LINKING COMPETITION AND NONSTRUCTURAL CARBON DYNAMICS IN AMERICAN CHESTNUT (*CASTANEA DENTATA)*
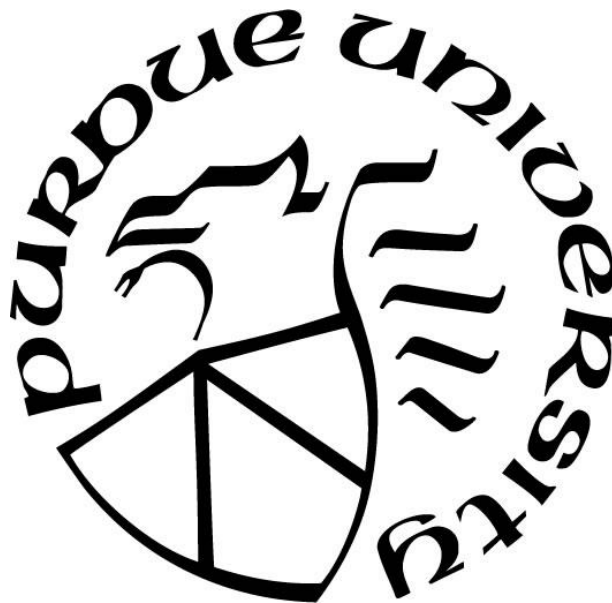
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Dedicated to my mom and dad.

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ABSTRACT

Nonstructural carbohydrates (NSC) are involved in essential plant functions and are closely associated with growth and mortality mechanisms. Quantifying NSC reserves can indicate a plant's resistance and resilience to stress or injury through damaging agents and environmental change. In this work, we studied seasonal NSC dynamics and responses to inter- and intra-specific competition in American chestnut (*Castanea dentata* (Marsh.) Borkh.), a species of high restoration interest. This research project is comprised of two experiments; first, we delineate seasonal NSC dynamics in mature chestnut to identify critical periods in NSC reserve dynamics that could help evaluate the condition of trees (Chapter 2). We used a custom-built allometric model to scale NSC concentration measurements into pool sizes. By assessing the magnitude and location of NSC pools, we found that chestnut stores most of its NSCs belowground and demonstrated an ability to mobilize root NSCs to fuel growth and metabolism. These results support the idea that chestnut could thrive under disturbance-based management. In the second experiment, we use the knowledge of chestnut biology gained in Ch. 2 to examine a fundamental question in the NSC literature: How does competition affect the internal balance of carbohydrate allocation to growth vs. storage? We studied the effect of intra- and inter-specific competition on American chestnut biomass accumulation and NSC reserves using mixtures of species with strong functional differences in a mature plantation experiment. Coarse root total NSC pools responded to density and species composition through their combined effects on NSC concentrations and tree biomass. There was a strong density $\times$ composition interaction: while NSC pools were largest in plots with the lowest planting density where chestnut was planted with black cherry or in a three-species mixture with black cherry and northern red oak, chestnut NSC pools were reduced when planted in monoculture or in a two-species mixture with northern red oak. Therefore, neighbor identity was more important than species richness for NSC pools in our system. Taken together, this body of work contributes to our understanding of the regulation of NSCs in temperate deciduous trees, and to our understanding of chestnut's unique biology in particular.

CHAPTER 1.   INTRODUCTION

1.1   Vulnerability and restoration of forest ecosystems in the eastern USA

Forests harbor most of Earth's terrestrial biodiversity and provide many ecosystem functions (FAO and UNEP 2020). Like other forests across the globe, forests of the eastern United States are impacted by the interacting disturbances of climate change and introductions of exotic pests (Fei et al. 2017; FAO and UNEP 2020). In the eastern USA, shifting patterns of temperature and precipitation have exposed forests to increasing moisture deficits on a nearly annual basis (Clark et al. 2016). Climate change has been associated with species-specific mortality, reductions in vigor, and range shifts translating to changes in forest composition and structure (Clark et al. 2016; Fei et al. 2017). Future forest vulnerability is further increased by projections of increased pest activity resulting from globalization and climate change (Rogers et al. 2017). The effects of introduced pests and pathogens have already been severe; for example, eastern hemlock (*Tsuga canadensis*) has been lost from much of its range due to the introduced hemlock wooly adelgid (*Adelges tsugae*), and American beech (*Fagus grandifolia*) populations have been restructured throughout in the northeastern US in response to beech bark disease (Ellison et al. 2005; Lovett et al. 2006). American chestnut (*Castanea dentata* (Marsh.) Borkh.), the focal species for this study, dominated a wide range of habitats until the non-native chestnut blight (*Cryphonectria parasitica*) reduced the species to an understory shrub (Wang et al. 2013; Dalgleish et al. 2016).

In light of the combined stressors of climate change and invasion of exotic pests, active forest restoration as well as managing forests for increased resistance and resilience has become a widely recognized imperative (Lake 2013; Nunez-Mir et al. 2015; FAO and UNEP 2020). A concerted effort to restore American chestnut using blight-resistant varieties is underway, which may serve as a model to restore other threatened plant species (Jacobs et al. 2013). Additionally, there has been a substantial effort to understand the potential linkages between forest diversity and resistance, the ability to function normally during a disturbance, or resilience, the capacity to recover normal function after the disturbance has occurred (Lake 2013; Ruiz-Benito et al. 2017; Grossman et al. 2018). Nonstructural carbohydrates (NSC; soluble sugars and starch) play a central, but understudied, role in these hypothesized relationships by fueling processes linked to

resistance (e.g. increased sugar concentrations to avoid damage from freezing) and to resilience (e.g. resprouting after topkill by fire) (Kozlowski 1992; Piper and Paula 2020).

1.2 The American chestnut story

American chestnut (*Castanea dentata*) was a dominant component of the eastern deciduous forest until the arrival of chestnut blight (*Cryphonectria parasitica*) in the early 20th century (Braun 1950; Keever 1953; Russell 1987). In 1900, American chestnut was present in nearly all states east of the Mississippi River (Fig. 1.1), comprising 25-50% of the basal area in many forests. Because of its commonness, colossal form, nutrient-rich leaves and nuts, and decay-resistant wood, chestnut is considered to have been a foundation species whose functional loss altered ecological community dynamics and nutrient cycling (Keever 1953; Ellison et al. 2005).

Before the arrival of the blight, chestnut was deeply embedded in American economies and cultures (Hawley and Hawes 1918; Wang et al. 2013). Chestnut trees produced an abundant annual nut crop that was an important food source for humans, livestock and wildlife (Emerson 1846; Hawley and Hawes 1918; Dalgleish and Swihart 2012). Native Americans included chestnut in their diet, medicine, and stories. For example, the Haudenosaunee people of present-day New York State mashed chestnuts to make pudding, dried and ground them into flour, and incorporated the species into legends and myths (Curtin and Hewitt 1918; Brewer 2017). Studies using pre-settlement land survey data (c. 1800 CE) in New York (Tulowiecki and Larsen 2015) and Pennsylvania (Black et al. 2006) indicate that Native Americans promoted chestnut along with other fire-tolerant, mast-bearing species like oak and hickory through their agroforestry practices. Chestnut could produce a straight stem 60-120 feet tall, and its high-tannin wood was durable and decay resistant (Braun 1950). White settlers in America used it for house-building, fencing, furniture, and as a source of tannin for the tanning industry (Emerson 1846; Braun 1950, Baxter 2008). Chestnut trees grow rapidly and have a remarkable capacity to re-sprout from the root collar; groves were coppiced to produce fuelwood before the arrival of the blight (Fig. 1.2; Hawley and Hawes 1918; Wang et al. 2013).

The fungal pathogen *Cryphonectria parasitica* was first discovered attacking American chestnut trees around New York City in 1904 (Murrill 1906). The blight fungus produces necrotic cankers that coalesce around the stem, destroying the vascular cambium and killing the aboveground portion of the tree (Roane et al. 1986). Chestnut blight spread rapidly from New

York, killing nearly every mature chestnut in the United States (roughly 4.2 billion trees) within a matter of decades (Gravatt 1949; Braun 1950; Hepting 1974). The root rot disease caused by *Phytophthora cinnamomi* Rands further contributed to chestnut decline, and root rot is still a damaging agent in the southern part of chestnut's range (Milburn et al.; Anagnostakis 2012). Today, chestnut is present in forests of the eastern United States as understory trees that are trapped in a cycle of blight, dieback, and re-sprouting; mature trees are very rare (Paillet 2002; Dalgleish et al. 2016). American chestnut is currently listed as an endangered species in Kentucky and Michigan and as a species of special concern in Maine and Tennessee (USDA Plants Database 2020).

Restoration efforts have been ongoing for over a century (Wang et al. 2013). Blight resistant trees have been produced through the combined efforts of the American Chestnut Foundation's long-standing backcross breeding program and the recent achievement of transgenic blight resistant trees at the State University of New York, College of Environmental Science and Forestry (SUNY-ESF) (Newhouse et al. 2017; Steiner et al. 2017). Genetically engineered American chestnut is the first transgenic plant specifically designed to spread in the environment (Newhouse and Powell 2020). There are several regulatory barriers and ethical questions that must be addressed before transgenic plants are deployed into nature. For example, SUNY-ESF, the primary research center for transgenic chestnut, is located in the center of historical and contemporary territory of the federally recognized Haudenosaunee Confederacy in New York State (Barnhill-Dilling and Delborne 2019). Genetic engineering is a contentious issue in this community (Rosen 2019). Some tribal members have expressed concern about a lack of meaningful tribal consultation, even though field trials and proposed release sites are within a few miles of sovereign nation borders and independent migration is the explicit goal of this program (Barnhill-Dilling et al. 2020). The broader American public shares some skepticism about releasing genetically engineered chestnut into the wild (Petit et al. 2021). These perspectives and concerns must be addressed to for responsible restoration to occur (Barnhill-Dilling et al. 2020). After these issues have been negotiated, the reintroduction of American chestnut through transgenic and/or traditional breeding is close at hand (Steiner et al. 2017).

