous research suggests that differential changes in turgor of the petiole and across the leaf lamina are responsible for the drooping and curling, respectively. It is possible that aquaporins facilitate water transport at a rate allowing these rapid leaf movements. This study aimed to understand the influence of aquaporin activity on leaf hydraulic resistance ($R_{\text{leaf}}$) under two temperature regimes, in two Rhododendron species native to the southern Appalachian Mountains. This included introducing aquaporin inhibitor ($\text{H}_2\text{O}_2+\text{FeSO}_4$) to R. catawbiense and R. maximum leaves at cold (0-10 C) and warm (20-30 C) temperatures and measuring $R_{\text{leaf}}$ using a hydraulic conductance flow meter. Results showed no effect of aquaporin inhibitor on $R_{\text{leaf}}$ at either temperature or species ($P>0.299$). Similarly, there was no difference in the effect of the inhibitor between species or temperature treatment ($P=1.33$). The results suggest that aquaporins are of little importance in water transport across Rhododendron leaves, even at temperatures near those inducing leaf reorientation. However, failure to meet benchmark values suggests data should be interpreted cautiously.

Keywords: Rhododendron, aquaporins, inhibitor, leaf curling, Appalachians
Introduction

The curling and drooping of *Rhododendron* leaves has been noted for centuries (Nilsen 1992) and mountaineers of the southern Appalachians have claimed to use the leaf movements of the abundant *R. catawbiense* and *R. maximum* to estimate the previous night’s temperature (Nilsen 1990), with colder temperatures leading to tighter curling and increased drooping. Indeed, it has been verified that leaf curling in these species is strongly negatively correlated with temperature, beginning to curl just under freezing. The rolling (and unrolling) of these species’ leaves has been reported to occur ca. 1-10 C (Nilsen 1985, 1987, 1991, Chen et al. 2013), and takes place in a matter of minutes (Harshberger 1899, Fukuda 1933, Nilsen 1987, 1992).

While the functional significance of this movement is still a matter of debate, it appears to be an adaptation of cold-tolerant *Rhododendron* species (Nilsen 1985, Boa and Nilsen 1988, Nilsen 1991, Peng et al. 2008, Wang et al. 2009, Nilsen et al. 2014), possibly enabling them to thrive in conditions with low light intensity, in the understory of deciduous forests, in the spring and summer followed by full light and freezing temperatures in the fall and winter. *R. catawbiense* is often found at elevations over 1000 m in open forest and on rocky outcrops, while *R. maximum* primarily grows in the understory of riparian forests along streams and rivers at elevations under 1000 m (Radford et al. 1968). It seems to be imperative that these species take full advantage of the increased sunlight they receive after temperatures warm in the early spring and before the deciduous canopy above them, *R. maximum* in particular, leafs out. Additionally, these changes in orientation may reduce leaf area exposed to nighttime skies which, when skies are clear, can result in leaf temperature dropping below air temperature, increasing
the likelihood of temperature-induced leaf mortality (Boa and Nilsen 1988, Germino and Smith 2001). These factors suggest that the leaf rolling and drooping exhibited by these species during periods of cold temperatures help to ensure that they remain near their photosynthetic and water status optimums (Russell et al. 2009).

These reorientations also constitute relatively rapid leaf movements (Nilsen 1992). The actual mechanisms of these movements are thought to be turgor-related, with reversible swelling of the parenchyma in the petiole cortex responsible for leaf drooping and differential turgor of axial and abaxial cells causing curling of the leaf blade (Nilsen et al. 2014). What is not well understood at this time is how such water fluxes required for these reorientations could occur so quickly. While we are only beginning to understand the important function aquaporins (AQPs; water channel membrane proteins) serve in a wide range of organisms, it has become apparent that they aid in rapid and significant water transport in plants, and may fill the gap in understanding how rapid leaf movements, such as those seen in these Rhododendron species, occur over short time periods (Moshelion et al. 2002, Uehlein and Kaldenhoff 2008, Heinen et al. 2009).

The objective of this study was to determine the impacts of AQP activity on leaf hydraulic resistance during changing temperatures in 2 cold-tolerant, evergreen species of the southern Appalachians (USA), R. catawbiense and R. maximum, which have been identified as strong curlers in previous research (Nilsen 1991). With an indication from molecular studies that AQPs may be important in rhododendron leaf curling (Chen et al. 2013), and evidence that colder temperatures may increase AQP expression (Aroca et al. 2005, Chen et al. 2013), the goal was to examine leaf hydraulic resistance at different temperatures, with and without AQP inhibitor. It was predicted that injection of the
inhibitor would have the greatest impacts at colder temperatures and in *R. catawbiense* (as it has been identified as the strongest curler; Nilsen 1991), indicating the highest level of AQP activity in those trials.

**Methods**

This study sought to improve the understanding of AQP function in leaf water transport of *R. catawbiense* and *R. maximum* in a laboratory setting. To do so, leaf hydraulic resistances ($R_{\text{leaf}}$) of both species were measured in water baths at two baseline temperatures, and 10 C higher water baths, with and without an AQP inhibitor.

**Plant material**

Plant material was collected from individuals of *R. catawbiense* and *R. maximum* growing in their natural habitat at Pilot Mountain State Park (Pinnacle, NC; 36°20’34.05”N, 80°28’57.89”W). *R. catawbiense* samples were taken from plants (n=3) at several locations along an upper ridge, while *R. maximum* collections were sampled from the single individual growing in the riparian zone along a tributary to Pilot Creek. Replicate terminal stem segments ca. 30 cm in length were taken from each individual, wrapped in damp paper towels, and sealed in large zip-top plastic bags. Samples were taken on a single day in May 2014, stored in a chilled cooler, and refrigerated until measurements were made (within 36 h).

**Leaf Hydraulic Resistance**

$R_{\text{leaf}}$ measurements were made using a hydraulic conductance flow meter (HCFM; Model Gen 3 HCFM-XP; Dynamax Inc., Houston, TX, USA). In preparation for measurements, healthy leaves at least 1 yr old, were removed from the collected stem segments at the base of the petiole using hand pruners. Petioles were wrapped with
several layers of Parafilm (to prevent damage to the vasculature due to the compression coupling), leaves were submerged in near-freezing distilled water (1 C), and a second excision, was made using a razor blade to remove a proximal portion of petiole. While still submerged, the leaf was fitted into the compression coupling while distilled, degassed water flowed through the coupling, to prevent introducing air bubbles into the vasculature. The leaves were sealed in plastic zip-top bags and returned to the water bath, where they remained for the duration of the treatment, in order to regulate leaf temperature. Measurements were made in the HCFM “Quasi-Steady State” mode with the lowest flow required to maintain adequate pressure for accurate measurements (approx. flow was 4E-07 kg/sec with a pressure ca. 115 kPa), under standard fluorescent laboratory lighting.

Data collection was initiated (Fig. 1), and measurements of resistance continued until several minutes after values stabilized (ca. 6.5 min on average). Next, with water flow and data recording continuing, leaves were transferred to an 11 C water bath. Again, the leaf temperature was given time to equilibrate and resistance measurements were recorded after values stabilized. While remaining in the 11 C water bath, an aquaporin inhibitor was injected with a syringe, spliced into the water line (before the compression sleeve) using a 3-way stopcock with a Leur-lock connection. The inhibitor solution was made of equal parts 0.6 mM H$_2$O$_2$ and a 3mM aqueous solution of FeSO$_4$ (Fenton reaction; Henzler et al. 2004, Ye and Steudle 2006, Maurel et al. 2008, Heinen et al, 2009, Kim and Steudle 2009), which inhibits aquaporin function. Before attaching the syringe, the stopcock was opened, allowing the inlet to fill with water and the air to be purged, to prevent introduction of air bubbles to the water line. The syringe was connected, the
stopcock was adjusted to allow injection into the line, and pressure was applied to the plunger for ca. 3 min. Approximately 1 ml of solution was injected into each leaf. The inhibitor was given at least 10 min to take effect and measurements were recorded until resistance values stabilized. This protocol was repeated with multiple leaves of each species (n=5) and at warmer temperatures. For the warm temperature treatment, the initial measurements were made in a water bath set to ca. 22 C. $R_{\text{leaf}}$ measurements were recorded when the values stabilized, and leaves were transferred to a second high temperature bath (32 C) for the increased temperature measurements. For each treatment, resistance was calculated as the mean of 60 s after values had stabilized.

