An ecological and evolutionary perspective on functional diversity in the genus *Salix*

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Dedication

I dedicate this dissertation to my grandparents because they laid the building blocks that made this possible, especially to my grandfather Arthur Brackett Hess (1923-2009) who really wanted to be here to see me graduate.

Table of Contents

List of Tables

List of Figures

List of Figures (cont.)

INTRODUCTION

Investigating the processes important to maintaining willow species (genus: *Salix*) distributions at two spatial scales

A central goal of ecology is to understand the mechanisms that determine species distributions and maintain species diversity at multiple spatial scales. In this research, I investigate how plant functional diversity impacts species distributions in willows and poplars (*Salix* and *Populus* (Salicaceae)) in local communities and across species' ranges. At Cedar Creek Ecosystem Science Reserve, a Long-Term Ecological Research site in eastern-central Minnesota, there are fifteen species in this plant family that occur within a 2,300 ha area. This high diversity is challenging to explain, as ecological theory predicts that closely related species should rarely co-occur since their shared ancestry often leads to physiological similarity, increased competition for resources and similar susceptibility to pests and pathogens (Elton 1946, MacArthur and Levins 1967, Webb et al. 2006, Gilbert and Webb 2007). In this dissertation, I present data from four experiments aimed at understanding the mechanisms that maintain local willow diversity both within habitats (α-diversity) and across the landscape (β-diversity) (Bazzaz 996, Whittaker et al. 2001, Silvertown 2006). I also address whether there is evidence that functional trade-offs limit species distributions both locally across the landscape and latitudinally across species' ranges.

In the first two chapters, I examine whether differences in species drought survival strategies could influence their habitat specialization and thus help maintain their high β-diversity across the landscape. I studied species drought tolerances because willows are known for being limited by water availability (Amlin and Rood 2002, Karrenberg et al. 2002, Pockman and Sperry 2000) and many plant species segregate across soil moisture gradients based on their drought tolerance (Brodribb and Hill 1999, Cavender-Bares and Holbrook 2001, Maherali et al. 2004). I compare the physiology of two groups of species that differ in their habitat affinities: wetland specialists (species that occur in perennially saturated habitats) and habitat generalists (species that occur in a variety of habitats that vary seasonally in water availability). In Chapter 1, I focus on differences in species' ability to protect their leaves from

photodamage under drought conditions. When water is limited, photosynthetic activity decreases, resulting in an excess of absorbed light that can be damaging to the leaf. One way that plants avoid damage to their photosystems is by up-regulating pigments such as xanthophylls, which can safely dissipate excess energy (Demmig et al. 1987, Adams and Demmig-Adams 1994). In this experiment, I tested whether species from drier, more seasonally variable habitats exhibited a greater capacity for xanthophyll-mediated thermal dissipation under drought conditions.

In Chapter 2, I examine differences in species water-use, senescence and overall drought survival strategies. Since willows are drought deciduous, differences in the timing of their stomatal closure and leaf abscission play a critical role in determining their ability to avoid potentially damaging tension in their xylem. Similar to Chapter 1, this chapter examines whether species with different habitat affinities exhibit unique responses to drought conditions. However, in this study, I also examine whether species demonstrate physiological divergence that could facilitate their co-occurrence and maintain α-diversity. If species temporally or spatially partition water resources in a manner that reduces their competitive interactions, species drought survival strategies could also be important in facilitating their co-occurrence within habitats when water is limited.

A mechanism that can minimize competitive interactions between species is niche differentiation. Niche differentiation occurs when there are functional trade-offs along resource gradients that prevent species from performing well across the entire gradient (Hutchinson 1957, Tilman 1982, Bazzaz 1996). In Chapter 3, I investigate whether there is evidence for niche differentiation across a water availability gradient in Minnesota, and consider whether this type of differentiation could explain the high diversity of willows and poplars across the landscape. I take both a functional approach, investigating the physiological basis of species distributions and determining whether there is evidence for important functional trade-offs, and an ecological approach, examining species patterns of co-occurrence and niche overlap in field plots. To further investigate the processes that influence species patterns of co-occurrence, I examine the extent to which species functional similarity, evolutionary trait lability, and phylogenetic community structure provide evidence for the role of environmental filtering in community assembly.

In the last chapter, I examine species distributions at a larger spatial scale to determine whether there

is evidence for functional trade-offs that could influence species' range limits. It has long been hypothesized that the biophysical costs associated with freezing tolerance cause a trade-off between freezing tolerance and growth rate that is critical to explaining species' northern and southern range limits (MacArthur 1972, Woodward and Pigott 1975, Loehle 1998). Despite the proposed importance of this trade-off, there has been relatively limited evidence for its existence and its applicability to different systems. In this study, I examine evidence for this trade-off in the willow family, and consider some of the complications with relating it to species distributions. This research has implications in understanding the relationship between species' ranges and climatic conditions, and serves as a starting point for investigating some of the ramifications of this potential trade-off.

Overall, my research addresses central to ecology relating to processes that influence species distributions and patterns of co-occurrence at multiple spatial scales. By focusing on a single plant family in a series of interconnected experiments, this research provides a comprehensive perspective on the physiology and ecology of willows and poplars. This approach also enables me to address questions related to the co-occurrence of closely related species. All of these experiments together provide a unique perspective on the ecology and physiology of an ecologically and economically important plant family.

CHAPTER 1

Willow species (genus: *Salix*) with contrasting habitat affinities differ in their photoprotective responses to water stress

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Although many Mediterranean and xeric plant species enhance their xanthophyll-mediated thermal dissipation under drought conditions, there has been limited research on photoprotective mechanism in droughted plants from other habitats. To investigate whether wetland plants utilize this mechanism under drought conditions, and whether species differ in their responses depending on their habitat affinities, we investigated the response of six willow (*Salix*) species to a short-term drought. In a greenhouse, 40 individuals per species were dried down over 4 weeks. Periodically during the drought, predawn and midday chlorophyll fluorescence measurements were taken and leaf discs were collected for pigment analysis with HPLC. Predawn water potential was also monitored throughout the experiment. All six species increased xanthophyll cycle activity and their capacity to dissipate excess energy during the drought by increasing their total de-epoxidized xanthophyll concentration and the concentration of zeaxanthin in proportion to chlorophyll. In general, habitat generalists had greater photoprotective responses than wetland specialists, while the wetland specialists had higher pre-drought nonphotochemical quenching. These differences are consistent with their contrasting photosynthetic rates. The observed variation in species drought responses suggests that their photoprotective strategies vary with habitat affinity

INTRODUCTION

Many plant species segregate along soil moisture and larger-scale precipitation gradients based on their drought tolerance, as a result of trade-offs in physiological and life history traits that prevent them from performing well under all environmental conditions (Whittaker 1956; Brodribb and Hill 1999; Silvertown et al. 1999; Cavender-Bares et al. 2004b). Although there has been substantial research investigating the role of xylem trade-offs in determining species distributions (Zimmermann and Brown 1977; Pockman and Sperry 2000; Maherali et al. 2004; Hacke et al. 2006), there has been less research on potential trade-offs relating to species' photoprotective mechanisms. These mechanisms are important in preventing irreversible damage to chloroplasts under drought conditions (Demmig et al. 1988; Flexas and Medrano 2002), and may play a role in determining species distributions across soil moisture gradients.

Under drought conditions, plants close their stomata, resulting in a build-up of carbon dioxide inside the leaf, and a reduction in photosynthetic activity. This results in an excess of absorbed light that can create reactive oxygen species and lead to chloroplast and photosystem damage. Plants utilize four different mechanisms to prevent photodamage and safely dissipate excess energy: the xanthophyll cycle (Demmig et al. 1987; Adams and Demmig-Adams 1994), photorespiration (Osmond and Grace 1995; Kozaki and Takeba 1996), Mehler reactions (Osmond and Grace 1995; Biehler and Fock 1996) and cyclic electron transport (Katona et al. 1992). Of these four mechanisms, the xanthophyll cycle is responsible for dissipating the majority of excess energy under drought conditions (Flexas and Medrano 2002; Demmig-Adams and Adams 2006). It is also used by plants to dissipate energy under high light, low nutrient availability, and during exposure to freezing temperatures (Adams and Demmig-Adams 1992; Demmig-Adams et al. 1995; Lovelock et al. 1995; Verhoeven et al. 1999; Cavender-Bares et al. 2005).

There is substantial evidence that Mediterranean (e.g. García-Plazaola and Becerril 2000; Kyparissis et al. 2000; Martínez-Ferri et al. 2000; e.g. Galmés et al. 2007) and xeric plant species (e.g. Balaguer et al. 2002; e.g. Barker et al. 2002) increase xanthophyll-mediated thermal dissipation under drought conditions, and that tropical species utilize these processes under high light conditions (e.g. Lovelock et al. 1994; Barker et al. 1997; Watling et al. 1997; Montgomery et al. 2008). However, there has

been limited research on the photoprotective responses of mesic and hydric species to drought conditions. Since photodamage is dependent on stomatal behavior, not necessarily the severity of a drought, these species may benefit from photoprotective mechanisms if they experience prolonged stomatal closure under high light conditions (Bota et al. 2004; Flexas et al. 2006). Furthermore, if there is a cost associated with increasing xanthophyll concentrations and maintaining xanthophyll cycle activity, species may vary in their photoprotective capacity depending on the frequency and longevity of water stress they encounter in their native habitats.

To address the importance of xanthophyll-mediated thermal dissipation in plants that occur in habitats with different seasonal water availability, we examined the drought responses of six willow species (genus: Salix) to a four week dry-down. Since willows are highly dependent on water availability (Amlin and Rood 2002; Karrenberg et al. 2002), and exhibit significant variation in habitat affinities along a soil moisture gradient (Morley 1969; Gleason and Cronquist 1991), they are an excellent study system for this research. The goal of this study was to address two key questions: (1) Do drought intolerant willow species exhibit enhanced xanthophyll-mediated energy dissipation under drought conditions? (2) Do species' photoprotective responses depend on their ecological habitat of origin?

MATERIALS AND METHODS

Species selection

We selected six willow species (genus: *Salix*) native to Minnesota for our study, including three wetland specialists and three broader habitat generalists, which we classified based on habitat descriptions from Morley (1969) and Gleason and Cronquist (1991). The three wetland species (*S. candida* Flueggé ex Willd., *S. pedicellaris* Pursh, and *S. pyrifolia* Andersson) primarily grow in fens, bogs and wet meadows in Minnesota, with *S. candida* inhabiting more alkaline wetlands and *S. pyrifolia* inhabiting more acidic wetlands. The three broader habitat generalists (*S. bebbiana* Sarg., *S. discolor* Muhl., *S. petiolaris* Sm.) occur in a variety of habitats including prairies, moist meadows, alluvial habitats and lakeshores. They tend

to occur in habitats that have more seasonal variation in water availability than the three wetland species.

Growth and dry-down conditions

In the spring of 2004, we propagated six native willow species from seed collected in south-central Minnesota at the Cedar Creek Ecosystem Science Reserve. We grew the willows in a greenhouse at the University of Minnesota, which was set to be 20°C year-round and achieved temperatures of 27°C on warm summer days. The plants were kept well-watered and fertilized for 2 years. By the summer of 2006, plant height and stem diameter (averaged across 10 individuals per species) were 89.3 ± 3.15 cm (one standard error) and 7.60 ± 0.27 mm (s.e.), respectively. Three weeks before the start of the experiment, we transplanted the plants into 6.25 L treepots. At this point we also measured the total leaf area of a subset of eight plants per species.

We began the dry-down treatment in June 2006. Plants were watered to field capacity and allowed to dry out over four weeks. We took measurements on six individuals per species, at three points in the experiment: pre-drought (day 0), mid-drought (day 15), and late drought (day 30). Plants were at field capacity during the pre-drought measurements, and were illuminated for 12 hours per day giving a midday light intensity of ~700 – 800 μmol on sunny days.

Predawn water potential measurements

We measured leaf water potential (Ψ_{PD}) using a Scholander pressure chamber (Soil Moisture Equipment Corp., Santa Barbara, CA) 2 hours before dawn each day of our measurements. We removed the leaves with razor blades, put them in plastic bags, and transferred them to the pressure chamber.

Chlorophyll fluorescence and gas exchange measurements

We measured chlorophyll fluorescence on dark- and light-acclimated leaves for six individuals per

species, using a pulse amplitude modulated chlorophyll fluorescence meter (LI-COR 6400–40, Li-Cor Inc., Lincoln, NE). We measured minimal dark-adapted fluorescence (F_o), and maximum dark-adapted fluorescence (F_m) (using a saturating pulse of 7000 μmol m^{-2} s^{-1} for 0.8 s), in the 2 hr period before dawn on one leaf per plant. We marked the spot on the leaf where the measurement was taken and measured steady-state fluorescence (F_s), and maximum fluorescence ($F_{m'}$) on the same spot on illuminated leaves between 1300 and 1500 hours the same day. A far-red pulse (740 nm) was then applied to excite PSI and thereby oxidizing PSII reaction centers for a measurement of $F_{o'}$. During the afternoon measurements, we also measured CO_2 assimilation at a light intensity of 1200 μmol m^{-2} s^{-1} (A_{1200}). We selected this light intensity because it was greater than ambient light levels and is known to saturate photosynthesis in some willow species (Robinson et al. 2004). We measured the first fully-expanded, living leaf on the main stem of each plant but were unable to follow the same leaves throughout the experiment because of leaf senescence. For our analysis, we calculated: maximum photochemical efficiency of PSII, F_v/F_m (where $F_v = F_m - F_o$); light-acclimated photochemical efficiency, $\Delta F/F_{m'}$ (where $\Delta F = F_{m'} - F_s$); electron transport, ETR (assuming a leaf absorbance of 0.8 and equal photon excitation of PSII and PSI); photochemical quenching, qP $[(F_{m'} - F_s)/(F_{m'} - F_o)]$; nonphotochemical quenching, qN $[(F_m - F_{m'})/(F_m - F_{o'})]$ (Schreiber et al. 1986); and Stern-Volmer nonphotochemical quenching, NPQ $[(F_m - F_{m'})/F_{m'}]$.

Pigment analysis

Immediately after taking each chlorophyll fluorescence measurement, we punched a leaf disc from a nearby leaf with an 8 mm diameter core borer. The discs were put in microcentrifuge tubes and dropped into liquid nitrogen. These samples were kept in a –80°C freezer until they were used for pigment analysis. Pigments were extracted according to Adams and Demmig-Adams (1992). Samples were analyzed by HPLC using an Allsphere ODS-1 (5 μm particle size, 250 × 4.6 mm) column (Alltech Chromatography, Deerfield, IL, USA). Solvents and method used are as described by Gilmore and Yamamoto (1991), however, midway through the analysis of samples the peaks began running together, so the A solvent was adjusted. The two A solvents both consisted of acetonitrile: methanol: 0.1 M Tris, pH 8.0 with a ratio of

78:8:3 for the first A solvent and 72:17:5 for the second A solvent. Adjusting the solvent altered the retention time of both chlorophylls, which were calibrated separately for each solvent. We calculated pigment concentrations on an area basis and the de-epoxidation state of the xanthophylls (DPS) as (Z + A)/(V + A + Z), where *Z* is zeaxanthin, *A* is antheraxanthin and *V* is violaxanthin.

Statistical analysis

We used repeated-measures ANOVA to analyze pigment, chlorophyll fluorescence and gas exchange measurements over time across species. Since there were only two individuals of *S. discolor* remaining on the last day of measurement, we presented the ANOVA analysis excluding this species. However, when we conducted the analyses including *S. discolor*, there were no qualitative differences in the results (data not shown). We used a t-test to examine the differences between habitat generalists and wetland specialists at each time interval (days 0, 15 and 30). We also used Tukey's multiple comparisons to check for differences between species within the same habitat classification. To investigate differences in species' stomatal conductance, we completed a multiple regression analysis on the relationship between stomatal conductance and predawn water potential. Since stomatal conductance is non-linearly related to predawn water potential, we first logged the two axes. We also completed a regression analysis on species' total leaf area and their decline in predawn water potential from day 0 to day 15. P-values <0.05 were considered significant and values <0.1 were considered marginally significant. All analyses were conducted with JMP 7.0 (SAS Institute, Raleigh, NC).

RESULTS

Progression of dry-down

Predawn water potential significantly decreased over time ($F = 56.5$, $df = 2, 18$, $P<0.0001$) during the dry-down (Fig. 1.1a), and this decrease corresponded with a decrease in stomatal conductance (g_s) in all

the species (F =118, df = 97, P<0.0001, Fig. 1.1b). Across species, there was also a correlation between the decline in water potential from day 0 to 15 and their total leaf area (Fig. 1.1c, F = 14.7, d f= 5, P = 0.019). By mid-drought, the habitat generalists had significantly lower predawn water potentials than the wetland specialists (Fig. 1.1a), but there were no significant differences between species within the two groups. Late in the drought, the wetland specialists had achieved predawn water potentials equivalent to the mid-drought measurements of the habitat specialists. There was no habitat effect in the log–log regression of predawn water potential on stomatal conductance.

All six willow species demonstrated drought-induced senescence in response to the dry-down. In several species, the senescence occurred rapidly and multiple plants lost all their leaves by day 30. This resulted in sample sizes of 4, 6, 2, 4, 5 and 6 in the late drought for *S. bebbiana, S. candida, S. discolor, S. pedicellaris, S. petiolaris* and *S. pyrifolia*, respectively. After the experiment, we dried-down a subset of individuals from each species until they lost their leaves and then rewatered them. Almost 60% of the plants resprouted after rewatering, but there was no difference between the resprouting ability of the habitat generalists and the wetland specialists (data not shown).

Carbon assimilation

All species demonstrated a significant decrease in light saturated carbon assimilation (A_{1200}, F = 44.6, df = 2, 18, P<0.0001) and stomatal conductance (g_s) over time (F = 39.3, df = 2, 18, P<0.0001). However, the habitat specialists closed their stomata more rapidly, resulting in significantly lower g_s, than the wetland specialists by the mid-drought. This resulted in lower A_{1200} in the habitat generalists despite their higher initial A_{1200} (Table 1.1).

Chlorophyll florescence

After the onset of the drought, photochemical quenching (qP, F =16.8, df = 2, 18, P <0.0001), light quantum efficiency ($\Delta F/F_{m'}$, F = 245.3, df = 2, 18, P<0.0001) and electron transport rates (ETR, F =

244.2, df = 2, 18, P<0.0001) declined in all species. Although habitat generalists had significantly higher ETR pre-drought, their ETR declined more rapidly than the wetland specialists (Table 1.1). A similar trend was observed in light quantum efficiency, as $\Delta F/F_{m'}$ was higher in habitat generalists than wetland specialists on day 0 but lower on day 15 (Fig. 1.2c & d). Meanwhile, qP demonstrated no difference between the habitat groups during the drought (Table 1.1).

The dark quantum efficiency (F_v/F_m) of PSII also significantly decreased during the drought (F = 9.6, df = 2, 18, P<0.0001, Fig. 1.2a & b) but the decrease was small. The pre-drought average across species was 0.823 ± 0.002 (± s.e., n = 36) and the late drought average was 0.754 ± 0.006 (± s.e., n = 26). Habitat generalists had slightly higher F_v/F_m before the drought but there were no significant differences between the two groups after the onset of the drought (Fig. 1.2c & d).

Across all species, there was a significant increase in nonphotochemical quenching calculated as NPQ (F = 7.3, df = 2, 18, P=0.005, Table 1.1) and as qN (F =11.6, df = 2, 18, P = 0.0006, Fig. 1.2e & f). Pre-drought, the wetland specialists had significantly higher NPQ and qN than the habitat generalists and this difference remained until the mid-drought in regards to qN ($\alpha = 0.05$). There were no significant differences in qN or NPQ between the species within each group.

Pigment analysis

All species exhibited a decline in the measured leaf pigments ($\alpha = 0.05$) during the drought and in the ratio of chlorophyll a/b (Table 1.2). Lutein, neoxanthin and chlorophyll were significantly different between habitat generalists and wetland specialists in the late drought. However, these differences disappeared when lutein and neoxanthin were considered in proportion to chlorophyll (μmol pigment/mol chlorophyll). The ratio of β-carotene/chlorophyll did not change over time, and the ratio of lutein/chlorophyll increased during the drought (F =14.8, df = 2, 18, P = 0.0002, Table 1.2). There was no evidence for the presence of lutein epoxide in any of the species.

Across all species, the total xanthophyll concentration (violaxanthin, antheraxanthin and zeaxanthin) in the willow leaves significantly changed over time (F = 9.5, df = 2, 18, P = 0.002, Fig. 1.3).

Additionally, both the de-epoxidation state of the xanthophylls (DPS, $F = 15.5$, $df = 2, 18$, $P = 0.0002$, Fig. 1.3) and the ratio of zeaxanthin/chlorophyll significantly increased ($F = 34.2$, $df = 2, 18$, $P<0.0001$, Fig. 1.4). The increase in DPS correlated linearly with an increase in qN (Fig. 1.5a) and NPQ (Table 1.1), and was greater in habitat generalists than in wetland specialists (Fig. 1.5b). Habitat generalists also had significantly higher ratios of zeaxanthin/chlorophyll and violaxanthin/chlorophyll (Fig. 1.4) during the mid-drought. However, the ratio of antheraxanthin/chlorophyll was only significantly higher in the habitat generalists late in the drought ($P = 0.03$, Fig. 1.4). The greater DPS values of the habitat generalists in both the mid-($P = 0.07$) and late drought ($P = 0.08$, Fig. 1.4) were slightly significant, indicating that the habitat generalists may have greater xanthophyll cycle activity than wetland specialists. This relationship was stronger when species were compared at similar predawn water potentials (habitat generalists, day 15 and wetland specialists, day 30) ($P = 0.02$).

When considered individually, the six willow species demonstrated distinct responses to the imposed drought treatment. While *S. discolor* exhibited the greatest increase in zeaxanthin, both in absolute concentration and concentration in proportion to total chlorophyll, *S. pyrifolia* and *S. pedicellaris* demonstrated relatively small changes in zeaxanthin concentration (Fig. 1.4). Three of the species (*S. bebbiana, S. discolor* and *S. candida*) also increased their total xanthophyll concentrations by mid-drought (Table 1.2).

DISCUSSION

Photoprotection and the xanthophyll cycle

This experiment demonstrated that six willow species which are relatively drought intolerant can dissipate excess energy through the xanthophyll cycle in response to drought conditions. During a four week dry-down, these species exhibited both an increase in their de-epoxidated xanthophyll concentrations (DPS) and their nonphotochemical quenching (qN, Figs 2, 3, 5). Furthermore, some of the species increased their capacity to dissipate excess energy by up-regulating xanthophyll production. This is

significant because leaf light absorption declines with chlorophyll level and an increase in the proportion of zeaxanthin to chlorophyll (Fig. 1.4) enhances a plant's photoprotective capacity.

All six of the species in this study minimized photodamage and maintain high maximum photosynthetic efficiency (F_v/F_m, Fig. 1.2a & b) during the drought in some of their leaves. However, most of the species also exhibited significant leaf loss and senescence. This is important to note because high light is known to shorten leaf life span in many species (Williams et al. 1989; Cavender-Bares et al. 2000), and there is evidence that photodamage may play a role in leaf senescence (Lovelock et al. 1994). It is, therefore, possible that the species in this study did experience photodamage during the dry-down but it went undetected because we do not have measurements immediately before senescence on individual leaves.

Leaf chlorophyll loss

All six species in this study demonstrated a decline in leaf chlorophyll content during the dry-down. Chlorophyll loss is common under drought conditions in other deciduous species (Munné-Bosch et al. 2001), and also some perennial grasses (Balaguer et al. 2002) and evergreen species (Martínez-Ferri et al. 2000; Munné-Bosch and Alegre 2000). Although chlorophyll loss can result from oxidative damage, there is increasing evidence that it can also result from enzyme-mediated processes (Matile et al. 1999). These processes may be advantageous, as they reduce light absorption and can limit the amount of damaging excess energy in the leaf (Adams et al. 1990; Munné-Bosch et al. 2001). We also note that all six willow species exhibited a slower degradation of chlorophyll a than chlorophyll b over time. This pattern has been observed in several other senescing species (Biswal 1995; Suzuki and Shioi 2004) but is not ubiquitous (Munné-Bosch et al. 2001).

Drought deciduousness and photoprotection

Xanthophyll cycle activity and chlorophyll loss played an important role in the photoprotective

responses of these six willow species during the dry-down, but these processes were only effective in preventing leaf damage under mild to moderate drought conditions. After four weeks of drought, many of the plants began to senescence. Although this senescence was not lethal, and many plants resprouted after rewatering, it did indicate that these species rely on other mechanisms to survive more severe droughts.

Plant drought response strategies are generally broken into two categories: drought avoidance and drought tolerance. Some plants 'avoid' drought conditions and high xylem tensions by rapidly closing their stomata, and other plants 'tolerate' drought conditions by maintaining function at low water potentials. Similar to other early successional species (Martínez-Ferri et al. 2000), the six willows in this experiment are drought avoiders and thus minimize photodamage by reducing their leaf chlorophyll levels, and limit water loss by dropping their leaves (Martínez-Ferri et al. 2000; Munné-Bosch and Peñuelas 2003; Munné-Bosch and Alegre 2004). Willows are also effective drought avoiders because they are avid resprouters (Newsholme 1992; Karrenberg et al. 2002), and some species can refill cavitated vessels (Utsumi et al. 1998).

The ecological significance of species' responses

The habitat generalists (*S. bebbiana, S. discolor*, and *S. petiolaris*) and the wetland specialists (*S. candida, S. pedicellaris,* and *S. pyrifolia*) demonstrated significant differences in their function even before the initiation of the drought treatment. The wetland specialists, on average, had lower pre-drought photosynthetic activity (A_{1200}), light-acclimated photochemical efficiency ($\Delta F/F_{m'}$) and overall leaf area, and higher qN and DPS than the habitat generalists (Table 1.1, Figs 1, 2). These differences may be indicative of contrasting growth strategies in the species. In general, plants from nutrient-limited systems such as wetlands are known to have more conservative growth strategies than species in more productive environments (Grime and Hunt 1975; Chapin 1980; Reich 1993). If this is the case with willows, then differences in species' pre-drought and drought physiology could be related to their distinct growth strategies. It is also possible that wetland plants specifically benefit from higher levels of qN and DPS under well-watered conditions because of the greater chance of waterlogging in their native habitats. Since

waterlogging causes a reduction in photosynthesis, it can also lead to photodamage in some plants (Close and Davidson 2003).

After the initiation of the drought, the habitat generalists and wetland specialists continued to diverge in physiological function. The habitat generalists' greater leaf areas likely contributed to their large declines in predawn water potential by mid-drought (Fig. 1.1c). At that point the habitat generalists were demonstrating more conservative water use and lower rates of stomatal conductance (g_s) than the wetland specialists. This resulted in lower A_{1200} and electron transport (ETR) in these species (Table 1.1). Although the wetland specialists maintained greater photosynthetic function into the mid-drought, it is possible that under drought conditions, willows benefit from dormancy. In general, the habitat generalists senesced before the wetland specialists and by the end of the dry-down, 39% of the habitat generalists had lost all of their leaves. When the plants were rewatered, there were no significant differences in the resprouting ability of the wetland specialists and the habitat generalists, however, we only investigated resprouting immediately after senescence and it is possible that species exhibit different responses after longer periods of dormancy.

Another difference in the drought responses of the habitat generalists and the wetland specialists involved their photoprotective activity. The habitat generalists, on average, exhibited the greatest photoprotective responses to the drought, as indicated by their larger increases in both *qN* and DPS (Fig. 1.5). Although the habitat generalists' lower predawn water potentials in the mid-drought can partially explain their higher photoprotective response, the wetland specialists still failed to increase their xanthophyll activity in the late drought when they had achieved comparable predawn water potentials. These results indicate that the wetland specialists exhibit smaller or slower photoprotective responses to drought than the habitat generalists, suggesting that there is a relationship between species' photoprotective ability and their habitat affinity.

The six willow species in the study also demonstrated differences in their carotenoid concentrations during the drought, but these differences did not correspond with their habitat classifications. Since many carotenoids such as lutein and β-carotene are involved in thermal quenching and scavenging of reactive oxygen species (Dall'Osto et al. 2006), differences in their concentrations may

be important to species' drought tolerance. Furthermore, some plants use other antioxidants, including salicylic acid, to minimize photodamage under drought conditions (Yang et al. 2004; Abreu and Munné-Bosch 2007). Therefore, it is possible other pigments and antioxidants besides those measured are important to these species' drought response, but further research is needed to better understand these processes in willows.

Conclusions - Willows generally inhabit mesic and hydric habitats and are considered drought intolerant compared with many other plant species. Although willows rarely encounter severe droughts in their native habitats, they often encounter changes in water availability that can reduce their stomatal conductance (Pockman and Sperry 2000; Amlin and Rood 2002), making them susceptible to photodamage under conditions of excess light. This experiment demonstrated that willows are capable of increasing their xanthophyll-mediated thermal dissipation under drought conditions, and that the rate and extent of their response appears to vary with their habitat affinity.

Table 1.1 – Average chlorophyll fluorescence and gas exchange parameters of six willow species during the dry-down. The parameter abbreviations are as follows: electron transport chain (ETR), photochemical quenching (qP), nonphotochemical quenching (NPQ), maximum photosynthetic capacity (A_{1200}) in mol CO_2 m^{-2} s^{-1}, and stomatal conductance (g_s) in mol H_2O m^{-2} s^{-1}. P-values are from t-tests comparing habitat generalist and wetland specialists for each parameter (NS = not significant). Values are reported ± one standard error.

		Habitat generalists			Wetland specialists			
	Day	*S. bebbiana*	*S. discolor*	*S. petiolaris*	*S. candida*	*S. pedicellaris*	*S. pyrifolia*	P-value
ETR	0	143±10.7	143±10.7	161±7.67	147±7.38	136±4.55	108±6.50	0.02
	15	51.0±5.04	38.1±6.07	76.3±8.85	91.0±6.59	95.1±3.96	69.7±5.72	<0.0001
	30	37.6±4.64	37.7±11.9	39.3±4.98	44.9±10.1	35.5±6.54	65.3±1.43	NS
qP	0	0.68±0.04	0.68±0.03	0.63±0.04	0.75±0.03	0.89±0.07	0.84±0.12	0.006
	15	0.74±0.16	0.26±0.16	0.75±0.21	0.66±0.07	1.00±0.27	0.83±0.29	NS
	30	0.22±0.14	0.00±0.00	0.27±0.27	0.37±0.13	0.06±0.06	0.46±0.20	NS
NPQ	0	2.29±0.17	2.33±0.17	1.92±0.18	2.41±0.18	2.94±0.14	2.81±0.21	0.001
	15	3.19±0.21	3.30±0.43	3.00±0.23	2.58±0.12	2.82±0.28	3.10±0.33	NS
	30	3.82±0.57	3.35±0.00	2.84±0.37	3.19±0.34	3.19±0.57	2.73±0.23	NS
A_{1200}	0	13.52±2.40	15.9±1.96	17.1±1.64	13.9±1.22	14.0±1.16	8.89±1.13	0.03
	15	0.07±0.27	-0.47±0.35	2.31±0.83	5.54±0.93	6.26±1.06	4.18±1.04	<0.0001
	30	0.76±0.56	-0.10±0.43	0.81±0.16	2.00±1.21	1.66±0.74	2.46±0.98	0.01
g_s	0	0.20±0.05	0.24±0.04	0.25±0.05	0.18±0.02	0.25±0.04	0.13±0.03	NS
	15	0.03±0.01	0.03±0.01	0.04±0.01	0.09±0.01	0.17±0.04	0.07±0.01	<0.0001
	30	0.02±0.01	0.01±0.00	0.02±0.01	0.04±0.02	0.05±0.02	0.05±0.02	0.002

Table 1.2 – Average leaf pigment concentrations of six willow species during the dry-down. Pigments are indicated by the following abbreviations: neoxanthin (neo), lutein (lut), chlorophyll (chl) and β – carotene (β-c). There were no significant differences between habitat generalists and wetland specialists in these pigment concentrations. Pigments that significantly ($\alpha = 0.01$) changed during the drought based on a repeated measures ANOVA where n = 26 are marked with an asterisk. Values are reported ± one standard error.

		Habitat generalists			Wetland specialists		
	Day	*S. bebbiana*	*S. discolor*	*S. petiolaris*	*S. candida*	*S. pedicellaris*	*S. pyrifolia*
neo/ chl	0	34.0±16.5	33.0±5.16	35.5±8.05	40.4±7.63	38.0±13.8	33.8±5.81
(μmol mol^{-1})	15	41.4±3.50	36.2±5.60	40.0±3.44	35.2±9.76	43.7±6.61	42.3±9.86
	30	53.8±15.3	51.0±9.01	59.7±13.2	46.1±4.52	59.5±27.4	55.1±17.0
lut/ chl*	0	116±19.5	85.7±9.11	91.8±15.7	86.3±17.7	98.9±14.6	101±13.7
(μmol mol^{-1})	15	125±11.0	119±6.96	111±16.3	103±13.1	124±34.7	121±9.85
	30	131±24.1	121±10.4	170±35.7	139±52.1	159±53.5	154±38.5
B -c/ chl	0	94.9±8.16	79.2±5.09	89.6±7.84	87.5±4.00	84.6±5.29	87.7±2.34
(μmol mol^{-1})	15	89.5±3.43	87.8±3.67	74.1±3.52	82.4±7.18	82.3±4.54	81.2±11.6
	30	92.4±7.50	73.7±2.2	103±11.7	91.2±16.4	81.7±12.3	88.5±6.33
chl*	0	36.6±6.90	57.3±7.51	55.9±6.92	45.1±4.09	49.0±7.61	32.7±4.05
(nmol cm^{-2})	15	37.2±7.67	34.4±2.90	48.2±5.97	47.6±2.29	40.7±7.32	29.6±3.65
	30	13.7±4.6	27.8±8.25	17.2±2.07	28.7±4.60	27.8±8.25	17.2±2.07
chl a/b*	0	2.66±0.20	2.77±0.08	2.67±0.38	2.78±0.35	2.77±0.12	3.18±0.16
	15	3.32±0.29	2.75±0.17	3.44±0.21	3.14±0.18	3.38±0.12	2.85±0.18
	30	3.89±0.40	3.55±0.01	3.37±0.14	3.63±.011	3.27±0.17	2.91±0.18

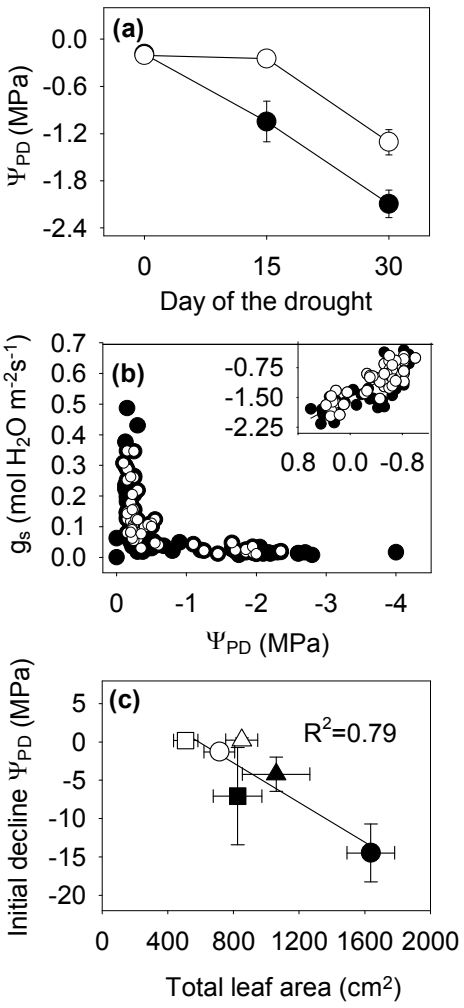

Figure 1.1 (a) Habitat generalists (closed symbols) demonstrated a more rapid decline in predawn water potential (Ψ_{PD}, MPa) than wetland specialists (open symbols) during the drought**. (b)** Ψ_{PD} correlated with stomatal conductance (g_s, mol H_2O m^{-2} s^{-1}) in both habitat generalists and wetland specialists. Each point represents measurements taken on one plant, one day during the drought. The inset graph is the log-log graph of the data. (c) The willow species with greater total leaf area (cm^2) demonstrated the greater losses in Ψ_{PD} during the drought. Species symbols are: ▲*S. bebbiana*, ● *S. discolor*, ■ *S. petiolaris*, Δ *S. candida*, ○ *S. pedicellaris*, and □ *S. pyrifolia*. Error bars are ± one standard error.