Successful reintroduction relies on understanding chestnuts' biology and ecology to identify favorable strategies for forest management (Griffin 2000; Jacobs 2007). However, information on chestnut's life history characteristics and adaptations to different environmental

conditions is lacking due to its absence throughout the last century of forest biology and ecology research (Jacobs 2007, Wang et al. 2013). Information on the ecology and management of American chestnut is derived largely from historical observations, studies on the growth of disease-induced stump sprouts, and a few naturalized plantings (Jacobs et al. 2013). Favorable conditions for growth and survival, such as abiotic site characteristics, stocking levels, and positive associations with other species, have yet to be identified. In particular, we have very little information on the nonstructural carbohydrate (NSC) strategy (e.g. conservative vs. growth-forward strategy, and important storage organs) of mature chestnut. While its prolific sprouting ability suggests that chestnut is adapted to frequent disturbances (Paillet 2002), this assumption has not been tested in mature trees. Data on saplings showed that chestnut had higher concentrations but smaller pools of both sugar and starch in the roots than *Quercus rubra,* suggesting that the species may be less amenable to disturbance-based management than *Q. rubra* (Belair et al. 2018). However, these results were based on relatively small individuals and might therefore not be scalable to larger trees due to differences in allometry, phenology, and the remobilization potential of stored reserves, which all complicate comparisons of NSC dynamics between seedlings and mature trees (Hartmann et al. 2018).
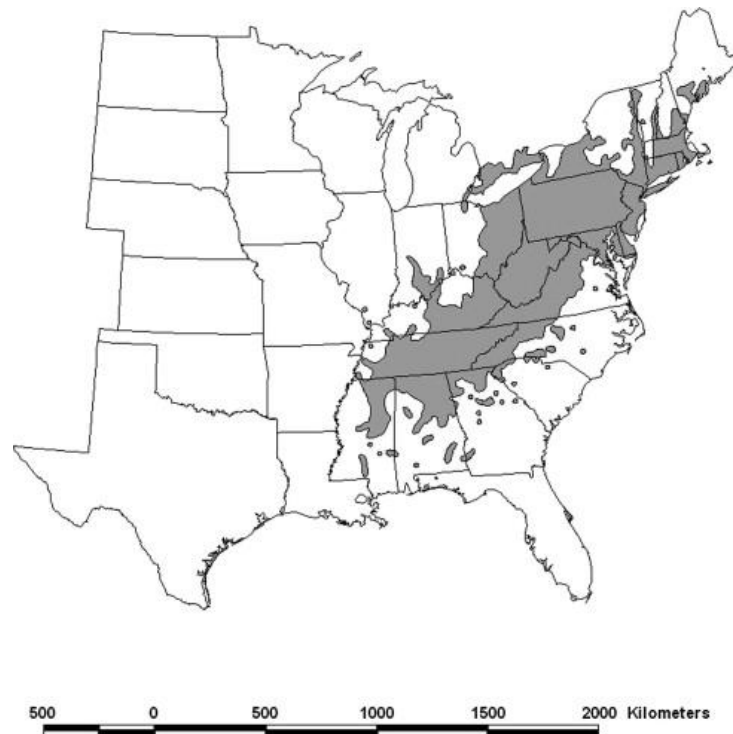
Figure 1.1. The pre-blight range of American chestnut, adapted from Little's Atlas of United States Trees, Vol. 4, minor eastern hardwoods (1977) by Jacobs (2007).

Figure 1.2. (a) Massive old-growth chestnut. (b) A typical clump of chestnut poles, with ash, basswood, and cherry in the background. Both images were taken on the Nantahala National Forest, NC circa 1910. Photos courtesy of the Forest History Society, Durham, NC.

1.3   Nonstructural carbohydrates in temperate deciduous trees

Plants fix carbon through photosynthesis, and the sugars they synthesize is used to build structural biomass or to fuel metabolic functions throughout the plant (Kozlowski et al. 1991). Sucrose, the primary transport sugar, is loaded into the phloem and transported by mass flow from the chloroplast to the mesophyll and the rest of the plant body (Kozlowski 1992). Sugar concentrations are probably held above some critical threshold to continue to support those functions (Martínez-Vilalta et al. 2016; Wiley et al. 2017; Weber et al. 2018). When carbon fixation is greater than consumption, carbon is also retained as reserves in organs and tissues either as soluble sugars or converted to more complex, water-insoluble (and therefore osmotically-inert) storage compounds such as starch and lipids (Chapin et al. 1990). Starch molecules, long chains of glucose molecules, can be rapidly converted back to glucose (which can be later converted to

fructose, both of which are needed to synthesize sucrose), or they can remain stored for varying timescales ranging from minutes to centuries (Richardson et al. 2015).

Nonstructural carbohydrates (NSCs) play a critical role throughout the lifespan of trees by fueling routine cellular processes under varying environmental conditions and by facilitating resistance to stressors. In temperate deciduous trees, stored carbohydrates fuel energetically expensive phenological changes like bud burst and early growth after a prolonged leafless period (Chapin et al. 1990). Stored carbohydrates also help prevent injury during drought and freezing events, and they support regrowth after disturbance (Kozlowski 1992). Because of these essential functions, NSC dynamics are often closely associated with growth and mortality mechanisms (Chapin et al. 1990; Landhäusser and Lieffers 2012; Adams et al. 2013; Sevanto et al. 2014).

Although NSCs are critical for stable plant function, basic questions regarding the regulation and function of NSC reserves for either growth or storage persist (Palacio et al. 2014). The difference between carbon acquisition and carbon demand can create either a net surplus or a deficit of NSCs, which ultimately results in either reserve accumulation (storage) or carbon starvation (McDowell 2011). Apart from seasonal and climatic conditions, life history strategies, wood characteristics, and tree health status can all affect NSC allocation dynamics (Kozlowski 1992; Hoch et al. 2003; Klein et al. 2016; Wiley et al. 2016; Furze et al. 2019). The limited availability of whole-tree NSC data and inconsistent patterns of allocation between species restricts our ability to predict whole-tree and organ-specific seasonal fluctuations of starch, sugar, and total NSCs with precision. Additionally, the degree to which NSC storage is moderated by intra- or inter-specific interactions, an essential element of restoration plantings, has yet to be determined.

1.4   Inter- and intra-specific interactions

Inter- and intra-specific interactions affect the productivity and survival of individual plants, and the cumulative balance of these interactions determines species abundance and ecosystem structure. Forest productivity (biomass accumulation) is thought to be positively related to increased diversity (Zhang et al. 2012; Liang et al. 2016), presumably because competition between individuals growing in mixed-species stands is less intense than competition among individuals growing among conspecifics (Forrester and Bauhus 2016). Neighboring plants compete for a common pool of above- and below-ground resources by adjusting biomass allocation

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and organ morphology (Poorter et al. 2012). Plants generally direct their growth towards organs that capture the most limiting resource; for example, plants facing nitrogen or water limitation increase biomass allocation to roots (Kozlowski et al. 1991). Competition intensity increases with forest stand density, although it can be ameliorated through complementary species mixtures (Forrester and Bauhus 2016).

Competition may be reduced in mixed-species stands due to complementarity stemming from facilitation or niche partitioning (Forrester and Bauhus 2016). Facilitation occurs when one species improves the resource availability or abiotic conditions for another species, thereby promoting its growth or survival. Deep rooted species can improve the soil moisture for shallow-rooted species through hydraulic lift, the passive, root-mediated transfer of water from deeper soil layers to the drier and shallower layer (Zapater et al. 2011). Niche partitioning also reduces competition intensity in mixed stands relative to monocultures. For instance, competition for light limits photosynthesis rates in suppressed individuals (Kozlowski et al. 1991). But a structural partitioning of the canopy results in increased light interception and a greater source of photosynthate at the stand level (Pretzsch 2014; Tatsumi 2020). In another study, European beech (*Fagus sylvatica* L.) deepened and intensified its root system in mixture with Norway spruce (*Picea abies* L.) to better exploit soil water resources (Schume et al. 2004), illustrating plastic biomass allocation in response to different competition regimes (del Río et al. 2019). Complementarity reduces competition for resources and promotes overyielding, when polycultures have higher structural biomass production than monocultures (Tilman et al. 2001; Forrester and Bauhus 2016).

While there is a substantial body of literature on the effect of species mixtures on the productivity and allocation of structural mass, research explicitly linking inter- and intra-specific interactions to nonstructural carbohydrate (NSC) dynamics, particularly for large trees, is scarce. Previous studies of these processes have mostly focused on herbaceous plants (Lacey et al. 1994; García-Cervigón et al. 2013) or tree seedlings in pot experiments (Guo et al. 2016, 2020; Wu et al. 2020). Guo et al. (2020) found that intra-specific competition modulated NSC storage in *Cunninghamia lanceolata* seedlings, where it increased root starch concentrations under drought, while starch concentrations were dramatically lowered when faced with added competition (Guo et al. 2020). Wu et al. (2020) found a positive relationship between competition (vs. single-grown seedlings) and starch concentrations in two *Larix* species, but there was no statistical difference in

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NSC dynamics between the seedlings grown in inter- vs. intra-specific competition; however both species had similar life history strategies and nearly indistinguishable morphology. We hypothesize that studying mixtures of species with strong functional differences (*sensu* Díaz and Cabido 2001) might be a more appropriate approach for evaluating the potential effects of interactions on productivity and NSC reserve storage.