Statistics

Intraspecific differences in $R_{\text{leaf}}$ caused by temperature and inhibitor treatments were compared using One Way Repeated Measures ANOVAs and Friedman Repeated Measures Analysis of Variance on Ranks, when data were non-parametric, efforts to control for high leaf-to-leaf variability in resistance measurements. One Way ANOVAs were used to compare interspecific responses to the treatments, $Q_{10}$ values (Nardini et al. 2010), and proportional changes after inhibitor injection.

Results

At 1 C, there was no difference between mean $R_{\text{leaf}}$ of $R$. *catawbiense* and $R$. *maximum*, $1.50 \times 10^5$ sec MPa/kg and $1.33 \times 10^5$ sec MPa/kg respectively, with high leaf-to-leaf variability in both species ($P=0.448$; Fig. 2). Similarly, $R_{\text{leaf}}$ increased in both species when the temperature was raised to 11 C, though neither increase was significantly higher than the baseline ($P>0.367$). Variable, and non-significant, changes in resistance occurred with the injection of aquaporin inhibitor in these cold temperature
trials, with no change in _R. catawbiense_ leaf resistance (P=0.367), and _R. maximum_ leaf resistance increasing by a factor of 0.29 (P>0.98; Fig. 3).

\( R_{\text{leaf}} \) was lower, with much greater leaf-to-leaf variation, in both species in the warm temperature trials (Fig. 2). Resistance in leaves of both species remained essentially unchanged when temperature was increased to 32 C (P>0.367). Similar to those in the cold temperature trials, injection of aquaporin inhibitor at higher temps had varying effects on leaf hydraulic resistance, causing an insignificant 0.43-factor increase in _R. catawbiense_ and a 0.05x decrease in _R. maximum_ (P>0.299; Fig. 3). \( Q_{10} \) values, for both species and treatments, were not significantly different from the 0.77x change expected in _R_{\text{leaf}}_ due to the decrease in water viscosity with the 10 C increase in water temperatures (P=0.055; Fig. 3), indicating no temperature dependence of _R_{\text{leaf}}_. Finally, there was no statistical difference between treatments or species in the proportional change of _R_{\text{leaf}}_ after the introduction of AQP inhibitor (P=1.33; Fig. 3), which ranged from a 0.95x to 1.29x increase across trials.

**Discussion**

Leaf curling and drooping in _R. catawbiense_ and _R. maximum_ are well documented (Harshberger 1899, Nilsen 1985, 1990, 1991, Wang et al. 2008, Wang et al. 2009, Nilsen et al. 2014); however, the functional significance of this reorientation remains a matter of some debate. Initially, it was thought that the curling may act to improve water stats of these plants, preventing potential hydraulic failure (Havis 1964, Havis 1965). This theory posited that curling inward created a humid microclimate within each leaf, reducing water lost through the stomata. This could be an advantageous adaptation, with soil moisture frequently trapped in the frozen ground during the cold
Appalachian winters, as any water loss could result in increased water stress and possible onset of cavitation of the xylem. Studies examining stomatal conductance in these species, however, found that stomata were rarely open during the winter months and that, while increasing this boundary air layer could help prevent water loss from leaky stomata, this leaf curling likely had little impact on plant water status (Nilsen 1985, Boa and Nilsen 1988, Nilsen 1990).

Another hypothesis is that leaf curling in *Rhododendron* is a means of protecting the leaves from exposure to potentially damaging, high intensity solar radiation, and associated increases in leaf temperature (Nilsen 1985, Boa and Nilsen 1988, Nilsen 1990, Wang et al. 2009), or from radiative heat loss to cold night skies which can result in leaf temperatures well below air temperatures (Boa and Nilsen 1988, Germino and Smith 2001). With *R. catawbiense* often growing on exposed outcrops and *R. maximum* under deciduous canopies, these species are exposed to high levels of solar radiation during the winter months. At the same time, low water availability and low stomatal conductance during this period prevent plants from releasing excess energy via transpiration, raising the likelihood that this increased radiation will damage photosynthetic machinery (Powles 1984). Damage to important photosynthetic structures can have amplified implications for these species. It is important for *R. maximum* to be at full photosynthetic capacity when temperatures increase in spring, and before the deciduous canopy has fully leafed-out, in order to take advantage of the available resources (Powles 1984, Russell et al. 2009, Nilsen et al. 2014). Leaves of curling *Rhododendron* species that were fixed in a flat, horizontal orientation where shown to suffer from damage to photosynthetic components and had much lower photosynthetic capability, resulting in lower carbon

Leaf curling and drooping in late fall and early winter when nighttime temperatures are low but daytime temperatures are relatively warm, however, could keep leaves from operating at full photosynthetic capacity. While leaves of these *Rhododendron* species do curl overnight during this part of the year, uncurling occurs as temperatures warm to above freezing, returning to their flat and horizontal orientation in about ten minutes (in a laboratory setting; Harshberger 1899, Nilsen 1987).

These rapid, turgor-mediated leaf movements are likely to require facilitation by some mechanism increasing water flux in leaf tissues to occur over such short time periods. AQPs operate in the transcellular water conduction pathway (Tyerman et al. 1999, Cochard et al. 2007) and are excellent candidates for permitting water transport at a rate required for these changes in orientation. Previous studies analyzing the role of AQPs in plant water transport often use comparative experiments, and have generally taken one of two avenues: the use of AQP inhibitors or antisense mutants. In this study, a reversible AQP inhibitor (H$_2$O$_2$ + FeSO$_4$) was used to create free radicals, which inhibit AQPs.

The finding that $R_{leaf}$ of both *R. catawbiense* and *R. maximum* were consistently unchanged after exposure to inhibitor was contrary to the hypothesis. Because the tested species exhibit the most extreme rolling of rhododendrons examined, and the documented role of AQPs in regulating the pulvini responsible for leaf movement in other species, it was expected that AQPs played some quantifiable and, potentially, major role in water
transport through the leaf. And, because leaf rolling most often occurs at lower temperatures in these species (Nilsen 1985, 1987, 1991, Russell et al. 2009), it was anticipated that greater AQP activity, and therefore greater increases in $R_{\text{leaf}}$ with inhibitor treatment, would occur in the lower temperature trials.

While there were no statistical differences between any $R_{\text{leaf}}$ before and after inhibitor exposure, there did seem to be greater effects in some treatments. The average change in $R_{\text{leaf}}$ post-inhibitor injection for $R. \text{ catawbiense}$ in the cold temperature treatment and $R. \text{ maximum}$ in the warm temperature treatment was ca. 1.0. However, each species, in opposing treatments, showed increases of 1.43 and 1.29 in $R_{\text{leaf}}$, respectively. These increases in $R_{\text{leaf}}$ may indicate greater involvement of AQPs in these treatments, but the results are inconclusive.

The findings suggest that, at both temperatures, AQPs have a limited role in the leaf water transport of these rhododendron species, supporting earlier findings in other species (Kaldenhoff et al. 2008). In contrast to previous studies (Sack et al. 2004, Ye et al. 2008, Nardini et al. 2010), it is possible water transport in the leaves of $R. \text{ catawbiense}$ and $R. \text{ maximum}$ is predominately apoplastic outside of the veins and less significant through the transcellular pathway where AQPs could be involved (Martre et al. 2002, Nardini et al. 2005, Hachez et al. 2008, Voicu et al. 2008). It is difficult to understand, however, how apoplastic water transport through the leaf could occur at rates fast enough to permit these rapid changes in orientation.

The lack of curling both in the field (at time of collection) and during the experiment is worth noting. Collected in late May, the plants were likely 1-2 months removed from cold temperature induced leaf curling. This may be of importance, as
AQP inhibitors have been shown to be upregulated as part of the cold acclimation process (Aroca et al. 2005, Chen et al. 2013). Additionally, Nardini et al. (2010) found AQP inhibitor had differing effects on leaf hydraulic conductance depending on season, suggesting that seasonal upregulation of AQP inhibitors would also lead to greater impacts of inhibitor on water transport. AQP expression has been shown to vary diurnally and seasonally in other studies as well (Moshelion et al. 2002, Nardini et al. 2005, Hachez et al. 2008). The fact that the plants were no longer experiencing near-freezing temperatures in their habitat and that leaves did not curl during cold temperature treatments, though they were exposed to that condition longer than required for curling in several previous studies (Harshberger 1899, Fukuda 1933, Nilsen 1987), may indicate that AQP inhibitors had been downregulated in the leaves tested. If this were the case, it would be expected that inhibitor treatment would have diminished effects on leaves.