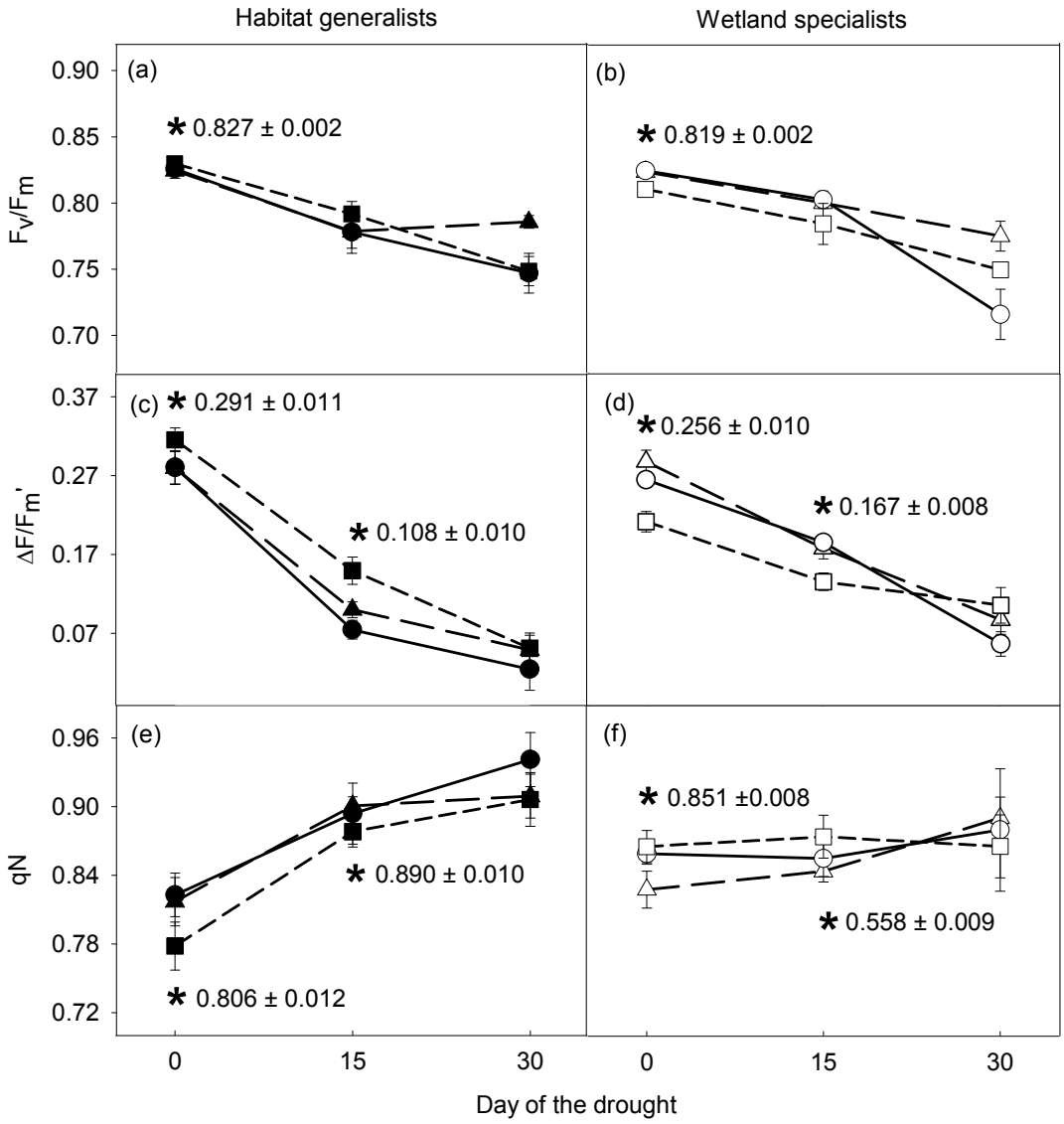

Figure 1.2 Maximum photochemical efficiency of PSII, F_v/F_m, **(a & b)**, light-acclimated photochemical efficiency, ΔF/Fm', **(c & d)** and nonphotochemical quenching, qN, **(e & f)** differed between wetland specialists (open symbols) and habitat generalists (closed symbols) during the drought. Error bars are ± one standard error and asterisks mark where the habitat generalists significantly differ from the wetland specialists ($\alpha = 0.05$). The reported values are the species' averages are ± one standard error. Species symbols are the same as Fig. 1.1.

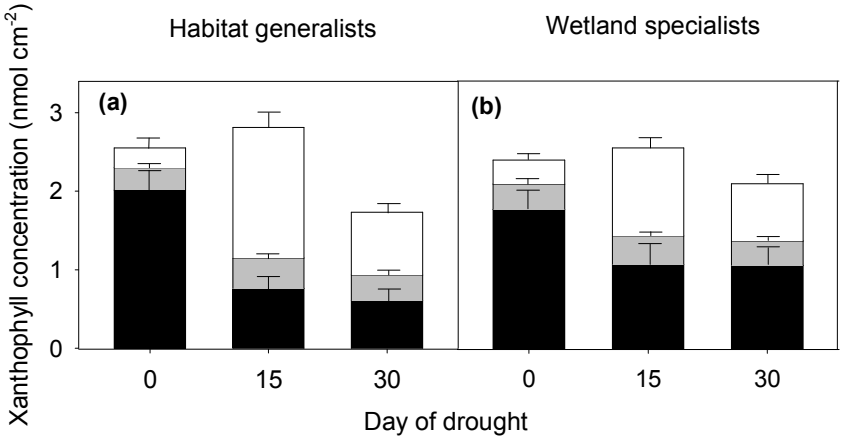

Figure 1.3 The de-epoxidation state (A + V)/(V + A + Z) of the xanthophylls increased during the drought in all species. The xanthophyll epoxidation states are labeled as follows: violaxanthin (V, black), antheraxanthin (A, gray) and zeaxanthin (Z, white). There was no significant difference between the xanthophyll pool size (nmol cm^{-2}) in the habitat generalists and the wetland specialists, although the generalists had slightly higher DPS values. Error bars are ± one standard error.

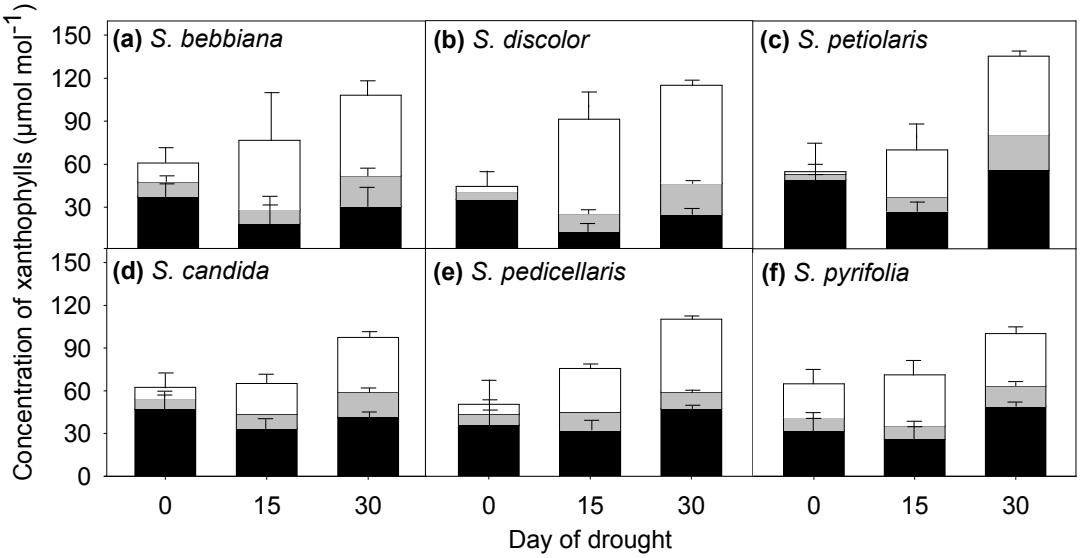

Figure 1.4 The concentrations of violaxanthin, antheraxanthin and zeaxanthin measured as µmol pigment per mol chlorophyll changed during the drought in all species. Zeaxanthin concentrations per unit chlorophyll were significantly higher in habitat generalist **(a-c)** than wetland specialists **(d-f)** on day 15 ($p = 0.03$). The xanthophyll epoxidation states are marked the same as in Fig. 1.3. Error bars are ± one standard error.

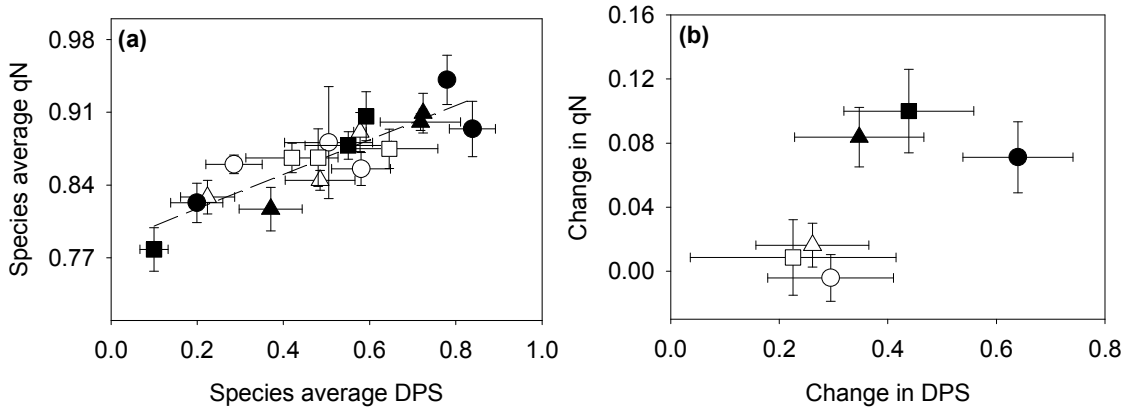

Figure 1.5 (a) Nonphotochemical quenching (qN) correlated with the de-epoxidation state of the xanthophylls (DPS) across all the species. Each point represents the average qN and DPS values for each species on each day of measurement. **(b)** Habitat generalists exhibited a greater increase of qN and DPS from day 0 to day 15 than the wetland specialists. Species symbols are the same as Fig. 1.1. Error bars are ± one standard error.

CHAPTER 2

Divergence in drought survival strategies of sympatric willow (genus: *Salix)* species

A central goal of ecology is to understand the mechanisms that determine species distributions and maintain high species diversity at multiple spatial scales. High levels of co-occurrence are especially challenging to understand among closely related species because they tend to share similar functional traits and be regulated by density-dependent effects such as competition and shared pest and pathogen susceptibility. To investigate whether traits related to willow species (genus: *Salix*) drought survival are important in maintaining species β-diversity across the landscape and α-diversity within habitats, we compared the response of six willow species from different habitats to an experimental dry-down. We found that species that occur in drier, more seasonally variable habitats have higher water-use efficiency, better stomatal control and faster growth rates than species from seasonally wetter habitats. However, the greatest physiological divergence occurred between two species from the same habitats in traits related to leaf senescence and hydraulic conductivity. These two species demonstrated distinct drought survival strategies that varied in their temporal use of water under drought conditions, in a manner that could minimize competition between these species when water is limited. We conclude that willow species growth patterns and drought responses may be important in driving habitat differentiation across a water availability gradient as well as causing temporal differentiation in resource utilization within habitats. Water-use strategies could therefore be important in the maintenance of both willow α- and β-diversity across the landscape.

INTRODUCTION

Across and within plant lineages, there is a strong correlation between species' hydraulic architecture and their distributions across soil moisture and larger-scale precipitation gradients (Brodribb and Hill 1999, Pockman and Sperry 2000, Cavender-Bares and Holbrook 2001, Maherali et al. 2004). Functional trade-offs associated with drought tolerance largely account for this correlation and are likely important in facilitating niche separation and maintaining species diversity across the landscape (β-diversity) (Silvertown et al. 1999, Cavender-Bares et al. 2004b, Choat et al. 2007). However, functional divergence can also be important in facilitating co-occurrence within habitats, especially if it leads to differential resource partitioning (Elton 1946, Tilman 1982). For example, in habitats experiencing periods of limited water availability, co-occurring species may compete heavily for water, and functionally distinct species that spatially or temporally partition water-use may be more likely to co-occur. Under these conditions, traits related to water-use, such as hydraulic architecture, may play a critical role in promoting both α-diversity (within habitats) and β-diversity (across habitats) across the landscape.

Throughout North America, species in the genus *Salix* (willows) occur in sympatry and have both high α- and β-diversity. Since willows are closely related to each other and share a recent common ancestor, they should be functionally similar and compete more heavily for limited resources than more distantly related species (Darwin 1859, Elton 1946). As a result, willows should demonstrate limited co-occurrence within habitats unless there has been significant divergence in functional traits related to resource use (Losos et al. 2003, Cavender-Bares et al. 2004, Fine et al. 2005). While willow species segregate along a water availability gradient based on their drought tolerance compared to more xeric species (Pockman and Sperry 2000), there has been limited research investigating the mechanisms important in determining habitat specialization within the genus.

Habitat specialization along a water availability gradient in many plant lineages is often attributed to a functional trade-off between drought tolerance and plant productivity (Hacke and Sperry 2001, Cochard et al. 2007). Under drought conditions, plants experience increased tension in their xylem, which can lead to cavitation, reduced hydraulic conductance and even hydraulic failure (Zimmerman 1983, Sperry

and Pockman 1993, Rice and McArthur 2004). Plants that have a high resistance to cavitation are more drought tolerant, but they also have greater wood density (Hacke et al. 2001) and tend to have a lower hydraulic efficiency because of their small xylem conduits (Zimmerman 1983, Cochard 1992, Hargrave et al. 1994, Hacke et al. 2006). This is likely because high wood density prevents xylem implosion, and it is less probable that a small conduit will have a large pit that could cause the spread of emboli in the xylem (Wheeler et al. 2005, Hacke et al. 2006, Christman et al. 2009). As a result, drought tolerant plants tend to be less productive than drought intolerant plants, and many plant species specialize in different habitats based on their vulnerability to cavitation (Brodribb and Hill 1999, Pockman and Sperry 2000, Maherali et al. 2004).

While species' vulnerability to cavitation can limit their distributions, it may play a smaller role in determining the distributions of closely related species because many closely related species share similar hydraulic architecture (Choat et al. 2007, Willson et al. 2008). There is also evidence that many co-occurring species differ in their vulnerability to cavitation (Martinez-Vilalta et al. 2003, Vilagrosa et al. 2003). One reason for this pattern is that some species do not require high cavitation resistance to survive drought conditions. For example, some species survive short-term droughts via controlled cavitation in organs such as leaves, distal shoots and fine roots (Rood et al. 2000, Froux et al. 2005). This type of hydraulic segmentation is considered a drought avoidance strategy, as it allows plants to isolate expendable organs and minimize runaway cavitation (Zimmerman 1983, Tyree and Ewers 1991) thus preventing water loss and maintaining lower tensions in the xylem under drought conditions (Munné-Bosch and Alegre 2004). This type of hydraulic segmentation may be particularly effective in habitats that experience seasonal droughts where species are not carbon-limited year round (McDowell et al. 2008).

To understand how phylogenetically-related species co-exist in habitats that differ in water availability and to investigate the extent to which co-occurring species differ in their water use, we compared the drought survival strategies of six willow species that specialize in different habitat types. The main objectives of this study were to examine the potential role of species' drought survival strategies in limiting their geographic distributions across the landscape and patterns of co-occurrence within the same habitats, and to better understand mechanisms that promote willows' high level of α- and β-diversity.

MATERIALS AND METHODS

Study species

The six willow species (*S. bebbiana* Sarg., *S. candida* Flueggé ex Willd., *S. discolor* Muhl., *S. pedicellaris* Pursh*, S. petiolaris* Sm. and *S. pyrifolia* Andersson) in this study are native to North America and are common in the northern United States and Canada. In this study, we categorized species as either wetland specialists (*S. candida, S. pedicellaris*, and *S. pyrifolia*) or habitat generalists (*S. bebbiana, S. discolor*, and *S. petiolaris*) based on descriptions of their native habitats (Gleason and Cronquist 1991, Smith 2008) and our own field observations. The wetland specialists primarily grow in fens, bogs and wet meadows, and the habitat generalists occur in a variety of habitats including wetlands, alluvial sites, and upland prairies that tend to experience more seasonal variation in water availability.

Characterizing species' native habitats

In the summer of 2007, we randomly-established twenty-four 10m × 30m plots at Cedar Creek Ecosystem Science Reserve, a long-term ecological research site (Cedar Creek LTER) in southeastern Minnesota. We selected plot locations using a random number generator and a coordinate grid, and only established plots when willows were present. In the center of each plot, we measured the approximate depth to the water table monthly for a year in a buried 1-1.5 m long PVC pipe (3.8 cm diameter). We also measured the height of water above the soil when sites were flooded and considered plots that were saturated or on floating sphagnum mats to have a water table depth of zero.

Growing and drought conditions

All the plants used in this study were propagated from seeds collected at Cedar Creek LTER in the spring of 2004. Plants were grown in a greenhouse with an average daily temperature of 20ºC and kept

well-watered and fertilized. We grew the plants for two years before the drought treatment, at which point the plants were approximately 0.8-1 m tall. Two weeks prior to the treatment, we transplanted all the plants into 6.25 liter treepots to guarantee that they were not root bound. For the drought treatment, plants were allowed to dry-out over a two and a half month period (June-Aug 2006) after being watered to field capacity. This experiment was conducted concurrently with another study on species' photoprotective responses to drought on a separate set of plants (Savage et al. 2009). In 2008, we conducted a follow-up experiment comparing the drought responses of ten individuals of *S. bebbiana* and *S. petiolaris*. These measurements were made on plants propagated from seeds in spring 2007 and grown under similar conditions to the first set of plants.

Growth and leaf area

Before the drought, we harvested eight plants per species to determine their root (RMF), stem (SMF) and leaf (LMF) mass fractions, and the average growth rate (GR) over the two year period. We calculated growth rate as $[\log(m_2 - m_1)/(t_2 - t_1)]$ where m_2 and m_1 are the mass of the plants at times t_1 and t_2.. For these measurements, we cleaned the roots with a sieve and rinsed them in water to collect as much fine root material as possible. Then we separated leaf, stem and root material and dried the plants in an oven at 70°C for 5 days. We also measured total leaf area on each plant (reported in Savage et al. 2009) and calculated average specific leaf area (SLA) based on four fully expanded leaves per individual. Leaves were scanned on a flatbed scanner, and leaf area was measured digitally using ImageJ (Abramoff et al. 2004).

Soil moisture and predawn water potential

During the dry-down, we monitored predawn water potential (Ψ_{PD}) before dawn using a Scholander pressure chamber (Soil Moisture Equipment Corp., Santa Barbara, CA) in ten plants per species. We also estimated soil moisture by weighing the pots and calculating the percent of water in the

soil after factoring out dry plant weight, pot weight, and soil dry weight (which were measured after the completion of the experiment). We measured Ψ_{PD} and estimated soil moisture on twelve days during the dry-down (days 0, 6, 10, 14, 18, 21, 25, 27, 30, 36, 57 and 75). For our analyses, we assumed that Ψ_{PD} did not decline after each plant dropped all its leaves.

Gas exchange and leaf relative water content

We measured gas exchange on seven plants per species using a portable photosynthesis system (LICOR 6400-40, Licor Inc., Lincoln, NE) on nine days during the drought and when the plants were at field capacity (pre-drought conditions). These measurements were taken on the first fully-expanded, living leaf on each plant until the plants dropped all their leaves. Measurements were taken at a light intensity of 1200 μmol m^{-2} s^{-1} (known to saturate photosynthesis in willows (Robinson et al. 2004)) between 800 and 1100, using ambient CO_2 levels (approximately 380 μmol mol^{-1}). As a result of differential dieback, we had the following number of gas exchange measurements per species: *S. bebbiana* n = 57, *S. candida* n = 63, *S. discolor* n = 48, *S. pedicellaris* n = 59, *S. petiolaris* n = 54, *S. pyrifolia* n = 63.

Five times after measuring gas exchange, we measured the relative water content (RWC) of one fully expanded leaf per plant. Leaves were collected, placed in a plastic bag and put in a cooler. Four leaf discs per plant (total of 1.5 cm^2) were cut using a #4 cork borer. We avoided taking tissue samples that included the midvein whenever possible. The samples were weighed, put in tubes with 1 ml of ddH_20 and left for four hours. Afterwards, the samples were blotted dry and their turgid weight recorded. The leaf discs were then dried (70°C for a day) to determine their dry weight. Relative water content was calculated as (wet weight − dry weight)/(turgid weight − dry weight).

Pre-drought plant hydraulics

Before the drought treatment, we measured Ψ_{PD} on six plants per species (except *S. pyrifolia*, n = 5). We then selected a pair of recent, terminal, fully-expanded leaves for afternoon measurements. One of

these leaves was covered with parafilm and aluminum foil before dawn so that it could equilibrate with the stem (Brodribb and Holbrook 2003). During a two-hour period after noon, we measured gas exchange on the transpiring leaf to determine transpiration (E) per unit leaf area. Immediately after these measurements, we removed the leaf and measured leaf water potential (Ψ_{leaf}). We also measured the water potential of the non-transpiring leaf to estimate stem water potential (Ψ_{stem}).

We used these measurements to calculate leaf (K_{leaf}) and leaf specific whole plant (K_{plant}) hydraulic conductivity using the following equations: $K_{leaf} = E/(\Psi_{leaf} - \Psi_{stem})$, and $K_{plant} = E/(\Psi_{leaf} - \Psi_{soil})$. These equations are based on an Ohm's Law analogy following previously described methods (Cavender-Bares et al. 2007). The Ohm's law analogy assumes steady-state transpiration, an assumption that is the most valid during the morning (Nardini and Salleo 2000). For the calculation of K_{plant}, it was also assumed that the plant canopy was in equilibrium with the soil and Ψ_{PD} was equal to soil water potential (Ψ_{soil}).

Pre-drought and drought stem hydraulics

Under pre-drought conditions (-0.22 ± 0.1 (s.e.) MPa), we gravimetrically measured stem hydraulic conductivity on eight plants per species. Then after the initiation of the drought, we measured both stem hydraulic conductivity and percent loss in conductivity (PLC) on 8 - 10 plants per species. To prepare the stem samples, we cut them under water with razor blades and sealed the leaf scars with Loctite super glue 409 (Henkel, Germany) (Cavender-Bares and Holbrook 2001). For the hydraulic conductivity measurements, we cut stem segments to 22 cm long. We determined this length based on a set of preliminary experiments where we incrementally shortened the stem segments and found that specific conductivity increased when stems were cut shorter than 20 cm long, indicating that a significant number of endwalls were removed (data not shown). To make the measurements representative of hydraulic conductivity in the stems, we cut the samples to 22 cm in length (Holbrook per. comm).

We measured hydraulic conductance by gravimetrically running 10 mmol KCL solution (dissolved in degassed millipore filtered water) through stem segments with a pressure head less than 2 kPa. We measured the flow rate on an analytical balance (Sperry et al. 1988) and made cross-sections of

the stem segments to measure the distal xylem area. For each stem, we calculated specific hydraulic conductivity (K_s = (stem length × hydraulic conductance)/xylem area), and measured the area of all the leaves above the stem segment to calculate leaf specific conductivity (LSC = (stem length × hydraulic conductance)/leaf area). We also calculated the Huber value (HV) for each stem segment by dividing the total leaf area by the xylem area. We repeated these measurements and calculations on a second set of plants (10 individuals of *S. bebbiana* and *S. petiolaris*) in 2008.

After measuring plants' native hydraulic conductivity, we recut the stems to 5 cm lengths to determine PLC. We measured stem hydraulic conductance and flushed the stems with a syringe at a pressure of approximately 100 kPa for two minutes following methods of Cavender-Bares (2005). Based on a preliminary analysis, this amount of pressure and time proved sufficient to remove all the emboli in the stem segments (data not shown). After flushing the stems, we measured hydraulic conductance and calculated percent loss in conductivity (PLC). By measuring PLC during the dry-down, we were able to generate vulnerability curves for the species and calculate that water potential at which they experienced a 50% decline in stem conductivity (PLC_{50}) using the following equation: $PLC = 100/(1 + e^{a(\Psi - b)})$, where Ψ is water potential, a is a constant and b is the water potential for PLC_{50} (Pammenter and Vander Willigen 1998). We were unable to produce a vulnerability curve for *S. candida* because of difficulty with getting hydraulic conductivity measurements after flushing.

Leaf dieback and leaf chlorophyll measurements

We marked twenty leaves, evenly spaced along a main stem and one side branch of ten individuals per species. On ten of our measurement days, we estimated the percent of each leaf that was living (0, 25%, 50%, 75% and 100%). We also collected four leaves from fifteen individuals per species and completed a regression analysis of leaf area on leaf length (data not shown, $P>0.0001$) (Cavender-Bares et al. 2007). We used these linear regressions to estimate the total leaf area based on lengths. Since leaf areas are inflated in smaller leaves (immature willow leaves tend to be narrower than fully expanded leaves), we did a separate set of regression analyses for leaves smaller than 3 cm^2. We then estimated the change in leaf area in each

plant throughout the drought and estimated the Ψ_{PD} where each species experienced 50% dieback (using the equation from Pammenter and Vander Willigen (1998)).

On the same set of plants, we measured relative leaf chlorophyll level. These measurements were taken on two fully expanded leaves per plant using a Minolta SPAD-502 (Spectrum Technologies, Inc., Plainfield, IL) eight times during the dry-down (days 9, 15, 21, 27, 36, 42, 49 and 57). We took average readings of four locations per leaf and followed the same leaves through time. We calibrated the SPAD meter by measuring leaf chlorophyll content in two leaves of four individuals per species. These leaves were frozen in liquid nitrogen immediately after collection. We used high-pressure liquid chromatography to determine leaf chlorophyll content on a leaf area basis (data not shown) (Savage et al. 2009). SPAD readings were linearly related to leaf chlorophyll content as described by the following equation: total leaf chlorophyll = -1.92 + 0.358 × SPAD reading ($F = 12.38$, $df = 47$, $P<0.0001$). There were no species or species * SPAD units effects ($\alpha = 0.01$).

Resprouting after the drought

We rewatered 18 individuals per species (except for *S. pyrifolia*, n = 15) after they had lost all of their leaves and monitored them for resprouting. It is important to note that plants lost leaves at different points in the drought. As a result, we determined whether or not the species can resprout and not which species are better at surviving a drought of a specific length.

Statistics

We compared species' rates of stomatal closure by conducting a multiple regression analysis on the initial linear portion of the relationship between Ψ_{PD} and stomatal conductance (g_s), when Ψ_{PD} was greater than -0.05 MPa. We considered stomatal closure to occur at 0.03 mol H_20 $m^{-2}s^{-1}$ (90% reduction in g_s from pre-drought measurement). After stomatal closure, we compared species' minimal stomatal

conductance (g_{min}) with an ANOVA. We also completed a multiple regression analysis of photosynthetic capacity (A_{1200}) on g_s and estimated water-use efficiency (WUE) as the slope of the regression line.

We used an ANOVA and Tukey HSD comparisons to compare species' pre-drought traits (RMF, LMF, SMF, A_{1200}, g_s, WUE, SLA, K_s, LSC, HV, K_{plant}, K_{leaf} and soil moisture). To determine whether species differed in their dry-down rates, we conducted a repeated measures MANOVA analysis on species' pot soil moisture over time. We tested the significance of a species by time interaction with the Wilks' Lambda statistic to see whether species exhibited different responses to soil moisture over time. Since many plants lost leaves and were subsequently rewatered, we only conducted this analysis on data from the first 30 days of the drought when we still had a full set of plants. We also examined the relationship between Ψ_{PD} and soil moisture using a multiple logistic regression analysis. We examined the effects of soil moisture, species and the interaction of soil moisture and species on Ψ_{PD} in this analysis. We conducted all the statistical analyses with the program JMP 7 (SAS Institute, Inc.) except the non-linear regression analyses which were completed in Sigmaplot 9.0 (Systat Software, Inc.).

RESULTS

Species' habitats

As predicted, the three wetland specialists (*S. candida, S. pedicellaris* and *S. pyrifolia*) occurred in plots that remained wet year round, and the three habitat generalists (*S. bebbiana, S. discolor,* and *S. petiolaris*) occurred in drier plots that varied seasonally in water availability (Fig. 2.1). These plot differences are consistent with our classification of the species as wetland specialists and habitat generalists.

Pre-drought physiology

There were clear differences in the pre-drought physiology of species with similar and different habitat affinities. First, the six willow species differed in their photosynthetic capacity (A_{1200}) before the initiation of the drought with the habitat generalist (*S. bebbiana, S. discolor* and *S. petiolaris*) having higher A_{1200} than the wetland specialists (*S. candida, S. pedicellaris* and *S. pyrifolia*) (Table 2.1). Second, the habitat generalists also exhibited higher growth rates. There were also significant differences in species' branch specific hydraulic conductivity (K_s), leaf conductivity (K_{leaf}) and Huber values (HV) but these differences did not directly relate to habitat type (Table 2.1). There were no significant differences in species' root, stem and leaf mass fractions (RMF, SMF, LMF), species' pre-drought leaf specific hydraulic conductivity (LSC), leaf specific whole plant conductivity (K_{plant}) and stomatal conductance (g_s) (Table 2.1).

The progression of the drought

In the beginning of the experiment, the pots were at field capacity and there was no significant difference in average pot soil moisture. However, during the dry-down, the habitat generalists (*S. bebbiana, S. discolor*, and *S. petiolaris*) lost water more rapidly than the wetland specialists (*S. candida, S. pedicellaris,* and *S. pyrifolia*, Fig. 2.2a). A concurrent study found that this decline in soil moisture was related to species' total leaf area (Savage et al. 2009). There is also evidence that species vary in their predawn water potentials (Ψ_{PD}) at any given soil moisture, as there were significant species ($F = 4.92$, $df = 5$, $P = 0.0002$) and species by soil moisture effects ($F = 3.17$, $df = 5$, $P = 0.008$) in the relationship between Ψ_{PD} and soil moisture (Fig. 2.2b). *Salix discolor* exhibited the greatest decline in Ψ_{PD} per unit of soil moisture, while *S. pedicellaris* exhibited the smallest decline. Since species dried out and lost leaves at different rates, the number of Ψ_{PD} and soil moisture comparisons for each species varied (*S. bebbiana* n = 69, *S. candida* n = 76, *S. discolor* n = 59, S. *pedicellaris* n = 75, *S. petiolaris* n = 70, *S. pyrifolia* n = 72).

Stomatal response to drought conditions

All species closed their stomata rapidly in response to the dry-down (Fig. 2.2c) before their relative leaf water content (RWC) dropped below 80% (Fig. 2.2d). There was a significant linear relationship between stomatal conductance and Ψ_{PD} in the beginning of the drought ($F = 160.7$, $df = 1$, $P < 0.0001$), and A_{1200} declined with stomatal conductance ($F = 231$, $df = 11$, $P < 0.0001$, Fig. 2.3) as the drought progressed. Although there was no significant species by Ψ_{PD} effect on g_s, species did significantly differ in their g_s ($F = 4.52$, $df = 5$, $P = 0.0001$, Fig. 2.2c). This effect was mainly driven by the difference between *S. discolor* and *S. pedicellaris* (Table 2.2). *Salix discolor*, a habitat generalist closed its stomata rapidly (-0.59 MPa) and *S. pedicellaris*, a wetland specialist closed its stomata more slowly (-0.71 MPa) during the drought. There was also a significant species effect on A_{1200} ($F = 6.98$, $df = 5$, $P < 0.0001$, Fig. 2.3), resulting in the habitat generalists having higher water-use efficiencies than the wetland specialist (Table 2.2). Additionally, the habitat generalist had lower minimum stomatal conductance (g_{min}, $F = 4.95$, $df = 5$, $P = 0.0004$, Table 2.1) than the wetland specialists.

Leaf senescence and dieback

All six species experienced drought induced leaf senescence during the dry-down (Fig. 2.4a, b, c & d). This dieback occurred sequentially with the oldest leaves exhibiting chlorosis and necrosis before the younger leaves. *Salix bebbiana*, a habitat generalist, maintained higher chlorophyll levels and maintained leaves at lower water potentials than the other five species (Table 2.2). There was significant variation in the rate of dieback between species with similar habitat affinity (Fig. 2.4).

Changes in hydraulic conductivity

The six willow species exhibited differences in their vulnerability to cavitation (Fig. 2.5a & b and Table 2.2) and these differences did not relate to species' habitat affinity (Table 2.3). The species with the

highest and lowest vulnerability to cavitation were both habitat generalists, and PLC_{50} correlated with branch K_s across species (Fig. 2.5b). The willow species in this study also differed in their leaf specific conductivity (LSC) during the drought (Fig. 2.6). *Salix bebbiana, S. candida*, and *S. pedicellaris* all maintained constant LSC, but *S. discolor, S. petiolaris* and *S. pyrifolia* all increased their LSC (at least in the initial stages of the drought).

Resprouting

The six willow species demonstrated variability in their ability to resprout after leaf shedding. Of the habitat generalists, *S. bebbiana* resprouted the least (38.9%). About 61.6% of the *S. discolor* and *S. petiolaris* plant resprouted. The wetland specialists (*S. candida, S. pedicellaris*, and *S. pyrifolia*), on the other hand, had resprouting percentages of 44.4%, 77.8% and 66.7%, respectively.

DISCUSSION

As a result of Hubbell's unified neutral theory (Hubbell 2001) and recent work on metacommunities (Leibold et al. 2004), there has been renewed interest in understanding the mechanisms that explain species distributions and coexistence across the landscape (McGill et al. 2006). In this study, we investigated the extent that traits related to species' drought survival differ between species with similar and different habitat affinities in ways that could be influential in maintaining both β-diversity across the landscape and α-diversity within habitats. While many willows segregate across the landscape according to their cavitation resistance when compared to more xeric species (Pockman and Sperry 2000), within the genus, hydraulic traits such as cavitation resistance differed more among co-occurring species than among species from different habitats (Fig. 2.5 and 2.6). While there is evidence that habitat generalists and wetland specialists differ in their physiology, the greatest physiological divergence in drought survival strategies occurred between two habitat generalists (Table 2.3). This divergence may provide an important

mechanism for partitioning water use and allowing these species to co-occur in habitats that experience seasonal changes in water availability.