1.5   Summary of objectives

This research project establishes baseline NSC dynamics for mature American chestnut and compares NSC reserves in chestnut trees growing under varying competition regimes in a hardwood plantation experiment. In Experiment I (presented in Chapter 2), whole-tree NSC storage dynamics were tracked through one full seasonal cycle. Our specific objectives for Ch. 2 were to: I) track seasonal variation in NSC concentrations (i.e. sugars and starch) in all major organs, and II) compare organ-specific pool sizes with one another to infer the relative importance of different organs for NSC storage and remobilization. The effect of competition on belowground NSC reserves was tested in Experiment II (Chapter 3), where our objective was to explore relationships between the type of interaction (intra- or inter-specific), tree productivity, and NSC dynamics in a plantation experiment that manipulated planting density and species composition. In the Appendix, I present original biomass data and allometric modelling that enabled us to quantify and compare NSC pools (NSC concentration x biomass) of important storage organs in our trees. Our findings have implications for resilience to modern forestry challenges like drought, flooding and pest outbreaks, and specific relevance for forest managers seeking to restore American chestnut to the landscape. These implications are discussed in Chapter 4 (Conclusions).

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CHAPTER 2.   ROOT SYSTEM DOMINATES CARBOHYDRATE RESERVE POOLS IN AMERICAN CHESTNUT (CASTANEA DENTATA): IMPLICATIONS FOR ECOLOGY AND MANAGEMENT

2.1   Abstract

Non-structural carbohydrates (NSCs) are critical for stable plant function, but the ability to predict NSC levels in specific forest tree species with precision is lacking. We evaluated seasonal and inter-organ NSC dynamics in American chestnut (*Castanea dentata*), a species of high restoration interest. Trees were sampled over the course of one year at different phenological stages and organ specific NSC concentration data was paired with biomass estimates from a bespoke allometric model to generate NSC pool sizes. Organ-level and whole-tree seasonal NSC concentrations and pools generally peaked at leaf fall (October) and were lowest during shoot expansion (June), although interactions between organ and collection period drove variation in pool sizes. Coarse root NSC reserves were replenished later in the growing season than aboveground organs. Coarse root reserves were also larger and more dynamic than in previous studies with other temperate deciduous trees, and they were the primary supplier of NSCs to support spring leaf-out. The tendency to store NSCs belowground and a demonstrated ability to mobilize root NSCs to fuel growth and metabolism, both support the idea that chestnut could thrive under disturbance-based management.

2.2   Introduction

Trees are long-lived, sessile organisms that must withstand biotic stressors and environmental variability (Petit and Hampe 2006). Nonstructural carbohydrates (NSCs) play a critical role throughout the lifespan of trees by fueling routine cellular processes under varying environmental conditions and by facilitating resistance to stressors. In temperate deciduous trees, stored carbohydrates fuel energetically expensive phenological changes like bud burst and early growth after a prolonged leafless period (Chapin et al. 1990). Stored carbohydrates also help prevent injury during drought and freezing events, and they support regrowth after disturbance (Payton and Brasch 1978; Kozlowski 1992). Because of these essential functions, NSC dynamics are often closely associated with growth and mortality mechanisms (Chapin et al. 1990;

Landhäusser and Lieffers 2012; Adams et al. 2013; Sevanto et al. 2014). Quantifying NSC reserves can therefore indicate a plant's ability to respond to and/or recover from stress or injury through damaging agents and environmental change (Canham et al. 1999; Landhäusser and Lieffers 2002; Wiley et al. 2019).

Plants fix carbon through photosynthesis, and most of the sugars they synthesize is used to build structural biomass or to fuel metabolic functions throughout the plant (Kozlowski et al. 1991). Sugar concentrations are probably held above some critical threshold to continue to support those functions (Martínez-Vilalta et al. 2016; Wiley et al. 2017; Weber et al. 2018). When carbon fixation is greater than consumption, carbon is also retained as reserves in organs and tissues either as soluble sugars or converted to more complex osmotically inert storage compounds such as starch and lipids (Chapin et al. 1990) that can be converted back to sugars or it can remain stored for timescales ranging from minutes to centuries (Richardson et al. 2015).

Although NSCs are critical for stable plant function, basic questions regarding the regulation and function of NSC reserves for either growth or storage persist (Palacio et al. 2014). The difference between carbon acquisition and carbon demand can create either a net surplus or a deficit of NSCs, which ultimately results in either reserve accumulation (storage) or carbon starvation (McDowell 2011). Apart from seasonal and climatic conditions, life history strategies, wood characteristics, and tree health status can all affect NSC allocation dynamics (Kozlowski 1992; Hoch et al. 2003; Klein et al. 2016; Wiley et al. 2016; Furze et al. 2019). For temperate deciduous trees, NSC (soluble sugars + starch) concentrations typically increase throughout the growing season and peak just before leaf fall, at the end of the photosynthetic period and when growth and respiration rates decrease (Kozlowski 1992). Total NSC concentrations tend to be lowest after spring leaf-out, after large quantities of starch that have been converted to soluble sugar for winter frost tolerance are being used to fuel cellular respiration and the production of new leaves (Kozlowski 1992; Hoch et al. 2003; Furze et al. 2019). However, the relative distribution and dynamics of NSC in different parts of a tree may change throughout the year owing to differences in the timing and duration of organ activities such as growth and flowering, or asynchronous root and shoot phenology (Abramoff and Finzi 2015). Rather than a uniform depletion of NCS reserves throughout the plant body of a large tree, most of the reserves used for growth and other metabolic functions appear to be sourced from localized and adjacent storage pools (Landhäusser and Lieffers 2003; Wiley et al. 2016).

While NSC concentration measurements gives information about the carbon status of individual organs at a limited scale, quantifying NSC pools (based on the mass allocation of the plant) allows the exploration of changes in plant physiological status and environmental cues at both whole-tree and inter-organ levels (Martínez-Vilalta et al. 2016). Moreover, pool size, not concentration, may be a better predictor of a plant's ability to recover from disturbance (e.g. resprouting ability, Landhäusser and Lieffers 2002; Wiley et al. 2019). Generating budgets for NSC pool requires comprehensive concentration data at the organ level paired with careful allometric scaling (Klein et al. 2016; Furze et al. 2019). For example, the difference between the annual maximum and minimum nonstructural carbon NSC pool (ΔNSC) likely largely represents the amount of carbon reserves that are required for a tree to fuel seasonal functions related to its phenology (Barbaroux et al. 2003; Hoch et al. 2003). Barbaroux et al. (2003) reported a 40-50% seasonal change in whole-tree NSC pools in *Quercus petraea* and *Fagus sylvatica*. While NSC concentrations were highest in the roots of both species, the largest NSC pools were stored in and mobilized from the stem. In contrast, Furze et al. (2019) found minimal seasonal depletion in whole-tree total NSC pools of five temperate species (*Quercus rubra*, *Pinus strobus*, *Acer rubrum*, *Betula papyrifera*, and *Fraxinus americana)*, and found that branch NSC reserves were largest and most dynamic of any organ. The limited availability of whole-tree NSC data and some conflicting results restricts our ability to predict whole-tree and organ-specific seasonal fluctuations of starch, sugar, and total NSCs with precision.

In this study, we evaluated the seasonal and inter-organ dynamics of NSC (soluble sugars and starch) pools on American chestnut (*Castanea dentata*) trees, a species of high restoration interest (Jacobs 2007). American chestnut was a dominant component of the eastern deciduous North American forests until the arrival of chestnut blight (*Cryphonectria parasitica*) in the early 20th century (Braun 1950; Paillet 2002). Through breeding and transgenics for blight-resistant trees, the reintroduction of American chestnut is likely (Wang et al. 2013; Steiner et al. 2017). A greater understanding of chestnut's biology and ecology is needed to prioritize deployment strategies for reintroduction (Griffin 2000; Jacobs 2007). Delineating seasonal NSC dynamics in mature chestnut might identify useful indicators for this species by recognizing critical periods in NSC reserve dynamics that could help evaluate the condition of trees. Assessing the magnitude and location of NSC pools also indicates chestnuts' potential competitive strategy (i.e., stress-tolerant or competitive, Grime 1977) and adaptation to disturbance.

American chestnut has a remarkable capacity to re-sprout from the root collar; groves were coppiced to produce fuelwood in the pre-blight era, and today the species exists mainly as sprouts <2.5 cm DBH trapped in a cycle of sprouting, infection with blight, and stem dieback (Hawley and Hawes 1918; Paillet 2002; Dalgleish et al. 2016). While this prolific sprouting ability suggests that chestnut is adapted to frequent disturbances (Paillet 2002), this assumption has not been tested in mature trees. Data on juvenile trees (saplings) showed that chestnut had higher root starch and sugar concentrations, but smaller root sugar and starch pools, compared to *Quercus rubra,* suggesting that the species may be less amenable to disturbance-based management than *Q. rubra* (Belair et al. 2018). However, these results were based on relatively small individuals and might therefore not be scalable to larger trees due to differences in allometry, phenology, and the remobilization potential of stored reserves, which all can complicate comparisons of NSC dynamics between seedlings and mature trees (Hartmann et al. 2018).

Our objectives were to: I) track seasonal variation in NSC concentrations (i.e. sugars and starch) in all major organs, and II) compare organ-specific pool sizes with one another to infer the relative importance of different organs for NSC storage and remobilization. To this end, we sampled major storage organs during important phenological stages throughout one year. Then, we paired organ specific NSC concentration data with biomass estimates from a bespoke allometric model to generate NSC pool sizes. We hypothesized that I) organ-level NSC concentrations and pool sizes would peak at leaf fall and be lowest during the early summer, in line with the prevailing theory of seasonal NSC dynamics in temperate trees (Kozlowski 1992). Given the potential importance of belowground reserves for chestnut described above, we further expected chestnut to have the highest NSC concentrations, and especially starch concentrations, in coarse roots. High coarse root concentrations may be overshadowed by aboveground organs when expressed in terms of pool size (Barbaroux et al. 2003; Furze et al. 2019). Therefore, we hypothesized that II) the largest NSC pools would be in aboveground organs (e.g. the stem or branches).