One notable concern about these results is the failure to record the expected decrease in $R_{\text{leaf}}$ with increases in leaf temperature. There should have been a ca. 0.77x reduction of $R_{\text{leaf}}$ (demarcated in Fig. 3) with each 10 C increase in temperature, due to the reduction in water viscosity. Unfortunately, this reduction was not measured in either species or temperature trial, suggesting that data should be interpreted with extreme caution. There are several possible complications that could have caused these issues. First, potential down regulation of AQP inhibitors under the increased temperatures of each set of trials (Aroca et al. 2005, Chen et al. 2013) could have offset the decline in $R_{\text{leaf}}$, due to decreases in water viscosity, by increasing resistance through the leaf. It is also possible that this issue was related to user error. Over tightening the compression coupling on the petiole connecting the leaf to the HCFM waterline may have restricted water movement.
into the leaf, resulting in artificially exaggerated $R_{\text{leaf}}$ measurements. If this were the case, the effects of temperature change and AQP inhibitor on $R_{\text{leaf}}$ could be severely moderated. Finally, and perhaps most likely, the hydraulic conductivity of the leaf may have deteriorated over the course of the experiment due to blockages in the vasculature. Though all measurements reported were made within 36 h of leaf sampling, in planning for this study, it was noted that ca. 48 h after being removed from the plant, there was essentially no water transport through the leaf (data not shown). Microscopic analyses indicated that this decline was due to development of tyloses in the vasculature. However, if this were the case, it would be expected that there would lower influence of these issues early in the experiment and greater impact later in the trials, a trend which is not seen (data not shown).

Overall, collected data suggest that AQPs have a limited role in water transport in the leaves of *R. catawbiense* and *R. maximum* under cold or warm temperature. However, complications which resulted in no change in $R_{\text{leaf}}$ with increases in temperature may have also diminished the effects of AQP inhibitor on leaf water transport in this study. Because of these factors, the data may be unreliable, and any conclusions drawn from the data presented here should be made with extreme caution.

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Figure Captions

Figure VI-1. An example $R_{\text{leaf}}$ curve ($R. \text{ maximum}$ leaf, under cold temperature treatment). The curve includes 1) equilibration of $R_{\text{leaf}}$ as the leaf rehydrates, 2) transfer of the leaf from the cold water bath to the higher temperature bath (11 C), 3) acclimation of the leaf temperature to water bath temperature, and 4) AQP inhibitor injection.

Figure VI-2. Mean $R_{\text{leaf}}$ of $R. \text{ catawbiense}$ and $R. \text{ maximum}$ under a) cold and b) warm temperature treatments. Bars show mean $R_{\text{leaf}}$ under control (open bars), increased temperature (hatched bars), and AQP inhibitor exposure (double hatched bars) conditions. Error bars show ± 1 sd.

Figure VI-3. Mean proportional change in $R_{\text{leaf}}$ of $R. \text{ catawbiense}$ (open bars) and $R. \text{ maximum}$ (hatched bars) between a) cold and b) warm temperature treatments. Bars show $R_{\text{leaf}}$ after the temperature increases and after inhibitor exposure. The dashed line marks the expected 0.77x change in $R_{\text{leaf}}$ accompanying at 10 C increase in leaf temperature. Error bars show ± 1 sd.
Figure VI-1
Figure VI-2
Figure VI-3
CHAPTER VII
CONCLUSIONS AND FUTURE RESEARCH

Conclusions

The research projects presented here are among the first to examine the water sources and seasonal use patterns of riparian vegetation in the southern Appalachian Mountains and surrounding foothills. By examining different species, under differing hydrological regimes, and at multiple locations, we were able to gain a more comprehensive understanding of variation in these processes across the region. More broadly, this work contributes to the small amount we currently know about the ecophysiology of these habitats in the mountains of the southeastern US. These findings also enable comparisons to other riparian systems from different regions of the US, particularly those from more arid habitats.

Chapters II and III present the first studies of plant water sources conducted in the temperate forest of the southeastern US. While conclusions from these projects were hampered by an apparent failure to sample all possible sources, they suggest that water sources utilized by riparian trees of the southeast appear similar to those of vegetation in the more arid southwestern US. Moreover, these findings seem to refute the theory of unreliability, or the avoidance of a dependence on water sources that are unreliable during a season or between years, at least for this region. The southeastern US should be an excellent place to test this hypothesis, sharing some of the same species, but with perennial streams and a more mesic environment. Further, the Jacob Fork River, along with where these studies were conducted, should serve as an excellent system within
which to ask this question. The Jacob Fork has been recognized for its high water quality, has historically high water levels, and was dammed just below the study reach—conditions that should create an ideal scenario for streamwater use if it were going to occur.

Monitoring changes in water sources in these species over a year demonstrated that streamwater likely never made substantial contributions to plant water, but that source usage varied over time. Interspecifically, isotopic compositions of plant water were similar through the year, suggesting similar source use strategies, and it appeared that the changes in the sources used were largely dictated by plant water demand and environmental conditions. Both species relied on deep ground water sources during the growing season when plant water demand was highest, but results indicated increased shallow soil water use after periods of increased rainfall.

In Chapter IV, the hypothesis that streamside trees would use little or no surface water, as shown in Chapters II and III, while those growing along the pond would use increased amounts of surface water was rejected. Results showed the opposite trend, with trees along the stream using more surface water than pondside trees which relied about evenly on soil and pondwater. There is no obvious reason why source use of streamside trees in this study would be different from that in Chapters II and III, as species and hydrologic conditions were similar at these sites. Regardless of the reasons for these differences, these findings lend some credence to the theory of unreliability. Understanding why pondside trees were not using more surface water is difficult to explain. It is possible that, because of the high water availability in the shallow soil layers, it was unnecessary for the trees to invest in structures able to absorb water from
the pond or deeper groundwater. Alternatively, because of the relatively low oxygen content of the ponds, it is possible that the hyporheic zones along the pond were anoxic and roots would not have been able to survive in those areas.

Chapter V investigated the impacts of differences in water source usage strategies on habitat preference in three *Rhododendron* populations distributed along an elevational gradient. The hypothesis, incorporating previous ecophysiological research on these species, was that the riparian *R. maximum* would be capitalizing on the abundant surface water available, *R. minus* would be exploiting the mesic shallow soil layers, and the more exposed *R. catawbiense* would be utilizing deep soil moisture in its drier habitat. The findings, which showed only minor differences in source use between *R. minus* and *R. catawbiense*, suggest that source use is likely only one of a suite of characteristics influencing habitat preference in these species. Another noteworthy result was the limited surface water use by *R. maximum*, which was found to rely primarily on upper soil layers. While hypotheses concerning high water availability in the soil and possible anoxic conditions in the hyporheic zones around the stream could apply here as well, this is another species growing along a perennial stream but not using the highly available source.

Finally, Chapter VI, somewhat of a departure from other research presented here, attempted to determine the influences of temperature on leaf water transport in *R. catawbiense* and *R. maximum*, as well as the role of aquaporins (AQP) in that transport at different temperatures. While these data suggest that AQPs play a very limited role in water movement into a leaf at increased temperatures, opposing the prediction made based on the leaf movements exhibited in these species, there is reason to be cautious
about the results. Based exclusively on the change in the viscosity of water that occurs with a 10 C temperature change, we expected to see a significant decline in resistance to leaf water transport with increased temperatures; this was not observed. The fact that this change did not occur serves as a red flag for interpretation of these results. The most likely scenario is that user error or the development of blockages in the plant vasculature artificially increased resistance and overshadowed any changes in transport that occurred due to temperature or AQP inhibitor exposure. There are possible alternative explanations, including lower AQP activity at warmer temperatures, but they are difficult to support with the high degree of variability in the results.

Overall, this work adds to the limited ecophysiological research conducted in the riparian forests of the southern Appalachians and increases our understanding of the plant-water relations in these habitats. We demonstrated that stable isotope methodology could be used in the mesic forests of the southeastern US. Additionally, we were able to characterize the water source use patterns of several species in different habitats, which varied between locations. While some seasonal variation in source usage was found, it was common for streamside plants to rely on sources other than streamwater, in agreement with research from plants in arid habitats. While we were unable to provide definitive evidence to support or reject theories put forth to explain the lack of surface water use by riparian plants, we were able to contribute significantly to the literature on that topic.