Stomatal regulation of xylem embolism

Many plant species close their stomata before xylem tensions get low enough to induce cavitation, leaving a safety margin between the point of stomatal closure and cavitation induction (Zimmerman 1983, Tyree and Sperry 1988). While safety margins can limit runaway cavitation and may prevent dieback under drought conditions (Sperry and Pockman 1993, Cochard et al. 1996), they also come at a cost because stomatal closure limits carbon assimilation. As a result, plants with small safety margins rarely occur in dry or variable habitats (Pockman and Sperry 2000, Martinez-Vilalta et al. 2002, Froux et al. 2005). In this study, none of the willow species demonstrated a safety margin between stomatal closure and cavitation (20 - 30% PLC, Table 2.2 and Fig. 2.5). However, these species may not require safety margins for drought survival because they are drought deciduous and can drop their leaves before reaching critical stem water potentials. On the other hand, the rate of stomatal closure in these six species consistently varied with water-use efficiency (Fig. 2.3 and Table 2.2) suggesting that species with higher water-use efficiency closed their stomata more rapidly.

Drought-deciduousness and leaf drop

Willows' main mechanism for drought avoidance is precocious leaf shedding and senescence (Rood et al. 2000, Amlin and Rood 2003, Savage et al. 2009). While leaf shedding is typically related to hydraulic failure in the leaf, petiole or stem (Nardini et al. 2001, Cochard et al. 2002, Hukin et al. 2005), it can be beneficial under drought conditions as it segments the vascular system and may prevent hydraulic failure in vital organs (Zimmerman 1983, Rood et al. 2000, Munné-Bosch and Peñuelas 2003, Munné-Bosch and Alegre 2004). It is also possible that since leaf shedding allows plants to maintain a larger water supply in their stems, it could facilitate more vigorous resprouting after drought (Brodribb and Holbrook

2003). Additionally, if senescence is sequential (occurring in older leaves first), then nutrient remobilization from older leaves could help younger, more photosynthetically active leaves last longer under drought conditions (Munné-Bosch and Alegre 2004, Milla et al. 2005). All six species in this study demonstrated sequential leaf senescence and a large decline in leaf chlorophyll prior to leaf drop (Fig. 2.4). However, the species with the most rapid (*S. discolor*) and the slowest (*S. bebbiana*) rates of dieback were both habitat generalists (Table 2.2). These rates of dieback were consistent with their vulnerabilities to cavitation, as the species with the highest vulnerability to cavitation, *S. discolor* had the fastest rate of dieback (Fig. 2.5a and Table 2.2).

Leaf water supply

In xeric and Mediterranean climates, many species maintain a large supply of water to their leaves by maintaining high Huber values (Mencuccini and Grace 1995, Maherali and DeLucia 2000, Ladjal et al. 2005, Choat et al. 2007). Leaf shedding can lead to a similar result under drought conditions if leaf drop occurs before stem cavitation. However, many species demonstrate cavitation simultaneously with or before leaf abscission causing their leaf water supply and their leaf specific conductivity (LSC) to either remain constant or decline under drought conditions (Shumway et al. 1991, Vilagrosa et al. 2003, but see Ladjal et al. 2005). Of the six willow species in this study, three maintained a constant LSC (*S. bebbiana, S. candida*, and *S. pedicellaris*) and three increased their LSC (*S. discolor, S. petiolaris,* and *S. pyrifolia,* Fig. 2.6) during the drought. These results suggest that *S. discolor, S. petiolaris,* and *S. pyrifolia* dropped their leaves at a faster rate than their stems embolized. This strategy would allow them to maintain a higher water supply in their stems and potentially limit their stem cavitation (Tyree and Ewers 1991, Mencuccini and Grace 1995). Interestingly, leaf drop only prevented hydraulic failure in *S. pyrifolia*, a wetland specialist, for a short period of time, while two of the habitat generalists maintained a high leaf specific hydraulic conductivity throughout the drought.

Vulnerability to cavitation and the efficiency trade-off

Similar to other willow and poplar species, the six species in this study were highly vulnerable to cavitation (Fig. 2.5a) (Hukin 2005, Pockman 2000, Rood 2000). Their limited variation in cavitation resistance compared to other woody species is not surprising, considering that closely related species tend to have similar stem hydraulic architecture (Wilson 2008). However, their vulnerability to cavitation did not relate to their habitat affinity, and the most and least vulnerable species were both habitat generalists (Fig. 2.5a). Instead, species vulnerability to cavitation appeared to vary consistently with a suite of traits, suggesting that species differ in drought survival strategies. For example, some species appeared to be good drought avoiders; these species were the most vulnerable to cavitation but they dropped their leaves early limiting loss in function (demonstrated by an increase in LSC, Fig. 2.6). The lack of a relationship between species' habitat specialization and their vulnerability to cavitation is especially interesting considering that we found evidence for a trade-off between xylem safety (as defined by PLC_{50}) and efficiency (as defined by K_s, Fig. 2.5b) in the genus. A study conducted on willow clones in Europe also found a similar result, as clones with higher cavitation resistance had lower productivity (Cochard et al. 2007). While this trade-off is often used to explain why species distributions correlate with their vulnerability to cavitation, in willows it might play an important role determining their drought response strategies.

Differences in the physiology of habitat generalists and wetland specialists

The three wetland specialists in this study had lower rates of photosynthesis (A_{1200}) and a slower growth rate than the habitat generalists (Table 2.1). While slow growth rates are not necessarily adaptive, they may arise from adaptive differences in species' resource allocation. For example, there is evidence that plants from nutrient limited-habitats invest more in defense and less in growth than species from more nutrient-rich habitats (Grime and Hunt 1975, Chapin 1980, Reich 1993). The habitats where the wetland specialists grow are not only nutrient-limited, but also frequently waterlogged. Species that survive in these habitats may depend on specific functional traits that reduce growth.

The habitat generalists and wetland specialists also differed in their response to the drought (Table 2.3). For example, the habitat generalists had higher water-use efficiencies than the wetland specialists (Table 2.2) indicating that they were more conservative in their water use. This is consistent with the habitat generalists ability to survive in drier, more seasonally variable habitats. Additionally, two of the habitat generalists exhibited lower minimum stomatal conductance after stomatal closure than the other species (Table 2.2), indicating that their stomata were either less leaky, or that they had lower epidermal conductance.

Differences in the drought responses of species with the same habitat affinity

The greatest functional divergence in this study occurred between two habitat generalists with distinct drought response strategies (Table 2.3). *Salix discolor*, on the one hand, was a true drought avoider. It closed its stomata rapidly (Table 2.2) and quickly dropped its leaves (Fig. 2.4), thus preventing concurrent stem cavitation (as indicated by an increase in LSC by mid-drought, Fig. 2.6). As a result, this species, which was the most vulnerable to cavitation prevented excessively negative water potentials in its xylem (Fig. 2.2b) and maintained a high resprouting ability (Table 2.2). On the other hand, *S. bebbiana* was more of a drought tolerator. It had the lowest vulnerability to cavitation (Fig.2.5a), maintained a greater margin between stomatal closure and induction of substantial stem cavitation and kept its leaves later into the drought than any other species (Fig. 2.4). When it finally dropped its leaves, stem cavitation occurred (as demonstrated by its constant LSC, Fig. 2.6), and it had the lowest resprouting of the habitat generalists. *Salix petiolaris*, the third habitat generalist, demonstrated an intermediate response. This species had the most dramatic increase in LSC (Fig. 2.6), and it was a good resprouter (Table 2.2), but it did not close its stomata or drop its leaves as rapidly as *S. discolor*.

These different drought survival strategies are associated with different costs and benefits. Since cavitation resistance comes at a cost to efficiency (Fig. 2.5b), *S. bebbiana*, the most drought tolerant species had the lowest rate of hydraulic conductance. *Salix discolor*, on the other hand, had the highest rate of hydraulic conductance but lost more leaf biomass in the early stages of the drought (Fig. 2.4). As a result,

S. discolor and *S. petiolaris* required less water during a drought than *S. bebbiana*. However, *S. bebbiana* maintained its leaves longer into the drought continuing to use water in the soil. While both of these strategies appear suitable for seasonally variable sites, variation in species' temporal use of water may play an important role in promoting their co-occurrence.

While the habitat generalists differed greatly in their drought responses, the three wetland specialists demonstrated limited functional variability and had more similar rates of dieback and vulnerability to cavitation (Fig. 2.4 and 2.5a). However, *S. pyrifolia* was unique in its initial increase in LSC (Fig. 2.6). The other two wetland specialists exhibited no change in LSC over time, dropping their leaves simultaneously with cavitation. Both *S. pedicellaris* and *S. pyrifolia* had high rates of resprouting after the drought, suggesting that they recover from leaf drop better than *S. candida*. The similarity in the wetland specialists' drought responses is not surprising, considering that they rarely experience drought conditions in their native habitats. As a result, competition under limited water-availability is likely not important in limiting their co-occurrence.

Conclusions - We found evidence for physiological divergence among closely related willow species that could be influential in their habitat differentiation, as well as important to their co-occurrence within habitats. Overall, the habitat generalists exhibited higher water-use efficiency and greater growth rates than the wetland specialists (Table 2.3). These differences are consistent with their drier, more seasonally variable habitats. However, the greatest divergence in drought response occurred between two habitat generalists. We hypothesize that this divergence could promote these species co-occurrence by allowing them to partition water temporally under drought conditions. While many studies have found differences in the physiology among co-occurring species that promote survival of short-term droughts (Pockman and Sperry 2000, Martínez-Vilalta and Piñol 2002, Martinez-Vilalta et al. 2003), divergence in drought survival strategies has not been observed among many closely related species (but see Ladjal et al. (2005)). Understanding physiological divergence among the co-occurring willows is an important step in understanding the mechanisms responsible for maintaining the high β and α-diversity in the genus *Salix* across the landscape.

Table 2.1 – Functional differences between willow habitat generalists and wetland specialists. Traits are root, stem, and leaf mass fraction (RMF, SMF, and LMF, g g^{-1}), growth rate (GR, g g^{-1} $month^{-1}$), specific leaf area (SLA, 10^2 * cm^3 g^{-1}), photosynthetic capacity (A_{1200}, mol CO_2 m^{-2} s^{-1}), stomatal conductance (g_s, mol H_20 m^{-2} s^{-1}), minimum stomatal conductance (g_{min}, mol H_20 m^{-2} s^{-1}), huber value (HV, $*10^{-4}$), branch and leaf specific hydraulic conductivity (K_s & LSC, g MPa^{-1} s^{-1} m^{-1}), and leaf and leaf specific whole plant conductivity (K_{leaf} & K_{plant}, mol MPa^{-1} s^{-1} m^{-2}). The averages are reported ± one standard error. All traits besides g_{min} were measured under pre-drought conditions. Bolded traits are significantly different among species at the following significance levels * 0.05, ** 0.01, and *** < 0.001. The results of the Tukey HSD multiple comparisons are indicated by letter superscripts (α = 0.05).

	Habitat generalists			**Wetland specialists**		
Traits	*S. bebbiana*	*S. discolor*	*S. petiolaris*	*S. candida*	*S. pedicellaris*	*S. pyrifolia*
RMF	0.62 ± 0.03	0.59 ± 0.02	0.56 ± 0.03	0.54 ± 0.03	0.51 ± 0.03	0.61 ± 0.03
SMF	0.22 ± 0.02	0.21 ± 0.01	0.26 ± 0.02	0.22 ± 0.02	0.19 ± 0.01	0.13 ± 0.03
LMF	0.16 ± 0.02	0.20 ± 0.02	0.18 ± 0.02	0.24 ± 0.02	0.30 ± 0.03	0.25 ± 0.02
GR***	**0.14 ± 0.01**^{a}	**0.16 ± 0.01**^{a}	**0.14 ± 0.01**^{a,b}	**0.13 ± 0.00**^{b}	**0.13 ± 0.01**^{b}	**0.12 ± 0.01**^{b}
SLA***	**1.6 ± 0.25**^{c}	**2.4± 0.18**^{a,b,c}	**1.4 ± 0.34**^{b,c}	**2.2 ± 0.36**^{a}	**2.0 ± 0.28**^{a,b}	**1.5 ± 0.16**^{c}
A_{1200}**	**19 ± 1.93**^{a,b}	**19 ±1.23**^{a,b}	**22 ± 0.55**^{a}	**16 ± 1.29**^{b,c}	**18 ± 0.94**^{a,b,c}	**13 ±1.38**^{c}
g_s	0.32 ± 0.04	0.27 ± 0.02	0.32 ± 0.03	0.32 ± 0.02	0.31 ± .03	0.23 ± 0.03
g_{min}***	**0.02 ± 0.00**^{a}	**0.02 ± 0.01**^{a}	**0.01 ± 0.0**^{a,b}	**0.04 ± 0.01**^{a,b}	**0.02 ±0.00**^{b}	**0.03 ± 0.01**^{b}
HV*	**5.73 ± 0.02**^{a,b}	**3.82 ± 0.05**^{b}	**4.53 ± 0.09**^{a,b}	**6.10 ± 0.10**^{a,b}	**8.48 ± 0.10**^{a}	**4.50 ± 0.10**^{a,b}
K_S**	**1.96 ± 0.40**^{b}	**3.56 ± 0.48**^{a}	**2.64 ± 0.37**^{a,b}	**2.19 ± 0.26**^{a,b}	**1.99 ± 0.20**^{b}	**2.84 ± 0.24**^{a,b}
LSC	0.26 ± 0.07	0.45 ± 0.09	0.33 ± 0.03	0.40 ± 0.9	0.53 ± 0.06	0.40 ± 0.09
K_{plant}	0.66 ± 0.08	0.89 ± 0.09	0.56 ± 0.11	0.79 ± 0.15	0.79 ± 0.14	0.68 ± 0.22
K_{leaf}*	**0.89 ± 0.11**^{b}	**1.60 ± 0.15**^{a}	**0.88 ± 0.11**^{b}	**1.20 ± 0.10**^{a,b}	**1.30 ± 0.07**^{a,b}	**1.4 ± 0.31**^{a,b}

Table 2.2 – Species-level drought response traits. Traits are water potential (MPa) at stomatal closure ($\Psi_{g=o}$), 50% dieback (DB_{50}) and 50% loss in hydraulic conductivity (PLC_{50}) and average water-use efficiency (WUE, mol CO_2 mol H_20^{-1}). WUE was defined as the slope of the regression line of A_{1200} on g_s, and stomatal closure was estimated as the intercept of the regression line of g_s on Ψ_{PD}. DB_{50} and PLC_{50} were calculated based on fitted sigmoidal curves. Asterisk marks which traits significantly differ between habitat generalists and wetland specialists ($\alpha = 0.05$).

	Habitat generalists			**Wetland specialists**		
Traits	*S. bebbiana*	*S. discolor*	*S. petiolaris*	*S. candida*	*S. pedicellaris*	*S. pyrifolia*
$\Psi_{g=o}$	-0.66	-0.59	-0.66	-0.63	-0.71	-0.64
DB_{50}	-2.39	-1.59	-1.78	-1.42	-1.44	-1.74
PLC_{50}	-1.72	-0.90	-1.07		-1.29	-1.03
WUE*	**56.6**	**69.5**	**66.5**	**55.8**	**49.2**	**55.3**

Table 2.3 – Traits that are similar and that differ between species with the same habitat affinity. Traits that did not differ between species with the same habitat affinity are reported in the first column. Traits that significantly differed between at least two species with the same habitat affinity are reported in column 2. Traits as the same as listed in Table 2.1 & 2.2.

Traits shared among species with similar habitat affinity	**Traits divergent among species with similar habitat affinity**
GR	SLA
A_{1200}	K_S
g_{min}	K_{leaf}
HV	$\Psi_{g=o}$
WUE	DB_{50}
	PLC_{50}

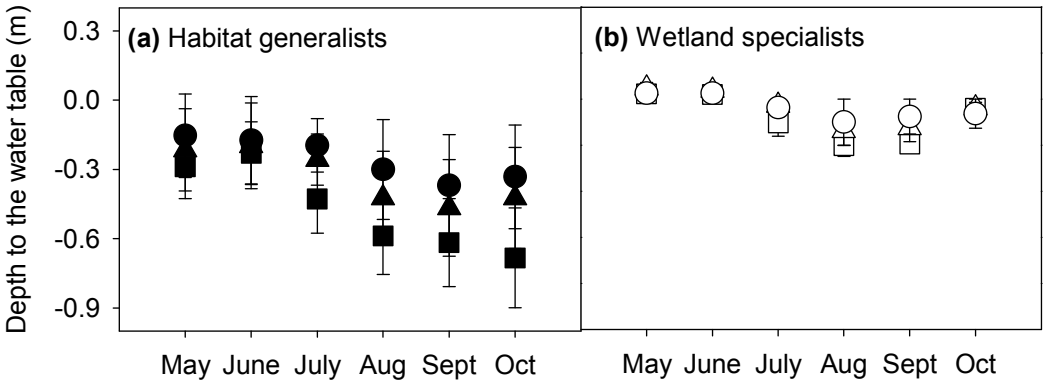

Figure 2.1 (a) Habitat generalists occurred in drier and more seasonally variable plots **(b)** than wetland specialists. The graph shows the average depth to the water table (m) measured in all plots where each of the species occurred. Species are represented by the following symbols: ■ *S. bebbiana,* ▲ *S. discolor,* ● *S. petiolaris,* □ *S. candida, Δ S. pedicellaris,* and ○ *S. pyrifolia.*

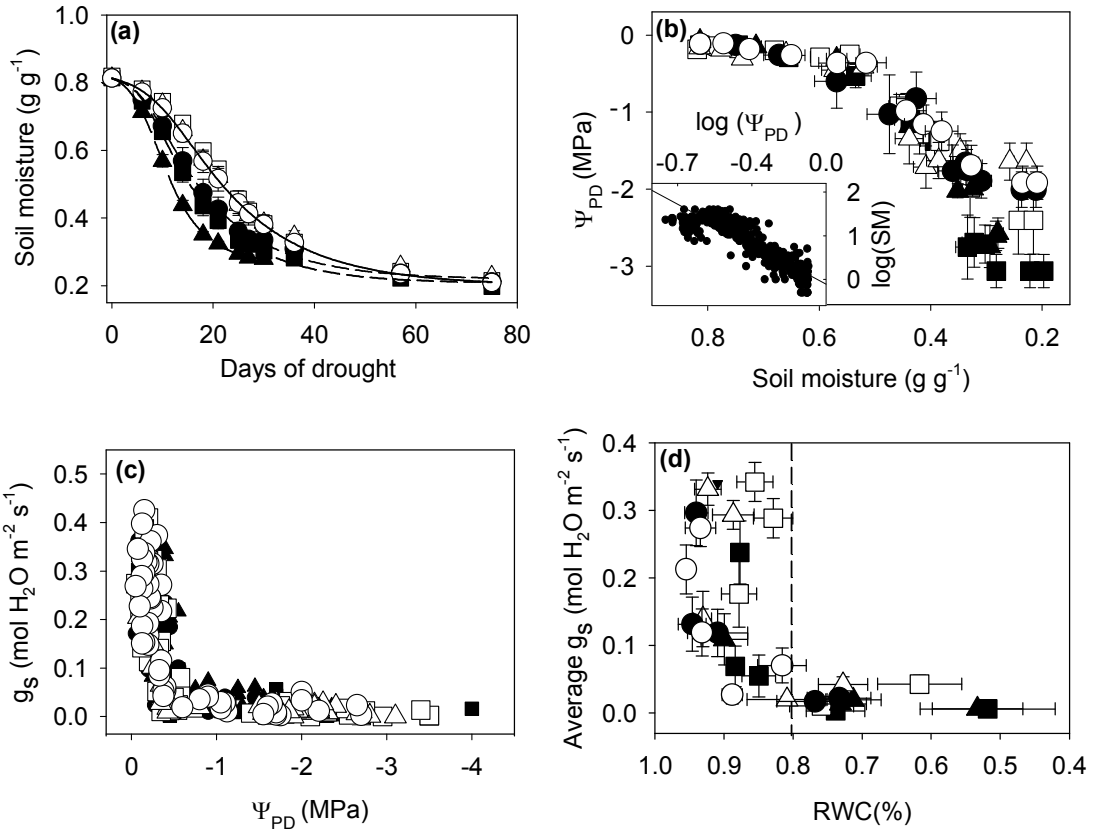

Figure 2.2 (a) The six species experienced different dry-down rates during the drought, with the habitat generalists drying out more rapidly than the wetland specialists. **(b)** Predawn water potential (Ψ_{PD}) was correlated with gravimetric soil moisture across species. The insert shows the log-log relationship used in comparing this relationship between species. **(c)** All six species exhibited a decline in stomatal conductance (g_s) with Ψ_{PD} and **(d)** leaf relative water content (RWC). All species had closed their stomatal before RWC was below 80% (indicated by dotted line) and data points represent average values for each species on each day of measurement (except for the inset in (b)). The symbols for each species are the same as in Fig. 2.1. Error bars are ± one standard error.

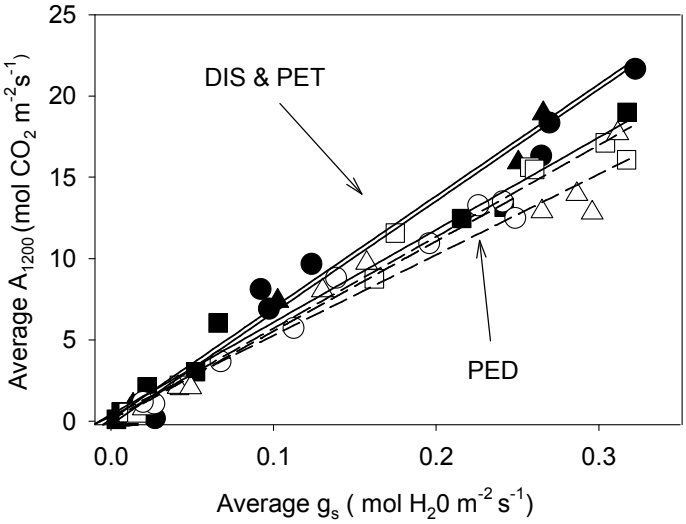

Figure 2.3 Species differed in their water-use efficiency during the drought, as defined by the slope of the linear regression between photosynthetic activity (A_{1200}) and stomatal conductance (g_s). *Salix discolor* (DIS) and *S. petiolaris* (PET) had the highest water use efficiencies, while *S. pedicellaris* (PED) had the lowest water-use efficiency. Data points represent average values for each species on each day of measurement. The symbols for each species are the same as in Fig. 2.1.

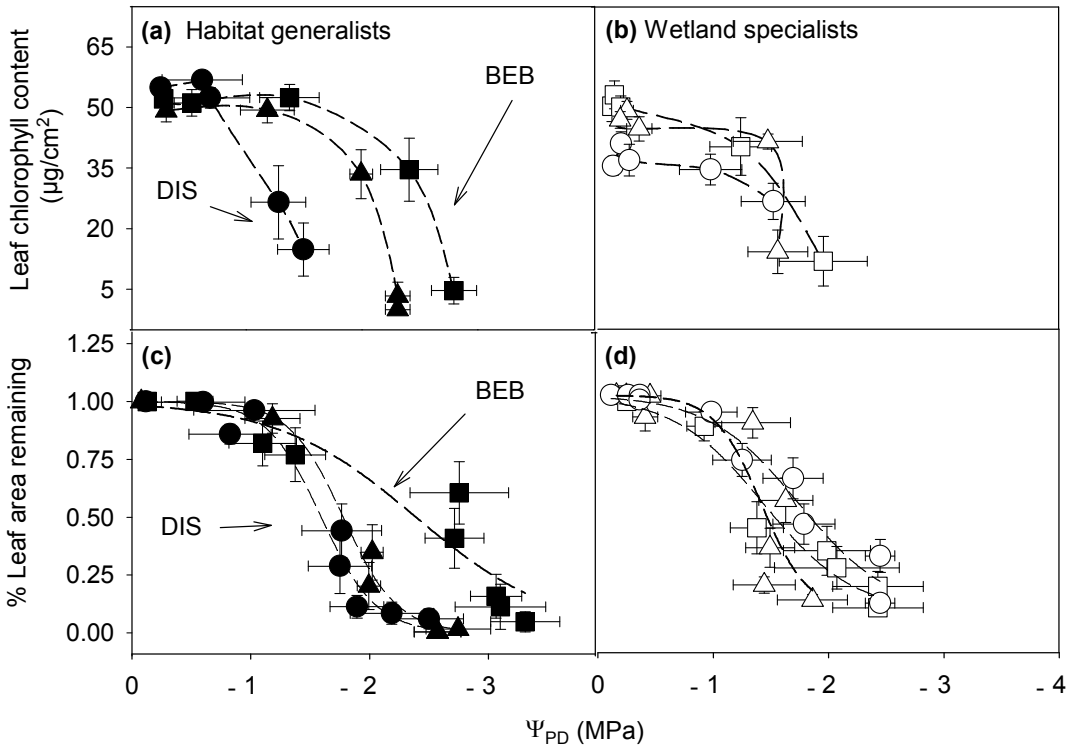

Figure 2.4 The habitat generalists demonstrated greater variation in their rate of leaf chlorophyll loss **(a, b)** and leaf dieback **(c, d)** during the drought than the wetland specialists. The greatest divergence between habitat generalists was between *S. bebbiana* (BEB) and *S. discolor* (DIS). Error bars are ± one standard error and the symbols for each species are the same as in Fig. 2.1.

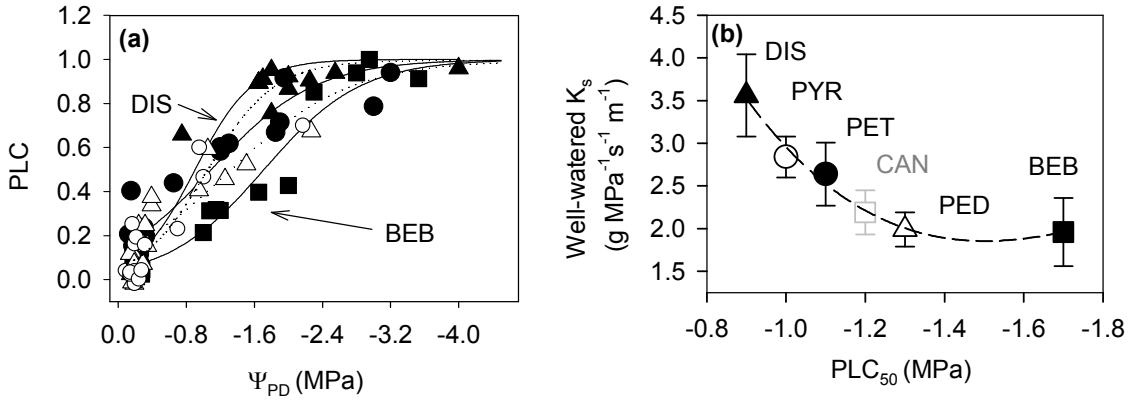

Figure 2.5 (a) Habitat generalists (solid lines) and wetland specialists (dotted lines) varied in their vulnerability to cavitation. The most (*S. bebbiana*, BEB) and the least (*S. discolor*, DIS) vulnerable species were both habitat generalists. Since the wetland specialists lost their leaves before reaching predawn water potentials lower than -2.4 MPa, we were only able to determine their vulnerability to less negative water potentials. Curves are fitted based on Pammenter and Vander Willigen (1998). Each point represents a different plant. **(b)** There was evidence for a trade-off between safety and productivity as indicated by the negative relationship between species' specific hydraulic conductivity (K_S) and their vulnerability to cavitation (described by PLC_{50}). We estimated PLC_{50} for *S. candida* (CAN) using the fitted curve and the measurement of plant K_s. The symbols for each species are the same as in Fig. 2.1. Error bars are ± one standard error.

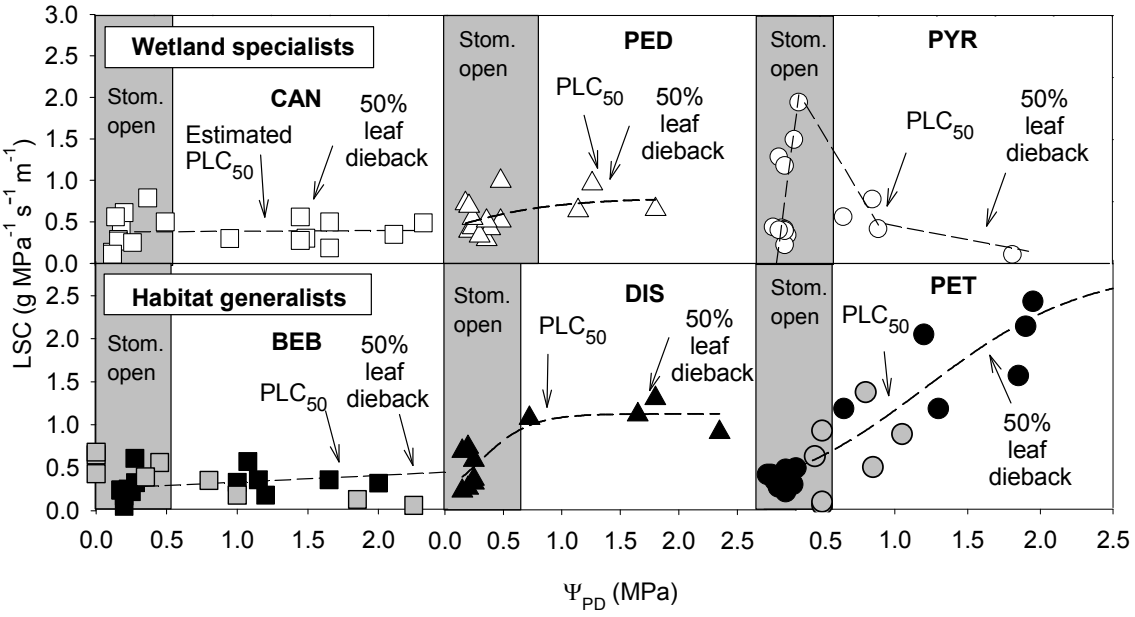

Figure 2.6 Two of the habitat generalists (*S. discolor,* DIS, and *S. petiolaris,* PET) exhibited consistent increases in their leaf specific conductivity (LSC) during the drought. This response was different than the other four species, which either demonstrated a small spike in LSC (*S. pyrifolia,* PYR) or a constant LCS during the drought (*S. candida,* CAN, *S. pedicellaris,* PED). On each graph, the estimated water potential for stomatal closure, PLC_{50}, and 50% leaf dieback occur are marked. Data from a follow-up experiment on BEB and PET is included in gray. The symbols for each species are the same as in Fig. 2.1.

CHAPTER 3

Niche differentiation and the role of trait lability in structuring willow (genus: *Salix*) and poplar (genus: *Populus*) communities in Minnesota

Thirteen willow (*Salix*) species occur in southeastern Minnesota and often co-occur within the same wetlands. This high local diversity is challenging to explain since closely related species are often functionally similar and density-dependent interactions such as competition and susceptibility to pests and pathogens should limit their co-occurrence. However, if willow species are partitioning resources, or if they are phylogenetically structured so that closely related species rarely co-occur, then the impact of these density-dependent processes could be reduced. In this study, I examined the role of niche partitioning in maintaining local willow diversity by documenting species distributions in plots across a water availability gradient and comparing species physiology in the field and greenhouse. By taking a phylogenetic approach, I also investigated whether willow communities exhibit phylogenetic community structure and whether there is evidence for environmental filtering. Overall, there was evidence that many willow species occur in guilds that exhibit niche partitioning across a water availability gradient. Within habitats, species exhibited phenotypic clustering, and across the landscape, species distributions correlated with traits associated with drought tolerance and recruitment. Traits associated with occurrence in drier habitats (i.e. high wood density and fast seedling root growth rate) were inversely related to relative growth rate suggesting that species from drier habitats may have a competitive disadvantage in wetter habitats. Additionally, willow phylogenetic community structure changed from being phylogenetically clustered in wet plots to being phylogenetically overdispersed in dry plots. I suggest that this pattern is a result of environmental filtering occurring on phylogenetically conserved traits in wet habitats and phylogenetically overdispersed traits in dry habitats. While this study finds evidence for niche differentiation across the landscape, it also reveals a high level of diversity within habitats. More research is needed to better understand the mechanisms important in maintaining diversity at this smaller spatial scale.

INTRODUCTION

The co-occurrence of closely related species is challenging to explain because their shared ancestry often results in functional and ecological similarity and increases their competition for shared resources (Darwin 1859, Elton 1946, MacArthur and Levins 1967, MacArthur 1958). While some have argued that competition is a major force in community assembly (MacArthur and Levins 1967, Tilman 1982), others claim that its role is minimal since species are functionally neutral (Hubbell 2001, Bell 2005). Additionally, there is growing evidence that multiple processes, including environmental filtering, density-dependent effects, stochastic events and dispersal limitation influence species distributions (Gotelli and McCabe 2002, Leibold et al. 2004, Tilman 2004). However, there is no consensus about the extent to which different processes dominate and interact in community assembly (Ulrich 2004, Ricklefs 2006, Myers and Harms 2009). Uncertainty about the importance of these processes has stimulated an increased interest in community assembly rules and research examining whether community structure can be predicted based on ecological principles (Diamond 1975, Weiher and Keddy 1995, Weiher et al. 1998).

A key component in understanding community assembly is determining whether species' functional differences affect their distributions. Correlations between plant functional traits and environmental gradients are often cited as important evidence for the role of environmental filtering and species sorting in community assembly (Ackerly 2003, Ackerly 2004, Cavender-Bares et al. 2004a, Kitajima et al. 2005, Poorter et al. 2005). Environmental conditions prevent species from surviving in communities for which they are maladapted, producing a strong association between species' functional traits and their habitat affinities. These correlations are particularly compelling when they have a strong physiological basis. For example, a correlation between species' drought tolerance and water availability supports the role of drought tolerance in limiting species distributions (Brodribb and Hill 1999, Maherali et al. 2004). Trait-environment relationships can also be reinforced by competition and other biotic interactions if functional trade-offs limit species' fitness across environmental gradients (Hutchinson 1957, Tilman 1982, Bazzaz 1996). Such trade-offs form the basis of niche theory, where competitive interactions

are minimized by resource partitioning within habitats (α-niches) and/or at a regional scale (β- niches) (Pickett and Bazzaz 1978, Silvertown et al. 2006).

Different community assembly processes can affect the similarity of traits within communities (Diaz et al. 1998, Weiher et al. 1998). When environmental conditions act as filters and thus limit species based on their environmental tolerances, species' functional traits should be more similar among co-occurring species (phenotypic clustering) than expected by chance (Weiher 1998, Webb et al. 2002, Cavender-Bares and Wilczek 2003). The same is also true in cases of facilitation and mutualism (Valiente-Banuet and Verdu 2007, Elias et al. 2008). On the other hand, ecological processes that limit species' similarity, such as competition (Elton 1946, MacArthur and Levins 1967, Stubbs and Wilson 2004) and pest-host interactions (Webb et al. 2006, Gilbert and Webb 2007) cause a greater divergence of traits among co-occurring species. These types of trait-community patterns can be tested by comparing species' pairwise trait differences with their level of co-occurrence (Cavender-Bares et al. 2004), and by comparing trait variance to a null model (Kraft et al. 2008, Cornwell and Ackerly 2009).