2.3   Methods

Site description

The study was carried out in a 13-year-old plantation experiment located at Martell Forest, a research site owned and operated by Purdue University in West Lafayette, Indiana (40°26'42"N, 87°01'47"W). The experiment is situated on a moderately well-drained site formerly used as an agricultural field. The main soil type is comprised of Rockfield silt loam (USDA NRCS 2017). Mean annual temperature in West Lafayette, IN is 11.4 C and mean annual precipitation is 996 mm (data from 1981-2018, National Climatic Data Center 2019).

American chestnut trees were planted in three replicate plots at a density of 2500 stems ha^{-1} (2 m spaced between trees). Each plot contained 30 trees (6 trees by 5 trees square). The seed origin of the chestnut trees were pure American chestnut trees greater than 100 years of age near Galesville, Wisconsin, USA.

Sample collection

We sampled the major organs from two trees in each of the three plots (total of six trees per collection time) during specific phenological stages throughout one full year. Different trees were sampled at each collection time, and sampling occurred when roughly 50% of the selected trees were at a given phenological stage. Collection times were determined by visual observation (except for the use of a sap flow meter (SFM1, ICT international) to determine times of peak sap flow in March 2020) (Figure 2.1). The seven sampling times were: 1) **leaf out** – when buds were breaking and leaves started to emerge (March 2019); 2) **shoot expansion** – when shoots were actively elongating, and leaf area was at a maximum (June 2019), **bud set** – late growing season, when long shoots had set bud but leaves were still green (August 2019), **leaf fall** – when leaves were yellow and 50% of the canopy remained intact (October 2019), **dormancy** – when trees were apparently inactive and the top layer of soil was frozen (January 2020), **sap flow** – when sap started to flow and buds were starting to swell (March 2020), and a repeat of **leaf out** (April 2020). Colder temperatures in the spring of 2020 delayed leaf out sampling relative to our leaf out sampling date in 2019.

Samples were obtained from different trees at each collection date to minimize the impact of sampling on individual stems. The size distribution of our study trees is provided in table 2.S1. Samples were collected from twigs (produced in the last year), shoots (~1 cm diameter), stem

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phloem, stem xylem, root collar, coarse roots (2-14 mm diameter), fine roots (< 2 mm) and leaves (when available) at each sampling date. We used a pole pruner to access leaf, twig and shoot samples. We used a hammer and chisel to obtain an inner bark sample (including outer and inner bark and phloem tissues) at a height of 1.5 m from the forest floor. The outer bark represented a small proportion of a sample as it was very thin (~1mm) and difficult to remove. Xylem (sapwood) samples were only collected at shoot expansion (June 2019) and leaf fall (October 2020) by chiseling a 2 cm wide by 2 cm long by 2 cm deep cube (with depth starting at the cambium) at the site of inner bark sampling. We also collected inner bark samples (including phloem tissue) from the root collar because chestnut is known to sprout vigorously from the root collar in response to the chestnut blight (Wang et al. 2013). Coarse root (2-14 mm diameter) and fine root (≤ 2 mm) samples were obtained by digging approximately half a meter from the tree and following the root back to the base of the tree. Tissue samples were frozen at -4 C until further processing in the lab.

Quantifying soluble sugars and starch

Tissue samples were ground in a ball mill (Retsch MM-400, Haan, Germany) and shipped to a laboratory at the University of Alberta for analysis. Briefly, water-soluble sugars were extracted with 80% hot ethanol from the tissue sample and starch concentrations were determined in the residue following the procedure of Landhäusser et al. (2018). The extracted sugars were quantified using phenol–sulfuric acid method with 2% phenol and concentrated H_2SO_4 and compared against a GFG (1:1:1, glucose, fructose, galactose) standard, absorbance was read at 490 nm. Interference of other compounds was corrected by running a parallel sugar assay without phenol. The residue was analyzed for starch using enzymatic digestion with a mixture of α-amylase and amyloglucosidase for 20 h, followed by a colorimetric quantification method for glucose hydrolysate with a peroxidase–glucose oxidase/odianisidine reagent. Absorbance was read at 525 nm after the addition of 75% H_2SO_4. A synthetic standard (Landhäusser et al. 2018) was analyzed in parallel with the tissue samples as a quality control check.
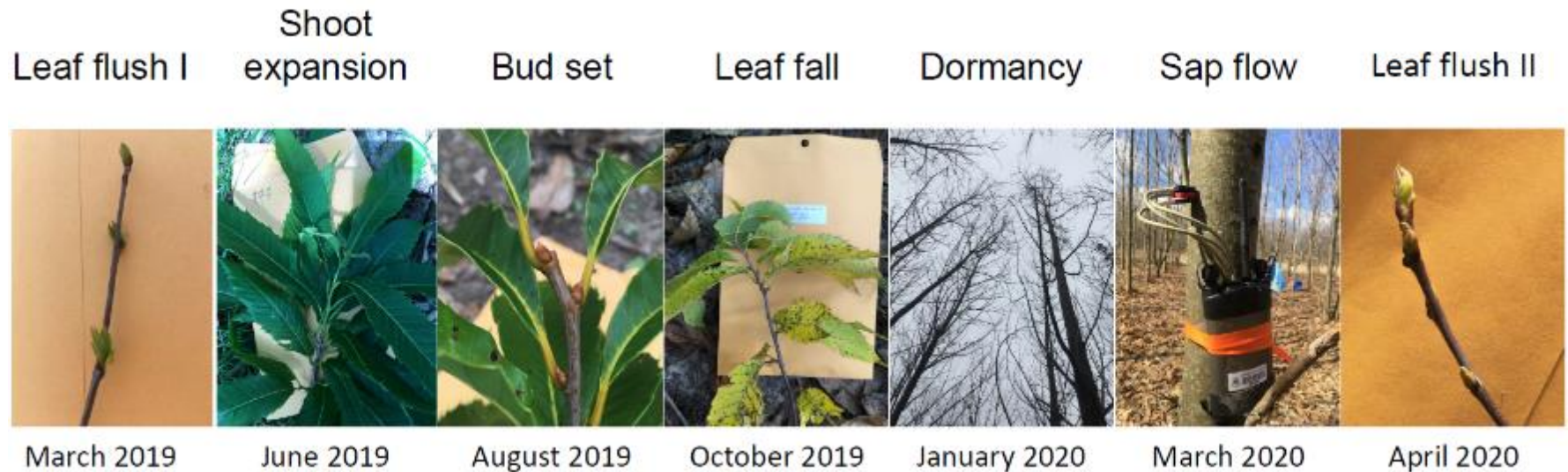
Figure 2.1. Annual sampling scheme aligned with phenological stages where NSCs are mobilized to support distinct life functions in temperate deciduous trees. 1) **leaf out** – when buds were breaking and leaves started to emerge (March 2019); 2) **shoot expansion** – when shoots were actively elongating, and leaf area was at a maximum (June 2019), **bud set** – late growing season, when long shoots had set bud but leaves were still green (August 2019), **leaf fall** – when leaves were yellow and 50% of the canopy remained intact (October 2019), **dormancy** – when trees were apparently inactive and the top layer of soil was frozen (January 2020), sap flow – when sap started to flow and buds were starting to swell (March 2020), and a repeat of leaf out (April 2020).

Seasonal analysis of NSC concentration data

Data analyses were performed in R (R Core Team 2017). We fitted linear mixed models (LMMs) to assess the effects of organ type (e.g. leaf, coarse root), collection period, and an organ × collection on starch, sugar and total NSC concentrations (Table 2.S2; Fig. 2.2). LMMs were fit using the *lme4* package (Bates et al 2015). The models also contained a random effect structure of 'tree ID' to account for potential effects of individual tree status on NSC concentrations. Concentration data were transformed with arcsine × [square root (*y*)] prior to statistical analyses to meet normality assumptions. Additionally, we compared the first and last NSC collections (leaf-out 2019 and leaf-out 2020) to test for trends in NSC dynamics not accounted for in our experiment design. Collection year did not have a significant effect on starch or total NSC concentrations at α = 0.05), although it did significantly affect sugar concentrations (Table 2.S3). With this information in mind, we pooled the data from Leaf-out 2019 and Leaf-out 2020 into a single leaf-out category for further analyses.

Allometry

We used novel biomass data to parameterize an allometric model used to scale NSC concentration data to organ-specific pool sizes. A comprehensive report of our sampling procedure can be found in Appendix **A**. Briefly, eight trees representing the range of DBH and height measurements in our focal experiment were excavated and measured during the summer of 2019 (Fig. A.1). Aboveground biomass measurements were taken following the guidelines of Picard et al. (2012). Trees were felled, and biomass components were separated into foliage, branches (current-year, canopy, scaffold), and bole. Belowground biomass was excavated using a commercial pneumatic system (Air Spade® Series 2000, Concept Engineering Group, Inc., Verona, Pennsylvania), which exploits air space in the soil to expose the root system (Lavigne and Krasowski 2007). We excavated roots to a maximum depth of c. 1 m. Not many roots grew deeper than 1 m; therefore, we did not correct for missing root mass. The excavated root system was partitioned into the stump and root diameter classes of <2mm and coarse roots > 2 mm.

We analyzed biomass data with a generalized linear mixed model (GLMM) with a Gamma distribution and log link using the *lme4* package in R. Our GLMM related the oven-dry biomass of each organ to organ type and the tree DBH and height, and tree ID was included as a random effect (Marginal R^2 = 0.99). More details on the model and its output are provided in Tables 2.S2 and A.2 and in Figures A.1-A.4.