*Future Research*

Having laid the foundation for water sourcing and other ecophysiological research, there are a number of areas that could and should receive attention in the future. First, a
comprehensive, and possibly long-term, study should be conducted on representative species to further our understanding of why plants utilize certain sources and how that affects overall plant health. This study should include water sourcing, gas exchange and water status measurements, as well as ecological assessment of individuals and the population. It would also be beneficial to investigate rooting habits of studied plants via excavation, allowing for comparison of root distribution and water sources used. This should be complemented by measurements of environmental variables, including soil water content at different depths. Taken together, these measurements would increase understanding about changes in source use with time, changes in plant phenology, and changes in environmental conditions, and the ecophysiological implications of those changes.

More applied projects should also be conducted. Using water sourcing to understand the impacts of disturbances, natural or manmade, on riparian forests has received little attention. The effects of flooding, damming, clearing of forests, invasive species, and especially water pollution (surface water or ground waters) on source use patterns should be assessed using stable isotopes and an effort should be made to determine the long-term impacts of these changes on riparian zones. The implications of inter- and intraspecific competition for water sources and the benefits of symbioses, including those with mycorrhizal fungi, in terms of sources also warrant much more investigation, especially in the southeastern US.

Finally, further research on the hydraulic characteristics of riparian trees and shrubs is needed. Coupling water sourcing, hydraulic, and water potential measurements should allow greater insight in these plants’ hydraulic strategy, vulnerability to hydraulic
failure, and likelihood of embolism repair should it occur. Understanding these hydraulic characteristics may also help explain confounding water source use patterns.
APPENDIX

FOLIAR UPTAKE, CARBON FLUXES, AND WATER STATUS ARE AFFECTED BY THE TIMING OF DAILY FOG IN SAPLINGS FROM A THREATENED CLOUD FOREST

Z. CARTER BERRY, JOSEPH C. WHITE, WILLIAM K. SMITH

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Abstract

In cloud forests, foliar uptake of water (FU) has been reported for numerous species, possibly acting to relieve daily water and carbon stress. While the prevalence of FU seems common, how daily variation in fog timing may affect this process has not been studied. We examined the quantity of foliar uptake, water potentials, gas exchange, and abiotic variation at the beginning and end of a nine-day exposure to fog in a glasshouse setting. Saplings of Abies fraseri and Picea rubens were exposed to morning, afternoon, or evening fog regimes to assess the ability to utilize fog water at different times of day and after sustained exposure to simulated fog. The greatest amount of FU occurred during morning fog (up to 50%), followed by afternoon fog (up to 23%), and then evening fog, which surprisingly had no FU. There was also a positive relationship between leaf conductance and FU suggesting a role of stomata in FU. Moreover, morning and afternoon fog lead to the greatest improvements in daily water balance and carbon gain, respectively. Foliar uptake is important for improving plant ecophysiology but is influenced by diurnal variation in fog. With climate change scenarios predicting changes to cloud patterns and frequency which will likely alter diurnal patterns, cloud forests that rely on this water subsidy could be affected.
Introduction

The importance of fog to ecosystems has been considered and debated for centuries (e.g. Hales 1757, Stone 1957a, b), but only in recent years has our development of new techniques such as high-precision sap flow, gas exchange, and stable isotopes allowed us to apply this to the water balance, carbon relations, and ecological processes of cloud forests (e.g. Dawson 1998; Bruijnzeel et al. 2011; Berry and Smith 2013a). Fog can enhance stomatal opening throughout a day, reduce extreme temperatures and radiative stresses, and increase plant water potentials via condensation drip and foliar uptake (FU) of water (Johnson and Smith 2006; Breshears et al. 2008; Reinhardt and Smith 2008; Limm et al. 2009; Berry and Smith 2012). The prevalence of fog has been functionally linked to the distributions of several ecosystems, including epiphytes and redwood trees of the Pacific Northwest, USA, the coffee forests of Angola, the Loma vegetation in Peru, many tropical montane cloud forests throughout central and South America, and the forests in this study, the relic spruce-fir forests of the southern Appalachian Mountains, USA (Weathers 1999; Cogbill and White 1991). While these ecosystem types vary in structure and species composition, the physiological mechanisms underlying the beneficial effects of fog seem to be similar. These include increases in leaf wetting leading to reduced transpirational water loss and/or reversed sap flow and foliar uptake (FU) (Burgess and Dawson 2004; Limm et al. 2009; Berry and Smith 2012; Eller et al. 2013; Gotsch et al. 2013).

While fog is a common occurrence in these ecosystems, it does not occur all the time. In fact, many of these forests experience dramatic seasonality in fog frequency (Dawson 1998; Holwerda et al. 2010). Additionally, there are times through a day where
certain ecosystems are more likely to experience fog. For example, in southern Appalachian spruce-fir forests most fog occurs during the morning hours, in the Peruvian Andes fog is frequently in the midday and afternoon hours, and in the coastal California redwood forests fog seems more common during the night (Limm et al. 2009; Berry and Smith 2012; M. Silman pers. comm.). This has led some to propose that plants in these ecosystems are not only adapted to fog, but adapted to utilize it at these particular times of day (Berry and Smith 2013a). To this point, we examined how fluctuation in the timing of daily fog regimes affects FU and physiological functioning during fog events.

While in some instances leaf wetting can hinder carbon exchange through stomatal pores, it can also significantly improve plant water status through reduced transpirational water loss and the addition of surface water into the leaf (Smith and McClean 1989; Brewer and Smith 1995; Simonin et al. 2009). Foliar uptake of water is the phenomenon whereby intercepted water on leaf surfaces is absorbed into the leaf and has now been demonstrated in over seventy species worldwide (Boucher et al. 1995; Martin and von Willert 2000; Gouvra and Grammatikopoulos 2003; Limm et al. 2009; Berry and Smith 2013b; Goldsmith et al. 2013). These species span a wide range of plant families, including some that do not occur in “cloud forests”, suggesting that this mechanism may be more ubiquitous than previously thought and simply requires leaf wetting events. Foliar uptake has been demonstrated for both species studied here (Abies fraseri and Picea rubens), contributing as much as 6.5 % of total leaf water content in a 24 hour simulated fog period. Under field conditions, fog water contributed up to 31 % and 24 % to total leaf water content in A. fraseri and P. rubens, respectively (Berry et al. 2013).
Southern Appalachian spruce-fir \([Picea \text{ rubens} \text{ Sarg.-Abies fraseri} \text{ (Pursh) Poir.}]\) forests experience fog 60-75\% of growing season days with 80\% of those days having morning fog (Berry and Smith 2012). These relic forests only exist above ~1500 m elevation at seven locations in Virginia, North Carolina, and Tennessee and have been suggested to co-occur with the prevalence of fog (Oosting and Billings 1951; Ramseur 1960; White 1984; Cogbill and White 1991). Moreover, current climate models for cloud forest regions suggest that, in general, clouds will become less frequent and cloud ceilings will rise with warmer air temperatures (Pounds et al. 1999; Still, Foster and Schneider 1999; Foster 2001; Richardson et al. 2003; Brient and Bony 2012). However, it is difficult for these models to predict changes in the seasonal or diurnal patterns that are currently unique to these cloud forests. It is, thus, important for scientists to examine how these species and their communities will be affected by changes in cloud regimes. Because these southern Appalachian forests harbor many endemics, including \textit{A. fraseri}, and they are constrained to a small elevational band on only a refugial few peaks, ecophysiological data linking their persistence to interactions with cloud regime may provide a more mechanistic insight that can be applied to a broader geographic scale.

The present study addresses how changes to the time of day of fog will affect FU, carbon balance, stomatal control, xylem water potentials, and leaf wetness of saplings of \textit{A. fraseri} and \textit{P. rubens}, the dominant canopy species in southern Appalachian spruce-fir forests. While fog occurs at all times of day in these cloud forests, it rarely occurs through the entire day. This variation leads to fog days with distinct diurnal patterns that can have differential effects on plant ecophysiology. Thus, in a glasshouse, we exposed saplings to a morning (MF), afternoon (AF), and evening (EF) fog regime and measured
the parameters above on the first day of fog and after nine days of exposure. Mechanistically, if foliar uptake varies with stomatal conductance, cellular photosynthetic activity, or light availability, an increase in foliar uptake (FU) during morning fog would be expected. Additionally, under foggy conditions in the field, morning periods have significantly greater values of leaf level photosynthesis and greater water potentials than clear days and thus we expected similar ecophysiological improvements in this study (Berry and Smith 2013a). Because fog regimes still aid in plant water conservation through reduced transpiration values, afternoon and evening fog both are likely to result in improvements in ecophysiological parameters over control plants as well, but not as strongly as the morning fog regime. Previous work (e.g. Berry and Smith 2013a) has reported a greater physiological response to fog in *A. fraseri* than *P. rubens* and, thus, we predicted a similar result in this experiment.