Studying trait evolution and phylogenetic relatedness within communities can also provide important insights into the mechanisms important in community assembly. Using this approach, it is possible to test for specific expectations based on ecological theory (for reviews see Webb et al. (2002), Cavender-Bares et al. (2009), Vamosi et al. (2009)). For example, environmental filtering favors the co-occurrence of species that are functionally similar in traits related to habitat specialization (Cavender-Bares et al. 2004b). If the relevant traits are phylogenetically conserved (shared between close relatives), then species should occur with their closest relatives (phylogenetic community clustering); if they are labile, then species should occur with more distantly related species (phylogenetic community overdispersion or evenness). Therefore, these methods allow us to examine whether these processes could be important in community assembly. However, since multiple processes can lead to the same patterns, it is important to complete these types of analyses in concert with more mechanistic and trait-based approaches (Cavender-Bares et al. 2009).

In this study, we investigated niche differentiation and phylogenetic community structure in willow communities in southeastern Minnesota. In this region, fifteen willow (genus: *Salix*) and poplar

(genus: *Populus*) species occur sympatrically and this represents high local diversity of closely related and thus potentially ecologically similar species. The main objectives of this research were to examine the mechanisms that could help maintain local diversity, and to investigate the major processes involved in willow and poplar community assembly. Since willows and poplars are highly dependent on water availability, and small changes in site hydrology can significantly alter their distributions (Amlin and Rood 2002, Denslow and Battaglia 2002, Rood et al. 2003), we examined whether they exhibited niche differentiation across a water availability gradient. Taking a physiological approach, we tested *a priori* hypotheses about specific trait-environment relationships. First, we examined whether traits related to drought tolerance and water-use correlated with species distributions (Brodribb and Hill 1999, Pockman and Sperry 2000, Cavender-Bares et al. 2004b, Maherali et al. 2004). Second, we investigated whether traits related to recruitment limit species distributions. Several studies on willows have found strong associations between seeding time and seasonal changes in water availability (Niiyama 1990, Pockman and Sperry 2000, Dixon 2003, Stella et al. 2006). There is also evidence that differences in seedling growth rates, root growth rates, and seed viability can limit species distributions in some systems (Karrenberg et al. 2002, Pax 2003, Markesteijn and Poorter 2009).

Since multiple traits associated with drought tolerance are known to trade-off with growth (Hacke and Sperry 2001, Cochard et al. 2007, Poorter et al. 2010), we examined whether such a trade-off was associated with species distributions, and whether there was evidence for it being a critical axis of niche differentiation in the system. Willows and poplars occur across a water availability gradient that extends from waterlogged habitats to sandy, upland habitats, therefore, we also discuss the possible influence of waterlogging tolerance on species distributions. Recent research has found evidence for a trade-off between waterlogging tolerance and drought tolerance (Silvertown et al. 2001, Niinemets and Valladares 2006) and such a trade-off could be important in this system.

To explore the potential processes involved in community assembly, we took both a trait-based approach comparing functional traits within and among communities, and a comparative phylogenetic approach investigating patterns of trait lability and species' phylogenetic relatedness. We investigated

differences between these two approaches and considered their importance in understanding deterministic processes in community assembly. The four main questions that this research addresses are:

1) Do willows and poplars exhibit niche differentiation across a water availability gradient?
2) Are species segregating across this gradient based on functional trade-offs associated with drought tolerance and recruitment strategies?
3) Is there evidence for environmental filtering and ecological sorting in willow community assembly?
4) How does trait and niche conservatism impact patterns of species co-occurrence in this system?

MATERIALS AND METHODS

Species distributions and patterns of co-occurrence

Field plots - Fifty 10 m × 30 m plots were randomly established in three preserves in the Anoka sand plain of eastern Minnesota: Cedar Creek Ecosystem Science Reserve LTER (Cedar Creek), Helen Allison Savannah Scientific and Natural Area (SNA), and Boot Lake SNA (plots also described in Chapter 2). Prospective plots were chosen by selecting grid cells on a map using a random number generator. Permanent plots were only established when there were willow and/or poplar species present. Since willows and poplars primarily occur in and near wetlands, we also randomly selected ten wetlands at Cedar Creek at which to establish plots. The plots were oriented to minimize environmental heterogeneity: for example, plots near bodies of water were positioned to run parallel to the water. When there were no obvious environmental gradients, the plots were oriented north to south. In each of the plots, we characterized species abundances by measuring their total basal area. For plants that had less than 12 stems, we measured the basal diameter of every stem. For plants with over 12 stems, we divided the stems into 4 mm size classes, measured six individuals in each size class, and counted the remaining stems. For the

three tree species our study, we measured dbh instead of basal diameter. In the study plots, there were a total of fifteen species: thirteen willow species and two poplar species (Table 3.1).

Environmental conditions - We measured depth to the water table monthly in a 1.5 m long PVC pipe (2.5 cm diameter) buried in the center of each plot for two years (Aug. 2007-2009). We did not take measurements during the winter when the soil was frozen and did not measure depth to the water table in plots that were perennially saturated. We estimated the burning frequency of the sites based on records for the last 30 years from Cedar Creek and the MN Department of Natural Resources. We also classified the plot habitats as upland, savanna, marsh, shrub swamp, peatland (bogs and fens) or "other wetland", based on site characteristics and the EPA classification system (http://www.epa.gov/owow/wetlands/types/). "Other wetlands" consisted of a variety of wetlands including wet meadows and seasonally flooded habitats. The two non-wetland habitats, savannas and uplands, were distinguished by fire frequency and vegetation type.

The plots were divided into three 10 m × 10 m subplots, and soil samples were collected in two subplots per plot in June 2008. We took ~15 cores per subplot (180.5 cm deep, 2.54 cm in diameter) and kept the samples in a cooler until we returned from the field. The samples were dried on trays in the sun, put through a 1.8 mm sieve and submitted to the University of Minnesota Analytical Laboratory within 48 hours of being collected. Each sample was analyzed for extractable phosphorus (Bray-1 method), exchangeable potassium and nitrate concentration along with pH and percent organic matter using standard protocols (NCR-13 Committee 1998). In plots that were perennially saturated or flooded, we estimated nutrient availability based on soil surveys (Grigal et al. 1974, Grigal and Cummins 1981). We also used these surveys to determine the soil type, and estimate total carbon, nitrogen and phosphorous in the top 1m of the soil.

Species distributions and patterns of co-occurrence – With the plot data, we (1) investigated the extent that different environmental conditions explain patterns of species abundances, (2) examined the extent that species patterns of co-occurrence are non-random, and (3) determined whether there was evidence for niche differentiation across multiple environmental gradients. To investigate the relationship between species abundances and environmental conditions, we did an indirect gradient analysis using a

non-metric multidimensional scaling (NMS) analysis in PC-ORD, v. 5.31 (MjM Software, Corvallis, OR). This is a non-parametric ordination analysis that uses iterative searches to find an ordination space that minimizes deviations based on plot dissimilarity. We assessed plot dissimilarity by calculating Sorensen distances between the plots based on species' basal areas. We double square root transformed the basal area data to down-weight abundant species and ran 250 iterations of the data to construct the ordination. These results were compared to the plot environmental data. We quantified plot water availability with three variables: (1) summer water availability - the average depth to the water table in Aug., driest month (WT_{dry}), (2) spring water availability - the average depth to the water table in May, wettest month (WT_{wet}), and (3) seasonal water variability - the maximum annual change in depth to the water table in each plot (ΔWT). Summer water availability gives an indication of the lowest water levels experienced in the plots (relevant to species' drought tolerance), and spring water availability indicates the highest water levels experienced in each plot (relevant to species' waterlogging tolerance). We also examined the correlation between plot environmental conditions and species composition, while controlling for distances between the plots with a partial Mantel test. For the environmental and basal area matrices, we used Sorensen distances, and for the geographic matrix, we used Euclidean distances. The analysis was conducted using the program zt (Bonnet and Van de Peer 2002) and the resulting Pearson's correlation coefficient was compared to a null distribution generated by permutating the rows and columns of the matrix 100,000 times.

To examine species patterns of co-occurrence and niche overlap, we used the program EcoSim 7.7 (Gotelli and Entsminger 2001). First, we estimated species pairwise co-occurrence with three difference indices: (1) c-score (Stone and Roberts 1990), which calculates the average number of checkerboard units (species pairs that do not co-occur and create 01 or 10 in the plot matrix) using the equation $C = (r_i - S)(r_j - S)$, where S is the number of sites containing both species, and r_i and r_j are the row totals for species i and j, (2) number of checkerboard species pairs in a matrix (Gotelli et al. 1997), and (3) the number of species combinations that occur (Pielou and Pielou 1968). All of these indices were calculated based on species presence and absence data and compared to a null model in which row and column totals were constrained, thus maintaining species occurrence frequencies and species richness at each site (Connor and Simberloff

1979, Gotelli 2000). The null model consisted of 1000 randomizations of the plot-species matrix with the Gotelli swap algorithm (Gotelli 2000). This model assumes the system is not dispersal limited and constrains the null community based on the actual measured data. While checkerboard patterns can result from processes (Diamond 1975, Connor and Simberloff 1983, Gotelli et al. 1997, Gotelli and McCabe 2002, Ulrich 2004, Bell 2005), they can serve as corroborative evidence for niche differentiation.

Second, we analyzed the field data to determine whether there was evidence for niche differentiation along a summer water availability gradient and a soil organic matter gradient. We defined species niches based on their response to their local environment (Hutchinson 1957, Bazzaz 1996, Whittaker et al. 2001) in terms of their basal area. Plots were organized into bins based on their environmental conditions, and bin numbers (h) were calculated using the Freedman-Diaconis equation, $h = 2(\text{IQR})/(n^{1/3})$, where IQR is the interquartile range and n is the sample number (Freedman and Diaconis 1981). We could not accurately estimate the frequency of the environmental bins in the field because we did not sample plots without willow and poplar species, and low plot numbers in some of the bins were not reflective of their availability across the landscape. Therefore, we assumed that the environmental bins occurred at equal probability. For this analysis, we calculated species niche overlap based on Pianka's index (Pianka 1973) and compared our results with a null model that maintained the matrix row and column totals. Pianka's niche overlap is calculated as: $O_{12} = (\sum(p_{2i} \times p_{1i})) / (\sqrt{\sum(p_{2i}^2 \times p_{1i}^2)})$ where p_{ji} is the proportion of species j that occur in bin i. The null model was based on 1000 randomization of the basal area-plot matrix. Since this test does not directly consider guild structure, we also investigated the extent that species are broken into different ecological guilds. We defined guilds as groups of species that utilize different portions of these environmental gradients, and considered species with high abundance in the same bins to be in the same guilds. We calculated the average c-scores of all the guilds and compared it to a null distribution generated by randomizing species into different guilds 1000 times. In this analysis, we tested whether species in the different guilds co-occur more often than expected by chance. We also ran the niche overlap analysis a second time using guilds instead of species to investigate whether guilds overlapped less than expected by chance.

Species' functional traits

We compared the functional traits of the species between plants in the field and a greenhouse common garden, focusing on traits related to drought tolerance and seedling establishment. We also measured traits related to productivity and growth to compare species growth strategies and test for a trade-off between drought tolerance and growth.

(1) Recruitment traits

Species' recruitment and phenology – For one growing season (2008), we monitored flower phenology weekly in three plots per species. When the fruits began to dehisce, we collected seeds. We cleaned and dried 500 seeds per species and estimated dry seed mass. We also measured seed viability weekly until they were no longer viable. For these tests, we placed 50 seeds per species on moist filter paper in six petri dishes and placed them in a well-lit window for four days. We quantified viability based on the percent of seeds that germinated. The seeds were kept in the refrigerator between tests. We were unable to measure seed viability on *P. tremuloides* because of low seed production and *S. serissima* because its seeds required cold stratification.

Seedling growth rate – After the germination experiment, we transplanted the germinated seeds into 2.5 cm diameter conetainers filled with potting soil (Sunshine Mix LG3, Sungro Horticulture, Bellevue, WA). We harvested half of the seedlings after four weeks and the remaining seedlings seven weeks, harvesting a total of 80 plants per species. During both harvests, we dried and weighed the above and below-ground biomass. We used these data to estimate relative growth rate as $(\log (M_2) - \log (M_1))/(t_2 - t_1)$ where M_2 and M_1 are the biomass at each time t_1 and t_2. During the first harvest, we also measured root length and calculated root growth rate over the first seven weeks. We cold stratified *S. serissima* seeds in the refrigerator for three months so that we could include them in the analysis but were unable to include *S. interior* in the study because it had very low seedling survival.

(2) Water-use and drought tolerance

Sampling and growth conditions – In a greenhouse common garden, we grew six to eight individuals of a subset of eleven species (*S. amygdaloides, S. bebbiana, S. candida, S. eriocephala, S. interior, S. lucida, S. nigra, S. pedicellaris, S. petiolaris, S. pyrifolia*, and *P. deltoides*) in a randomized design from cuttings collected the summer of 2007. Growth conditions were the same as in the temperate treatment described earlier in the dissertation (Chapter 4). We were not able to measure all the functional traits on four of the species (*S. amygdaloides, S. bebbiana, S. pedicellaris* and *S. petiolaris*) because of variation in cutting survival.

In the field, we sampled plants from a subset of plots that exhibited different levels of water availability. We divided the plots into four categories based on their depth to the water table in Aug. 2008. These categories were: (1) saturated, (2) water within 0 - 0.5 m of the surface, (3) water within 0.5 – 1 m of the surface and (4) dry without water within 1m of the surface. We sampled each species in two plots per water availability category. The only exceptions were the tree species (*S. amygdaloides, S. nigra,* and *P. deltoides*) and the clonal species (*S. interior* and *P. tremuloides*), which we sampled in more than two plots because the tree species occurred at low densities, and we wanted to ensure that we sampled unique genotypes of all the clonal species.

Stomatal traits and cuticle thickness – We measured leaf stomatal density and average stomatal aperture on four to six individuals per species in the greenhouse and six individuals per species in the highest and lowest water availability categories where they occurred in the field. Leaf stomatal traits were measured on impressions made of the abaxial and adaxial sides of leaves using clear nail varnish. On each peel, we measured stomatal density in three spots and aperture on nine stomata using SPOT Advanced software (Diagnostic Instruments, Sterling Heights, MI) and an Olympus BX50 microscope (Cavender-Bares et al. 2007). Since many willow and poplar species are amphistomatous, we calculated stomatal density as a cumulative density of the abaxial and adaxial sides of the leaf. We also calculated an index of species' stomatal area per unit leaf area (SPI) as stomatal density × (stomatal aperture)^2 (Sack et al. 2003). In the field, we measured leaf cuticle thickness on six individuals per species per water availability

category. We made cross-sections of fresh leaves, dyed them with Sudan IV and analyzed their cross-sections with SPOT Advanced and ImageJ (National Institute of Health, Bethesda, MD).

Leaf pressure-volume curves - We compared species' leaf turgor dynamics using pressure volume curves (Tyree and Hammel 1972, Hinckley et al. 1980). In the greenhouse, we sampled three leaves per species for all the species except *S. pedicellaris* and *S. petiolaris.* Stem samples were collected predawn to make sure that they were turgid. In the field, we sampled six species from two plots in the lowest and highest water availability categories where they occurred. These stem samples were collected in the morning and allowed to rehydrate in water for 3 hrs before we took measurements. We removed one mature leaf per sample under water and measured its turgid weight. We allowed the leaf to dry-out under ambient conditions and alternated between measuring leaf water potential (Ψ_{PD}) with a pressure chamber (SAPS, model 3115, ICT International Pty Ltd, Australia) and leaf mass (Turner 1981). We did not measure leaves that had an initial water potential lower than −0.2 MPa. We calculated leaf relative water content (RWC) by comparing each leaf's dry mass (M_{dry}) with its wet mass (M_{wet}) based on the equation: $(M_{wet} - M_{dry})/(M_{turgid} - M_{dry})$, where M_{turgid} is the leaf mass when it was originally removed from the stem. We considered the inflection point of the graph of $1/\Psi_{PD}$ versus RWC to be an estimate of each leaf's turgor loss point (TLP). We were unable to get water potential measurements from leaves of *S. candida* collected in the field because of complications associated with dense wooly hairs that absorbed water.

Wood density – We collected one to two year old distal branches from six plants per species in the field and the greenhouse in Aug. 2008. We cut 4 cm segments, removed all of their bark and pith and measured their fresh volume using water displacement. Dry weight was measured after drying at 65°C for three days, and density was calculated as dry weight per unit of fresh volume (Hacke et al. 2000).

(3) Traits related to productivity and growth

We measured a suite of leaf functional traits related to plant productivity and growth including specific leaf area (SLA), leaf area (LA), leaf nitrogen (leafN) and maximum photosynthetic capacity (A_{1500}). For SLA and LA, we sampled three fully-expanded leaves from eight individuals per species in the

greenhouse (all species except *S. amygdaloides* & *S. bebbiana*) and ten individuals per species in the field in each water availability category. Leaves were dried in a drying oven for two days at 65°C, and leaf area was measured digitally using Image J. To measure leaf nitrogen, we sampled leaves from six individuals per species in each water availability category in the field, dried 1.5 g of their leaf material at 65°C and sent it to the University of Minnesota Analytical Laboratory. The laboratory estimates total nitrogen on a mass basis using the Dumas combustion method (Simone et al. 1994, Matejovic 1995). In the summer 2008, we also measured gas exchange on five individuals per species in the greenhouse (all species except *S. pedicellaris* and *S. petiolaris*) and six individuals per species in the driest and wettest water availability category where they occurred in the field. For these measurements, we used a portable photosynthesis system (LICOR 6400-40, Licor Inc., Lincoln, NE) and a light intensity of 1500 μmol m^{-2} s^{-1} (a light intensity that saturated photosynthesis in these species based on preliminary analysis). We measured the first fully-expanded leaf on the main stem of each plant using ambient CO_2 levels. We also used these data to calculate water-use efficiency as photosynthetic capacity divided by stomatal conductance (g_s).

(4) Trait correlations and phylogenetically independent contrasts

To understand whether species' functional divergence could explain their distributions and patterns of co-occurrence, we examined the relationship between species' functional traits and their distributions with three different analyses. First, we described species distributions based on the average environmental conditions in the plots where they occur, weighted by their normalized basal area in each plot. We used these variables to examine the relationship between species' functional traits and their distributions with a set of regression analyses using *a priori* hypotheses from the literature (i.e. that traits associated with drought tolerance would be negatively correlated with water availability). All of the regression analyses were completed in JMP 8.0 (SAS Institute Inc., NC). We also tested whether traits and environmental conditions were correlated across the phylogeny using phylogenetically independent contrasts (PIC) (Felsenstein 1985, Garland et al. 1992) implemented in R (R foundation for statistical computing, Austria) using the picante package (Kembel et al. 2009) and the maximum likelihood

phylogenies described below. This analysis tests whether changes in habitat are correlated with changes in functional traits across the phylogeny. P-values were corrected for multiple comparisons and branch lengths were transformed according to Garland et al. (1992). Second, we examined whether traits related to drought and recruitment came at a trade-off with productivity and growth. These trade-offs were analyzed directly and using phylogenetically independent contrasts. Third, we compared the similarity of species' traits within plots to determine whether co-occurring species exhibited phenotypic clustering or overdispersion. For this analysis, we compared species' trait differences with their pairwise co-occurrence using a Mantel test in the program zt. The correlations were compared to a null distribution (similar to the one described above) that was based on 100,000 random permutations of the matrices (by row and column). Co-occurrence (c_{ij}) was calculated as $c_{ij} = 1 - 0.5 \times \Sigma (p_{ij} - p_{hj})$ where p is the proportion of total basal area in species i and h in plot j (Schoener 1970), following Cavender-Bares et al. (2004).

Phylogenetic analysis

We estimated a phylogeny of the 15 species in our study based on sequences of the nuclear alcohol dehydrogenase gene (*ADH*). We selected this gene because it is variable in willows (Belyaeva 2008) and has been used to identify cases of allopolyploidy in other plant species (Sang et al. 2004, Slotte et al. 2006). Since hybridization and polyploidy are common in Salicaceae, and there are four known tetraploids in our study (*S. discolor, S. lucida, S. serissima* and *S. humilis*) (Suda and Argus 1968, Dorn 1976, Löve 1982), it is possible that these species have an allopolyploid origin.

We collected leaf material from Cedar Creek LTER and deposited voucher specimens of the sampled plants in the University Minnesota Herbarium. We extracted DNA from the leaves of two individuals per species using a Dneasy Plant Mini Kit (Qiagen, Valencia, CA). We used two sets of primers designed by Jim Clarkson (Belyaeva, I., 2008) to PCR-amplify a 1450 bp region of the *ADH* gene. The PCR reaction mixture consisted of 10-20 μg genomic DNA, 0.12 μM each primer, 0.1 mM dNTP, 0.9 × Taq buffer, 1.8 mM MgCl2, 50 U/mL Taq polymerase (Sigma, St. Louis, MO, USA). The thermal cycling was 94°C (1 min), 50-54°C (1 min), 72°C (2 min) for 38 cycles and a final extension of 72°C (7 min) (modified from

Belyaeva, 2008). We cloned the polyploid species using a TOPO TA cloning kit (Invitrogen, Carlsbad, CA, USA) and cleaned the plasmid DNA using a QIAprep Miniprep kit (Qiagen). We sequenced 8-10 clones per PCR product. We also cloned one individual of two diploid species (*S. amygdaloides* and *S. petiolaris*) to test whether there was evidence for multiple gene copies. The remaining diploids were cleaned using a GenElute PCR Clean-up kit (Sigma) and directly sequenced using an ABI Prism 3730xl Prism Analyzer (Applied Biosystems Inc., Foster City, CA, USA).

The sequences were manually aligned in the program Se-Al v. 2.0 (Rambault 2001). Since the two primer sets had a 150 bp overlap that had between 6-20 polymorphisms, we were able to align the DNA segments for all the cloned alleles. Clones that were identical, allowing for known Taq error frequencies (Tindall and Kunkel 1988), were excluded from the analysis. We analyzed the species phylogeny using both parsimony and maximum likelihood optimality criteria with the program PAUP 4.0b7 (Swofford 2001). For the parsimony analysis, we weighted characters equally and treated gaps as missing data. We reconstructed trees using TBR branch swapping and a starting tree obtained by stepwise addition. For the maximum likelihood analysis, we determined the most likely model of evolution for our data using Modeltest 3.7 (Posada and Crandall 1998). We used this model and performed a heuristc search for the most likely tree using the TBR branch swapping algorithm. Branch support was determined using bootstrapping.

We assessed monophyly in each of the plant species by examining both the 50% majority-rule consensus tree from the parsimony analysis and the best scoring maximum-likelihood tree. Polyploids with allele copies that occur in different parts of the phylogeny were considered to be allopolyploids. We pruned the phylogeny, retaining one individual per species and multiple alleles from polyploids that appeared to have different parents. To accommodate uncertainty in the ancestry of the potential allopolyploids, we used derivative phylogenies that represented all possible combinations of the multiple alleles in our subsequent analyses.

Community phylogenetic structure and trait conservatism

We examined the relationship between trait evolution and phylogenetic community structure using the picante package in R. First, we determined the phylogenetic signal of species' traits and habitat affinities using the K statistic (Blomberg et al. 2003). The K statistic is a measure of phylogenetic signal that determines when trait evolution deviates significantly from a Brownian model of evolution. We compared the K statistics of species' traits and habitat affinities to a null model based on 999 randomizations of the species across the tips of the phylogeny. Traits were considered conserved when the observed variance of the independent contrasts was significantly lower than expected based on the null model. Higher K values (closer to 1) indicate greater phylogenetic signal.

Second, we examined the phylogenetic relatedness of species within plots using three different metrics. The first metric was NRI (net relatedness index), which is calculated as $-(\text{MPD} - \text{MPD}_{\text{null}})/\text{sd}(\text{MPD}_{\text{null}})$, where MPD and MPD_{null} are the mean pairwise phylogenetic distance between species in the plots and in the null model, respectively (Webb 2000). A negative NRI indicates phylogenetic overdispersion and a positive NRI indicates phylogenetic clustering. For this analysis, we used the same null model as for the K statistic. The second metric we used was PSV (phylogenetic species variability) (Helmus et al. 2007). This metric ranges from 0 to 1, with 1 indicating there is a star community phylogeny and a high phylogenetic variability, and with 0 representing a low variability and a high phylogenetic clustering. The third metric was average taxonomic distinctiveness (Δ+), which is calculated as $\Delta+ = [\Sigma\Sigma_{i<j} d_{ij}/[s(s-1)/2]$, where d_{ij} is the phylogenetic distance between species pairs (i and j) summed over the total number of species s (Clark and Warwick 1998). Higher Δ+ values indicate a higher level of phylogenetic diversity. This metric of phylogenetic diversity is unbiased with relation to species richness (Schweiger et al. 2008). For all of these analyses, we used maximum likelihood branch lengths and reported the range of values that resulted from the all the derivative species phylogenies.

RESULTS

Species distributions and patterns of co-occurrence

Species abundances (as described by their basal areas) differed among plots depending on water availability and soil characteristics. When species abundances were compared using an NMS ordination analysis, the major ordination axes correlated with multiple environmental variables (Table 3.2). The strongest correlations ($r>0.5$) occurred with total soil carbon (C), total soil nitrogen (N), soil organic matter (OM), and depth to the water table in the summer (WT_{dry}) and in the spring (WT_{wet}). In this analysis, plots classified as the same habitat type were also clustered in ordination space (Fig. 3.1). The relationship between species' basal areas and plot environmental conditions was significant when accounting for geographic distances between plots, using a partial Mantel test ($r = 0.19$, $P<0.0001$). However, many of the environmental variables were highly correlated in the field such that their association with species distributions could not be distinguished (Appendix 1 - Table S1.1).

Species demonstrated non-random patterns of co-occurrence based on all three co-occurrence indices (Table 3.3) and they exhibited greater variation in niche overlap than expected by chance along two environmental gradients (a summer water availability and a soil organic matter gradient, Table 3.4). However, species grouped into distinct guilds along both of these gradients (Fig. 3.2). When guilds were defined as groups of species that utilize different portions of these environmental gradients (species that demonstrate high abundance in the same environmental bins), species within the same guild demonstrated a higher level of co-occurrence than expected by chance (based on c-score, $\alpha = 0.01$). There was also significantly less niche overlap than expected by chance between guilds in terms of summer water availability and soil organic matter (Table 3.4).

Trait-environment correlations

In general, species' functional traits related to their distributions across a summer water availability gradient in the predicted manner. First, species from drier habitats exhibited higher water-use efficiencies (WUE) and more negative leaf turgor loss points (TLP) (Fig. 3.3a & b). These two traits are important because they often indicate a higher drought tolerance. TLP was correlated with species distributions when measured in both the greenhouse and in the field, but WUE was not significantly correlated with species distributions across a water availability gradient when measured in the greenhouse (α=0.05). There were two traits related to species' recruitment (seed viability and seedling root growth rate (seedling root GR)) that correlated with species distributions. These correlations demonstrated that species from drier habitats had seeds that remained viable for a longer period of time ($F=7.5$, $df = 11$, $P = 0.02$, Fig. 3.3e) and had faster seedling root GR than species from wetter habitats ($F = 14.6$, $df = 11$, $P = 0.003$, Fig. 3.3f).

Cuticle thickness, flowering time and seeding time did not correlate species distributions across the measured environmental gradients (Appendix 1- Table S1.3 and Fig. S1.2) and stomatal pore index (SPI) demonstrated a correlation in the opposite direction to our expectation ($F = 7.97$, $df = 13$, $P = 0.01$, Fig. 3.3d). However, there was a significant positive relationship between specific leaf area (SLA) and mean weighted soil phosphorus ($F = 8.30$, $df = 13$, $P = 0.01$), and leaf nitrogen and soil nitrate ($F =13.1$, $df = 13$, $P = 0.003$) when these traits were measured in the field. There was no relationship between SLA and mean weighted nutrient availability when SLA was measured in the greenhouse (Appendix 1- Fig. S1.3).

A few species in the study exhibited intraspecific variation in their stomatal density ($F = 2.29$, $df = 14$, $P = 0.003$), wood density ($F = 1.61$, $df = 24$, $P = 0.04$) and TLP ($F = 1.90$, $df = 17$, $P = 0.03$) in the field. In general, species demonstrated higher stomatal density and less negative TLPs in wetter plots than individuals from drier plots ($\alpha = 0.05$, Appendix 1 – Fig. S1.4), which is consistent with the trends observed across species. However, in contrast to the trend across species, *S. bebbiana* and *S. petiolaris* exhibited higher wood density in plots with the higher levels of water availability in the summer ($\alpha = 0.05$, Appendix 1- Fig. S1.4). There were also significant differences between the functional traits measured in

the field and those measured in the greenhouse. The plants in the greenhouse had lower WUEs and were able to maintain turgor to lower water potentials than species grown in the field, and the plants in the greenhouse had lower SPI and stomatal density than plants in the field (Fig. 3.3 and Appendix 1- Table S1.3).

Similar trait-environment relationships were found using phylogenetically independent contrasts (PIC), although some showed somewhat weaker relationships (e.g. 50% seed viability). On the other hand, wood density, which is a trait that is often associated with higher drought tolerance, showed a stronger correlation with species distributions using PIC. This can happen if there are important differences in wood density between subclades, but within each subclade the magnitude and direction of the evolutionary change in wood density parallels that of species' habitat affinities. Since there was limited variation in the PIC based on the phylogenies estimated in this study, we only presented the PIC correlations for one of the phylogenies in Figure 3.3. The statistics for the other phylogenies are presented in Appendix 1 (Table S1.2).

Phenotypic clustering

There was a high level of phenotypic clustering within plots in traits related to plant water-use, as seedling RG, SPI, wood density and SLA correlated with species pairwise co-occurrence (Table 3.5). Seedling RGR also correlated with species co-occurrence across plots. These results are consistent with the strong correlations observed between these traits and species distributions across a summer water availability gradient (Fig. 3.3 & 3.4).

Functional trade-offs

Two of the traits that correlated with summer water availability, seeding GR ($F = 17.04$, $df = 11$, $p = 0.002$) and wood density ($F = 5.63$, $df = 13$, $p = 0.04$) were inversely related to seedling relative growth rate (RGR), and RGR was inversely related to depth to the water table in the summer ($\alpha = 0.05$, Fig. 3.4).

These relationships were significant when comparing species directly and when considering phylogenetically independent contrasts (α=0.05, Fig. 3.4 and Appendix 1- Table S1.2).

Salix phylogeny

The thirteen willow species in this study appear to occur in two clades based on the maximum likelihood *ADH* phylogeny (Fig. 3.5, Appendix 1- Fig. S1.1). This ML tree is also one of the 596 most parsimonious trees (remaining trees not shown). According to this tree, four species in the subgenus *Salix* (*S. nigra, S. amygdaloides, S. serissima,* and *S. lucida*) formed a well-supported clade (Clade 1). The remaining species formed a less supported clade (Clade 2). The strict consensus of the maximum parsimony trees exhibited less resolution in clade 2 than the best scoring maximum likelihood tree and had 41 out of 54 nodes resolved in the whole tree. This limited resolution is consistent with the lack of bootstrap support for this clade (Fig 3.5). Both *S. serrissma* and *S. lucida* appear to be allopolyploids and contain alleles that are related to species in different subgenera. The simplest explanation for the observed pattern is that there was one polyploidy event in a common ancestor of these two species. The origins of the polyploidy events in *S. discolor* and *S. humilis* are more difficult to determine. All of the alleles of *S. humilis* clustered in the maximum-likelihood and maximum parsimony phylogenies, but with no bootstrap support and without greater species sampling it is unclear whether the species is an allo- or autopolyploid. *Salix humilis* also had at least seven copies of the *ADH* gene, whereas we expect a maximum of four alleles in a tetraploid. These results indicate that *S. humilis* is either an octoploid or has undergone *ADH* gene duplication in addition to polyploidization. *Salix discolor*, on the other hand, had only four copies of the *ADH* gene, but these copies were paraphyletic with respect to *S. bebbiana*. There was no evidence for more than two alleles in the two diploid species (*S. petiolaris* and *S. amygdaloides*) cloned in this study.

Trait evolution and phylogenetic community structure

The majority of traits measured did not deviate from a Brownian motion model of evolution, indicating a lack of phylogenetic conservatism (Table 3.6). The only traits that demonstrated a potentially significant phylogenetic signal were SPI and stomatal aperture. Species niches, as described by their distribution across a summer water availability gradient (in terms of WT_{dry}), were phylogenetically conserved across species as indicated by high K values showing greater phylogenetic signal than expected by Brownian motion evolution. Across plots, phylogenetic overdispersion (evenness) correlated with water availability (Fig. 3.6 and Appendix 1 – Table S1.2). This correlation was apparent when phylogenetic dispersion was measured using the net relatedness index (NRI), phylogenetic species variability (PSV), and taxonomic distinctiveness (Δ+). In general, the wettest plots demonstrated the greatest clustering and the driest plots demonstrated the greatest overdispersion. Since soil organic matter was correlated with water availability, plots with higher organic matter content also demonstrated greater phylogenetic clustering. However, there was no relationship between species richness and water availability or soil organic matter across the plots, indicating that the change in phylogenetic community structure is not associated with a concomitant change in species diversity (Fig. 3.6c).

DISCUSSION

This study provides strong evidence for the role of niche differentiation in maintaining willow and poplar diversity in southeastern Minnesota, and demonstrates the role of trait lability in structuring plant communities. Niche differentiation appears to occur across two primary environmental gradients, a water availability gradient and a soil organic matter gradient (Table 3.3). Part of this differentiation results from differences in species' drought tolerance and recruitment strategies (Fig. 3.3) and the observed trait-environment relationships may be reinforced by several functional trade-offs (Fig 3.4). While niche differentiation can account for some of the local diversity across habitats (β-scale), there is still a high level of co-occurrence within communities (α-scale). At this spatial scale, species are phenotypically clustered,

indicating that co-occurring species are functionally similar (Table 3.5). There was also a significant relationship between phylogenetic community structure and water availability such that wet plots were more phylogenetically clustered than dry plots. Interestingly, traits associated with dry habitats (e.g. TLP, wood density, seedling root growth rate) were evolutionarily labile and the one trait associated with wet habitats (e.g., SPI) was phylogenetically conserved (Table 3.6). This result suggests that the observed trend between phylogenetic community structure and water availability (WT_{dry}, Fig. 3.6) resulted from differences in trait lability and demonstrates that differences in trait lability can impact patterns of co-occurrence among closely related species.

Niche differentiation across a summer water availability and soil organic matter gradient

The fifteen species in this study appeared to segregate across the landscape based on water availability and soil characteristics. These trends are evident from the NMS ordination analysis that compared plot dissimilarity based on species abundances (Fig. 3.1). In this analysis, multiple environmental variables related to water and nutrient availability correlated with the major ordination axes (Table 3.2). Most species also demonstrated distinct unimodal distributions across a summer water availability gradient (Fig. 3.2). These data indicate that species are likely specializing for habitats with contrasting environmental conditions. The only exception was *P. deltoides,* which had a bimodal distribution, however, this species was also underrepresented in the plots. The observed level of habitat specialization in this system was consistent with several other studies that have found willow and poplar species tend to segregrate across the landscape based on water availability and soil conditions (Schnitzler et al. 1992, Pockman and Sperry 2000).