NSC pool size calculation

Since the collection of NSC samples was not done repeatedly on the same trees, tree sizes of sample trees varied among and within the different collection periods (Table 2.S1). While DBH did not have a significant effect on starch concentrations, it did have a positive effect on sugar and total NSC ($P<0.05$; Table 2.S5). However, the magnitude of size-based differences in sugar and NSC concentrations were minor. Averaged across organs, sugar concentrations for trees in the largest 25% of size classes were less than 2% higher than for trees in the smallest 25%, and this difference was less than 3% for NSCs (Fig. 2.S2). Therefore, to reduce the variation associated with tree size, we calculated organ NSC pool sizes based on an average-sized tree derived from our sampled trees for our experiment (Fig. 2.3; median DBH=10.61 cm, whole-tree biomass=43.75 kg $\pm$ 4.56 SE).

Starch, soluble sugar, and total nonstructural carbohydrate pools were estimated by multiplying organ-specific concentration and biomass estimates at each collection time. Whole-tree NSC pools were determined by summing organ-level NSC pools for the shoot expansion and leaf fall collections when all organ types were sampled. The error associated with our NSC concentration and organ biomass estimates was incorporated into the standard error for each of our pool size calculations following standard procedures for propagation of error in multiplication. Leaves were the only strictly annual organ sampled for this study, and the seasonal change in leaf mass was not accounted for in the allometric model. Leaf pools presented here represent maximum estimates because biomass sampling occurred at the time of peak leaf area in 2019.

Analysis of NSC pool size data and ΔNSC

Generalized linear mixed models with a Gamma distribution and log link were built using *lme4* in R to analyze the starch, sugar, and total NSC pool size across organs and phenology stages. Organ, collection, and organ $\times$ collection interaction were used as discrete predictors, tree ID and plot number were included as nested random effects, and tree DBH was included as a covariate (Table 2.S6). Our final analysis was to compute the ΔNSC pools (kg) for each organ. Δstarch, Δsugar, and ΔNSC are the difference between the maximum and minimum starch, sugar, and total NSC pools over the course of one year (Fig. 2.4). Estimates of xylem pools were available for shoot expansion and leaf out collections, and ΔNSC pools were not estimated for leaf pools because of uncertainty around the change in leaf biomass throughout the year. Whole-tree ΔNSC pools were not estimated due to the uncertainty around leaf and xylem pools. To avoid inflating

type I error due to our large sample of estimated pool sizes (74 trees at each collection), we calculated ΔNSC for each organ of our median-sized tree, and compared estimates visually using 95% confidence intervals (CI), which incorporate error from both the maximum and minimum pool sizes stemming from our concentration and allometric models (Fig. 2.4). When comparing two means, $P < 0.05$ when the overlap of 95% CI is less 50% or less, and $P < 0.01$ when there is no overlap between CI (Cumming and Finch 2005).

2.4 Results

Organ-specific and seasonal variation in NSC concentrations

Overall starch, soluble sugar, and total NSC concentrations varied significantly in response to collection period; however, the seasonal variation differed for the different tissue types (tissue × collection interactions $P<0.001$; Table 2.S2; Fig. 2.2.). Apart from the inner bark samples of the stem, similar patterns in starch and total NSC concentrations suggest that starch dynamics drove most of the seasonal variation in total NSCs in this species (Fig. 2.2).

A basic seasonal pattern in total NSC concentrations was conserved across organs, although the prevalence of one NSC fraction over the others changed based on organ type (for example, the starch:sugar ratio). In that pattern, total NSCs tended to dip from winter dormancy until well into the growing season, and then to accumulate in all organs from leaf-out until leaf-fall. Total concentrations were generally high during dormancy due to the peak in soluble sugars. Starch concentrations were very low (<1%) in all organs except coarse and fine roots at dormancy and into the sap flow period (Table 2.S7). From dormancy to sap flow, sugar concentrations in the inner bark and the root collar bark tissue stayed high while a concurrent decrease in sugar and an increase in starch were observed the twigs, branches, and coarse roots (Fig. 2.2). Coarse roots were the only organ with higher mean starch than mean sugar concentrations at sap flow. Total NSCs dropped in all perennial organs between sap flow and leaf out.

While sugar concentrations didn't change substantially between leaf out and leaf fall in any organ, starch dynamics depended on organ during this time. Starch concentrations in the twigs, branches, and inner bark fell slightly until bud set, at which point they rose monotonically until leaf fall. In contrast, coarse root starch concentrations continued falling until leaf fall, when they sharply increased to their annual maximum (mean 15.6% ± 3.5 SD). Leaves had a separate pattern,

wherein starch concentrations rose steadily from leaf out until bud set and then dropped between bud set and leaf fall (Fig. 2.2).

NSC pool sizes

Starch, sugar, and total NSC pools also responded to tissue, collection, and tissue $\times$ collection interactions ($P<0.001$; Table 2.S6; Fig. 2.3). Our estimated starch, sugar, and total NSC pool sizes are the product of seasonally varying concentration data and static biomass estimates. Therefore, pool sizes inevitably followed similar seasonal patterns as the concentration data described above.

Averaged across phenological stages and our experimental trees, coarse roots had the largest estimated starch, sugar and total NSC pools (starch: 1.15 ± 0.86 kg, sugar: 1.01 ± 0.69 kg, total NSCs: 2.16 ± 1.41 kg; uncertainty figures are ± 1 SD). Despite having the largest biomass of any organ (Fig. 2.S1), low total NSC concentrations in the xylem reduced its importance as an NSC pool relative to other organs such as the branches and even the leaves at shoot expansion (Table 2.S8). Average whole-tree starch, sugar, and total NSC pools increased from shoot expansion (SE, June) to leaf fall (LF, October), the periods for which we have comprehensive organ-level data (Fig. 2.5, Table 2.S8).

ΔNSC

Overall, starch and total NSC concentrations showed a greater amplitude in seasonal variation than sugar concentrations which also were more similar among organs (Fig 2.4). The seasonal maxima and minima differed for each organ: aboveground tissues (twigs, branches, and inner bark) and fine roots had the smallest NSC pools at shoot expansion. The root collar had minimum NSC at leaf out, and coarse roots had minimum total NSC pool at bud set. Twigs, inner bark, and fine roots had the highest NSC concentrations at dormancy, while branches, coarse roots, and the root collar peaked at leaf fall. Xylem increased from shoot expansion to leaf out, the two collections during which it was measured.

Coarse roots had the largest Δstarch, sugar, and NSC values; the 74 trees in our experiment saw an average of 0.886 kg (± 0.586, 95% CI) change in the estimated NSC reserves in the coarse roots (Fig. 2.4). While the 95% CI for coarse roots overlapped the CI of branches and xylem by more than 50%, coarse root Δstarch was significantly larger than any other organ (Fig. 2.4). Branches, twigs, and coarse roots had larger mean Δstarch than mean ΔNSC, and the twigs and