**Materials and Methods**

We conducted a glasshouse experiment to examine the responses of *A. fraseri* and *P. rubens* to three daily fog regimes, morning (MF), afternoon (AF), and evening (EF). Saplings of each species were randomly assigned to one of four groups: six hours of fog at the designated times or a control with no fog. We measured photosynthetic responses, plant water potentials, and stable isotopic values of sapling needles both at the beginning and end of a nine day period. By controlling the isotopic composition of cloud and soil water we were able to determine if the isotope composition of needle water was affected by exposure to fog water. Previous field studies have demonstrated that morning hours are the most common period for fog in these, and other, temperate cloud forests. This
study addressed whether the time of day of fog influenced ecophysiological parameters in these species.

Species

*Abies fraseri* (Pursh) Poir. (Fraser Fir) and *Picea rubens* Sarg. (Red Spruce) grow in threatened spruce-fir communities of the southern Appalachian Mountains which experience cloud immersion on 60 to 75 percent of all days during the growth season (Saxena and Lin 1990; Mohnen 1992; Baumgardner et al. 2003; Berry and Smith 2012). Cloud immersion and rainfall both occur consistently throughout the year with no distinct seasonality (Berry and Smith 2012). *Abies fraseri* is endemic to these temperate cloud forests which only remain on seven mountaintop areas in the southern Appalachians (Ramseur 1960). Because of this endemism and the rarity of temperate cloud forest communities, these studies are of particular interest in understanding sapling responses to changes in climatic factors such as fog.

Saplings were collected from three different sites on Mt. Mitchell, NC (Mt. Mitchell State Park, 35°45'53"N, 82°15'54"W), the highest point in the eastern United States (2037 m), and transported back to a glasshouse with their root masses (~0.3 m$^3$ soil collected) contained in plastic bags lined with damp cloths in a cooler. All saplings used in this study were between 0.30 and 0.45 m tall, estimated to be between 5 and 10 years old, and had not yet reached a reproductive age. Saplings were transplanted into 7.3 liter pots filled with Metro Mix 360 potting soil (Sun Gro Horticulture, Vancouver, Canada). Saplings acclimated for eight weeks to glasshouse conditions of daytime highs 20-25°C, daily absolute humidity averaging ~5 g/m$^3$, and midday PPFD around 1500 µmol m$^{-2}$s$^{-1}$. Pots were watered with approximately 500 ml of water every 3 days to keep soil
volumetric water content above 25% similar to field conditions (K. Reinhardt pers. comm.). Watering ceased once the fog experiment began.

Experimental Setup

Four chambers were constructed with clear, polyvinyl sheeting, measuring 0.9 x 1.6 x 0.8 m, and equipped with two electric, water proof fans to circulate fog throughout the chamber (Adda AQ series, Brea, California). Four saplings of each species (A. fraseri and P. rubens) were randomly assigned to one of four chambers and to a position within the chamber. Chambers were monitored before the onset of fog treatments to ensure no variation in temperature or sunlight between chambers due to location within the greenhouse. Each chamber was assigned one of four treatments: morning fog (700 to 1300), afternoon fog (1200 to 1800), evening fog (1800-0000), or no fog (control). These were chosen as all three fog regimes here are seen in the field throughout the growing season. All fog treatments ran for nine days. In the cloud chambers, fog was generated using a five-disk ultrasonic fog-generating device (Chaoneng Electronics, Nanhai, Guangdong, China) sitting in a 5 liter distilled water reservoir. Each reservoir was supplied with water from the same 200 liter reservoir located centrally to all chambers using ½ inch plastic tubing and a passive control valve (Hudson Valve, Bakersfield, California). The chambers were equipped with HOBO Pro v2 sensors and data loggers (Model U23-001; Onset, Bourne, MA) to monitor air temperature and humidity every 15 minutes. The sensors had previously been calibrated with ventilated and shielded, fine wire thermocouple psychrometers (36 ASW gauge). To measure photosynthetic photon flux density (PPFD; μmol m⁻² s⁻¹, 0.4-0.7 μm wavelengths) each chamber was equipped with a Photosynthetic Light-Smart Sensor (Model S-LIA-M003; Onset, Bourne, MA)
connected to a four-channel HOBO Micro Station Data Logger (Model H21-002; Onset, Bourne, MA) programmed to log every 15 minutes. PPFD sensors were matched against a LICOR quantum sensor (model 190S).

*Leaf level gas exchange, xylem water potentials, and leaf wetness*

Leaf gas exchange was measured using a Li-Cor LI-6400 model portable photosynthesis system using the clear conifer chamber (Li-Cor, Lincoln, Nebraska) on day two and eight of the fog treatments. These days were chosen to examine stomatal responses after one day of each fog regime and then again after a week of fog exposure to determine if extended periods of fog resulted in differential responses. Gas exchange was measured at 900 h, 1200 h, and 1500 h on mature shoots at mid-sapling heights on all four saplings of each species in each chamber. When gas exchange measurements were made during fog treatments, each chamber was rigged with a port to allow the individual shoot outside of the chamber, thoroughly blotted dry with tissue paper and immediately inserted into the cuvette. Leaf conductance values were discarded if conductance seemed extraordinarily high, probably due to moisture still on the surface of the shoot.

Additionally, air temperature and relative humidity inside the leaf chamber were regulated to be within ±5 % of ambient values by setting relative humidity to that value within the chamber, controlling the humidity of the air into the cuvette, and adjusting the flow rate accordingly. Total PPFD varied with time of day, generally below 400 µmol m⁻² s⁻¹ during morning measurements and between 500 and 900 µmol m⁻² s⁻¹ during midday and afternoon measurements. Total leaf area in the cuvette was calculated by counting the number of leaves in the chamber and using a mean total leaf area determined by leaf area measurements (as described in Smith et al. 1991).
Xylem water potentials of terminal shoots were measured using a Scholander type pressure chamber (PMS Instruments, Corvallis, Oregon; model 1000) on days 1, 5, and 9 of the fog treatments. Water potentials were taken at 700 (predawn), 1100 (morning), and 1500 (afternoon) on one shoot of all four saplings per species in each chamber. Two leaf wetness sensors (Model S-LWA-M003, Onset, Bourne, MA) were mounted on opposite sides of each chamber, connected to a four-channel HOBO Micro Station Data Logger (Model H21-002; Onset, Bourne, MA), and set to log every 15 minutes. Sensors were calibrated before the experiment to determine the relative threshold sensor value that resulted in leaf wetness in each species. Values presented are the mean leaf wetness for each chamber.

Isotope experiment and foliar uptake of water

We examined the influence of each fog regime on the isotopic composition of needle tissue with a focus on the potential for FU on day one of experimental fog exposure and after nine days of exposure. To separate soil water isotopic composition and fog isotopic composition, each pot was wrapped entirely with polyvinyl plastic and attached with a waterproof adhesive to the primary shoot at the soil surface. We measured the change in the isotopic composition of both hydrogen ($\delta^{2}$H) and oxygen ($\delta^{18}$O) of each sapling from before fog exposure to immediately after termination of the fog treatment. To ensure enough needle tissue for the water extraction process, ~0.6 g of P. rubens and ~1.0 g of A. fraseri were taken from each sapling (four saplings per species per chamber). Needles were clipped from multiple locations on each plant, rinsed with deionized water to remove residual fog water on the plant surface, thoroughly hand-dried,
immediately sealed in a 60 ml glass vial reinforced with laboratory film, and placed into a -10 °C freezer until water extraction occurred (Limm et al. 2009).

Fog water was enriched before the experiment with a calculated quantity of 99.9% Deuterium oxide (Sigma Aldrich, St. Louis, Missouri) to approximately 72‰ δ2H (Vienna standard mean ocean water-V-SMOW), well above the likely δ2H of any saplings in the experiment. Burgess and Dawson (2004) had previously confirmed that the ultrasonic fog generating device does not cause significant fractionation. To ensure that the isotopic composition of the fog and soil water did not change during the course of the experiment, we took samples of both before and after each measurement period. Fog water (3.5-ml vials) and soil (30 ml vials) were collected and stored at -10°C until extraction or spectroscopy measurements occurred. While water was not collected off of the leaf surface immediately before uptake, we are confident that fog and leaf surface water isotope ratios were nearly identical due to the saturated environment (relative humidity 100%) and presence of constant, dense water particles in the air. This led to a saturated equilibrium between fog and leaf surface water where the values of the two were likely very similar or within the error of uncertainty.