Species patterns of co-occurrence and niche overlap provide evidence for species niche differentiation based on soil organic matter and summer water availability (Table 3.3 and 3.4). Across the plots, there were significantly more checkerboard species pairs (pairs of species that do not co-occur in plots) than expected based on a null model, indicating that multiple species rarely co-occur in field. While checkerboard patterns can result from multiple ecological and historic processes, including habitat

specialization, vicariance and stochastic processes (Diamond 1975, Connor and Simberloff 1983, Gotelli et al. 1997, Gotelli and McCabe 2002, Ulrich 2004, Bell 2005), their presence can serve as strong corroborative evidence for niche differentiation.

While there were many pairs of species that did not co-occur within plots, there were also several groups of species that demonstrated a high level of co-occurrence and exhibited similar habitat specialization (Fig. 3.2). We considered these species to be in the same ecological guilds because they demonstrated high abundance in the same summer water availability and soil organic matter bins. Species within guilds had a greater co-occurrence (based on c-score) than expected by chance. Since guild structure can mask patterns of niche differentiation, leading to greater variation in niche overlap (Winemiller and Pianka 1990), we investigated the level of niche overlap both among species, and among guilds. With this analysis, it became apparent that although niche overlap is highly variable between species, it is also lower than expected by chance between guilds (Table 3.3). These results support our hypothesis that niche differentiation is occurring across these environmental gradients, but also indicate that not all species are occurring in unique niches.

The functional basis of niche differentiation

There was strong evidence that species distributions across a summer water availability gradient correlated with traits related to drought tolerance and recruitment (Fig. 3.3a-f). In general, species from drier habitats had a higher drought tolerance than species from wetter habitats, as demonstrated by their higher water-use efficiencies (WUE), more negative leaf turgor loss points (TLP), and higher wood densities. All of these traits are known to be related to plant drought tolerance and often have predictable associations with species distributions based on water availability (Fischer and Turner 1978, Hacke and Sperry 2001, Lenz et al. 2006). In terms of recruitment, there were two traits that related to species distributions (Fig. 3.3e &f). First, species from drier habitats exhibited longer seed viability. Since willow seeds only germinate under saturated conditions (Niiyama 1990, Scott et al. 1996, Woods and Cooper 2005), it is possible that seeds that remain viable for longer periods of time are more likely to germinate in

sites where water availability is low and sporadic. Second, species from drier habitats exhibited faster seedling root growth rates (GR) than species from wetter habitats. This is likely important in allowing these species to access deeper water supplies, thus minimizing the risk of seedling desiccation. Based on phylogenetically independent contrasts, there was also evidence for correlated evolution between species habitat specialization and several physiological and functional traits (TLP, SPI, and seedling root GR, Fig. 3.3g, j & l). These correlations provide strong support for an adaptive interpretation of the observed trait-environment relationships, and provide evidence for the importance of these traits in species niche differentiation.

While some of the observed trait variation in the field appeared driven by plasticity, especially in terms of SLA (which only significantly correlated with species distributions in the field), other traits showed significant correlations when measured both in the field and in a greenhouse common garden (e.g. TLP and SPI, Fig. 3.3). These results provide important evidence that interspecific variation in these traits is genetically-based. Although we did not investigate trait plasticity under common garden conditions, it is also possible that differences in species' function under different growing conditions are important to their habitat specialization. In a previous study, a subset of willow species from drier habitats had higher WUE than species from wetter habitats under drought conditions (Chapter 2). If this trend is reflective of a larger pattern across the genus, differences in species' ability to increase their water-use efficiency may influence their ability to survive and reproduce under drier conditions.

The three traits that did not correlate with species distributions as predicted were cuticle thickness, SPI and time of seed production. While willow species in drier habitats in the Athabasca sand dunes exhibited thicker cuticles (Cooper and Cass 2003), cuticle thickness did not differ substantially among species in this study (Appendix 1 – Fig. S1.3). One possible explanation for these results, is that the chemical and structural characteristics of the cuticle are more important in preventing water loss than its thickness (Riederer and Schreiber 2001). Cuticles also play an important role in plant defense against common pathogens such as rusts and mildews (Wynn 1976, Carver et al. 1990), and it is possible that these functions are more tightly related to cuticle thickness than water loss. In terms of SPI, we predicted that plants from drier habitats would have lower SPI, consistent with a more conservative water-use strategy.

However, species from drier habitats exhibited higher SPI than species from wetter habitats (Fig. 3.3d). One explanation for this trend is that wetland plants benefit from lower stomatal area because waterlogging can reduce the function of root water transporters (aquaporins), causing water stress (Tournaire-Roux et al. 2003). Under these conditions, low SPI could limit water loss and prevent high tensions from occurring in the xylem. Lastly, while other studies have shown that the timing of seeding is an important factor in determining willow species distributions (Vansplunder et al. 1995, Dixon 2003, Woods and Cooper 2005, Stella et al. 2006), we found no relationship between seed production time and water availability. This is likely due to the nature of the local water availability gradient in the Anoka sand plain, where plots varied more in their level of water availability than in the timing of changes in water availability.

One of the main requirements for niche differentiation is the existence of functional trade-offs that prevent species from performing well under all environmental conditions (Hutchinson 1957, Tilman 1982, Bazzaz 1996). In this study, we found evidence for two trade-offs, one between seedling root GR and seedling relative growth rate (RGR), and one between wood density and seedling RGR (Fig. 3.4). Since both of these traits are related to species distributions along a summer water availability gradient (Fig. 3.3 and Table 3.5), these trade-offs could play a critical role in maintaining niche differentiation across this gradient. It is also interesting to note that seedling RGR was inversely related to species distribution across a summer water availability gradient (WT_{dry}). While this trend could be a result of the previously mentioned trade-offs, it could also be indicative of a larger trade-off between functional strategies required in different habitats.

Possible mechanisms that facilitate species co-occurrence

While some of the local willow and poplar diversity in the study is explained by niche differentiation (β-niches), this differentiation cannot explain the high co-occurrence of species within the same habitats. It is therefore important to consider how diversity is maintained at smaller spatial scales. One possibility is that species are partitioning resources within plots (α-niche differentiation). For example, there is evidence in some systems that co-occurring plant species differ in their rooting depth in the soil

(Parrish and Bazzaz 1976, Bartelheimer et al., Clarkson et al. 2009). It is also possible that species differ in their temporal use of resources (Hutchinson 1958, Grubb 1977). In a previous study, we found that three co-occurring willow species demonstrated a divergence in their drought survival strategies, which likely results in a shift in their temporal dependency on water under drought conditions (Chapter 2). These differences could be important in facilitating co-occurrence when water availability is limited. Another possible explanation for high α-diversity is that there is a high level of disturbance that reduces the impact of density-dependent processes in these systems (Grime 1973, Connell 1978, Huston 1979). Disturbance may be especially important in seasonally flooded habitats and/or habitats that have a high fire frequency. Another unexplored factor in this system is the potential for a trade-off between colonization and competition (Skellam 1951, Tilman 1994) or between fecundity and stress tolerance (Muller-Landau 2010). Since willows and poplars are early successional species and tend to be short lived, their co-occurrence could be maintained by the co-occurrence of dispersers and competitors/stress tolerators. Lastly, it is possible that species co-occur within plots because they are functionally neutral. A high level of functional similarity can allow for stable co-occurrence in saturated communities (Scheffer and van Nes 2006), but the role that these mechanisms play in species co-occurrence is still highly debated (Nee and Colegrave 2006).

Phenotypic clustering, environmental filtering and phylogenetic community structure

Most of the traits that were significantly correlated with species distributions clustered within plots (Table 3.5), providing strong evidence for environmental filtering in this system. This phenotypic clustering is particularly compelling because there are physiological and functional reasons to expect these trait-environment associations. The importance of genetically-based variation in these traits is reinforced by the strength of the clustering when traits were measured in a common garden. However, three of the traits (TLP, WUE and seed viability) that correlated with water availability did not demonstrate phenotypic clustering within plots. These results suggest that while these traits do differ across a larger water availability gradient, there is still significant trait variation within plots. This variation could help explain

the high diversity of Salicaceous species at smaller spatial scales, but more research is needed to further explore this possibility.

Since environmental filtering favors the co-occurrence of functionally similar species (Table 3.5, Fig. 3.3), it is possible to investigate the effects of this process on community assembly by examining phylogenetic community structure and trait lability. The expectation is that environment filtering leads to closely related species co-occurring (phylogenetic clustering) when traits are conserved, and not co-occurring (phylogenetic overdispersion/evenness) when traits are labile. In this study, most of the traits that related to species distributions across a summer water availability gradient (e.g. WUE, TLP, wood density and seedling RG) did not deviate from a Brownian motion model of evolution, indicating that they were not phylogenetically conserved (Table 3.6). A similar pattern was found when we examined species habitat affinities, as described by plot environmental conditions weighted by species abundance. Considering these patterns of trait lability, if environmental filtering is acting in this system, we would expect willow and poplar communities to be overdispersed, or to demonstrate a random phylogenetic structure. However, across all the plots, there was a significant correlation between phylogenetic relatedness within plots (as determined by NRI, PSV and $\Delta+$) and water availability (Fig. 3.6). While this trend initially seems counterintuitive, this pattern could be the result of differences in the functional requirements for surviving in seasonally dry versus by seasonal waterlogged habitats.

The correlation observed between species' drought tolerance and their distributions in this study provides strong support that drought is an important environmental filter in this system. However, this study also contains a large number of more hydric sites that are either perennially saturated, or that experience seasonal waterlogging. Waterlogging is a major factor in limiting species distributions in other systems (Shumway and Banks 2001), and its importance in this system is suggested by the low SPI observed in species from wetter habitats (Fig. 3.3). Since the water availability gradient in the field extends into wetland habitats, it may not be appropriate to consider water availability only in terms of its implications for drought stress. When we examined water availability temporally (spring (WT_{wet}) and summer (WT_{dry})), it is possible to separate out the majority of the effects of these two divergent stresses since waterlogging occurs in the spring, and drought is typically important in the late summer. Based on the

K statistic, species distributions described by WT_{dry} are phylogenetically labile, while their distributions described by WT_{wet} are phylogenetically conserved (Table 3.6). These results are consistent with the observed lability in traits associated with drought tolerance (e.g., TLP, wood density, and seedling root GR) and the observed conservatism of the one trait that was associated with specialization in wet habitats (SPI). Therefore, patterns of phylogenetic community structure could be explained by differences in the lability of traits required for specializing in different habitats, as environmental filtering would act on conserved traits in wet habitats (e.g., SPI), leading to phylogenetic clustering, and on labile traits in dry habitats, leading to phylogenetic overdispersion/evenness. However, more research is needed to fully explore this possibility and to test whether multiple traits associated with drought tolerance are phylogenetically conserved.

The implications of reticulate evolution and species hybridization

It is evident from this and other research (Brunsfeld et al. 1991, Hardig et al. 2000, Palme et al. 2003, Hamzeh and Dayanandan 2004) that willow species diversity is in part a product of hybridization and reticulate evolution, which violates a key assumption of phylogenetic analysis. We completed our trait and community analyses using phylogenies that placed the allopolyploid species in different parts of the tree, which is not an accurate representation of allopolyploid genetic history. Since homology in hybrids is difficult to assess because phenotypes often result from interactions between parental genotypes (McDade 1990), studying trait evolution in a lineage that has experienced reticulate evolution can be problematic. However, allopolyploidy and hybridization are common in many plant lineages and issues regarding reticulate evolution reflect a larger need to adjust current phylogenetic methods account for more web-like genetic interactions (Arnold 1997, Vriesendorp and Bakker 2005, McBreen and Lockhart 2006, Baum 2007). Further research is needed to better understand the impact of reticulate evolution on the analyses in this study.

One interesting implication of reticulate evolution and hybridization is that gene flow can allow for the spread of adaptive alleles between species (Anderson 1949, Arnold 2004, Bouck et al. 2005, Martin et al. 2006). If this type of gene transfer has occurred in willows, it could account for the lability observed

in traits related to habitat specialization and also explain the strength of the PIC correlations (Fig. 3.3). It has also been hypothesized that phylogenetic community structure can be influenced by interspecific hybridization, the idea being that genetic assimilation between close relatives would make communities appear overdispersed/even (Cavender-Bares et al. 2009). It possible that such a mechanism is important in structuring some willow communities.

It is important to emphasize that the inferred phylogeny in this study is only based on one gene, and lineage sorting and segregation during sexual reproduction can lead to discrepancies between gene trees and the true species phylogeny (Pamilo and Nei 1988, Doyle 1992). Since there have been relatively few phylogenetic studies of willow and poplar species (Leskinen and Alstrom-Rapaport 1999, Azuma et al. 2000, Hamzeh and Dayanandan 2004, Carstens et al. 2005), and no large-scale treatment of the genus, additional research is needed to better understand the phylogenetic relationships between the species in this study.

Conclusions – We present evidence that willows and poplars demonstrate niche differentiation across a water availability gradient, and traits associated with drought tolerance and recruitment trade-off with growth. Species not only exhibit limited distributions based on summer water availability, but some species also demonstrate less niche overlap than expected by chance. Both the comparative trait and the comparative phylogenetic approaches indicate that environmental filtering is important in community assembly. We also found evidence that the level of trait conservatism differs between traits important for specializing in wet versus dry habitats. However, more research is needed to better understand the physiological basis of species' specialization in wet habitats, and to determine whether multiple traits associated with this type of specialization are phylogenetically conserved.

Table 3.1 – Willow (*Salix*) and poplar (*Populus*) species, their typical habitats and their distributions across a water availability gradient in the study area. Species distributions are described both in terms of their weighted average depth to the water table (m) in the wettest (WT_{wet}) and the driest month (WT_{dry}) of the year.

Species	Typical habitat	WT_{wet}	WT_{dry}
P. deltoides Bartram ex Marsh.	Upland	-0.77	-0.86
P. tremuloides Michx.	Upland	-0.89	-1.08
S. amygdaloides Andersson	Wetland	-0.17	-0.41
S. bebbiana Sarg.	Shrub swamp	-0.18	-0.46
S. candida Flueggé ex Willd.	Marsh	0.01	-0.3
S. discolor Muhl.	Shrub swamp	-0.03	-0.52
S. eriocephala Michx.	Shrub swamp	-0.17	-0.64
S. humilis Marsh.	Savanna	-0.83	-1.1
S. interior Rowlee	Upland	-1.02	-1.17
S. lucida Muhl	Peatland	0.07	0
S. nigra Marsh.	Wetland	-0.01	-0.24
S. pedicellaris Pursh	Peatland	0.04	-0.14
S. petiolaris Sm.	Shrub swamp	-0.11	-0.38
S. pyrifolia Andersson	Peatland	0.01	-0.04
S. serissima (L.H. Bailey) Fernald	Marsh	0.02	-0.2

Table 3.2 – Correlations between species composition and plot environmental conditions based on a NMS ordination analysis. The NMS ordination axes represent the ranking of the dissimilarity between field plots based on species' basal area (Fig. 3.1b). Correlations are shown as Pearson's correlation coefficients (<0.1). Strong correlations (>0.30) are marked in bold. The environmental variables are: N (total nitrogen, g/m^3), C (total carbon, g/m^3), OM (organic matter), pH, P (total phosphorus, g/m^3), K (exchangeable potassium, ppm), NO_3^- (nitrate, ppm), eP (exchangeable phosphorus, ppm), ΔWT (change in water table during the year, m), WT_{dry} (depth to the water table in Aug., m) and WT_{wet} (depth to the water table in May, m).

Variable	Axis 1	Axis 2
Fire	**-0.348**	0.022
C	**0.405**	**0.689**
N	**0.380**	**0.678**
P	-0.263	-0.292
eP	0.242	-0.200
K	**-0.316**	-0.251
OM	0.149	**0.693**
WT_{dry}	**0.594**	**0.492**
WT_{wet}	**0.572**	**0.481**
ΔWT	-0.234	**-0.344**

Table 3.3 – Average plot co-occurrence values based on three different indices. P-values indicate when the number of checkerboard units and C-score values are significantly greater than expected by chance, and when the number of species combinations is significantly lower than expected by chance. These are the expected conditions when there is niche differentiation and/or habitat specialization. Analyses were completed in EcoSim and the null model is described in the text.

	Observed	**Simulated**	**P-value**
Checkerboards	40	29.4	**<0.0001**
C-score	43.2	41.2	**0.03**
Number of combinations	39	42.1	0.06

Table 3.4 – Species and guild niche overlap across a water availability gradient and a soil organic matter gradient. Niche overlap was calculated based on Pianka's index. Plots were organized into five water availability bins based on their weighted depth to the water table in the driest month (WT_{dry}) and into four soil organic matter bins. Significant p-values ($\alpha = 0.05$) are bolded and indicate when the observed mean was significantly smaller, and observed variance was significantly greater than in the null model. Smaller niche overlap than expected by chance indicates niche separation. Greater variance suggests guild structure indicating that some species frequently utilize the same resources and others utilized different resources (Gotelli and Entsminger 2001). Analyses were completed in EcoSim. Null model described in text.

	Mean Overlap			**Variance**		
	Observed	Simulated	P-value	Observed	Simulated	P-value
Water availability						
Species	0.358	0.349	0.70	0.109	0.000	**0.01**
Guilds	0.194	0.355	**0.05**	0.102	0.003	0.81
Soil organic matter						
Species	0.414	0.373	0.87	0.134	0.000	**0.02**
Guilds	0.284	0.428	**<0.0001**	0.112	0.001	0.95

Table 3.5 – Phenotypic clustering within field plots. Phenotypic clustering was determined by comparing species pairwise trait differences to their level of co-occurrence (based on Mantel tests) and determining whether the correlation was more significant than expected from a null model (described in the text). Negative slopes indicate clustering and positive slopes indicate overdispersion/evenness. Species pairwise co-occurrence was determined based on Schoener's co-occurrence index. Significant p-values (α = 0.05) are bolded. The traits are seedling relative growth rate (seedling RGR, g g^{-1} day^{-1}), root growth rate (seedling root GR, cm cm^{-1} day^{-1}), specific leaf area (SLA , cm^2/g), stomatal pore index (SPI), leaf turgor loss point (TLP, MPa), wood density (g cm^{-3}) and water-use efficiency (WUE, A g^{-1})

Functional traits	**Field**		**Greenhouse**	
	r	P-value	r	P-value
Seedling RGR	NA	NA	-0.21	**0.03**
seedling root GR	NA	NA	-0.19	**0.05**
50% seed viability	NA	NA	-0.01	0.51
SLA	-0.18	**0.04**	-0.05	0.40
SPI	-0.09	0.17	-0.38	**0.01**
TLP	-0.16	0.07	-0.10	0.27
Wood density	-0.13	0.08	-0.25	**0.05**
WUE	0.04	0.34	-0.24	0.10

Table 3.6 – Phylogenetic signal of phenotypic traits and niche axes. For each variable, the range of values determined based on derivative phylogenies is reported. Variables were considered conserved when some of the phylogenies had significantly ($\alpha = 0.05$) less variation than expected from a Brownian motion model of evolution as indicated by K values approaching 1.

Traits	K	Range P-values	Median P-value	Phylogenetic Signal
Distributions across environmental gradients				
WT_{dry}	0.31- 0.35	0.06 - 0.09	0.07	No significant trend
WT_{wet}	**0.52 - 0.62**	**0.02 - 0.09**	**0.04**	**Conserved**
Nitrate	0.16 - 0.20	0.12 - 0.19	0.18	No significant trend
NT	0.08 - 0.09	0.48 - 0.51	0.49	No significant trend
OM	0.16 - 0.21	0.21 - 0.26	0.22	No significant trend
Species' traits				
Seedling RGR	0.09 - 0.10	0.40 - 0.47	0.45	No significant trend
Seedling root GR	0.13 - 0.14	0.25 - 0.29	0.22	No significant trend
Seed viability	0.22 - 0.26	0.23 - 0.31	0.25	No significant trend
SLA	0.24-0.25	0.30 - 0.33	0.34	No significant trend
SPI	**0.62 - 0.70**	**0.01 - 0.03**	**0.01**	**Conserved**
Stom. Aper.	**0.85 - 1.0**	**0.03 - 0.06**	**0.05**	**Conserved**
TLP	0.11 - 0.12	0.37 - 0.44	0.42	No significant trend
Wood density	0.03 - 0.04	0.87 – 0.91	0.9	No significant trend
WUE	0.12 - 0.14	0.46 - 0.50	0.45	No significant trend

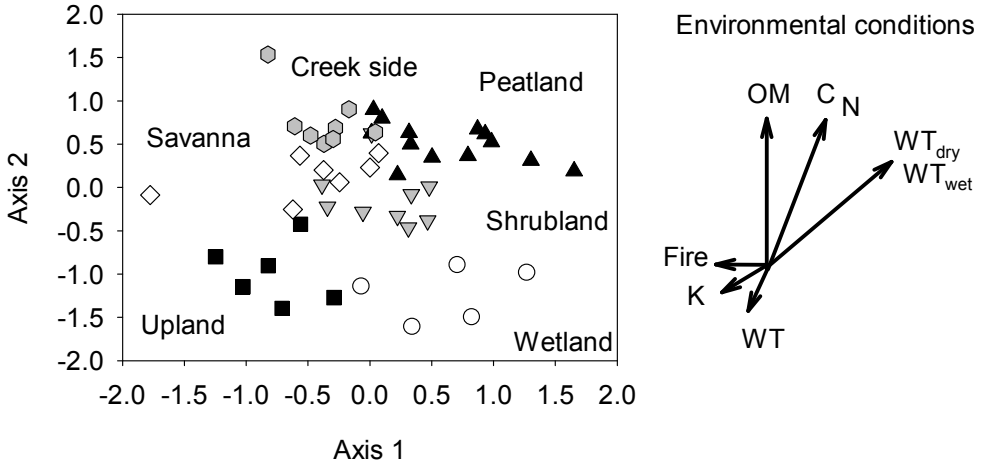

Figure 3.1 The NMS ordination of willow and poplar species abundances in field plots provide evidence for species' habitat specialization as indicated by the observed correlation between the ordination axes and multiple environmental variables. Each point marks a field plot and the symbols indicate habitat type. Distances between points indicate the dissimilarity between plots in terms of species composition. The ordination axes correlate with several environmental variables ($r > 0.3$), which are pictured as vectors. The environmental variables include N (total nitrogen, g/m^3), C (total carbon, g/m^3), OM (organic matter), P (total phosphorus, g/m^3), K (exchangeable potassium, ppm), WT_{dry} (depth to the water table in Aug., m), WT_{wet} (depth to the water table in May, m) and fire frequency (fires $year^{-1}$). The vector indicates the strength (increased by a factor of 2/3) and direction of the correlations with the ordination axes.

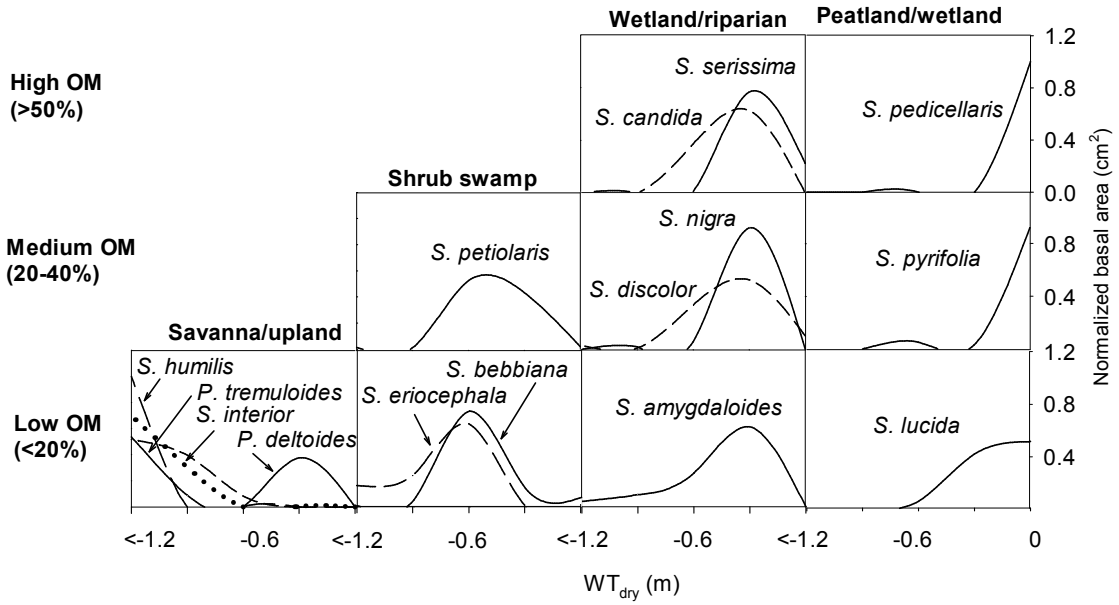

Figure 3.2 The fifteen species in this study demonstrated relatively limited distributions across the water availability gradient sampled in the field. Species distributions are described by their normalized basal areas across field plots. Graphs are stacked horizontally based on the water availability bins, and vertically based on the soil organic matter (OM) categories where they are the most abundant (for simplicity only three bins are shown for OM). Since depth to the water table could only be measured to −1.2 m, the figures indicate that the left side of the water availability gradient contains plots where the water table was <−1.2 m. The habitats that occurred within the water availability bins are noted above the graphs. It is important to note that there were very few individuals of *P. deltoides* found in the plots and this could account for the species' bimodal distribution.

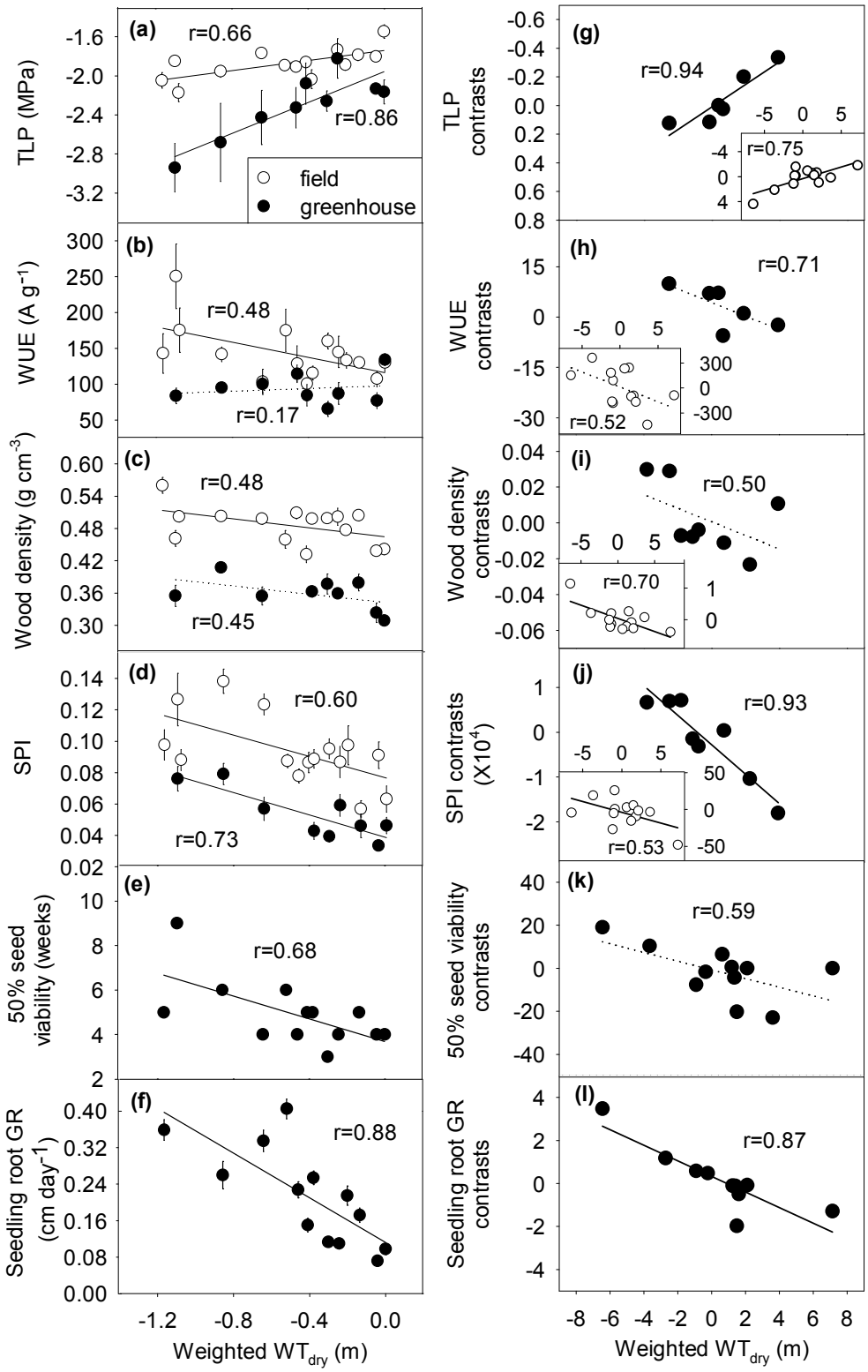
(a)
r=0.66
r=0.86
field
greenhouse
TLP (MPa)
(b)
r=0.48
r=0.17
WUE (A g-1)
(c)
r=0.48
r=0.45
Wood density (g cm-3)
(d)
r=0.60
r=0.73
SPI
(e)
r=0.68
50% seed viability (weeks)
(f)
r=0.88
Seedling root GR (cm day-1)
Weighted WTdry (m)
(g)
r=0.94
r=0.75
TLP contrasts
(h)
r=0.71
r=0.52
WUE contrasts
(i)
r=0.50
r=0.70
Wood density contrasts
(j)
r=0.93
r=0.53
SPI contrasts (X10-4)
(k)
r=0.59
50% seed viability contrasts
(l)
r=0.87
Seedling root GR contrasts
Weighted WTdry (m)

Figure 3.3 Traits related to **(a-d)** water-use, and **(e-f)** traits related to seedling recruitment correlated with species distributions across a water availability gradient (described by weight average depth to the water table in the summer (WT_{dry})). Phylogenetically independent contrasts for the same traits are graphed in **(g-l).** Correlations between contrasts indicate when changes in habitat and traits values are correlated across the phylogeny. The graphed contrasts are based one of phylogenies and correlations based on the other phylogenies are described in Appendix 1 (Table S1.2). Traits that were measured in the field are marked with open circles, and traits measured in a greenhouse common garden are marked with closed circles. Error bars are ± one standard error. Significant regressions are indicated with a solid line and non-significant regression are indicated with a dotted line ($\alpha = 0.05$).

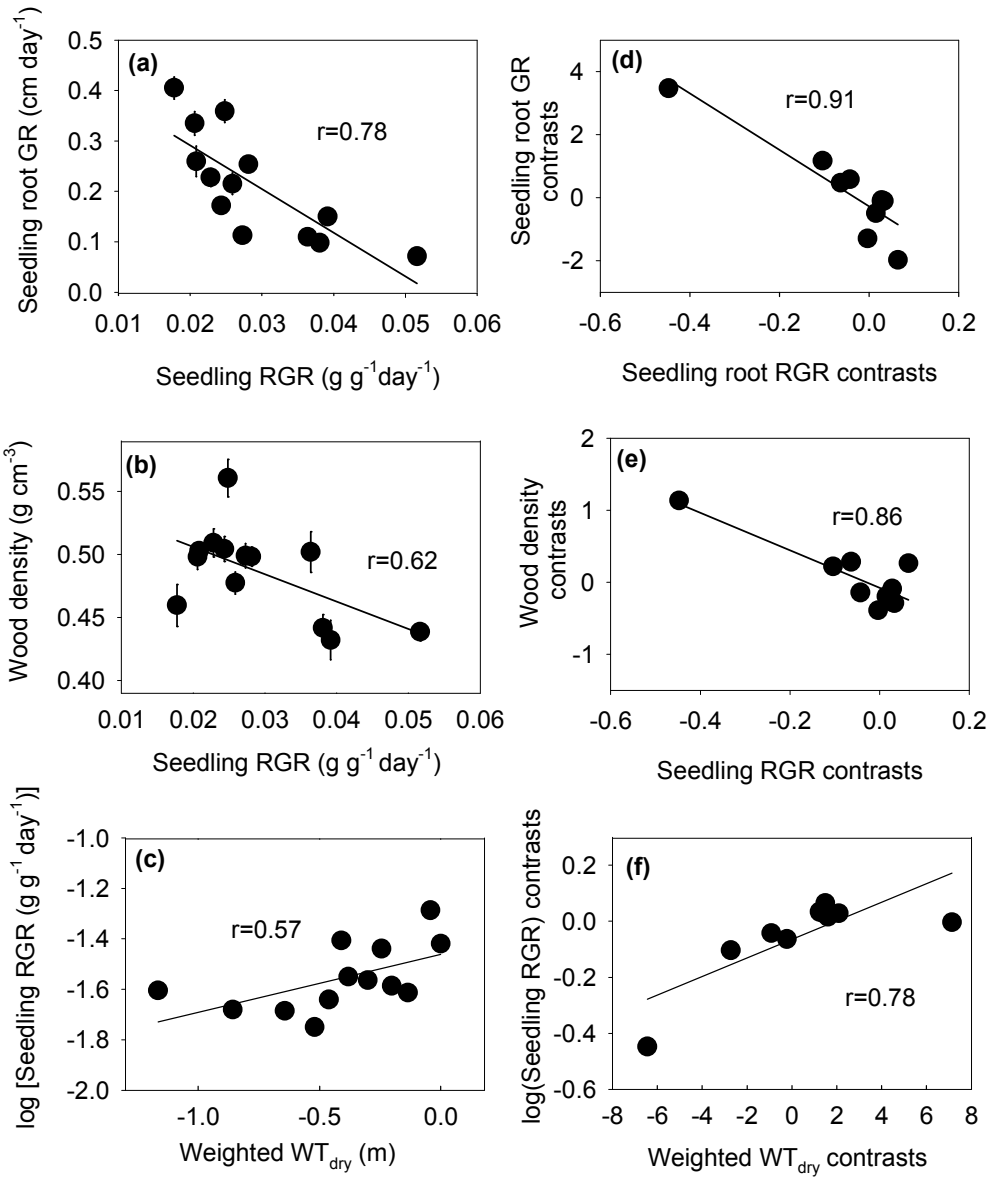

Figure 3.4 – (a) Seedling root growth rate (cm day^{-1}) and **(b)** wood density (g cm^{-3}) were inversely related to seeding RGR (g g^{-1} day^{-1}), and **(c)** log(seedling RGR) was postively associated with depth to the water table across species. **(d-f)** All of these correlations were also significant in terms of phylogenetically independent contrasts (PIC) indicating that these traits exhibited correlated evolution with species' habitat affinity. The graphed contrasts are based on one of the phylogenies and correlations based on the other phylogenies are described in Appendix 1 (Table S1.2). Circles represent species averages and error bars are ± one standard error.