inner bark had larger Δsugar than ΔNSC. While branches and inner bark had similar Δsugar and ΔNSC, branches had significantly larger Δstarch than the inner bark (Fig. 2.4).
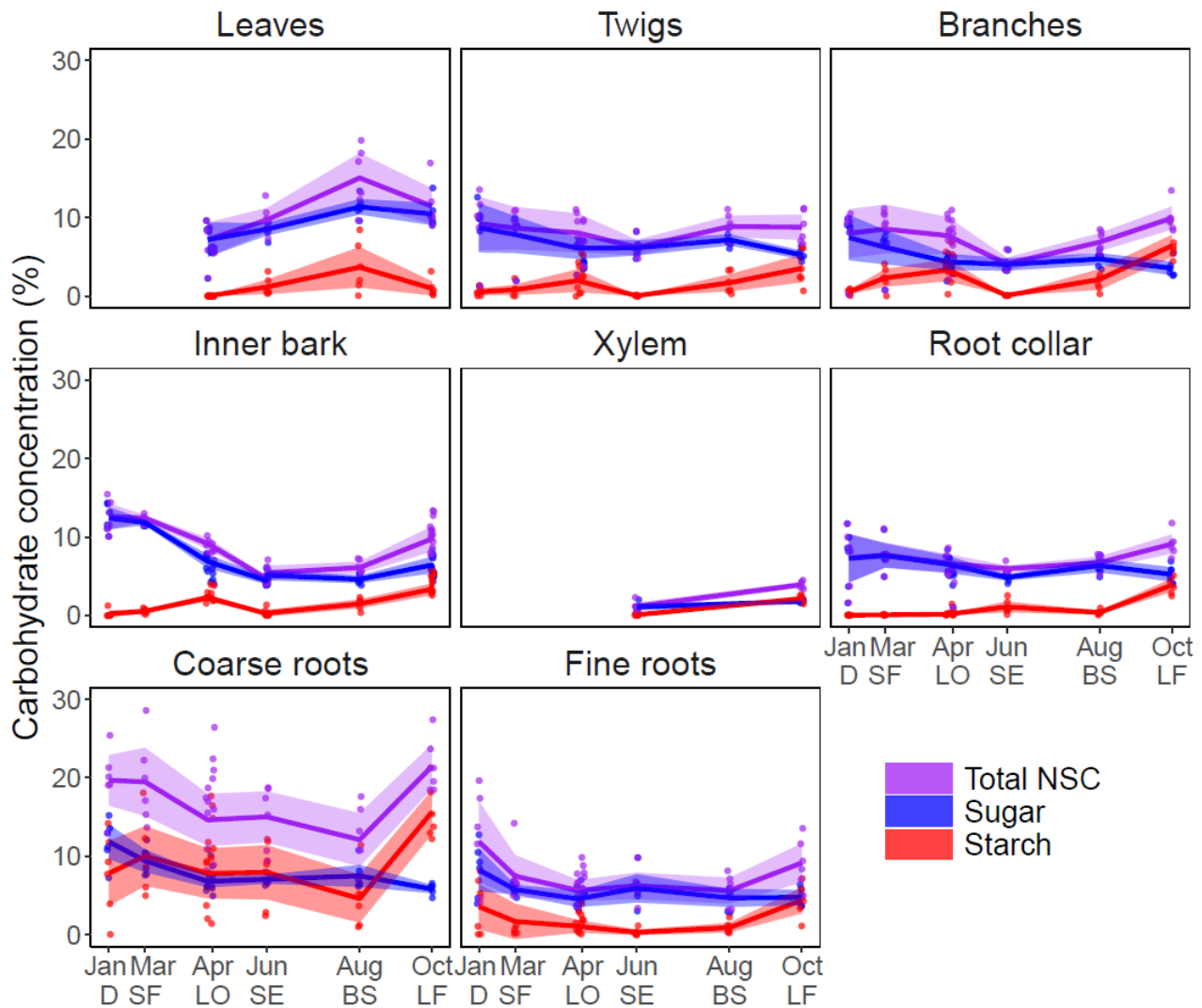
Figure 2.2. Seasonal changes in nonstructural carbohydrate concentrations in American chestnut *(Castanea dentata)*. Shaded bands are 95% confidence intervals around the means at each collection. Tissue samples were collected at dormancy (D, January), sap flow (SF, March), shoot expansion (SE, June), bud set (BS, August), and leaf fall (LF, October).
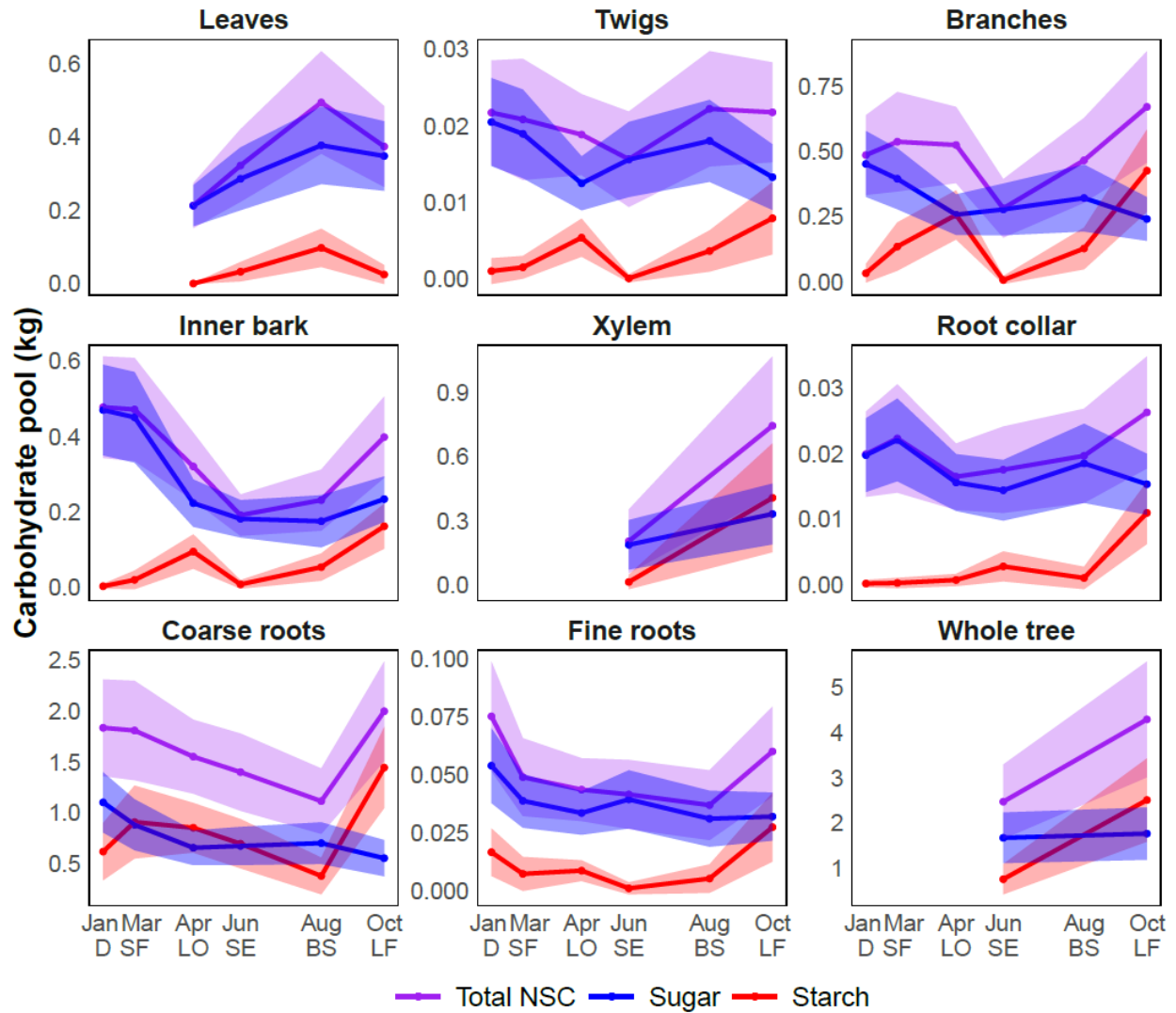
Figure 2.3. Estimated NSC pool sizes for 14-year old American chestnut (*Castanea dentata)* trees. Pool sizes are presented in terms of the median-sized tree for our experiment (median DBH=10.61 cm, corresponding to Ht=10.3 m and whole-tree biomass= 43.75 kg ± 4.7 SE). Shaded bands are 95% confidence intervals around means. Note the different scales used on the y axis.
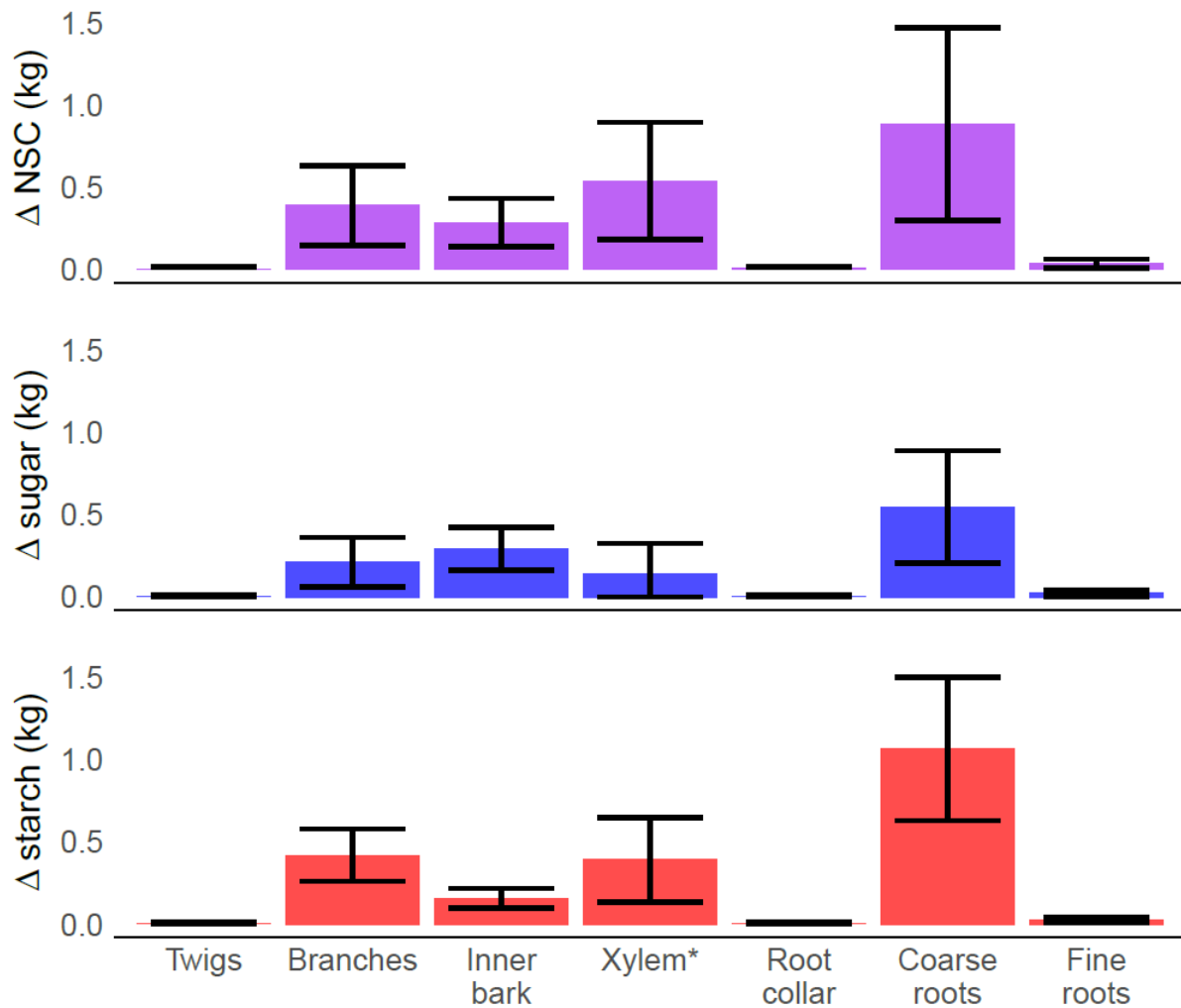
Figure 2.4. Annual fluctuation in NSC, sugar, and starch total pools for our median-sized tree. Estimates of xylem pools were available for shoot expansion and leaf out collections. Error bars are 95% CI around the estimated ΔNSC for the median-sized tree in this study.