Water was extracted from needle and soil samples using cryogenic vacuum distillation at the SIRFER lab at the University of Utah (Ehleringer et al. 2000). Four replicate injections of each sample were analyzed using isotope-ratio infrared spectroscopy by introduction into a wavelength-scanned cavity ring-down spectrometer water analyzer (model L1102-I; Picarro, Sunnyvale, CA, USA). Laser-based isotopic analysis can be sensitive to error introduced by certain secondary organic contaminants (West et al. 2010). To minimize any possible effects, the isotopic enrichment of fog
water was well above any possible measured offsets from plant stable isotope ratios and reported data are the average of the third and fourth injections. Three laboratory reference materials were also run through the spectrometer, calibrated to the Vienna Standard Mean Ocean Water (VSMOW), and used to calibrate sample measurements. The average uncertainty from the reference materials was 1.77 ‰ for δ²H and ± 0.17 ‰ for δ¹⁸O. All isotopic compositions are expressed in delta notation (δ²H ‰) relative to the V-SMOW standard.

**Data Analysis**

To determine the relative proportion of leaf water contributed by fog, a linear, two-source mixing model was applied (Dawson et al. 2002; Gotsch et al. 2013):

\[ f_A = \frac{\delta_t - \delta_B}{\delta_A - \delta_B} \]  

(1)

where \( f_A \) is the fraction of water contributed by fog, \( \delta_A \) is the isotopic ratio of the enriched fog water, \( \delta_B \) is the isotopic ratio of water in the leaf immediately before the onset of fog, and \( \delta_t \) is the isotopic ratio of water in the leaf immediately following fog. If the isotopic value decreased, which would lead to a negative value from the mixing model, the relative contribution of fog water to plant water is stated as zero. All comparisons of ecophysiological data were tested for statistical significance using a two–factor ANOVA examining the effects of fog treatment and species following appropriate evaluations of normality and equality of variance (Zar 1999). In the text, p-values are reported only if the relationship was statistically significant. Correlations were tested between ecophysiological variables and the change in isotopic ratios between before and after fog treatments. All analyses and figures were generated using JMP (SAS, Cary, NC) or SigmaPlot v. 12 (Systat Software, San Jose, CA).
Results

Temperature, humidity, PPFD, and leaf wetness

Although all three experimental chambers received six hours of fog, the change in time of day significantly altered the diurnal fluctuations of abiotic variables. Mean daily air temperatures ranged from 13.7 ± 0.49 °C during MF to 14.8 ± 0.45 °C during EF, similar to temperatures seen in field conditions (Berry and Smith 2013a). Mean and maximum daily temperatures were greater during EF and AF than control and MF chambers (Table 1). Similarly, and as expected, the mean and maximum daily humidity was greater in the experimental chambers over the control chambers. Maximum absolute humidity was almost double the control chamber (10.92 ± 0.67 g/m³) in experimental chambers, ranging from 18.70 ± 1.12 g/m³ in the EF chamber to 20.94 ± 1.29 g/m³ in the AF chamber. The MF and AF chambers had the lowest PPFD as fog occurred through midday when PPFD is typically greatest. There were strong differences in the number of hours of leaf wetness. Complete leaf wetness began within 15 minutes of fog onset in all three treatments, similar to observed wetness in the field. In the MF chamber, moisture on leaves evaporated within thirty minutes of the cessation of fog (average 5.89 ± 0.09 hours per day). In the AF and EF chambers, leaf wetness persisted for 15.63 ± 1.37 and 13.06 ± 0.91 hours, respectively, presumably due to the fact that fog ceased during periods with lower atmospheric water demand.

Foliar uptake of water

Foliar uptake of water occurred in both *A. fraseri* and *P. rubens* as evidenced by the change in leaf water δ²H in the direction of the enriched fog water (Figure 1). The enriched fog water δ²H was 71.6 ± 1.2 ‰, while the soil water was -8.7 ± 2.2 ‰. All
needle δ²H values were between these two values when the experiment began and increased following fog exposure when FU occurred. The δ²H of soil water did not change significantly during the experiment (0.27 ± 3.6 ‰) indicating that there was no leakage of condensed fog water into the soil or isotopic enrichment. Using a mathematical mixing model, we found that the timing of fog exposure had a dramatic effect on the relative contribution of fog water to needle water (Figure 1). Foliar uptake was greatest during MF on day one of the experiment with fog water contributing 46.6 ± 8.7 and 55.0 ± 1.9 percent of needle water in *A. fraseri* and *P. rubens*, respectively. With the exact same duration of fog, the contributions to needle water were less in the AF treatment, contributing about 23 % in both species (Figure 1). Surprisingly, there was no FU during EF. When FU was measured again, after nine days of fog exposure, fog water contributed less to leaf water. In *A. fraseri*, fog contributions during MF dropped to 29.5 ± 3.5%, still making a significant contribution to leaf water. The contribution of fog in AF remained similar to day one, but with greater variation, suggesting that there were different responses between individuals. For *P. rubens*, saplings in both MF and AF had strong decreases in the contribution of fog water to 8.1 ± 9.0% and 8.5 ± 2.7%, respectively (Figure 1). On day 9, there was again no contribution of fog water to needle water when plants experienced fog in the evening. Because of the strong association with time-of-day for fog occurrence, we tested the relationship between leaf conductance and FU. There was a significant positive relationship (F₁,₂₄ = 14.78, p < 0.001, r² = 0.38) between these two variables, suggesting a possible influence of leaf conductance on foliar uptake (Figure 2). There was no relationship between FU and leaf water potentials or between leaf conductance and water potentials during fog events (data not shown).
leaf gas exchange

Using a two-factor ANOVA, there was a significant effect of the timing of fog on net photosynthesis ($F_{7,178}=6.34, p<0.001$), leaf conductance ($F_{7,178}=5.40, p=0.001$), and transpiration ($F_{7,178}=12.26, p<0.001$) (Figures 3 and 4). Additionally, there was an overall species effect on photosynthesis only ($F_{1,178}=10.93, p=0.001$) with *P. rubens* having greater photosynthesis. There were no interaction effects between fog treatment and species or time of measurement across all subsequent analyses presented. While there was no difference in morning (900 h) photosynthesis or leaf conductance, there was a significant decrease in transpiration ($F_{3,54}=118.27, p<0.001$) driven largely by the very low transpiration rate in the MF chamber (Figure 3). Consistent with field measurements for understory saplings (e.g. Berry and Smith 2013a), morning net photosynthesis ranged from 1.10-2.55 µmol m$^{-2}$s$^{-1}$ for *A. fraseri* and 2.09-3.99 µmol m$^{-2}$s$^{-1}$ for *P. rubens*. Maximum daily leaf conductance occurred only during morning measurements and was similar for both species, ranging from 0.24-0.35 mol m$^{-2}$s$^{-1}$. The presence of fog did not inhibit maximum photosynthesis or leaf conductance values. Morning transpiration values were high (1.77-2.67 mmol m$^{-2}$s$^{-1}$) for all chambers except for the saplings experiencing morning fog treatment which, due to the lower vapor gradient from the leaf to the air, transpired at 0.17-0.44 mmol m$^{-2}$s$^{-1}$ (Figures 3 and 4).

There were also significant differences between fog regimes in midday (1200 h) net photosynthesis ($F_{3,54}=8.56, p<0.001$) and transpiration ($F_{3,54}=46.00, p<0.001$), but not midday leaf conductance (Figure 3). Saplings experiencing AF had greater midday net photosynthesis than all other plants. Saplings in MF still had very low transpiration rates at midday relative to all other chambers driven by the presence of cloud mist still in the
chamber (Figure 4). There was a significant effect of the day of the experiment with significant increases in midday net photosynthesis in all three fog regimes after eight days of fog (morning fog $F_{1,12}=52.17$, $p<0.001$; afternoon fog $F_{1,12}=22.22$, $p<0.001$; evening fog $F_{1,12}=15.29$, $p=0.012$, control chamber not significant). These increases ranged from 0.9-1.8, 0.8-1.8, and 0.8-1.3 µmol m$^{-2}$s$^{-1}$ in the MF, AF, and EF treatments, respectively (Figures 3 and 4). This corresponded with no increases in control plant net photosynthesis or changes in midday leaf conductance or transpiration rates.