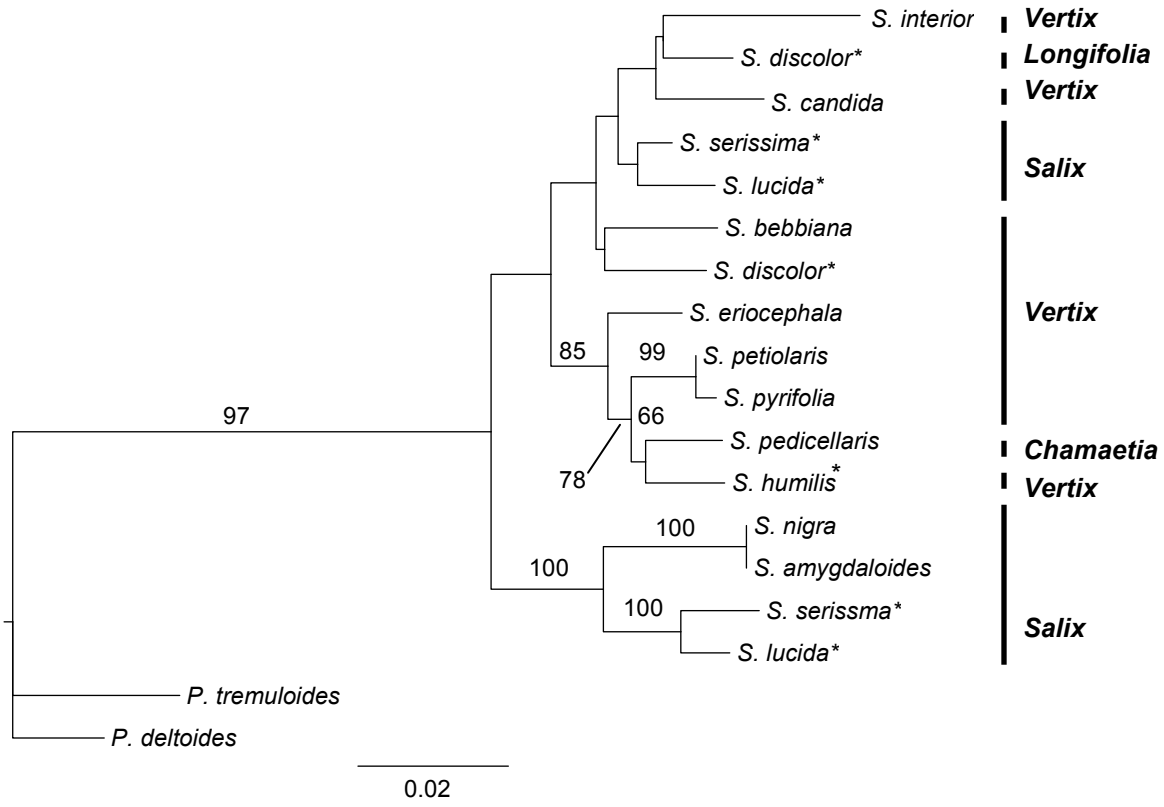

Figure 3.5 Phylogeny of *Salix* species based on the *ADH* gene. This species tree was obtained by pruning the maximum likelihood gene tree shown in Appendix 1 (Fig.S1.1). Branch lengths were estimated under a HKY + G model of sequence evolution. Bootstrap values for are shown for all nodes that have over 50% support, and asterisks mark polyploid species. Subgenus classification are listed to the right of the tree (based on Argus (1997)). There is evidence for one allopolyploid event involving the ancestral parents of *S. lucida* and *S. serissima.* History of polyploidization is unclear in *S. humilis* and *S. discolor* because bootstrap support is lacking. However, the number of alleles found in *S. humilis* suggest that it is an octoploid in Minnesota.

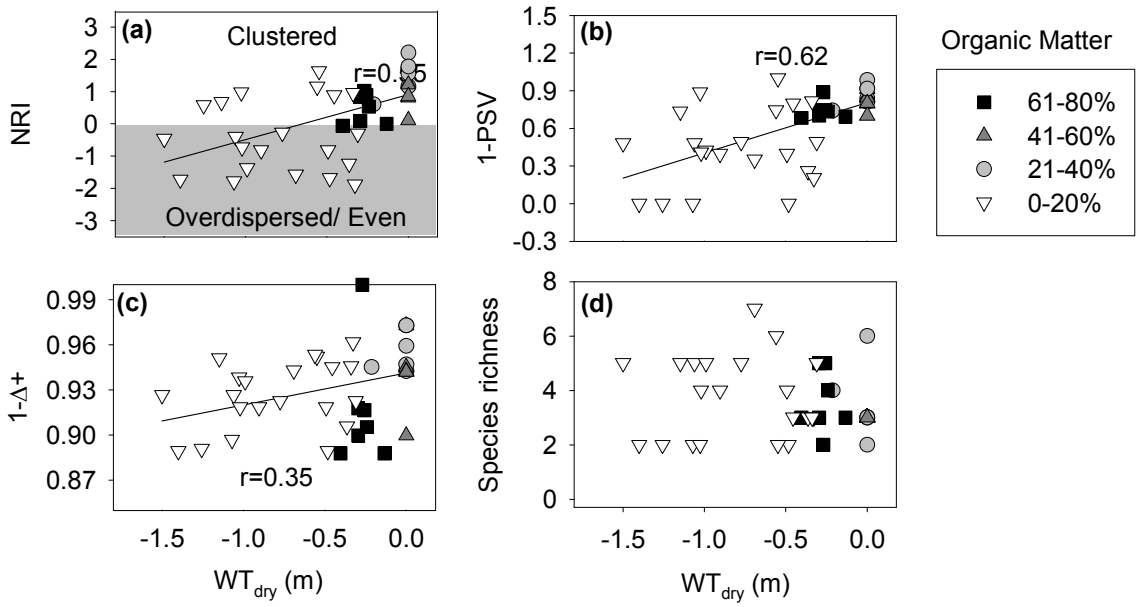

Figure 3.6 Plots with higher water summer availability (WT_{dry}) exhibited **(a)** a greater phylogenetic clustering as indicated by their higher net relatedness index (NRI), **(b)** their lower phylogenetic species variability (graphed as 1 - PSV for consistency) and **(c)** their lower taxonomic distinctness (graphed as 1 - Δ+ for consistency). All of these correlations were significant with $\alpha = 0.05$. **(d)** There was no relationship between species richness within plots and summer water availability. The graphed phylogenetic metrics are based on one of the phylogenies and correlations based on the other phylogenies are described in Appendix 1 (Table S1.2).

CHAPTER 4

Photoperiod and timing of cold acclimation modify a trade-off between freezing tolerance and growth rate in the willow family (Salicaceae)

It has long been hypothesized that the biophysical costs associated with freezing tolerance cause a trade-off between cold tolerance and growth rate that is critical to explaining species northern and southern range limits. Despite the proposed importance of this trade-off, there has been relatively limited research on its existence and its applicability to different systems. In this study, we examined evidence for a freezing tolerance-growth rate trade-off in the willow family (Salicaceae). We grew twenty-four willow and poplar species that were collected across North America in a greenhouse common garden under temperate and subtropical climate treatments. We described species distributions based on maximum entropy models and used these models to estimate climate parameters for each species. We then measured a wide range of traits relating to freezing tolerance including senescence, budburst and susceptibility to different temperature minima at multiple times during the years. We used these data to compare species freezing tolerances to their climate distribution envelopes. We also compared species growth rates in the different climate treatments. We discovered that species from colder climates exhibited higher freezing tolerance and slower growth rates than species from warmer climates. However, the northern species exhibited fast growth rates in a longer photoperiod treatment indicating that their slower growth rates were not a result of a slower growth capacity. Instead these results indicate that species from northern latitudes may be limited in warmer climates because of their dependence on long day lengths for the up-regulation of growth in the summer. Taken together these results indicate that the observed trade-off between freezing tolerance and growth is mediated by species photoperiod requirements for growth and may involve a suite of traits associated with freezing tolerance that influence species overall cold tolerance strategies. However, more research is needed to examine whether this trade-off is applicable to species range limits.

INTRODUCTION

With heightened concern about the ecological impact of global climate change, there is growing interest in exploring how climate influences species' range limits and the extent that species' ranges shift with changes in climate conditions (Rehfeldt et al. 2006, Iverson et al. 2008, Thuiller et al. 2008). This interest has led to the development of species distribution models that describe species' current and future ranges based on a range of environmental variables (for reviews see Guisan and Zimmermann (2000) and Guisan and Thuiller (2005)). Since the mechanisms that maintain species' boundaries are largely unknown, these models often rely upon correlative relationships between occurrence data and current climatic conditions without prior knowledge of the biotic and abiotic factors important in maintaining species distributions (Gaston 2003, Case et al. 2005, Goldberg and Lande 2007, Morin et al. 2007, Holt and Barfield 2009). However, species-level physiological and ecological information can strengthen these models (Schenk 1996, Davis et al. 1998) and improve our understanding of the ecological implications of global climate change.

Freezing temperatures are known to be important in limiting many plant species distributions (Parker 1963, George et al. 1974, Guy 2003, Cavender-Bares 2005) and these limitations are the subject of some of the earliest research on species distributions (Merriam 1894, Hutchinson 1918). Since freezing stress increases with decreasing minimum temperature (Larcher et al. 1973, Korner and Larcher 1988, Ball et al. 2006, Cavender-Bares 2007), many species are believed to be limited along their northern boundaries by their susceptibility to temperature minima (Larcher and Bauer 1981, Pockman and Sperry 1997). It has also been observed that many species do not exhibit a greater freezing tolerance than is required in their native ranges (Sakai and Weiser 1973, Pither 2003). For plants with tropical ancestry, this trend is straightforward because there is no selective pressure for them to acquire tolerance of temperatures lower than those that occur within their range limits. However, the trend is more difficult to explain in species with temperate or boreal ancestry, since these species have to lose freezing tolerance as they migrate south. Therefore, it has been hypothesized that freezing tolerance compromises individual plants' growth rates resulting in a trade-off between freezing tolerance and growth (MacArthur 1972, Woodward and Pigott

1975, Loehle 1998). This trade-off theoretically prevents northern species from colonizing warmer climates because they cannot compete with faster growing southern species and is appealing because it could account for the association between species' range limits and their freezing tolerance.

There are many physiological mechanisms that could explain a trade-off between freezing tolerance and growth. For example, many plants undergo physiological changes during acclimation that allow them to maintain function under cooler growing conditions and survive freezing temperatures. One such change is an increase in photoprotective pigments in their leaves (Anderson et al. 1992, Adams and Demmig-Adams 1994). These pigments minimize damage from excess light under freezing conditions by safely dissipating excess energy (i.e. xanthophyll pigments) or by acting as antioxidants and reducing the number of free radicals in the leaves (i.e., carotenoids) (Adams et al. 1995, Verhoeven et al. 1996, Perez-Torres et al. 2004). Many species also invest in structural modifications, which result in denser tissue and increased lignin and pectin in their cell walls (Huner et al. 1981, Wisniewski and Davis 1995, Kubacka-Zebalska and Kacperska 1999). Sometimes these investments are further supported by the build-up of sugars (Sakai and Yoshida 1968, Guy 1990), antifreeze proteins (Griffith et al. 1992) and supercooling compounds (Kasuga et al. 2008) that prevent ice nucleation during freezing events. Since most of these physiological changes come at a carbon and nutrient cost, freezing tolerant species would not be able to invest as much in growth compared to non-freezing tolerant species.

Another potential mechanism for the hypothesized trade-off is that a constraint on xylem anatomy could lead to slower growth rates in freezing tolerant species (Wang et al. 1992). In the xylem, wide conduits are less freezing tolerant than narrow conduits because their greater diameter allows for the cavitation of larger bubbles after freeze-thaw events (Sperry and Sullivan 1992, Davis et al. 1999, Cavender-Bares and Holbrook 2001). Since larger bubbles are more likely to expand, block conduits, and reduce vascular function, species from colder climates tend to have narrower conduits (Pockman and Sperry 1997). However, narrower conduits have lower hydraulic conductivity and do not support fast growth rates (Brodribb and Feild 2000, Sperry 2000, Brodribb et al. 2002). As a result, it is possible that species with high freezing tolerance do not achieve high growth rates because of their xylem anatomy.

Although the freezing tolerance-growth trade-off has long been hypothesized (Woodward and Pigott 1975, Loehle 1998), surprisingly little evidence exists for it in the literature, and complexities involved in applying it to species distributions have remained relatively unexplored. One of these complexities is that freezing tolerance can vary seasonally, and differences among species in their seasonality can have consequences for their distributions (Sakai and Larcher 1987). For example, species that survive extremely cold temperatures in the winter can be limited in their northern distributions by whether they acclimate before the first autumn frost (Smithberg and Weiser 1968, Sakai and Weiser 1973). In general, there are four components of species' cold tolerance that can limit their distributions: their rate of acclimation in the autumn, their phenology (leaf senescence, growth cessation, budburst and flowering), their cold-acclimated freezing tolerance, and their rate of de-acclimation in the spring (Sakai and Larcher 1987). If species distributions are influenced by different components of species' freezing tolerance, it is important to know which components are related to growth and the implications of these different physiological strategies on species distributions.

Another complexity associated with the hypothesized trade-off is that both freezing tolerance and growth can be plastic. For example, some arctic species can increase their average xylem vessel diameters when grown under warmer conditions, leading to a decrease in their freezing tolerance (Gorsuch and Oberbauer 2002). If freezing tolerance and growth depend on environmental conditions, the question that arises is whether the costs associated with freezing tolerance are only incurred when freezing tolerance is expressed. A trade-off between freezing tolerance and *in situ* growth rate might not have any effect on species distributions if species could maintain higher growth rates at their southern boundaries without compromising their freezing tolerances at the northern range edge. Under these conditions, freezing tolerance would not prevent species from invading regions offering warmer climates. However, if there is a cost associated with maintaining the ability to express freezing tolerance, then freezing tolerant species may have slow growth rates regardless of the level of freezing tolerance they express under different climatic conditions.

The last major issue regarding the hypothesized trade-off is that its expression may differ depending on the biogeographic and evolutionary history of different plant lineages. For example, if it is

more costly to acquire freezing tolerance than to maintain it, lineages originating from warmer climates might demonstrate a much steeper trade-off than those from colder climates. Another possibility is that some lineages may have evolved unique mechanisms to survive freezing temperatures that are not as costly to growth. Either of these scenarios could create lineage-specific variation that could impact how this hypothesized trade-off is manifested across and within lineages.

To address some of these issues and to test the validity of this hypothesized trade-off, we examined the relationship between freezing tolerance and growth rate in a group of twenty-four North American species in the genera *Salix* and *Populus* (family Salicaceae). These genera span North America from the Arctic Circle to Mexico and dominate many early successional communities (Argus 2007). Salicaceae is a good study system for investigating this hypothesized trade-off because it provides the opportunity to explore some of the complexities surrounding this putative trade-off. Willows (*Salix* spp.) and poplars (*Populus* spp.) are known for being highly plastic in their growth rates (Lennartsson and Ogren 2002) and differing in their phenology and rates of cold acclimation (Ogren 1999, Lennartsson and Ogren 2004). Additionally, since these lineages' migration patterns into North America are understood (Sakai 1970, Taylor 1990, Collinson 1992, Skvortsov 1999), it possible to investigate this trade-off with specific consideration to the history of the lineage and its ancestry.

MATERIALS AND METHODS

Selecting and propagating species

We selected 20 willow species and four poplar species from a broad range of biomes (the arctic tundra to subtropical forests), including two species with wide latitudinal distributions (*S. interior* and *S. exigua*) (Table 4.1). In the spring and summer of 2007, we collected cuttings from each species in the middle of their latitudinal ranges. The only exceptions were the two widespread species (*S. interior* and *S. exigua*), which we collected at multiple locations so we could investigate intraspecific variation in their phenology and growth. We deposited a set of voucher specimens of each species in the University of

Minnesota Herbarium. We propagated all the species by stem cutting and transplanted them into 6.25 L treepots in a soil mixture of 50% sand, 30% compost and 20% peat. Before starting the climate treatments, we grew the plants with a day length of 15.5 hours at an average temperature of 23–27°C. The plants were well-watered and fertilized for the duration of the study.

Growth conditions and climate regimes

The majority of plants were grown in one of two primary climate treatments; a temperate and a subtropical treatment. In each of these treatments, there were 6–12 unique genotypes (individuals) per species. We used clones from the same genotypes in the two climate treatments whenever possible, and each climate treatment was evenly split into two replicate greenhouse rooms. The temperate treatment simulated the seasonal temperatures and photoperiod of Franklinville, NY (42.34°N,-78.46°W), where the greenhouse was located (Fig. 4.1). In this treatment, the plants were kept in the greenhouse during the first winter and only allowed to cool to 2°C. During the second winter, we moved the plants outside the greenhouse and let them experience subzero temperatures (Fig. 4.1). The subtropical treatment simulated the seasonal temperatures and photoperiod of Morelia, Mexico (19.77°N, 101.19°W), which is near the southern range limit of some of the species in this study. Since we were unable to decrease the summer photoperiod in the greenhouse, the summer photoperiod was greater in this treatment than in Morelia (Fig. 4.1). We started these treatments in November 2007 and ran them until August 2009. We also had an additional treatment, which we used to investigate the role of summer photoperiod in determining growth rates. This photoperiod treatment was conducted on a subset of 5–6 individuals for each of 10 species that covered the same range of climates as the full species set (Table 4.1). It had a temperature regime identical to the temperate treatment but had a summer photoperiod of 20.5 hrs (similar to the photoperiod in Fairbanks, Alaska where the most northern species were collected).

Characterization of species' ranges

We characterized species' ranges based on range maps from multiple sources (USGS 1999, Argus 2007) and by modeling their climate distribution envelopes. For the modeling analysis, we used the program Maxent version 3.3.1 (Philips 2004), which determines the probability that a habitat will be suitable based on a maximum entropy model that is constrained by the occurrence data for each species. We got climate information from the Worldclim climate dataset (resolution of 2.5 arc-minutes) (Hijmans et al. 2005) and used herbarium records as our occurrence data (accessed using the NatureServe Central Database, GBIF data portal, www.gbif.org/datasets/resource/607, 2009-03-08). For quality assurance, we did not include herbarium records that fell outside of published species' ranges (Argus 2007) and records that did not contain precise latitude and longitude information. We only included samples that had enough information to confirm species' identity (i.e. subspecies classification for species that have been reclassified). We found between 73 and 1200 collections per species that met our criteria (see Appendix 2 – Table S2.1 for a list of herbaria accessed).

We ran the model with a random test set containing 25% of the data points and did a jackknife analysis to compare the relative importance of different climatic variables. We assessed the performance of the model with a threshold-independent receiver-operating characteristic analysis (ROC). We considered good performing models to have AUC (area under the curve) greater than 0.9 (Swets 1988). We then estimated the minimum temperature (Min T) in species' modeled climate distribution envelopes, which we defined as the zenith of the average minimum temperature probability curve. We also determined several other climatic variables described in Appendix 2 (Fig. S2.1). For the range map analysis, we compared range maps to climate conditions using DIVA-GIS (Hijmans et al. 2001) and the Worldclim climate data (Hijmans et al. 2005) and we estimated the maximum summer photoperiod both at species northern range limits and in the middle of their ranges (Geographical Services Division 1987, NOAA 2009), and several other variables relevant to species' cold acclimation and freezing tolerance (See Appendix 2 – Fig. S2.1). We also determined the shift in photoperiod that species experienced during the experiment by calculating

the differences between the maximum day lengths in species native ranges and the different greenhouse treatments.

Species' growth, survival and photosynthetic capacities

To investigate whether or not species from colder climates had slower growth rates than species from warmer climates, we measured plant height and basal area three times during the study in the subtropical, temperate, and long summer photoperiod treatments (May, June and August 2008). We sampled 6–10 individuals per species in the temperate and subtropical treatment, and 4–6 individuals per species in the photoperiod treatment. We calculated plant growth rate as $[\ln(g_2) - \ln(g_1)]/(t_2 - t_1)$ where g_1 and g_2 are the growth measurements at times t_1 and t_2 (Hunt 1982). Since many of the species in this study are shrubs, we also monitored branch elongation to get an estimate of species' lateral growth. For this measurement, we recorded the length of three branches per plant in the spring and summer. We also determined the percent mortality in each treatment during the summer 2008. Plants were considered dead if they had no leaves and had not grown all year. As a result of mortality, we could not measure the growth rates of *S. arbusculoides* and *S. petiolaris* in the subtropical treatment.

We measured photosynthetic capacity (A_{max}) and specific leaf area (SLA) in the temperate treatment in May 2008 because these traits often correlate with relative growth rate (Poorter et al. 1990, Cornelissen et al. 1996, Reich et al. 1999). We measured gas exchange on 4–6 individuals per species using a portable photosynthesis system (LICOR 6400-40, Licor Inc., Lincoln, NE). We used a light level of 1500 $\mu mol\ m^{-2}\ s^{-1}$ because we determined that this light level was able to saturate photosynthesis in a subset of species (data not shown). We used a large mixing volume to keep CO_2 levels close to ambient (approximately 380 $\mu mol\ mol^{-1}$) and took all the measurements between 8:00 am and 10:30 am over a three day period. We calculated specific leaf area on three fully-expanded leaves per plant in Sept. 2007.

Comparison of species' freezing tolerance

To examine whether traits related to freezing tolerance relate to species distributions, and whether any of these traits come at the expense of growth, we compared species' (1) cold acclimation, (2) cold-acclimated freezing tolerance, and (3) timing of budburst.

(1) Cold acclimation

Phenology and growth cessation – We compared the timing of two phenological changes associated with cold acclimation: growth cessation and leaf senescence. We examined growth cessation by comparing the decline in species' growth rates from their maximum growth rates in the spring as a proportion of their maximum growth rates. We also calculated the percent of individuals that experienced senescence (all the leaves on the plant were yellow or had dehisced) during each week of the autumn and winter of 2007-2008.

Autumn and early winter freezing tolerance – While the plants were cold acclimating in October and November 2008, we conducted five freezing experiments in the temperate treatment (Table 4.2). We measured leaf freezing tolerance at −5.5°C in Oct. and Nov., stem freezing tolerance at −5.5°C and −10°C in Oct., and stem freezing tolerance at −10°C and −15°C in Nov. Since we had limited plant material, we were not able to include all of the species in each of the freezing experiments (Table 4.2). To measure leaf freezing tolerance, we cut a 6 cm stem segment from a distal branch on each plant. The samples were cut and recut under water, and we kept at least two fully-expanded leaves on each sample. To test stem freezing tolerance, we used 5 cm segments that were cut 6 cm from the end of the branches and we removed all their leaves. We put the stem samples in rose tubes with distilled micropore filtered water (particle size < 0.2 μm) for all the freezing experiments. Each set of experiments was conducted over a one week period using 6–8 samples with leaves and 5-10 stem samples without leaves, per species.

For the freezing experiments, we used a modified freezer that was controlled with a programmable chiller (Cole-Palmer Instrument Co., Vernon Hills, IL) and cooled with coils filled with circulating Dynalene heat transfer fluid (Daynalene, Inc., Whitehall, PA) (Cavender-Bares 2007). We cooled the samples at a rate of 4°C hr^{-1} and kept the samples in the freezer at a set minimum temperature for three hours. We used the freezer to warm up the leaf samples at a rate of 10°C per hour and we warmed up the stem samples by putting them in a refrigerator for one hour and then leaving them on a lab bench for one hour. To ensure that the conditions in the freezer were homogenous and that the samples were cooling at the expected rate, we monitored the temperature of a subset of stems during the experiment using copper-constantan thermocouples and a 23X Campbell datalogger (Campbell Scientific, Inc., Logan, UT).

We quantified species' freezing tolerance by measuring their predawn chlorophyll fluorescence using a Hansatech Pulse-Modulated Fluorometer (Hansatech Instruments LTd., Norfolk, England). We measured F_o, which is the minimum dark-acclimated chlorophyll fluorescence (measured when samples are exposed to very low light levels) and F_m, which is the maximum dark-acclimated chlorophyll fluorescence (measured when samples are exposed to a saturating pulse of 7000 μmol $m^{-2}s^{-1}$ for 0.7 s), before and after the freezing tests. On the leaves, we also measured chlorophyll fluorescence 3, 7, and 25 hours after each freezing test, and on the stems 25 hours after each test. All the samples were kept in the dark during the experiment and all measurements were taken in the same spot on each sample. These chlorophyll fluorescence measurements were used to calculate maximum photosynthetic efficiency (F_v/F_m) with the equation $F_v/F_m = (F_m - F_o)/F_m$. Since photodamage causes irreversible declines in F_v/F_m (Krause 1988, Boorse et al. 1998a), this metric is a good indicator of tissue damage in many plant species including willows (Smillie and Hetherington 1983, Fisker et al. 1995, Lennartsson and Ogren 2002). As a control, we measured chlorophyll fluorescence on a set of plants that were kept in a dark cabinet during the experiment. We also examined whether cambial death occurred in the stem samples after each set of freezing tests. For this assessment, we placed the stem segments in plastic bags with wet paper and kept them in the refrigerator for two weeks. We then examined longitudinal cross-sections of the segments with a dissecting scope and determined whether the segments exhibited cambial death.

(2) Cold-acclimated freezing tolerance

We compared the ability of cold-acclimated plants to survive freezing at −55°C in the late winter (February 2009) after the plants had been outside for three months (Table 4.2). For the first part of the freezing cycle, we used a freezer setup that was identical to the one used in the autumn and early winter experiments. For this experiment, we cooled samples at a rate of 4°C hr^{-1} until the samples reached −20°C. We then transferred them to a −55°C freezer in an insolated polystyrene foam container, which allowed the samples to cool gradually at a rate of 4°C per hour until they reached −55°C. At that point, they were held at a constant temperature for three hours. We controlled their rate of warming by moving them first to a −20°C freezer, then to a refrigerator and finally to the lab bench. Since the samples were kept in a closed insulated container, their warming was buffered and they warmed at an approximate rate of 16°C per hour. To confirm that the samples were cooling at the expected rate during all the stages of the experiment, we also monitored the temperature of a subset of samples throughout the experiment with copper-constantan thermocouples and a 23X Campbell datalogger. This experiment was completed over a two week period.

We assessed freezing damage using an electrolyte leakage technique (Burr et al. 1990, Friedman et al. 2008). Electrolyte leakage techniques are based on the assumption that when cells lyse because of freezing injury, their electrolytes are released, causing an increase in electrical conductivity that is proportional to cell mortality. While these measurements are not sensitive to cell recovery and their success can vary depending on species (Calkins and Swanson 1990, Boorse et al. 1998a), previous research on willows and poplars suggest that they are an effective measure for assessing freezing damage in these genera (Lennartsson and Ogren 2002, Friedman et al. 2008). To measure electrolyte leakage, we put two 1-cm stem samples from each plant into individual test tubes with 1 mL of water. One of these tubes was frozen and the other was kept in a water bath in the refrigerator. After the freezing cycle, we added 4 mL of water to both the frozen and unfrozen samples and put them in a shaking water bath at room temperature. We waited 20 hours and then measured electrical conductivity in each test tube with a conductivity meter (Oakton 510, Oakton Instruments, Vernon Hills, IL). The samples were then autoclaved to lyse all cells in both samples, and placed back in the water bath. We took a final set of conductivity measurements after 20

hours and calculated the index of injury (*It (%)*) based on Flint's equation (Flint et al. 1967): *It (%)* = 100 × $(R_f - R_o)/(1 - R_o)$, where R_f is the relative conductivity of the frozen sample divided by the relative conductivity of the frozen sample after it has been heat killed, and R_o is the relative conductivity of the non-frozen sample divided by the relative conductivity of the non-frozen sample after it has been heat killed. We also assessed cambial death for each plant the same as in the autumn and early winter. We sampled 6–8 individuals per species and cut 1-cm long stem segments for the electrolyte leakage measurements, and 5-cm segments for the cambial mortality assessment. Protocol for sample collection was the same as described earlier.

In addition to comparing species' cold-acclimated freezing tolerance, we also monitored the temperature at which freezing occurred (as indicated by an exotherm) in the stems of a subset of species (*P. fremontii, S. hookeriana, S. lucida, S. nigra* and *S. pyrifolia*). For this test, we froze 5-cm long stem samples of five individuals per species to −10°C. The samples were cooled at the same rate as in the other freezing experiments. We monitored stem temperature with copper-constantan thermocouples inserted into the pith of each stem. We logged the temperature every minute using a 23X Campbell datalogger and considered exotherms to occur when there was a spike in temperature greater than 1°C (Cavender-Bares et al. 2005). We used these data to get an average exotherm temperature across this subset of species and examine whether there were significant differences between the timing of species' exotherms.

(3) Budburst

In the spring of 2009, we monitored bud activity on all the plants that were outside in the temperate treatment. We monitored 5–10 individuals per species but did not include *S. pseudomonticola* or *S. pulchra* because of their small samples sizes. We recorded the date of first budburst (the appearance of green tissue emerging from the bud) of each plant and used it to calculate its growth degree days (GDD). GDD is calculated as $\Sigma\ (T_{max} - T_{min})/2 - T_{threshold}$, where T_{max} and T_{min} are the maximum and minimum temperature of each day between January 1, 2009 and the date of budburst, and $T_{threshold}$ is a threshold temperature for growth. We did not include days that had an average temperature less than the threshold

temperature, and selected a threshold temperature of 1°C based on research conducted on willows in Europe (Lennartsson and Ogren 2004). We determined the maximum GDD for each species and compared this value to its distribution.

Potential trade-off between vessel diameter and growth

One explanation for a potential trade-off between freezing tolerance and growth is that xylem anatomy prevents freezing tolerant species from having a fast growth rate. Since narrow xylem vessels are more freezing tolerant than wide vessels, high freezing tolerance could result in slow growth rates because of the limited hydraulic conductivity of narrow vessels (Sperry and Sullivan 1992, Davis et al. 1999, Cavender-Bares and Holbrook 2001). Therefore, we investigated whether species' vessel diameters positively correlated with their distributions and negatively correlated with growth across species. For this analysis, we made cross-sections of one year old branches in the late winter 2009 in the temperate treatment. The cross-sections were made by hand and analyzed with Image J (Abramoff et al. 2004). We measured the area of the vessels in one quarter of each cross-section (over 100 vessels per sample), calculated the average vessel area and estimated the diameter of each vessel. We also calculated stem hydraulic conductivity (K_s) based on the Hagen-Poiseuille equation: $[(\pi\rho/128\eta)\Sigma d^4]/A$, where ρ is the density of water, η is the viscosity of water, d is the diameter of each vessel, and A is the area of the xylem measured. We compare species' average vessel diameters, their K_s and their growth rates using linear regression analyses.

Statistical Analysis

Since we used clones in the different climate treatments, we were able to compare species' functional and phenological traits between the treatments with two-sided, matched t-tests. We also used an ANOVA to determine whether species differed in their traits within treatments and t-tests to determine whether populations within the widely distributed species were significantly different within treatments.

We completed several linear and non-linear regression analyses to examine the relationships between climatic variables and estimates of species' functional traits, phenological traits and growth rates. When analyzing growth rates, we did a multiple regression analysis on the effects of summer day length at species northern range limit and their modeled Min T on growth. We also used a logistic regression with a binomial distribution to compare the viability measurements (chlorophyll fluorescence and electrolyte leakage) used for assessing freezing damage to cambial survival. After compiling all the data on species' average freezing tolerance (including phenological traits and freezing susceptibility at different times during the year), we completed a principal components analysis (PCA) on species in trait space. We then used the primary axis of the PCA to describe species' general freezing tolerance and completed a regression analysis of PCA1 on species' growth rates. We completed this analysis using species' growth rates in the subtropical treatment because it was the most representative of climate conditions south of the species current distributions. We used JMP 8.0 (SAS Institute Inc., Car, NC) for all the statistical analyses in the paper except for the non-linear regression analyses, which were completed in Sigmaplot 9.0 (Systat Software, Inc., San Jose, CA) and the PCA which was completed in PC-ORD 5.0 (MJM software design, Gleneden Beach, OR).

RESULTS

Species' climate distribution envelopes

The Maxent niche models described species distributions with a high specificity and sensitivity (AUC of greater than 0.9) and corresponded well with species' range maps (Argus 2007). The test samples also fit the data with only two species having test AUC's less than 0.9 (*S. alaxensis* – 0.89, *P. balsamifera* – 0.88). Based on the jackknife analyses, the most informative climate variables for 17 of the species were related to temperature maxima (i.e. maximum annual temperature and mean temperature of the warmest month). However, these variables were only marginally more informative than other variables because all of the climatic variables were highly correlated (Appendix 2 – Fig. S2.1).

The relationship between species' growth rates and their distributions

Species native to warmer climates exhibited faster height growth rates than species from colder climates in both treatments (Fig 4.2a & b) and height growth rate positively correlated with basal growth rate across species ($F = 130.5$, $df = 22$, $P<0.0001$, $r^2 = 0.86$, Appendix 2 - Fig. S2.2). Overall, variation in species' annual growth rate (growth from May to Aug.) in the temperate treatment was better explained by differences in species' native photoperiods because modeled Min T did not significantly correlate with species' growth rates when maximum photoperiod (at species' northern range limits) was considered in the multiple regression analysis (Table 4.4). Across species, growth rate also correlated with the difference between the maximum photoperiod in species' native range and the photoperiod in the treatment, such that species exhibited slower growth rates when they were grown under day lengths shorter than observed in their native ranges (Fig. 4.2c). During the first year of growth, many species from colder climates also exhibited greater mortality in the subtropical treatment than the temperate treatment (Table 4.3).

In the long summer photoperiod treatment, species from colder climates exhibited faster height growth rates than in the temperate treatment (Fig. 4.2d). In fact, species from colder climates (latitudes above 55°N) had the highest growth rates and demonstrated the greatest increase in growth rate in the long photoperiod treatment compared to the short photoperiod, whereas species from warmer climates and lower latitudes (below 55°N) demonstrated a more variable response. As Fig. 4.2d demonstrates, there were significant species ($F = 6.0$, $df = 9$, $P<0.0001$), photoperiod ($F = 28.4$, $df = 1$, $P<0.0001$), and species by photoperiod effects ($F = 20.9$, $df = 9$, $P<0.0001$) on growth rate when comparing the long summer photoperiod treatment to the shorter summer photoperiod treatment. In general, species grew better in the summer photoperiod more similar to the one in their native range. A similar trend was observed in terms of species' basal growth rates (data not shown).

While species significantly differed in their photosynthetic capacity (A_{max}, $F = 4.07$, $df = 23$, $P<0.0001$) and specific leaf area ($F = 1.84$, $df = 23$, $P = 0.02$), there was no association between these traits and species' climate distribution envelopes. However, these two traits were correlated across species ($F =$

16.7, df = 22, P = 0.0005, $r^2 = 0.42$), and there was a weak correlation between SLA and basal growth rate in the long summer photoperiod treatment (F = 6.5, df = 8, P = 0.04, $r^2 = 0.45$, Appendix 2 – Fig. S2.2).

Species' cold acclimation and their climate distribution envelopes

Phenology and growth cessation – In the autumn, species from colder climates exhibited a greater decline in growth than species from warmer climates (Fig. 4.3a). These results are consistent with the earlier senescence observed in the more northern species (Fig. 4.3b). Since all of the species collected in Alaska senescenced before we began monitoring them in the autumn, we were unable to document differences in their timing of senescence.

Budburst and winter dieback – Species from colder climates exhibited budburst earlier than species from warmer climates in the winter of 2008-2009 after exposure to outdoor winter conditions (Fig. 4.3c). A greater proportion of the species from warmer climates also demonstrated dieback in the winter. This dieback could have contributed to their late budburst because they had to resprout from their roots. On average, 57%±8 (s.e.) of the individuals from eight of the species native to the warmest climates (*S. caroliniana, S. exigua, S. gooddingii, S. hookeriana, S. nigra, S. sitchensis, P. fremontii,* and *P. trichocarpa*) experienced dieback during the winter.