2.5   Discussion

We hypothesized that organ-level NSC concentrations (and, therefore, our pool sizes) would peak at leaf fall and be lowest during the early summer, in line with the prevailing theory of seasonal NSC dynamics in temperate trees (Kozlowski 1992). Additionally, given the potential importance of belowground reserves for chestnut, we expected to find high NSC concentrations, and especially starch concentrations, in coarse roots. Our predictions regarding seasonal and inter-organ NSC dynamics were mostly met (Fig. 2.2). However, the relative importance of different organs for NSC storage differed from our hypothesis, which predicted that the mass differences in

aboveground vs. belowground organs (Fig. 2.S1) would mostly drive differences and would therefore make the aboveground portion of the tree (i.e. stem and/or branches) the largest NSC pool. Instead, we found that the coarse roots were the largest and most dynamic NSC pool.

The classic model of NSC dynamics in temperate deciduous trees predicts a steady decrease in NSC concentrations and pools throughout the leafless period (leaf-fall through leaf-out) as source-limited starch reserves are hydrolyzed to sugars, which serve osmotic functions or are utilized for respiration locally or in distal organs since soil temperatures are often not below freezing and root functions are maintained (Kozlowski 1992). We did not observe a marked decline in total NSCs between sap flow and leaf out, except in the fine roots (Fig. 2.2). Twig and branch NSC reserves did not change between sap flow and leaf out, while coarse root reserves decreased, and stem inner bark increased during this time, suggesting that coarse root reserves may have been mobilized to support spring leaf out (Loescher et al. 1990). The dip in branch and twig starch concentrations and pools between leaf out and shoot expansion does however support the model of canopy reserves supporting early-season growth (Klein et al. 2016). This depression could also reflect preferential allocation of NSC towards growth over storage, as starch concentrations approached 0% during shoot expansion.

Some sugar reserves are typically converted back to starch at the end of the dormant season (at leaf-out), presumably because high sugar concentrations are no longer needed to suppress the freezing point of plant tissues (Kozlowski 1992). Starch and sugar concentrations in the study trees had a nearly inverse relationship throughout the year in the twigs, branches, root collar, and coarse root, suggesting starch-sugar interconversion in response to environmental cues as described above. Total NSC concentrations were stabilized during periods of interconversion (i.e. reduced variability in NSC relative to starch or sugar; Table 2.S7, Fig. 2.2).

Following leaf out, NSCs are expected to accumulate throughout the growing season through to leaf-fall Kozlowski 1992). While starch and total NSC reserves in the branches, twigs, and inner bark rebounded after shoot expansion (June), coarse root reserves continued to monotonically decline until the end of the growing season (Fig. 2.3). This pattern could be linked to the physical distance between roots and the photosynthetic machinery, which contributes to a sink hierarchy wherein tissues closer to the canopy receive a prioritized share of the NSCs (Wardlaw 1990; Landhäusser and Lieffers 2012). The later minima in root reserves may also be related to differences in timing of phenological events between aboveground organs, roots, and

root-associated organisms. While we did not track the timing of root growth for this study, a global meta-analysis found that peak root grown occurs an average of 28 ± 12 d after peak shoot growth in temperate biomes (Abramoff and Finzi 2015). Furthermore, as much as 20-40% of a plant's fixed carbon are exported via fine roots as root exudates (Badri and Vivanco 2009). As the structure and composition of mycorrhizal communities may also change seasonally (Dumbrell et al. 2011), fluctuations in sink strength for belowground organs may be tied to a complex set of factors outside the scope of this study.

Our objective for analyzing NSC pool sizes was to compare the magnitude of NSC dynamics across organs to infer the relative importance of different organs for NSC storage. While our expectation was that larger biomass in aboveground organs would outweigh the root system in terms of importance for NSC storage, we found that coarse roots were the largest reservoir of starch, sugar and total NSCs (Table 2.S8, Fig. 2.3). Coarse roots held at least twice as much total NSCs as any other organ. High total NSC storage in coarse roots is partially attributable to its greater accumulation of starch; coarse roots were the only organ in which starch concentrations and pools were consistently similar to or greater than soluble sugars (Fig. 2.5). Starch pools in the coarse roots also responded most dramatically to seasonality, as indicated by our Δ NSC calculations (Fig. 2.4).

Differences in ΔNSC in canopy vs. root reserves, particularly at spring leaf out, may indicate that branches play a more minor role in NSC storage and remobilization in our 14-year-old chestnut trees than has been found in other studies using older, larger trees (Klein et al. 2016; Furze et al. 2019). For example, Furze et al. (2019) found maximum NSC pools and changes of NSCs in the branches, and minimal changes in NSC pools in the coarse roots. This discrepancy in the relative importance of organ specific NSC pools could be due to several factors. First, the trees measured by Furze et al. were 61-111 years old and weighed an estimated 300-1500 kg, making them considerably older and larger than our 14-year-old chestnuts (median whole-tree biomass=43.75 kg $\pm$ 4.56 SE). The proportion of total biomass in branches decreased with size in the eight chestnuts excavated for this study (Appendix A, Fig. A.4). Furthermore, root to shoot ratios (RSR, belowground biomass/aboveground biomass) typically decrease with tree size (Poorter et al. 2012), and we found a negative relationship between DBH and RSR in our excavated trees (Appendix A; Figs. A.3-A.4; P=0.09). A smaller RSR would mean that larger trees must have higher root NSC concentrations to arrive at the same relative pool size as smaller (or younger)

trees, although Furze et al. (2019) do not report concentration data to compare with. Another study using juvenile trees found higher root sugar and starch concentrations in American chestnut than northern red oak (Belair et al. 2018). The two species had similar NSC pools when expressed on a content basis because northern red oak allocated more biomass to roots (34.7±1.5% RSR for chestnut, 43.8±1.0% for oak; Belair et al. 2018). Our mean chestnut RSR for 14-year-old chestnut trees was not substantially different (32.45±2.9%; Appendix A).

Large and dynamic belowground pools in chestnut may reflect preferential allocation of carbohydrates to the roots as a secure storage site. Belowground NSC reserves are important resources for resprouting after disturbances such as surface fires (Landhäusser et al. 2012; Clarke et al. 2013). Additionally, large and accessible NSC reserves in the coarse roots may have prevented the total extinction of chestnut in forests of the eastern USA by fueling resprouting despite the endemic chestnut blight (Paillet 2002). Belowground NSC reserves could also play a role in warding off other pathogens such as root rot (ink disease). The root rot fungus *Phytophthora cinnamomi* has affected American chestnut populations in the Piedmont region of the southeastern United States since the 1850's, and presents an added barrier to restoration (Clark et al. 2012; Wang et al. 2013). *P. cinnamomi* is borne on soil water and causes necrosis of the large roots and root collar area, usually killing the plant (Wang et al. 2013). Given the importance of root reserves for chestnut, achieving resistance to this secondary disease of chestnut will be vital to give the species a fighting chance at restoration. To our knowledge, the association between NSC reserves and resistance to *P. cinnamomi* has not been tested. This relationship deserves further study because NSC reserves fund chemical defenses and the NSC status of a plant affects its reaction to damaging agents such as fungal infection and herbivory (Goodsman et al. 2013; Najar et al. 2014).

We suggest that promoting root development in chestnut may be a key to realizing productive and resilient restoration plantings. Vigorous root development can be encouraged at many points during the production of planting stock, starting with selecting genotypes or rootstocks with a large RSR. In the nursery, careful root undercutting of bareroot seedlings or investing in a containerized system may be used to stimulate root development an improve outplanting success (Davis and Jacobs 2005; Landis 2008). Finally, interplanting chestnut with other species that promote belowground allocation of structural biomass and NSCs is an unexplored but promising method of modifying chestnut allometry (del Río et al. 2019).

2.6   Conclusions

We established a detailed model of seasonal NSC dynamics at the organ and whole-tree level in American chestnut, a species of high restoration interest (Jacobs 2007). Seasonal dynamics in NSC concentrations generally conformed with models of other temperate deciduous species. Patterns of fluctuation varied by organ type, probably alluding to the unique metabolic roles for different organs within the tree. Scaling from concentrations to pool sizes allowed us to interpret seasonal NSC dynamics of each organ as part of the entire tree. Building a custom allometric model with data from trees harvested from our study site reduced the uncertainty (vs. using data from trees grown from different genetic stock, on different soil types, etc.) around these calculations.

Coarse roots were a remarkably important storage site for starch, sugar, and total NSCs. The size and dynamic nature of the coarse root pool suggests that concentrating sampling efforts on coarse roots could serve as a useful indicator of whole-tree NSC status in future studies. Additionally, the tendency to store NSCs belowground, and a demonstrated ability to mobilize root NSCs to fuel growth and metabolism, supports the idea that chestnut could thrive under disturbance-based management (Wang et al. 2013; Belair et al. 2018). Furthermore, the target seedling for chestnut should emphasize robust root development.