There were significant differences between fog regimes in afternoon net photosynthesis ($F_{3,54}=7.21$, $p<0.001$), leaf conductance ($F_{3,54}=14.11$, $p<0.001$), and transpiration ($F_{3,54}=54.01$, $p<0.001$). All experimental fog chambers had greater photosynthesis than control saplings with the difference being more pronounced after eight days of fog (Figures 3 and 4). Morning and afternoon fog treatments had greater afternoon net photosynthesis – always above 1.5 µmol m$^{-2}$s$^{-1}$ and up to 3.6 µmol m$^{-2}$s$^{-1}$. Afternoon net photosynthesis was greater on day eight compared to day two. Leaf conductance remained above 0.2 mol m$^{-2}$s$^{-1}$ for all plants exposed to fog, while afternoon conductance in control plants was below 0.07 mol m$^{-2}$s$^{-1}$. Afternoon transpiration was greatest in MF and EF saplings driven by the high afternoon stomatal conductance. Afternoon transpiration was very low (below 0.5 mmol m$^{-2}$s$^{-1}$) in control and AF saplings.

**Water potentials**

There was a significant effect of fog treatment ($F_{3,271}=50.91$, $p<0.001$) on xylem water potentials with all fog chambers experiencing greater water potentials than control plants. Morning (1100 h) and afternoon (1500 h) water potentials were significantly greater in plants that experienced MF and AF (Figure 5; morning $F_{3,85}=32.17$, $p<0.001$, afternoon $F_{3,85}=57.21$, $p<0.001$).
afternoon $F_{3,85}=70.82$, $p<0.001$). Water potentials during MF remained above -1.0 MPa for the entire day throughout the experiment. On day one, plants in AF decreased to -1.2 to -1.5 MPa by midday, but by day nine morning and afternoon water potentials had improved and remained above -1.0 MPa for the entire day (Figure 5). Saplings in EF also had significantly greater water potentials than controls, although the magnitude of this effect was smaller than for MF and AF. After nine days, predawn, morning, and afternoon water potentials in control plants were significantly lower than all cloud-immersed plants. Minimum daily water potentials in control plants reached -1.75 to -2.0 MPa. There was also a significant species effect on water potentials ($F_{1,271}=8.23$, $p=0.005$) with *A. fraseri* having greater values. There were no differences between treatments in predawn water potentials on day one indicating that water potentials were similar for all plants at the beginning of the experiment.

**Discussion**

*The importance of daily immersion timing on foliar uptake of water*

For saplings from a fog-adapted conifer ecosystem, foliar uptake of fog water was dependent on the time of day that the fog experience occurred. Foliar uptake (FU) occurred during morning (MF) and afternoon (AF) hours, while no significant FU occurred during evening (EF) hours. During MF, as much as half of leaf water came from foliar uptake which declined to a quarter of total leaf water during AF. The fact that no FU occurred during EF was surprising because uptake has been found to occur at night for other species (Limm et al. 2009; Eller et al. 2013; Gotsch et al. 2013). In southern Appalachian cloud forests, cloud immersion occurs most frequently during morning hours (Berry and Smith 2012), perhaps suggesting that both species are highly
adapted to utilize fog water during the morning fog periods or that the utilization is different in our study species. The largest contributions of fog water were in saplings experiencing low water stress following eight weeks of glasshouse acclimation and no exposure to cloud immersion, thus no previous exposure was necessary for FU to occur. However, the fact that there was variation across fog treatments suggests that the utilization of this subsidy is not necessarily universal. Fog contributed significantly less to leaf water on day nine in MF and AF treatments. While FU has been demonstrated in a growing number of species across a wide range of phylogenies and growth habits (Goldsmith et al. 2013), this study suggests that the utilization of fog water does not simply occur when leaves are wet or plants are water-stressed and may be an active, selective process. There are likely a complex set of interactions between numerous variables including environmental factors (VPD, temperature, and time of day), the plant’s need for an additional water subsidy, and an adaptive mechanism that drive the utilization of fog water.

During fog conditions in the field and glasshouse, needles of both species stay wet despite the fact that these species (and conifers generally) are thought of as having hydrophobic needle surfaces (Reed and Smith 2012). Contact angles for both species across multiple sites average over 90° which is generally considered non-wettable (Aryal and Neuner 2010; Reed and Smith 2012). Thus, during high fog periods where water particles are intercepted by leaf surfaces, these “non-wettable” surfaces still retain sufficient water for foliar uptake to occur. Saplings in the MF treatment had high leaf wetness throughout the fog treatment but this surface water was absorbed by the leaf or evaporated following cessation of fog, likely driven by the high temperature and sunlight
in the middle of the day (Table 1). Saplings in the AF and EF treatments had much greater leaf wetness durations (15.63 ± 1.37 and 13.06 ± 0.91, respectively) driven by similar processes of lower quantities of FU and less evaporative demand at the times where fog ceased. Thus a prolonged leaf surface wetness does not result in greater FU in our study species, further reaffirming the idea that this process may be selective. While foliar uptake did not occur at night, prolonged leaf wetness through nighttime hours may aid in plant hydraulic maintenance by limiting the nighttime transpiration that can occur (Eller et al. 2013).

The pathway for FU likely varies across species but has been difficult to demonstrate to this point. Some have proposed specialized structures such as absorbent trichomes and hydathodes (Benzing et al. 1978; Martin and von Willert 2000) while in the absence of these structures there is debate over whether it is stomatal or cuticular (Jagels 1991; Limm et al. 2009; Goldsmith et al. 2013; Eller et al. 2013). The crux of this conversation lies in fundamental physical tenets of plant ecophysiology. If the pathway were stomatal, water film would have to cover stomata, severely limiting CO$_2$ uptake (Smith and McLean 1989). Alternatively, leaf cuticles are adapted to be selectively permeable. The permeability of leaf surfaces is based on the chemical composition of protective waxes and varies across the surface (Shepherd and Wynne Griffiths 2006). In this study we found a positive relationship between leaf conductance and the uptake of fog water (Figure 2). While this does not demonstrate stomatal uptake, it does suggest that stomatal opening may play some role, likely in concert with the cuticle, in facilitating foliar uptake. We believe at this point that with the present data, neither pathway can currently be excluded. In *A. fraseri*, stomata occur in two distinct depressions on the
underside of the needle and in a small bunch on the top of the needle at the tip (Reed and Smith 2012). Most leaf wetting events in this system occur on the top of needles and when the needle is inclined, water frequently collects at the tips (Berry pers. obs.). Theoretically, FU could occur through the cuticle or stomata at needle tips while stomata on the underside still easily take in CO$_2$. Alternatively, the correlation between leaf conductance and FU could be explained by other processes that vary diurnally such as such as numerous light- or temperature-limited cellular processes. Although in this study no relationship was found between FU and water potentials, leaf wetness, or PPFD (data not shown). Presently, elucidating the pathway (or multiple pathways) of this entry into the leaf and understanding the drivers that dictate FU are pressing questions that are pivotal to understanding the evolution of this trait and its adaptive significance.

*Foliar uptake reduces water loss and improves carbon gain*

The presence of fog and occurrence of FU resulted in significant improvements in plant water status and carbon uptake. For *A. fraseri* on day 1, following two months of no fog exposure, plant water potentials were greater than control plants in the MF and AF regimes (Figure 5a). Morning fog resulted in the greatest water potentials throughout the day, (remaining mostly above -1.0 MPa) and following nine days of fog exposure, all plants had greater water potentials than control plants. The afternoon water potentials were greatest in plants that experienced the most FU to that point in the day (MF and AF; Figure 5). The high afternoon water potentials in MF saplings is likely driven by the fact that these plants also had the greatest FU (~50% on day 1) and the lowest daily transpirational water loss (Figure 3 and 4). Morning fog exposure also resulted in the lowest morning and midday transpiration values due to the reduction in the leaf-to-air
vapor pressure difference, even when leaf conductance was greatest. Water potentials for evening-fogged plants remained greater than control plants despite no FU during the experiment. This suggests that some nighttime transpiration could be occurring on clear nights which can be alleviated by fog and leaf wetness. The low transpiration and improved water potentials in response to fog are additional ways that cloud-immersed environments improve plant functioning.