Autumn and early winter freezing tolerance – Species differed in their leaf freezing tolerance in the autumn. In general, species native to colder climates (Min T<−5°C) demonstrated little to no decline in maximum photosynthetic efficiency after freezing to −5°C, while species native to warmer climates (Min T>−5°C) demonstrated losses that ranged from 0.1 to 0.7 in response to freezing at −5.5°C (Fv/Fm, Fig. 4.4a). By November, all of the species from colder climates had dropped their leaves. The species that still had leaves exhibited an increase in their leaf freezing tolerance (T = −7.6, df = 58, P<0.0001) and all but one species (*S. sitchensis*) was able to maintain an F_v/F_m within 0.2 of their prefreezing level (Fig. 4.4a). The reported values of F_v/F_m were measured 25 hours after freezing. The same trend was observed after 0,

3, and 7 hours. None of the leaves demonstrated recovery from their initial declines in F_v/F_m and instead continued to decline in efficiency over time (data not shown). The unfrozen control samples also did not demonstrate any significant change in F_v/F_m over time (data not shown).

In contrast to the leaf samples, the stem samples exhibited minimal decline in F_v/F_m after freezing at −5.5°C in October (average decline −0.17±0.01, Fig. 4.4b). Freezing at −10°C was required before significant freezing injury in the stems was detectable. At this temperature, the species from warmer climates demonstrated a greater loss in function than species from colder climates (Fig. 4.4b). By November, all the plants that remained green exhibited a lower decline in F_v/F_m than in October, indicating that they have undergone some acclimation (T = 5.93, df = 37, P<0.0001). In general, stem F_v/F_m correlated with cambial death, and a decline of 0.25 in F_v/F_m resulted in cambial death in 50% of the stem samples (LR χ^2 = 111.2, df = 1, P<0.0001). While most species from warmer climates showed no cambial death after freezing at −10°C in November, many exhibited significant damage after freezing at −15°C (Fig. 4.4b and c).

Cold-acclimated freezing tolerance – In the late winter, species from warmer climates exhibited a higher index of injury after freezing at −55°C (in terms of electrolyte leakage) than species from colder climates (Fig. 4.4d). Stem index of injury also positively correlated with cambial mortality across species (LR χ^2=93.5, df=1, p<0.0001). We found that in the winter, species exhibited an exotherm at −5.17°C ± 0.18, on average, and there were no significant differences between species in their exotherm temperatures.

Xylem anatomy – As expected, species from colder climates had smaller vessels than species from warmer climates when grown under common conditions (Fig. 4.5a). While there was a relationship between species' average vessel diameter and their stem specific hydraulic conductivity (K_s, Fig. 4.5b), neither species' K_s (Fig. 4.5c) nor their average vessel diameter was significantly related to species' growth rates in the cold treatment.

Intraspecific variation in growth and phenology

There was some evidence for population-level variation in growth and phenology within the two species collected at multiple locations (*S. interior* and *S. exigua*). The Alaskan population of *S. interior* demonstrated a lower growth rate than the Minnesotan population both in terms of plant height ($T=6.01$, $df=5$, $p = 0.002$, Table 4.5 and basal area ($T=4.18$, $df=5$, $p<0.01$). The Alaskan population of *S. interior* also demonstrated earlier senescence than the Minnesota population (Table 4.5). However, *S. exigua*, a widespread species with a narrower latitudinal distribution than *S. interior*, demonstrated no population-level differences in growth or senescence. There were no significant population-level effects on the timing of budburst in either of the two willow species.

Evidence for a trade-off between freezing tolerance and growth

Species' height and basal growth rates in the subtropical treatment ($F = 67.4$, $df = 22$, $P<0.0001$, Fig.4.6 a & b) correlated with species' freezing tolerance providing evidence for a trade-off between freezing tolerance and growth in willows. For this analysis, species' freezing tolerance was described by the primary axis of the PCA of species' freezing traits in the temperate treatment. This axis explained 61.9% of the variation observed in all physiological and phenological traits. Each of these traits was also correlated with species' growth rates individually (Appendix 2 – Fig. S2.3).

DISCUSSION

In an effort to better understand the factors that influence species distributions, we examined whether there was evidence for a trade-off between freezing tolerance and growth in the family Salicaceae and investigated the extent that such a trade-off could explain species' range limits. Across species, there was evidence for a trade-off between freezing tolerance and growth (Fig. 4.6), and both species' freezing tolerance (Fig. 4.4) and their growth rates (Fig. 4.2) correlated with species' climate distribution envelopes.

However, species from colder climates exhibited higher growth rates when they were grown under photoperiods more similar to those experienced in their native ranges (Fig. 4.2d). These results indicate that species have different photoperiod cues for growth and that the observed trade-off is not necessarily driven by differences in species' inherent growth capacities. Further research is needed to determine whether differences in species' photoperiod requirements for growth could be influential in determining their range limits.

The relationship between species' freezing tolerance and their distributions

Cold acclimation and freezing tolerance varied in four generalizable ways based on species distributions. First, species from colder climates experienced earlier cold acclimation than species from warmer climates. This is indicated by their early decline in growth rate and their early leaf senescence in the autumn (Fig. 4.3). These traits are important to cold acclimation because actively growing tissue is very susceptible to freezing damage (Lennartsson and Ogren 2002), and a frost event can lead to significant nutrient loss if it occurs before senescence and nutrient translocation (Larcher et al. 1973, Sakai and Larcher 1987). Second, species from colder climates demonstrated higher freezing tolerance in the early autumn than species from warmer climates (Fig. 4.4). For example, species from colder climates demonstrated minimal loss in leaf function when frozen at −5.5°C, while species from warmer climates (Min T> −5°C) did not display this level of resilience until the late autumn (Fig. 4.4a). Third, species from colder climates exhibited higher cold-acclimated freezing tolerance suggesting that species differ in the temperature they can acclimate to in the winter (Fig. 4.4d). Fourth, when grown outside in a temperate climate, willows and poplars from colder climates experienced earlier budburst than species from warmer climates (Fig. 4.3c). This earlier budburst is likely important in northern latitudes because of the shorter growing season.

While willow and poplar species from different climates differ in their freezing tolerance, the ecological relevance of this correlation is challenging to interpret. Overall, our results suggest that species are able to acclimate to colder temperatures than they would on average experience in their native ranges.

For example, many species from intermediate climates (Min T > −15°C) exhibited limited susceptibility to freezing at −55°C in the late winter (survival >50%). While we used average minimum temperature in our analysis instead of actual minimum temperature, the species in this study appeared to demonstrate a higher freezing tolerance than would be expected, even when considering the lowest recorded temperature at each site in the last 100 years (data not shown). These results are consistent with Sakai's (1970) research, which found that multiple species could survive freezing at temperatures below -60°C. He also found that some species could acclimate to colder conditions after growing in the field for a couple of years. If this is the case with the species in this study, it is possible that they could achieve higher levels of cold-acclimated freezing tolerance than observed in our experiments.

There are several explanations for why these species appear to demonstrate a higher freezing tolerance than expected based on their geographic distributions. First, species' ability to survive freezing to different temperature minima is dependent on the response of multiple organs to freezing, including ones not measured in this study (Sakai and Larcher 1987). For example, root cold tolerance may be important in areas that experience permafrost (Wan et al. 1999, Starr et al. 2004). A second explanation for the observed trend is that species' ranges may be more limited by the timing of acclimation and de-acclimation than their cold-acclimated freezing tolerance. Studies conducted in willow plantations on Europe have found that one of the major limiting factors to plant productivity is their ability to acclimate early enough in the autumn and late enough in the spring to avoid freezing damage (Verwist et al. 1996, Christersson et al. 1983). Several studies have also found that willows and poplars transplanted from warmer climates often experience dieback associated with frost damage because of late growth cessation (Mohn and Pauley 1969, Christersson et al. 1983, Larsson 1998). In this study, the large differences observed in the timing of cold acclimation could play a critical role in limiting species' northern range limits regardless of their cold-acclimated freezing tolerance (Fig. 4.3 & 4.4).

In general, there are many physiological and phenological traits that could be important in limiting species' productivity and survival in cold climates. While any one of the measured traits could theoretically limit species distributions, the strong correlations observed between each of the traits and species' modeled climatic envelopes (Fig. 4.3 & 4.4) indicate that species' climatic tolerances are likely the result of cold

tolerance strategies involving multiple physiological and phenological traits. However, more research is needed to better understand the extent that cold acclimation is mediated by temperature and photoperiod and to determine whether the observed differences in species' freezing tolerances are the result of differences in their rates of acclimation or their non-acclimated freezing tolerance.

The relationship between species' growth rates and their distributions

Species from colder climates exhibited slower height growth rates than species from warmer climates when grown in both the temperate and the subtropical treatments (Fig. 4.2a & b). While the positive relationship between species climatic envelopes and their growth rates appears consistent with the hypothesis that growth rate limits species southern distributions, it does not appear driven by differences in species' growth capacities as previously hypothesized (Loehle 1998). When species from colder climates (i.e. from northern latitudes) were grown under a longer day lengths for a growing season, some of them exhibited higher growth rates than species from warmer climates (Fig. 4.2d). These results suggest that species' growth rates in the spring and summer are limited by day length, not because longer days allow for a longer period of photosynthesis, but because long days serve as a physiological cue for the up-regulation of growth. This is consistent with research on the European willow, *S. pentandra* that found that long days are required to stimulate the production of gibberellins and promote stem elongation in the summer (Junttila and Jensen 1988, Olsen et al. 1995). Therefore, we propose that the positive relationship between species' growth rates and the minimum temperature in climatic envelope is likely influenced by variation in their photoperiod requirements for growth.

Another factor that may limit species' growth under different climatic conditions is their temperature requirements for de-acclimation and budburst. Many temperate species require a period of chilling before they de-acclimate in the spring (Farmer 1968, Heide 1993, Chuine 2000). If they do not experience chilling temperatures, they may not experience budburst, but if they do, they may not produce flowers or vigorous growth the following year (Petri and Leite 2004). In this study, many of the species from colder climates did not leaf out and eventually died in the subtropical treatment (Table 4.3). This

mortality and relative inactivity could be a result of not attaining the required chilling temperatures. At least one species in this study that occurs in colder climates, *S. pulchra,* is known to have a chilling requirement for growth (Pop et al. 2000). If this is a widespread requirement for many of the northern species, it could limit species southern distributions and explain their slower growth rates in the temperate and subtropical treatments.

Both ecological models (Case et al. 2005, Price and Kirkpatrick 2009) and empirical studies (Woodward and Pigott 1975, Gross and Price 2000) suggest that competition can limit species distributions at larger spatial scales. It has also been hypothesized that competition is greater in warmer, less stressful environments (Bertness and Callaway 1994, Callaway et al. 2002). As a result, competition may be the greatest along species' southern range limits and may be a major determinant of species' survival under warmer climatic conditions. Although we did not directly measure species' competitive performance in this study, willows and poplars are generally shade intolerant, and it is likely that height growth rate is a critical component of their establishment at different sites. However, the growth rates observed in these treatments may not be reflective of growth rates experienced in species' native ranges. In this study, the relationship between species' growth rates and their distributions was influenced with the extent that species were displaced from their native environment (Fig 2c). As a result, the species from the coldest climates were grown under climate conditions that were the most divergent from those in their native ranges. Both provenance studies and greenhouse experiments have demonstrated that some plant species exhibit higher growth rates in climates and photoperiods more similar to those experienced in their native ranges, and a significant decline in growth rate when they are grown under drastically different climate conditions (Vaartaja 1959, Giertych 1979, Oleksyn et al. 1992, Reich and Oleskyn 2008). Without further research it is not possible to determine whether the observed decline in species' growth rates occurs at a spatial scale relevant to species' ranges or whether it only occurs when plant are grown well beyond their range limits. It is also possible that ecotypic variants of species maintain higher growth rates at their southern range limits and prevent any growth rate depression along the extent of species ranges.

The freezing tolerance-growth trade-off

Across species, there was a significant negative correlation between species' freezing tolerance and their growth rates, supporting the hypothesis that there is a negative relationship between freezing tolerance and growth in the plant family Salicaceae (Fig. 4.6 and Appendix 2 – Fig. S2.3). This trade-off was apparent when considering both species' height growth rates and basal growth rates. While the observed relationship was significant, it is likely mediated by species' photoperiod requirements for growth and therefore the result of each traits strong association with species distributions.

There was no evidence that xylem function played a role in the observed trade-off. While vessel diameter correlated with species distributions, and average vessel diameter positively correlated with stem specific hydraulic conductivity (K_s), there was no relationship between species' vessel anatomy and their growth rates in the temperate treatment (Fig. 4.5). These data indicate that species from colder climates have lower vulnerability to freeze-thaw cavitation, but species' growth rates are not solely dependent on their hydraulic capacity. This is unexpected because multiple studies have found that stem hydraulic conductivity positively correlates with species' growth rate (Poorter et al. 2010, Chave et al. 2009). While it is possible that the relationship between these variables is obscured because we compared closely related species that have very similar function, it is also possible that this discrepancy is a result of the observed differences between species' maximum growth rates and their growth rates in the different treatments. In this study, many of the species from colder climates did not achieve their highest growth rates when grown under a mid-latitudinal photoperiod (Fig. 4.2). As a result, the growth rates in the temperate treatment were not representative of species' maximum growth rates and therefore they would not necessarily be expected to relate to species' hydraulic capacity.

Lineage specific implications of the trade-off

The existence of a trade-off between freezing tolerance and growth in the willow family has interesting implications because of the biogeographic and evolutionary history of the lineage. It is believed

that freezing tolerance is ancestral in willows and that all of the North American species arrived on the continent by traveling across the Bering Strait when climate conditions were temperate (Taylor 1990, Collinson 1992, Skvortsov 1999). This implies that the observed trade-off resulted from species losing freezing tolerance as they migrated south, and thus supports the hypothesis that there is a selective disadvantage to maintaining freezing tolerance in warmer climates. Another interesting implication of this trade-off is that previous research suggests that willows and poplars exhibit faster growth rates than expected from their freezing tolerance compared to other woody species (Loehle 1998). In fact, Loehle's analysis of this trade-off found that willows and poplars were outliers compared to other species because they exhibited both high growth rates and high levels of freezing tolerance. His results in concert with this study suggest that the trade-off space for willows and poplars may be offset from that of other woody species and/or that loss of freezing tolerance has not had sufficient time to occur in willows. While it is possible that this is a result of their temperate ancestry, it is also possible that willows and poplars have lineage specific traits that influence their freezing tolerance and growth, such as their ability to vitrify their cells under freezing conditions (Hirsch et al. 1985).

Other factors that influence range limits

Understanding the complexities associated with species distributions is challenging on many levels, and there are several important issues to consider when interpreting the results from this study. One is that most analyses in this study compare species-level traits and do not consider intraspecific variation. With widely distributed species, genetically-based variation among populations could be a major determinant in the persistence of species under different climatic conditions (Boorse et al. 1998b, Etterson and Shaw 2001, Olsson and Agren 2002). In this study, we found that two populations of *S. interior* differed in growth rates and the timing of their senescence under temperate climatic conditions. These differences provide evidence for genetically-based variation among populations in species' phenological cues (Table 4.4). If widely distributed species demonstrate significant ecotypic variation, especially in their

photoperiod requirements for growth up-regulation, species may exhibit higher growth rates at their southern boundaries than predicted from this study.

Another important consideration in interpreting the trade-off between freezing tolerance and growth is that species distributions are the result of multiple ecological and evolutionary processes. While this study presents evidence that there is a trade-off between freezing tolerance and growth, it does not negate the importance of other factors. Willow and poplar species are known for being limited by water availability (Pockman and Sperry 1997, Amlin and Rood 2002, Savage et al. 2009) and are intolerant to the combination of drought and freezing temperatures (Sakai 1970). This may explain why their distributions in Europe are tightly correlated with average summer temperature and average minimum temperature (Myklestad and Birks 1993). Herbivory is also known to influence willow species distributions (Bach 2001, Maschinski 2001) could influence their ranges, since herbivore-plant interactions often change latitudinally (Pennings and Silliman 2005, Schemske et al. 2009). There is also evidence that phenology plays a major role in determining species distributions including the distributions of several willow and poplar species (Chuine and Beaubien 2001).

Conclusions – This study presents evidence for a trade-off between freezing tolerance and growth in 24 willow and poplar species. However, differences in species' growth rates appear to be the result of variation in their photoperiod cues for growth and not necessarily a result of differences in their inherent growth capacities as previously hypothesized (Loehle 1998). This indicates that freezing tolerance does not directly prevent species from having fast growth rates, and that the observed relationship between species' freezing tolerance and growth is likely the result of selection for specific trait combinations in different climates (i.e. a dependency of growth rate on photoperiod and a high freezing tolerance). Since species' growth rates were highly correlated with the photoperiod in their native ranges, further research is needed to understand whether this trade-off occurs at a scale that is relevant to species distributions or whether it is purely the result of the more northern species being plant well beyond their range limits.

This study serves as a starting point for revisiting this hypothesized trade-off and considering the complexities associated with its application to species distributions. Further research is needed to better understand whether differences in species' growth rates can affect their competitive ability and whether

species demonstrate slower growth rates near southern range limits. Since aspects of this trade-off could be lineage specific, it is important to do further research on the applicability of this trade-off to other woody species. Research on trade-offs such as this one can increase our understanding of how species distributions relate to climatic conditions, and better educate us as to the ecological consequences of global climate change.

Table 4.1 – The 24 willow (*Salix*) and poplar (*Populus*) species in this study and information on their collection sites. Species that were grown in the longer summer photoperiod treatment are marked with †.

Species	Abbr.	State	Lat. & Long.
S. alaxensis† (Andersson) Coville	ALA	AK[1]	64.8°N, 147.8°W
S. arbusculoides† Andersson	ARB	AK[1]	64.8°N, 147.8°W
S. candida Flueggé ex Willd.	CAN	MN[2,3]	44.9°N, 93.1°W / 48.6°N, 93.4°W
S. caroliniana† Michx.	CAR	FL[4]	29.7°N, 82.3°W
S. eriocephala Michx.	ERI	MN[2,3]	44.9°N, 93.1°W / 48.6°N, 93.4°W
S. exigua Nutt.	EXI	OR[5] NV[6] AZ[7]	44.1°N, 123.2°W / 36.2°N, 115.1°W 34.9°N,109.8°W
S. fuscescens† Andersson	FUS	AK[1]	64.8°N, 147.8°W
S. gooddingii† C.R. Ball	GOO	NV[6]	36.2°N, 115.1°W
S. hookeriana† Barratt ex Hook.	HOO	OR[5]	44.1°N, 123.2°W
S. interior Rowlee	INT	MN[2,3] AK[1]	44.9°N, 93.1°W / 48.6°N, 93.4°W 64.8°N, 147.8°W
S. lasiandra Benth.	LAS	OR[5]	44.1°N, 123.2°W
S. lucida† Muhl	LUC	MN[2,3]	44.9°N, 93.1°W / 48.6°N, 93.4°W
S. nigra Marsh	NIG	MO[8]	39.1°N, 94.6°W
S. pedicellaris Pursh	PED	MN[2,3]	44.9°N, 93.1°W / 48.6°N, 93.4°W
S. petiolaris Sm.	PET	MN[2,3]	44.9°N, 93.1°W / 48.6°N, 93.4°W
S. pseudomyrsinites† Andersson	PMY	AK[1]	64.8°N, 147.8°W
S. pseudomonticola C.R. Ball	PSM	AK[1]	64.8°N, 147.8°W
S. pulchra Cham.	PUL	AK[1]	64.8°N, 147.8°W
S. pyrifolia Andersson	PYR	MN[2,3]	44.9°N, 93.1°W / 48.6°N, 93.4°W
S. sitchensis† Sanson ex Bong.	SIT	OR[5]	44.1°N, 123.2°W
P. balsamifera L.	BAL	AK[1]	64.8°N, 147.8°W
P. deltoides Bartram ex Marsh.	DEL	MN[2,3]	44.9°N, 93.1°W / 48.6°N, 93.4°W
P. fremontii Watson	FRE	NV[6]	36.2°N, 115.1°W
P. trichocarpa Torr. & A. Gray ex Hook.	TRI	OR[5]	44.1°N, 123.2°W

[1] Bonanza Creek Experimental Forest LTER, and along the Tanana River, Fairbanks, AK

[2] Cedar Creek Ecosystem Science Reserve LTER, East Bethel, MN, permit #RP801

[3] Boot Lake, and Savage Fen Scientific and Natural Area (SNA), MN, Dept. of Natural Resources, permit #2007-5R

[4] San Felasco and O'Leno State Parks, FL, Dept. of Environmental Protection, District 2, permit #10290712 and private land

[5] Willmamette and Suislaw River Valleys, OR, U.S. Bureau of Land Management, permit #S1-07

[6] U.S. Bureau of Land Management Land, southern NV

[7] Apache National Forest, AZ, U.S. Department of Agriculture Forest Service, permit #FS-2700-25(03/06)

[8] Private land

Table 4.2 – **Description of freezing experiments**. Species full names are listed in Table 4.1.

Date	Tissue	Freezing Min. T (˚C)	Viability measurement	Species
Early Oct.	Leaf	-5.5	Chlorophyll fluorescence	CAN, CAR, DEL, ERI, EXI, FRE, GOO, HOO, INT, LAS, LUC, NIG, PED, PET, PYR, SIT, TRI (17 spp.)
Early Oct.	Stem	-5.5 & -10.0	Chlorophyll fluorescence & mortality	ALA, BAL, CAN, CAR, DEL, ERI, EXI, FRE, FUS, GOO, HOO, INT, LAS, LUC, NIG, PED, PYR, SIT, TRI (19 spp.)
Late Nov.	Leaf	-5.5	Chlorophyll fluorescence	CAR, EXI, FRE, GOO, HOO, LAS, SIT, TRI (8 spp.)
Late Nov.	Stem	-10.0 & -15.0	Chlorophyll fluorescence & mortality	ALA, ARB, BAL, CAN, CAR, DEL, ERI, EXI, FRE, FUS, GOO, HOO, INT, LAS, LUC, NIG, PED, PET, PMY, PUL, PYR, SIT, TRI (23 spp.)
Early Feb.	Stem	-55.0	Electrolyte leakage & mortality	BAL, CAN, CAR, DEL, ERI, EXI, FRE, GOO, HOO, INT, LAS, LUC, NIG, PED, PYR, SIT, TRI (17 spp.)

Table 4.3 – Variation in percent of mortality in the tropical and temperate treatments. Species are ordered based on their modeled climate envelopes with the species native to the coldest climates listed first. Values are reported as the percent of each species that died before the summer 2008. Bold values indicate when mortality for a given species is greater than the average mortality for the treatment. Species with * have a greater mortality in the tropical treatment than the temperate treatment. Species full names are listed in Table 4.1.

Species	Temperate	Subtropical	Difference
ALA	**27%**	**25%**	-0.02
ARB	**40%**	**50%**	0.10*
FUS	0%	**33%**	0.33*
PSM	0%	**25%**	0.25*
PUL	**38%**	**40%**	0.03*
PMY	**27%**	**57%**	0.30*
PYR	0%	**38%**	0.38*
PET	11%	**43%**	0.32*
BAL	17%	11%	-0.06
LUC	**22%**	**57%**	0.35*
PED	18%	**20%**	0.02*
ERI	**29%**	0%	-0.29
INT	13%	**56%**	0.42*
CAN	**23%**	**29%**	0.05*
DEL	0%	14%	0.14*
TRI	0%	0%	0.00
LAS	18%	11%	-0.07
NIG	**27%**	0%	-0.27
EXI	17%	0%	-0.17
CAR	0%	0%	0.00
GOO	**29%**	0%	-0.29
SIT	0%	0%	0.00
FRE	0%	0%	0.00
HOO	0%	0%	0.00

Table 4.4 – The relationship between species' growth rates, and the maximum photoperiod and modeled min T (°C) in their native ranges. Growth rates are presented as height and basal growth rates. P-values are from a multiple regression analysis and values that are significant to 0.05 are bolded.

	Tropical treatment		Temperate treatment	
Effects	Height growth	Basal growth	Height growth	Basal growth
Min T	**<0.0001**	**0.0004**	0.6032	0.9924
Photoperiod	0.8078	0.2245	**0.0006**	**0.0001**
Min T * Photo	**0.0003**	**0.0023**	**0.0151**	0.1132

Table 4.5 – Evidence for ecotypic variation in *S. interior* and *S. exigua*. Growth rates are expressed as height growth rate (cm cm^{-1} day^{-1}) and basal growth rate (mm^2 mm^{-2} day^{-1}). Species populations are labeled based on the state where they were collected. Bold values indicate when populations were significantly different (α=0.05). Averages are reporting ± one standard error.

	S. interior		*S. exigua*	
	AK population	MN population	OR population	NV population
Height growth rate	**0.0004 ± 0.0001**	**0.0157 ± 0.0025**	0.00195 ± 0.0026	0.0114 ± 0.0021
Basal growth rate	**0.0009 ± 0.0006**	**0.0142 ± 0.0031**	0.0194 0 ± 0.0025	0.0161 ± 0.0027
Date of senescence[†]	Before 4 Oct	By 2 Nov	After 20 Nov	After 20 Nov

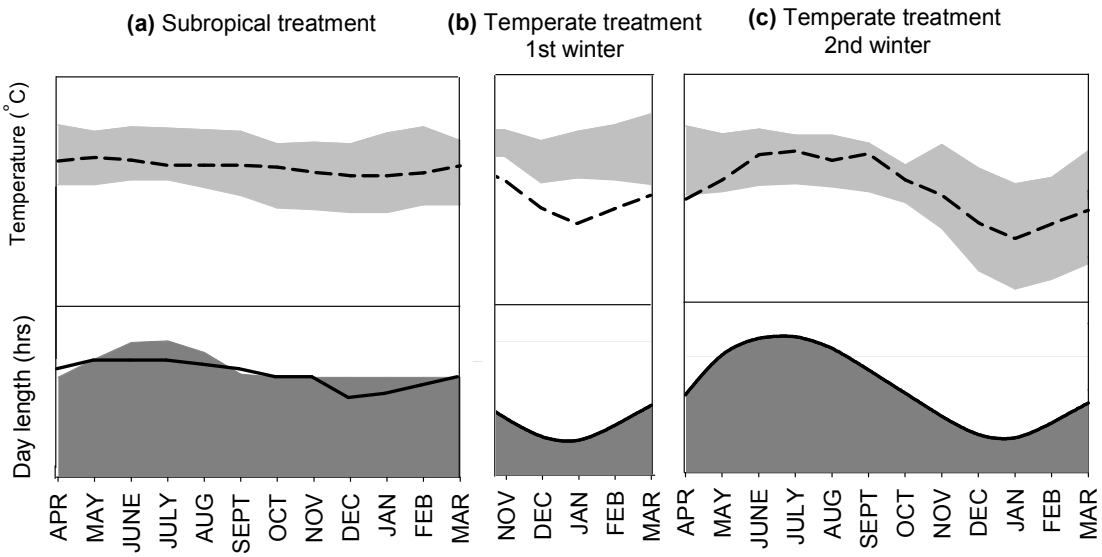

Figure 4.1 - Plants were grown in two climate treatments: **(a)** a subtropical treatment, which had a monthly temperature and day length similar to Morelia, Mexico and **(b & c)** a temperate treatment, which had a similar monthly temperature and day length to Franklinville, NY. During the first winter of the temperate treatment, the plants were kept in the greenhouse (2007-08) and during the second winter, they were placed outside (2008-09). The maximum and minimum temperatures and day lengths in the treatments are marked with light and dark gray shading, and the average monthly temperature and day length at the target sites are noted with dashed lines and solid lines, respectively.

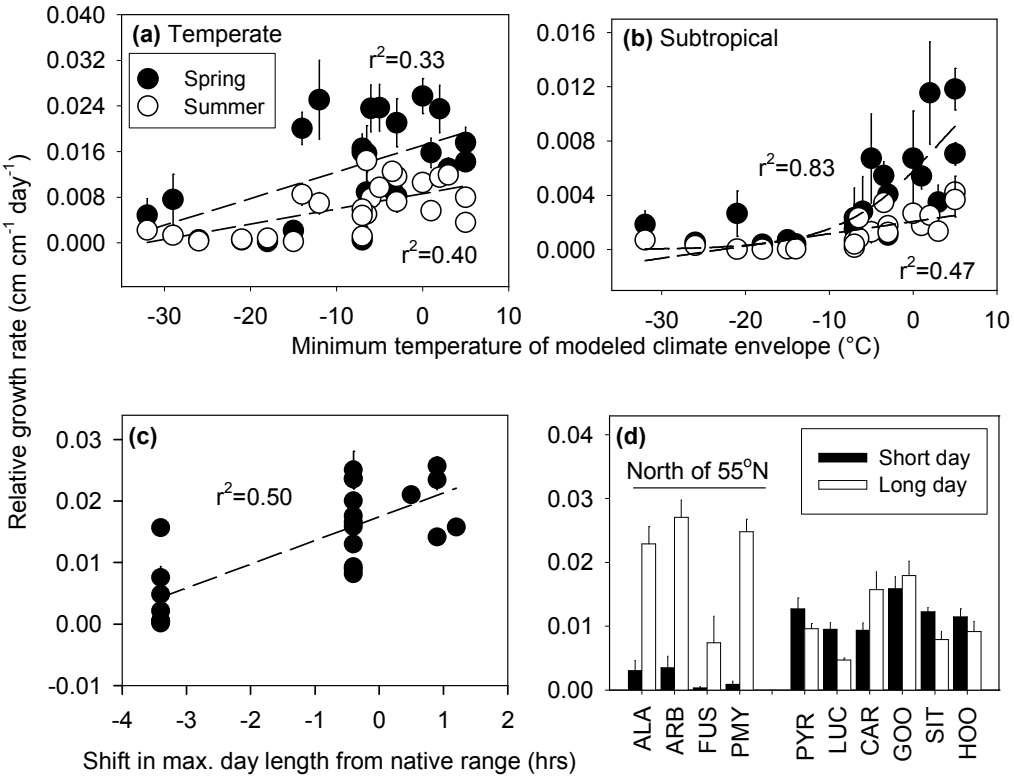

Figure 4.2 (a) (b) In the temperate and the subtropical treatments, species from colder climates exhibited slower height growth rates than species from warmer climates in both the spring (closed circles) and the summer (open circles). **(c)** There was also a relationship between species' growth rates and the photoperiod shift between species' native ranges and the temperate treatment. The shift was calculated as the difference between the maximum day length at the collection site and the maximum day length in the treatment. **(d)** In the long photoperiod treatment (long day, white bars), the northern species (north of 55°N) had faster height growth rates than in the regular temperate treatment (short day, black bars), while species with southern ranges (south of 55°N) exhibited more variable results. Species are ordered based on their climatic envelopes with the species from the coldest climates on the left. Species' averages are reported in the graphs and error bars are ± one standard error.

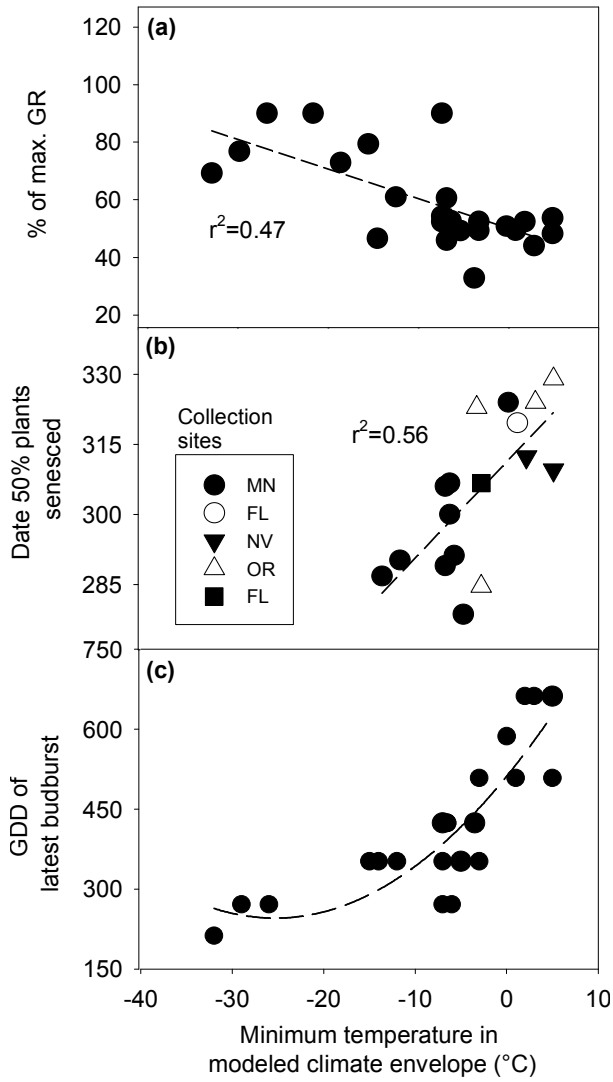

Figure 4.3 Species demonstrated substantial variation in their seasonal phenology as species from colder climates exhibited **(a)** a greater decline in growth rate in the early autumn, **(b)** earlier senescence and **(c)** earlier budburst in the spring than species from warmer climates. Growth rate (GR) in the autumn is reported as a percent of maximum spring growth rate [$(GR_{spring} - GR_{fall})/GR_{spring}$]. GR values were arcsin-square root transformed. Senescence is graphed as the date when 50% of the plants in each species had senescenced. The symbols in (b) indicate the state in which the species were collected. Budburst is described as the growing degree day (GDD) when the last plant in each species experienced budburst. Each point represents a species' average and error bars are ± one standard error.

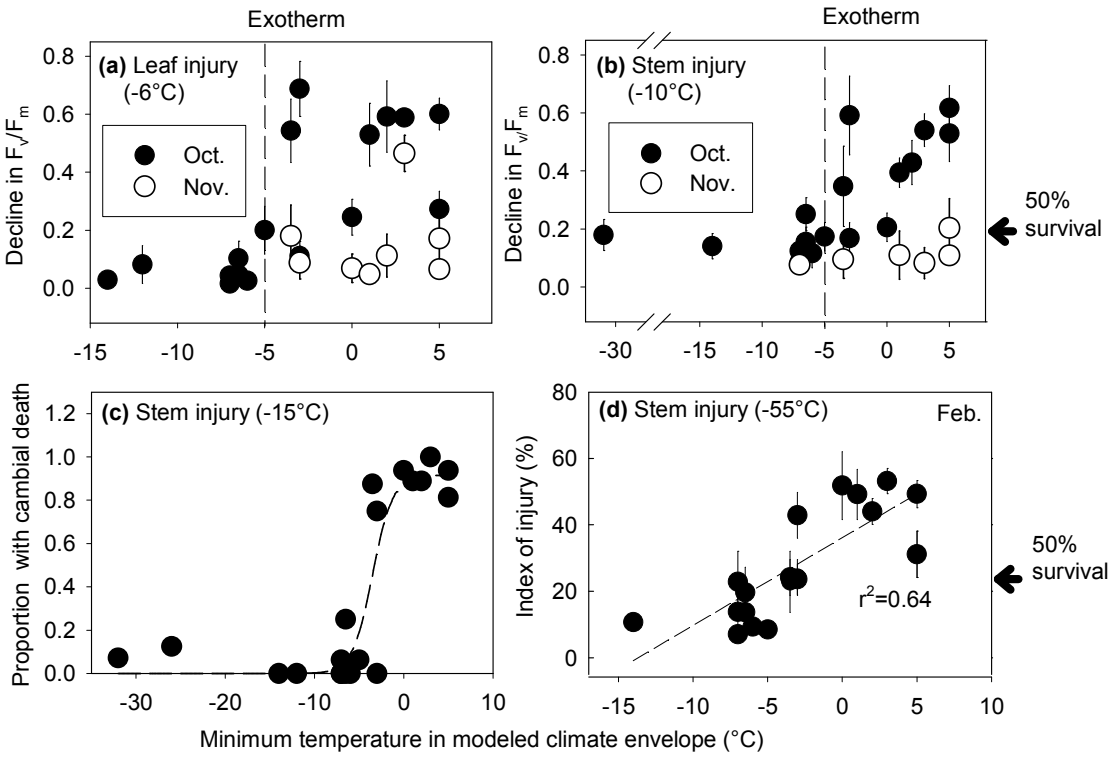

Figure 4.4 (a) & (b) In October, willow and poplar species (closed circles) from cold climates demonstrated limited loss in photosynthetic efficiency (F_v/F_m) when their leaves and stems were frozen at −5.5°C and −10°C, respectively, in the temperate treatment. Species from warmer climates demonstrated greater loss in function and more variability in their responses, and exhibited a greater tolerance to the same freezing temperatures in November (open circles). Dashed lines mark the temperature at which there was an exotherm, indicating ice formation in the tissue. Species that rarely experience temperatures below this point may not typically freeze in their native ranges. **(c)** A smaller proportion of individuals native to colder climates exhibited cambial damage than those from warmer climates after freezing at −15°C in November. **(d)** These species also exhibited lower levels of injury in response to the freezing at −55°C in the late winter. Each circle represents species' averages, and the error bars are ± one standard error.