In addition to improvements in water status, fog improved plant carbon gain, particularly in the afternoon (Figures 3 and 4), possibly associated with greater leaf conductance for all experimental fog treatments. Saplings in the AF treatment had the greatest midday and afternoon net photosynthesis for both species. Moreover, afternoon leaf conductance was greater in both species than control plants no matter what time of day fog occurred (panels (c) and (d), figure 3 and 4). This greater leaf conductance led to greater midday and afternoon photosynthesis particularly on day eight in midday and afternoon measurements. In theory, cloud immersion could limit photosynthesis by drastically reducing incident sunlight or by water film formation that could restrict stomatal CO$_2$ uptake (Smith and McLean 1989). This study builds on previous research demonstrating that due to the low light saturation points in _A. fraseri_ and _P. rubens_ (less than 400 µmol m$^{-2}$ s$^{-1}$) and the improved plant water status, maximum photosynthetic capacity can readily occur during cloud immersion (Johnson and Smith 2006; Reinhardt and Smith 2008; Berry and Smith 2013a). Enhanced photosynthesis during cloud immersion is also explained by the increased diffuse light that penetrates deeper into understories and is effectively utilized by the unique needle arrangement in _Picea_ and
Abies (Ishii et al. 2012). Additionally, cloud immersion eases photoinhibitive stresses from high sunlight and temperature (Johnson and Smith 2006; Reinhardt and Smith 2008).

Conclusions – climate change, cloud forests, and foliar uptake

Based on regional climate models of the United States, southern Appalachian temperatures are projected to increase between 2.7 °C and 4.4 °C by the year 2100 (IPCC 2007; U.S. Global Change Research Program 2009), depending on the emissions scenario. Many climate models also predict higher cloud ceilings and a reduction in cloud frequency through mid-latitudes, likely reducing the exposure of cloud forests to frequent fog (Still et al. 1999; Foster 2001; Richardson et al. 2003; IPCC 2007). The increase in temperature and reduction of fog will lead to non-analog climates by 2100, if not sooner. Less frequent cloud immersion will lead to a lesser reliance on fog water during periods of increased temperature and plant water demand. This study demonstrates that a reduction in cloud frequency may not be necessary for plants to have negative responses. Simple changes in the diurnal or seasonal frequency could negatively affect FU and associated ecophysiology. How Abies fraseri and Picea rubens are able to adapt to this warmer and dryer climate will likely dictate if we see further retreat to higher elevations or even potentially a loss of the entire ecosystem.

This paper has demonstrated that FU only occurred when fog occurred during daytime hours and that more fog water was utilized if fog occurred during morning hours. While FU is now believed to be a widespread phenomenon, this study suggests that utilization of this direct water source may be reliant on additional interactive factors. Addressing what factors drive the direct utilization of fog water will be important in the future. Foliar uptake has now been demonstrated in over seventy species across
numerous growth habits, phylogenies, morphologies, and biomes (Goldsmith et al. 2013). In many of these environments, FU seems like a strategy to alleviate water or temperature stresses for species adapted to milder and wetter climates, but this is not universal. Many of the suggested species are not from frequently foggy environments and, thus, simply having periods of leaf wetness may be a primary prerequisite. Foliar uptake and its associated ecological impacts should be examined across a greater number of species and ecosystems, as well as from an evolutionary perspective.

**Acknowledgements**

We would like to thank Ben Robb and Daniel McCall for glasshouse assistance, Brad Erkkila and Abby Howell-Dinger at the University of Utah SIRFER lab for valuable insight into isotopic procedures and for sample processing, to Mt. Mitchell State Park for project insight and cooperation, and to Mary Jane Carmichael and Katherine D.H. Berry for glasshouse assistance and manuscript advice. Financial support was provided through the National Science Foundation (IOS 1122092) and a Vecellio grant to Z.C. Berry through the Biology Department, Wake Forest University.
References


Reed JE, Smith WK (2012) Stomatal frequency, distribution, and needle hydrophobicity in cloud forest spruce and fir, Southern Appalachian Mountains. RURALS 7: 3.


Figure Captions

Table A-1. Air temperature, absolute humidity, and number of hours per day of leaf wetness in the control chamber (no fog) and each of the three experimental fog chambers.

Figure A-1. The percent contribution of leaf water derived from foliar uptake calculated using a linear, two-source mixing model for *Abies fraseri* and *Picea rubens*. Saplings of each species were exposed to fog during morning, afternoon, or evening hours and the relative contribution of fog water measured on day one of fog exposure and after nine days of fog exposure. Bars represent standard error.

Figure A-2. Relationship between leaf level conductance during fog conditions and the change in δD ‰. This figure includes all plants from the morning and afternoon chambers where foliar uptake occurred. The $r^2$ for this relationship is 0.38.

Figure A-3. Net photosynthesis (a, b), leaf conductance (c, d), and transpiration (e, f) values during morning (0900 hours), midday (1200 hours), and afternoon (1500 hours) for saplings of *Abies fraseri*. Saplings were exposed to morning fog (open circles), afternoon fog (closed triangles), evening fog (open triangles), or no fog (control, closed circles) and measurements conducted on day two, following one day of fog exposure, and on day eight. Bars represent standard error.

Figure A-4. Net photosynthesis (a, b), leaf conductance (c, d), and transpiration (e, f) values during morning (0900 hours), midday (1200 hours), and afternoon (1500 hours) for saplings of *Picea rubens*. Saplings were exposed to morning fog (open circles), afternoon fog (closed triangles), evening fog (open triangles), or no fog (control,
closed circles) and measurements conducted on day two, following one day of fog exposure, and on day eight. Bars represent standard error.

Figure A-5. Xylem water potentials for saplings of *Abies fraseri* (a, b, c) and *Picea rubens* (d, e, f) measured at 700 h, 1100 h, and 1500 h. Saplings were exposed to morning fog (open circles), afternoon fog (closed triangles), evening fog (open triangles), or no fog (control, closed circles) and measurements conducted on day one, five, and nine of the experiment. Bars represent standard error.
<table>
<thead>
<tr>
<th></th>
<th>Mean daily temperature (°C)</th>
<th>Maximum daily temperature (°C)</th>
<th>Mean daily absolute humidity (g/m³)</th>
<th>Maximum daily absolute humidity (g/m³)</th>
<th>Maximum daily PPFD (µmol m⁻² s⁻¹)</th>
<th>Leaf wetness hours per day</th>
</tr>
</thead>
<tbody>
<tr>
<td>No fog</td>
<td>13.76 ± 0.49</td>
<td>22.90 ± 1.12</td>
<td>8.93 ± 0.56</td>
<td>10.92 ± 0.67</td>
<td>643 ± 36</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>Morning fog</td>
<td>13.69 ± 0.49</td>
<td>22.74 ± 0.74</td>
<td>11.94 ± 0.42</td>
<td>19.59 ± 1.02</td>
<td>365 ± 12</td>
<td>5.89 ± 0.09</td>
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<tr>
<td>Afternoon fog</td>
<td>14.29 ± 0.47</td>
<td>25.13 ± 1.05</td>
<td>11.89 ± 0.54</td>
<td>20.94 ± 1.29</td>
<td>459 ± 19</td>
<td>15.63 ± 1.37</td>
</tr>
<tr>
<td>Evening fog</td>
<td>14.82 ± 0.45</td>
<td>24.91 ± 0.89</td>
<td>12.26 ± 0.57</td>
<td>18.70 ± 1.12</td>
<td>573 ± 49</td>
<td>13.06 ± 0.91</td>
</tr>
</tbody>
</table>
Figure A-4
Figure A-5
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PUBLICATIONS

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**White JC** and WK Smith. Water source partitioning in three *Rhododendron* species along an elevational gradient, southern Appalachian Mountains, USA. *Plant and soil*, in review.

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Berry ZC, JC White, and WK Smith. 2014. Foliar uptake, carbon fluxes, and water status are affected by the timing of daily fog in saplings from a threatened cloud forest. *Tree Physiology*, 34: 459-470.


**GRANTS AND CONTRACTS**

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White, JC and WK Smith. “Seasonal variation in water sources of the riparian tree species *Acer negundo* and *Betula nigra* in the southern Appalachian foothills, USA.” 75th Annual Meeting of the Association of Southeastern Biologists, Spartanburg, South Carolina (April 4, 2014).

White, JC and WK Smith. “Seasonal variation in water sources of the riparian tree species *Acer negundo* and *Betula nigra* in the southern Appalachian foothills, USA.” 111th Annual Meeting of the North Carolina Academy of Sciences, Raleigh, North Carolina (March 29, 2014).


White, JC and WK Smith. “Seasonal variation in water sources of the riparian tree species *Acer negundo* and *Betula nigra* in the southern Appalachian foothills, USA.”


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