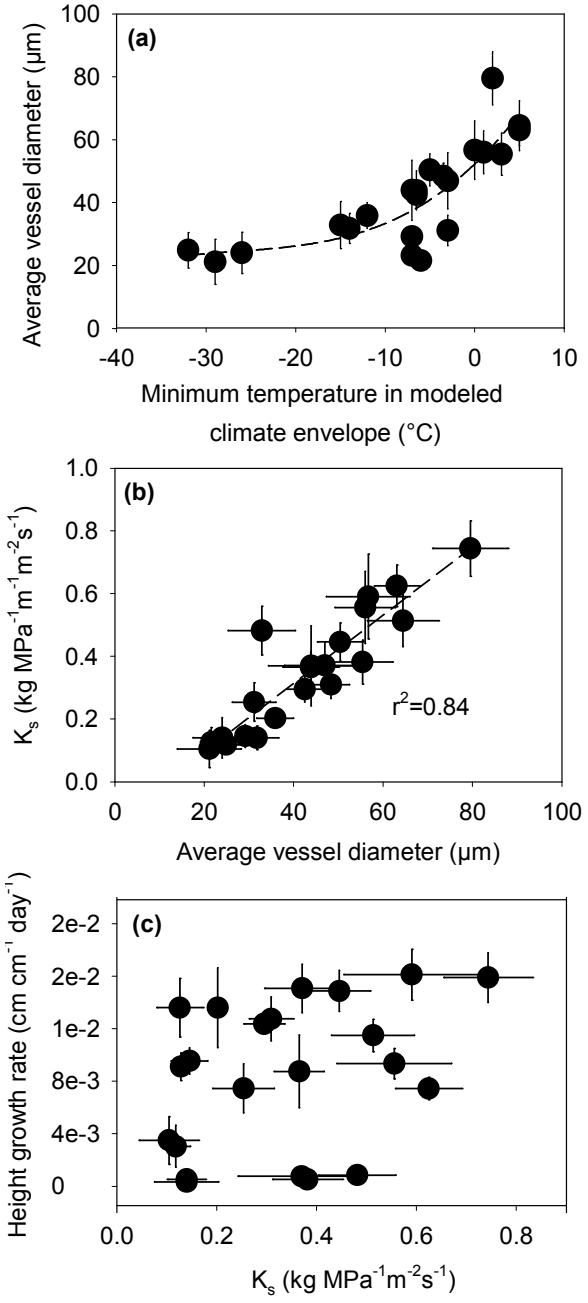

Figure 4.5 (a) Species from colder climates had larger average vessel diameters than species from warmer climates. **(b)** Average vessel diameter correlated with stem specific hydraulic conductivity across species. **(c)** However, stem specific hydraulic conductivity did not significantly correlate with height growth rate. All points represent species' averages and error bars are ± one standard error.

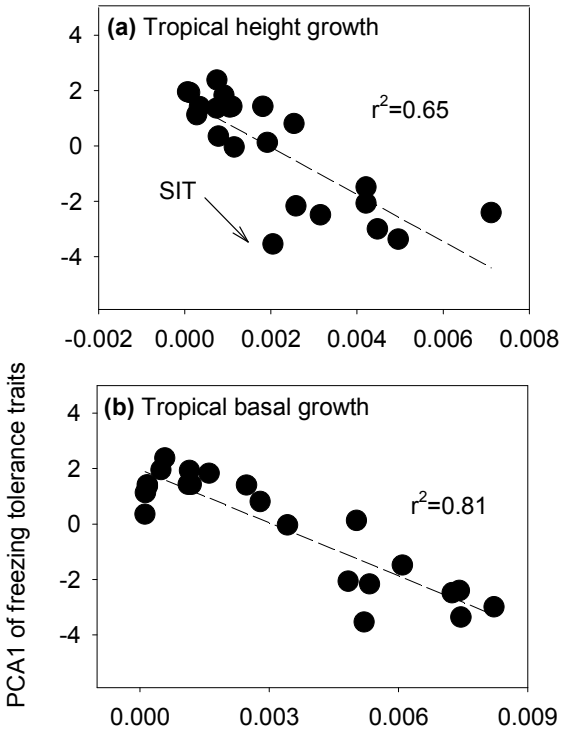

Figure 4.6 Across species, there was evidence for a trade-off between species' freezing tolerance and growth rates in the subtropical treatment both in terms of species' **(a)** height growth rates and **(b)** basal growth rates. Freezing tolerance is based on the primary axis of a PCA of the measured traits associated with freezing tolerance. Each point represents a species' averages. *Salix sitchensis* (SIT) is marked because it experienced greater lateral growth than height growth during the experiment.

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APPENDIX 1 – Supplemental material for Chapter 3

Figure S1.1 – Best scoring maximum likelihood tree of Minnesotan *Salix* and *Poplar* species based on the *ADH* gene. This phylogeny was inferred using a HKY + G model of sequence evolution. The *ADH* gene was sequenced for two individuals per species. The polyploid species were cloned (*S. discolor*, *S. humilis*, *S. lucida* and *S. serissima*) and 8-10 clones were sequenced per individual. All unique alleles were included in the phylogenetic analysis. Bootstrap values for nodes that had over 50% support are listed on the tree. There was evidence that all but three of the diploid species (*S. amygdaloides, S. bebbiana* and *S. nigra*) were monophyletic. *Salix bebbiana* did not have strong bootstrap support, and *S. amygdaloides* and *S. nigra* demonstrated limited divergence in their *ADH* sequences. Two of the polyploids (*S. lucida* and *S. serissima*) had parents that were in different clades, indicating that there was likely an allopolyploid event in the ancestor of these species. However, the history of the other two polyploids, *S. discolor* and *S. humilis* is difficult to interpret because of limited bootstrap support.

Table S1.1 – Co-variation of environmental conditions in the plots. Correlations between environmental variables in plots are shown in terms of Pearson's correlation coefficient. Environmental variables that demonstrated significant positive and/or negative relationships (r>0.3) are bolded. The environmental variables are: N (total nitrogen, g/m^3), C (total carbon, g/m^3), OM (organic matter), pH, P (total phosphorus, g/m^3), K (exchangeable potassium, ppm), NO_3^- (nitrate, ppm), ΔWT (change in water table during the year, m), WT_{dry} (depth to the water table in Aug., m), WT_{wet} (depth to the water table in May, m), eP (exchangeable phosphorus, ppm).

	C	N	P	pH	eP	K	OM	NO_3^-	ΔWT	WT_{dry}
N	**0.99**									
P	**-0.54**	**-0.56**								
pH	0.14	0.14	-0.27							
eP	0.06	0.10	-0.16	0.06						
K	**-0.52**	**-0.50**	**0.52**	-0.15	-0.06					
OM	**0.87**	**0.88**	**-0.62**	**0.30**	-0.09	**-0.46**				
NO_3^-	0.14	0.11	-0.25	-0.01	-0.01	**-0.32**	0.10			
ΔWT	**-0.41**	**-0.39**	0.18	0.07	0.00	**0.41**	-0.25	-0.14		
WT_{dry}	**0.75**	**0.72**	**-0.42**	0.16	0.08	**-0.47**	**0.60**	0.22	**-0.50**	
WT_{wet}	**0.73**	**0.71**	**-0.42**	0.26	0.08	**-0.42**	**0.63**	0.19	-0.20	**0.92**

Table S1.2 – (a) Phylogenetically independent contrasts (PIC). Pearson's correlations coefficients along with p-values are reported for trait correlations between the listed traits and species weighted depth to the water table in the summer (WT_{dry}). Since there were eight derivative phylogenies, a range of values is given. Bolded traits were significant in some of the phylogenies ($\alpha = 0.5$).

Traits	r	P-values
Seed viability	-0.59 – - 0.68	0.03 – 0.06
Seedling root GR	-0.81 – -0.87	<0.001– 0.004
Seedling RGR	-0.52 – -0.60	0.02 – 0.05
SPI	-0.53 – -0.92	0.006 – 0.06
TLP	0.66 – 0.75	0.03 – 0.07
Wood density	-0.50 – -0.70	0.35 – 0.45
WUE	-0.35 – -0.71	0.35 – 0.52

(b) The relationship between summer water availability (WT_{dry}), phylogenetic dispersion (NRI and PSV) and taxonomic distinctness (Δ +). The correlation coefficients and p-values for the regression analyses of NRI, 1-PSV and 1 – Δ+ on summer water availability are reported. Since there were eight derivative phylogenies, a range of values are given.

Metric	r	P-values
NRI	-0.39 – -0.47	0.002-0.01
1 – PSV	-0.60 – -0.63	<0.0001
1 – Δ+	-0.34 – -0.37	0.009-0.03

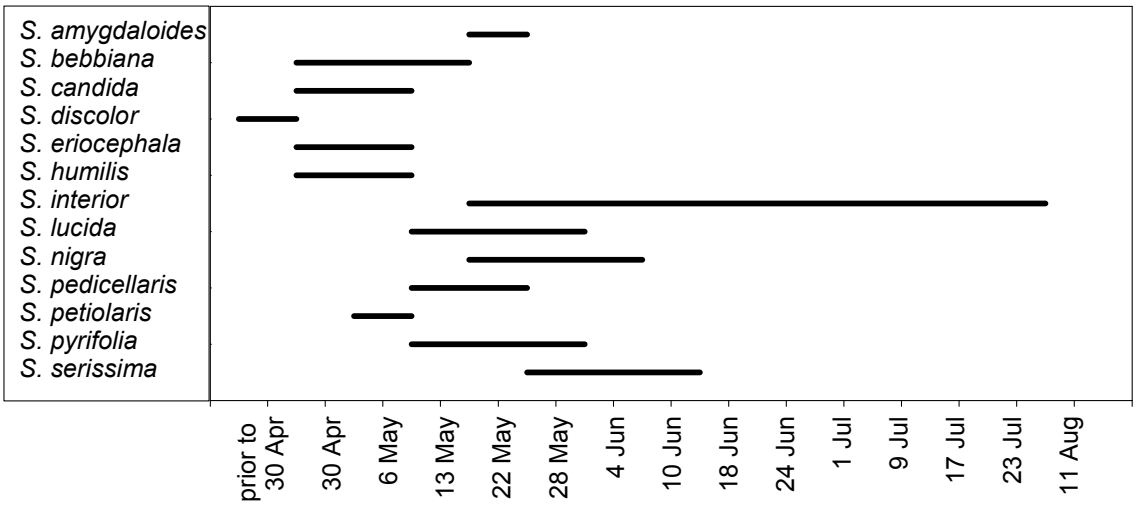
Table S1.3 – (a) Additional functional traits measured in the field. Traits include seed mass (g), stomatal aperture (μm), stomatal density (stomata mm^{-2}), abaxial and adaxial cuticle thickness (μm) and maximum photosynthetic capacity (A_{1500}, mol $CO^2g^{-2}s^{-2}$). Species are abbreviated to the first three letters of the specific epithet. Average values for traits are reported ± one standard error.

Species	Seed mass	Stomatal aperture	Stomatal density	Abaxial cuticle	Adaxial cuticle	A_{1500}
AMY	7.90E-05	15.0 ± 0.8	0.04 ± 0.00	43.6 ± 3.6	62.5 ± 4.7	7.68 ± 0.66
BEB	9.29E-05	7.7 ± 0.4	0.14 ± 0.01	NA	NA	9.03 ± 0.58
CAN	1.32E-04	9.4 ± 0.4	0.11 ± 0.00	37.6 ± 2.4	46.7 ± 2.8	12.28 ± 1.25
DIS	1.07E-04	22.8 ± 0.3	0.03 ± 0.00	21.3 ± 1.1	41.1 ± 5.9	10.14 ± 0.61
DEL	6.43E-04	16.1 ± 0.5	0.03 ± 0.00	48.7 ± 3.4	47.3 ± 2.6	9.84 ± 0.68
ERI	8.30E-05	14.4 ± 0.9	0.06 ± 0.01	33.8 ± 3.0	32.1 ± 1.9	9.23 ± 1.20
HUM	1.45E-04	12.2 ± 0.6	0.07 ± 0.00	37.2 ± 1.9	54.7 ± 2.6	11.76 ± 1.38
INT	2.63E-05	8.6 ± 0.5	0.17 ± 0.01	34.3 ± 2.9	45.3 ± 3.5	22.32 ± 2.15
LUC	4.47E-05	18.2 ± 1.4	0.02 ± 0.00	38.9 ± 2.7	41.0 ± 2.4	11.54 ± 0.36
NIG	8.25E-05	14.4 ± 0.6	0.04 ± 0.01	31.0 ± 2.3	42.5 ± 1.6	11.66 ±1.48
PED	2.34E-04	12.2 ± 0.7	0.04 ± 0.00	40.4 ± 3.7	37.9 ± 2.2	14.62 ±1.57
PET	2.46E-04	9.6 ± 0.4	0.10 ± 0.01	56.2 ± 2.8	38.9 ± 2.1	10.96 ± 0.90
PYR	5.58E-05	8.1 ± 0.4	0.14 ± 0.01	NA	NA	7.95 ± 0.83
SER	1.67E-04	20.4 ± 0.3	0.03 ± 0.00	46.4 ± 4.1	45.7 ± 3.5	9.96 ± 1.19
TRE	NA	20.5 ± 0.7	0.02 ±0.00	45.1 ± 2.2	41.3 ± 2.0	8.94 ± 1.0

(b) Additional functional traits measured in the greenhouse. Traits are the same as listed above.

Species	Stomatal aperture	Stomatal density	A_{1500}
AMY	NA	NA	12.06 ± 2.4
BEB	NA	NA	8.27 ± 0.1
CAN	11.4±0.8	0.03±0.00	9.91 ± 3.3
DIS	NA	NA	9.19 ± 1.4
DEL	20.0±0.4	0.02±0.00	NA
ERI	12.1±1.0	0.04±0.00	9.36 ± 0.6
HUM	NA	NA	NA
INT	14.9±1.2	0.04±0.00	18.5 ± 8.7
LUC	16.5±0.8	0.02±0.00	8.52 ± 1.1
NIG	15.1±1.0	0.03±0.00	10.8 ± 0.71
PED	11.9±1.0	0.03±0.00	NA
PET	10.7±1.0	0.04±0.00	NA
PYR	8.4±0.2	0.05±0.00	7.36 ± 0.7

(a) Female flowers receptive and/or male flowers producing pollen



(b) Timing of seed production

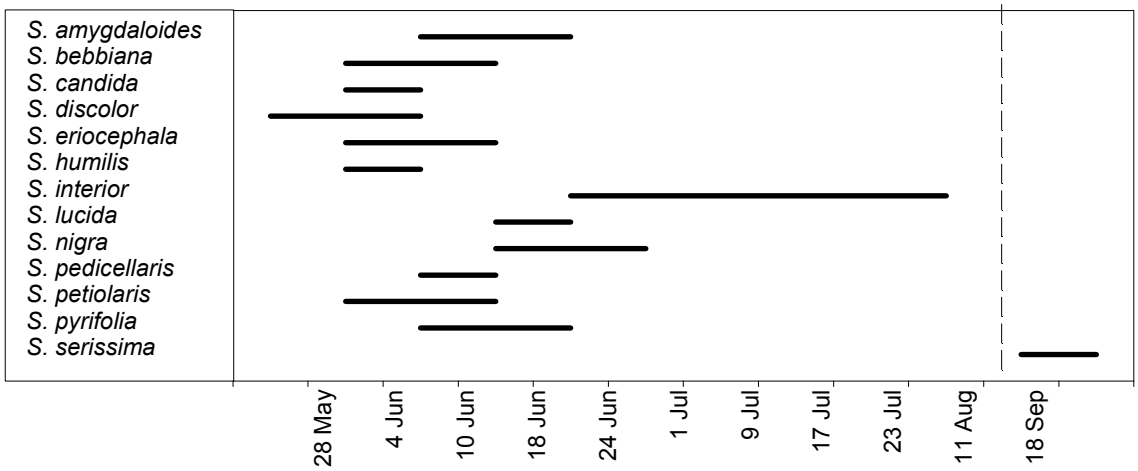


Figure S1.2 – Graphs indicate the timing of species flowering and seed production in the monitored plots in the spring and summer 2008.

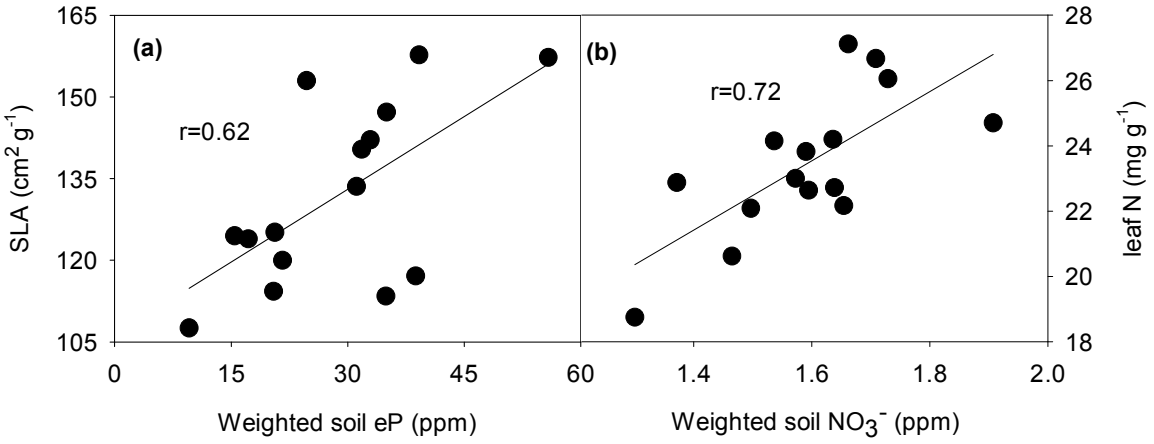


Figure S1.3 (a) Across species SLA (cm^2g^{-1}) correlated with weighted soil phosphorus (eP, ppm) and **(b)** leaf nitrogen (Leaf N, mg g^{-1}) correlated with weighted soil nitrate (NO_3^-, ppm) when these traits were measured in the field. There was no relationship between SLA and soil phosphorus when SLA was measured in the greenhouse.

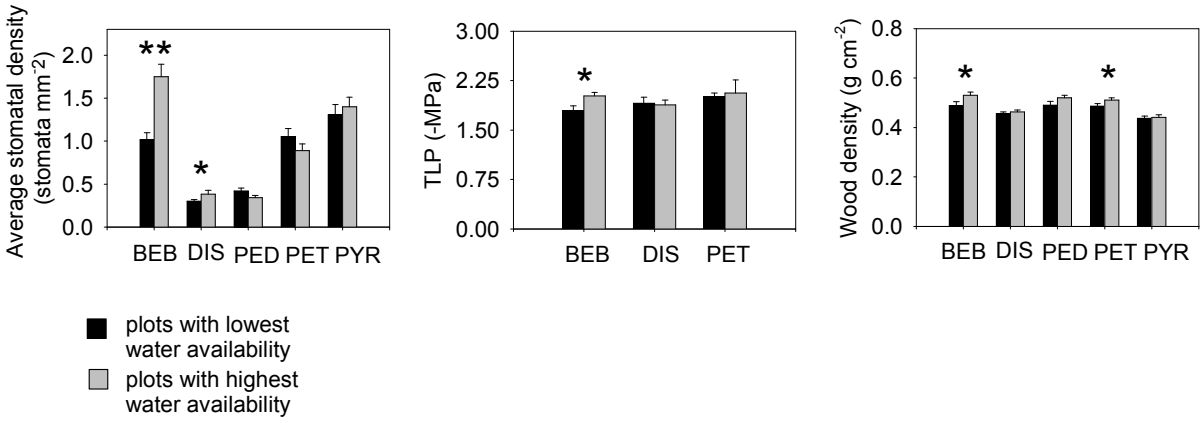


Figure S1.4 In the widely distributed species, functional traits were measured in plots with both high and low water availability. Stomatal density, TLP and wood density all demonstrated significant plot-level effects based on an ANOVA. Species traits that significantly differed between plots are noted with ** for α=0.01 and * for α=0.05.

APPENDIX 2 – Supplemental material for Chapter 4

Table S2.1 - Herbarium collection sources (accessed through GBIF data portal)

Arizona State University, Herbarium, USA
Australian National Herbarium (CANB), Australia
BeBIF Provider, Belgium
Berkeley Natural History Museums, USA
Bernice Pauahi Bishop Museum, USA
Biologiezentrum der Oberoesterreichischen Landesmuseen, Austria
Bishop Museum Natural History Specimen Data, USA
Bodensee-Naturmuseum Konstanz, Germany
Botanic Garden and Botanical Museum Berlin-Dahlem, Germany
Botanical Society of the British Isles - Vascular Plants Database, UK
Bronx River Bioblitz, USA
Bundesamt für Naturschutz, Germany
Burke Museum, USA
California State University, Chico, USA
Canadian Biodiversity Information Facility, Canada
Canadian Museum of Nature Herbarium, Canada
Colorado State University Herbarium (CSU), USA
Comisión nacional para el conocimiento y uso de la biodiversidad, Mexico
Conservatoire botanique national du Bassin parisien, France
European Environment Agency, UNIS
Fairchild Tropical Botanic Garden, USA
Finnish Museum of Natural History, Finland
Forest Research Institute, Department of Natural Forests, Poland
GBIF, New Zealand, Spain & Sweden
GEO-Tag der Artenvielfalt, Germany
Harvard University Herbaria, USA
Haus der Natur Salzburg, Austria
Herbaria of the University and ETH Zürich (Z+ZT), Switzerland
Herbario de la Universidad de Sevilla, SEV, Spain
Herbario SANT, Universidade de Santiago de Compostela, Spain
Herbarium of Oskarshamn (OHN), Sweden
Herbarium of the Białowieża Geobotanical Station, Poland
Herbier de l'Université Louis Pasteur, France
Icelandic Institute of Natural History, Iceland
Inatura - Erlebnis Naturschau Dornbirn, Austria
Institute of Dendrology PAS, Poland
Internation Botanical Collections (S), Sweden
Jyväskylä University Museum - The Section of Natural Sciences, Finland
Korea National Arboretum, Korea
Lousiana State Herbarium, USA
Lund Botanical Museum (LD), Sweden
Marine Science Institute, UCSB, USA
Missouri Botanical Garden, USA
Museo Nacional de Costa Rica, Costa Rica
Muséum national d'histoire naturelle et Réseau des Herbiers de France, France

Table S2.1 (cont.)

Museum of Natural History, Wroclaw University, Poland
National Herbarium of New South Wales, Australia
National Museum of Natural History, USA
National Museum of Nature and Science, Japan
Natural History Museum, University of Oslo, Norway
Natural History Museum, Vienna - Herbarium W, Austria
New Mexico Biodiversity Collections Consortium, USA
NLBIF, Netherlands
Oklahoma Vascular Plants Database Provider, USA
Oregon State University, USA
Royal Botanical Gardens Herbarium, CBIF, Canada
Service du Patrimoine naturel, Muséum national d'Histoire naturelle, Paris, France
Specimen Database of Colorado Vascular Plants, USA
Steiermärkisches Landesmuseum Joanneum - Herbarium GJO, Austria
SysTax, Germany
The Danish Biodiversity Information Facility, Denmark
The Danish Royal Veterinary and Agricultural University's Arboretum, Denmark
The New York Botanical Garden, USA
Tiroler Landesmuseum Ferdinandeum, Austria
UK National Biodiversity Network, UK
US National Plant Germplasm System Collection, USA
University Museums of Norway (MUSIT), Norway
University of Alabama Biodiversity and Systematics, USA
University of Alaska Museum of the North, USA
University of Arizona Herbarium, USA
University of California - Davis, USA
University of Colorado Museum, USA
University of Connecticut, USA
University of Kansas Biodiversity Research Center, USA
University of Tennessee, Knoxville, USA
University of Vienna, Institute for Botany Herbarium, Austria
USDA PLANTS Database, USA
Utah State University - USU-UTU Specimen database, USA
Utah Valley State College Herbarium, USA
Vascular Plant Collection - University of Washington Herbarium (WTU), USA
Yale University Peabody Museum, USA

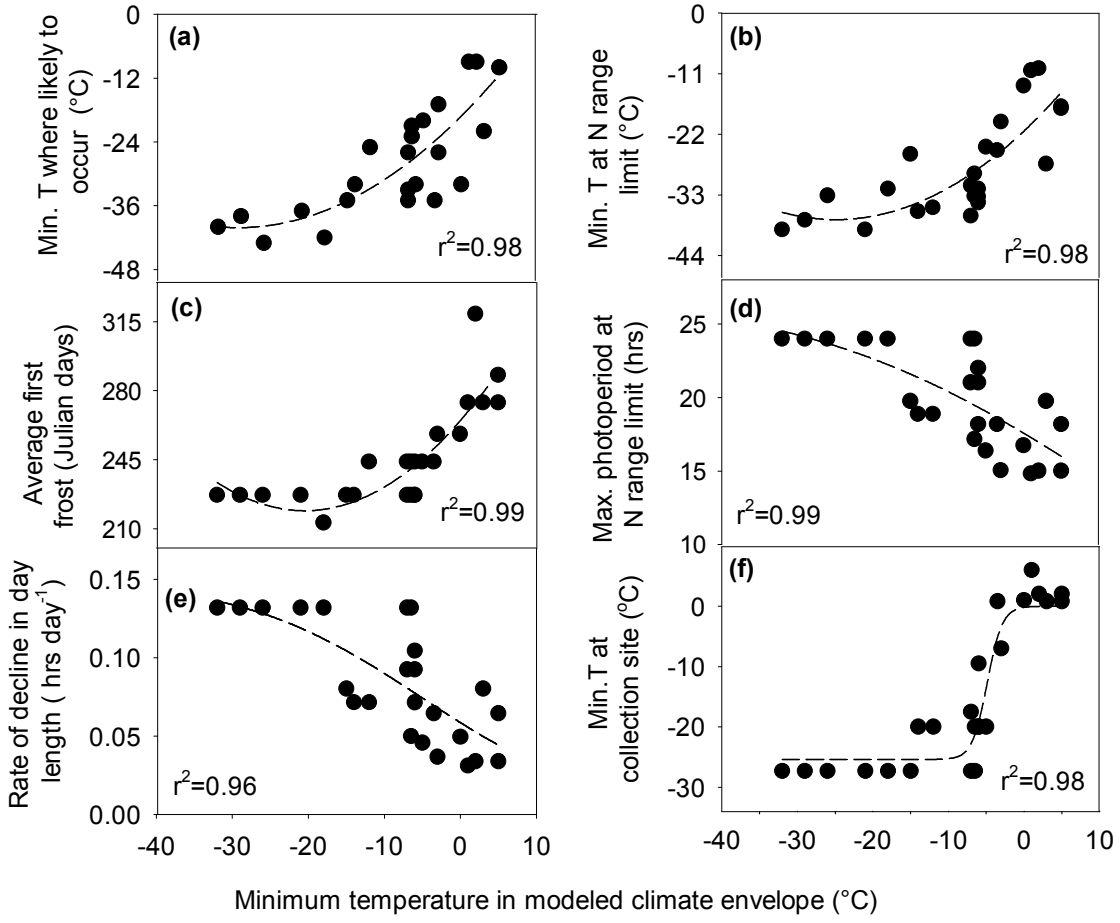


Figure S2.1 – We estimated four additional climate variables that are relevant to plant cold acclimation and freezing tolerance: **(a)** the lowest minimum temperature where each species is probable to occur (the average min. temperature where the probability of occurrence drops below 0.05 based on the Maxent model), **(b)** average minimum temperature of the coldest month (°C) at northern range limit, **(c)** most probable date of first frost at N range limit (90% probability that frost occurs after given date) (NOAA 1951-1980, Geographical Services Division 1957), **(d)** the maximum day length at species' N range limit, **(e)** the rate of decline in day length during late summer and winter (hrs day^{-1} between summer and winter solstice), and **(f)** the average minimum temperature at species' collection sites. Since all of these variables correlated with minimum temperature in species' modeled climate envelopes (Min T), we used Min T in all our analyses in the study.

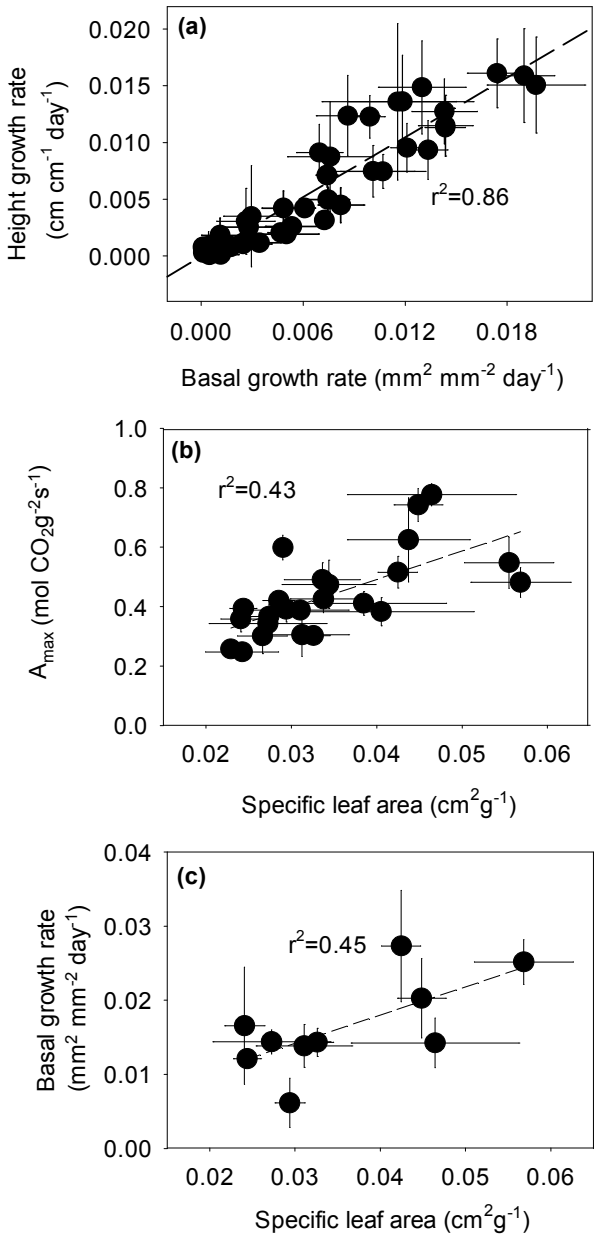


Figure S2.2 (a) Across species, height growth rate positively correlated with basal growth rate. Each point represents a species in either the temperate or the tropical treatment. **(b)** Across species, maximum photosynthetic capacity (mol CO_2 $g^{-1}s^{-1}$) correlated with specific leaf area (cm^2g^{-1}). **(c)** Species' basal growth rates (measured in the long summer photoperiod treatment) also positively correlated with specific leaf area. Points represent species' averages and error bars are ± one standard error

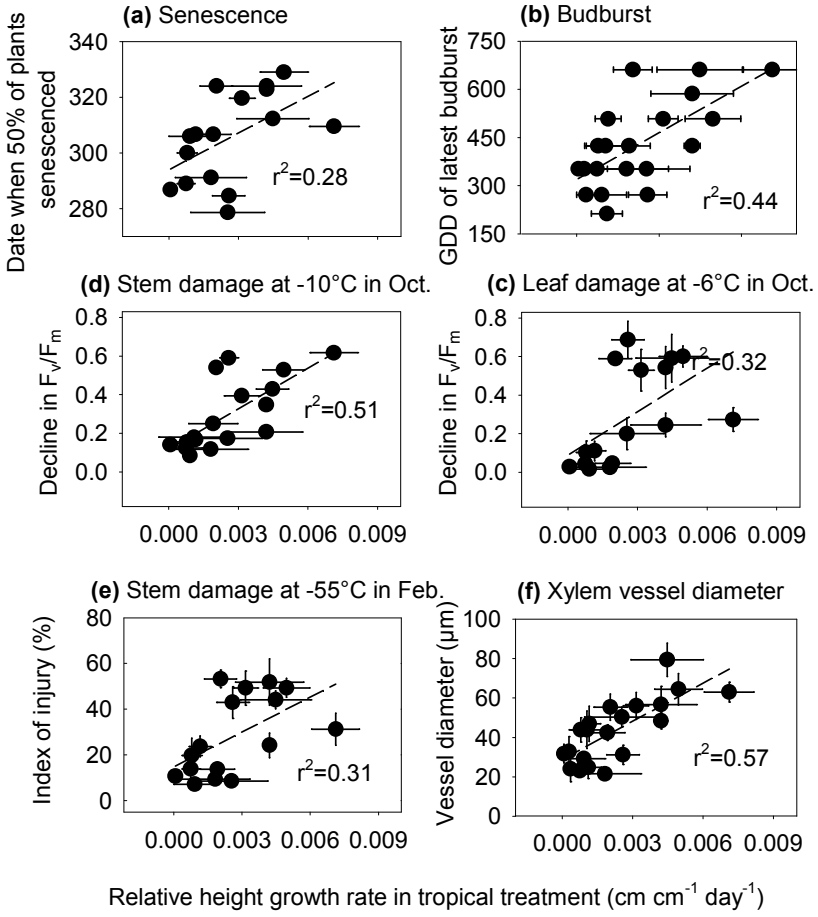


Figure S2.3 – Species that exhibited slow growth rates in the tropical treatment demonstrated **(a)** earlier senescence and **(b)** earlier budburst than faster growing species in the temperate treatment. These phenological differences are likely beneficial in more northern, colder climates. The slower growing species also demonstrated greater freezing tolerance than the faster growing species, as demonstrated by **(c)** their limited damage after their leaves were frozen at -6°C in the autumn, **(d)** stems frozen at -10°C in the autumn, and **(e)** stems frozen at -55°C in the late winter. **(f)** Average xylem vessel diameters in the temperate treatment were also correlated with growth rates in the tropical treatment. All points represent species' averages (except for timing of senescence and budburst) and error bars are ± one standard error.