2.7   References

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2.8   Supplementary Materials for Chapter 2

Figure 2.S1. Organ-specific and whole-tree biomass calculated for the median-sized tree in our experimental plantation (n=74; median DBH=10.61 cm, whole-tree biomass=43.75 kg $\pm$ 4.56 SE). Error bars are 95% confidence intervals for biomass estimates generated from the allometric model. Biomass data was collected at shoot expansion, the time of maximum leaf area.
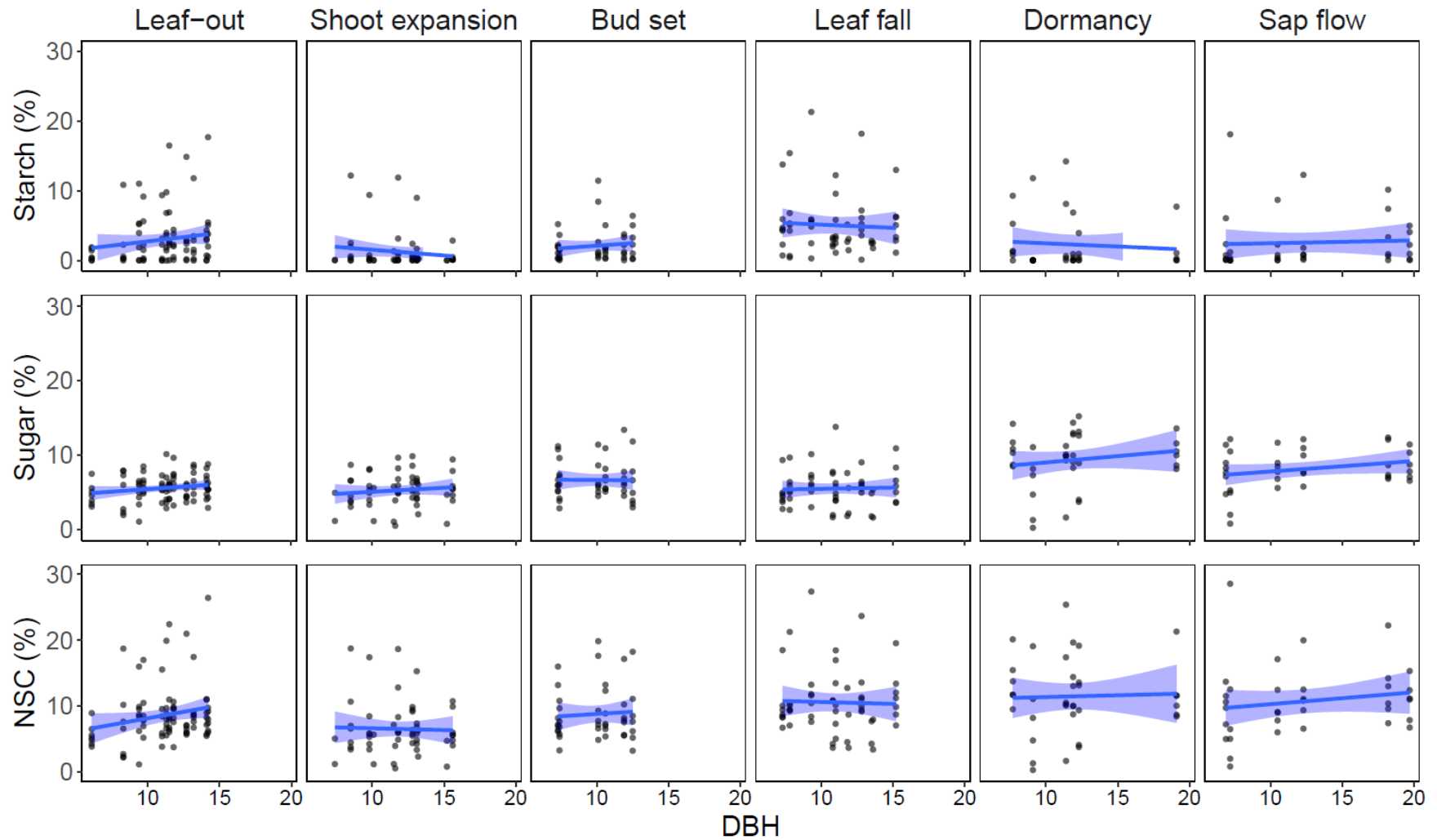
Figure 2.S2. Relationship between tree size (DBH) and starch, sugar, and total NSC concentrations. DBH did not affect starch concentrations, but it did have a significant effect on sugar and total NSC at $\alpha=0.05$.

Table 2.S1. DBH and estimated total biomass characteristics of the 42 trees sampled for NSC concentrations.

Collection	n	mean DBH (±SD)	mean whole-tree biomass (±SD)
1 – leaf out	6	10.87 ± 2.83	89.21 ± 86.60
2 – shoot expansion	6	10.93 ± 2.53	91.80 ± 50.20
3 – bud set	6	9.97 ± 2.20	48.58 ± 18.97
4 – leaf fall	6	10.57 ± 3.05	75.15 ± 60.75
5 – dormancy	6	11.92 ± 3.94	115.48 ± 145.49
6 – sap flow	6	12.47 ± 5.44	179.00 ± 220.01
7 – leaf out II	6	11.35 ± 2.16	87.60 ± 63.10

Table 2.S2. NSC concentration models for starch, sugar, or total NSC (starch + sugar) concentrations. Our analyses are based on 305 starch, sugar, and total NSC samples from 54 trees.

NSC fraction	Parameter	Chisq	Df	*P* value	Pseudo R^2
Starch	Whole model	426.3	41	< 0.001	0.723
	Tissue	245.361	7	< 0.001	—
	Collection	66.899	5	< 0.001	—
	Tissue × collection	165.714	29	< 0.001	—
	Random: tree	141.857	1	< 0.001	—
Sugar	Whole model	310.42	41	< 0.001	0.06
	Tissue	39.083	7	< 0.001	—
	Collection	17.516	5	0.002	—
	Tissue × collection	90.826	29	< 0.001	—
	Random: tree	299.761	1	< 0.001	—
Total NSC	Whole model	355.74	41	< 0.001	0.651
	Tissue	126.202	7	< 0.001	—
	Collection	16.352	5	< 0.001	—
	Tissue × collection	88.109	29	0.001	—
	Random: tree	359.117	1	< 0.001	—

Table 2.S3. Results of likelihood ratio tests on the effect of collection year (2019 or 2020) on starch, sugar, or total NSC concentrations at leaf-out. For each carbohydrate fraction, both models included tissue type as a fixed effect and individual tree was included as a random effect; models differed in their inclusion of collection year as a fixed effect.

		Starch		Sugar		Total NSC	
Parameter	**Df**	**Chisq**	*P* **value**	**Chisq**	*P* **value**	**Chisq**	*P* **value**
Collection year	7	7.54	0.375	20.804	**0.004**	11.953	0.102

Table 2.S4. Allometric model fit from excavation data (8 trees). We used a Gamma distribution and log link. The model converged with a maximum gradient of 0.024.
Model statement: biomass ~ DBH × tissue + Ht + (1|tree).

Parameter	**Chisq**	**Df**	*P* **value**	**Pseudo R^2**
Whole model	5.5847	18	< 0.001	0.989
DBH	36.945	1	< 0.001	—
Height	96.928	1	< 0.001	—
Tissue	352.084	8	< 0.001	—
Tissue × DBH	11.758	8	< 0.001	—
Random: tree	32.133	1	< 0.001	—

Table 2.S5. Results of likelihood ratio tests on the effect of DBH on starch, sugar, or total NSC concentrations. For each carbohydrate fraction, both models included tissue type × collection as fixed effects and individual tree was included as a random effect; models differed in their inclusion of DBH as a fixed effect.

		Starch		Sugar		Total NSC	
Parameter	**Df**	**Chisq**	**_P_ value**	**Chisq**	**_P_ value**	**Chisq**	**_P_ value**
DBH	1	1.571	0.21	8.177	**0.004**	**8.22**	**0.004**

Table 2.S6. Linear mixed-effects models for starch, sugar, and NSC pools.

NSC fraction	**Parameter**	**Chisq**	**Df**	**_P_ value**	**Pseudo R^2**
Starch	Whole model	20313	42	< 0.001	0.997
	Tissue	36.2249.42	7	< 0.001	—
	Collection	73045.17	5	< 0.001	—
	DBH	496.35	1	< 0.001	—
	Tissue×collection	341226.86	29	< 0.001	—
	Random: Block/tree	716.75	1	< 0.001	—
Sugar	Whole model	32714	42	< 0.001	0.992
	Tissue	87088.80	7	< 0.001	—
	Collection	1868.71	5	< 0.001	—
	DBH	496.31	1	< 0.001	—
	Tissue×collection	5411.83	29	< 0.001	—
	Random: Block/tree	715.18	1	< 0.001	—
Total NSC	Whole model	16943	42	< 0.001	0.994
	Tissue	109621.52	7	< 0.001	—
	Collection	2514.18	5	< 0.001	—
	DBH	496.32	1	< 0.001	—
	Tissue×collection	8778.38	29	< 0.001	—
	Random: Block/tree	490.96	1	< 0.001	—

Table 2.S7. NSC concentrations across tissues and collection times.

Collection	Tissue	Starch mean	sd	Sugar mean	sd	NSC mean	sd	Starch:sugar ratio
Dormancy	Branches	0.005	0.003	0.075	0.036	0.080	0.039	0.103
Dormancy	Coarse roots	0.078	0.052	0.118	0.028	0.197	0.040	0.764
Dormancy	Fine roots	0.036	0.036	0.083	0.034	0.119	0.064	0.364
Dormancy	Inner bark	0.002	0.005	0.124	0.018	0.126	0.021	0.015
Dormancy	Root collar	0.000	0.000	0.073	0.039	0.073	0.039	0.010
Dormancy	Twigs	0.005	0.005	0.087	0.039	0.092	0.042	0.055
Sap flow	Branches	0.023	0.014	0.062	0.027	0.085	0.039	0.326
Sap flow	Coarse roots	0.100	0.047	0.094	0.018	0.195	0.054	1.080
Sap flow	Fine roots	0.017	0.028	0.058	0.008	0.075	0.034	0.269
Sap flow	Inner bark	0.005	0.003	0.118	0.004	0.124	0.005	0.046
Sap flow	Root collar	0.001	0.000	0.076	0.019	0.077	0.020	0.010
Sap flow	Twigs	0.008	0.008	0.078	0.029	0.087	0.034	0.093
Leaf out	Branches	0.041	0.020	0.038	0.011	0.079	0.025	1.087
Leaf out	Coarse roots	0.099	0.053	0.070	0.015	0.169	0.055	1.468
Leaf out	Fine roots	0.015	0.012	0.050	0.012	0.066	0.019	0.306
Leaf out	Leaves	0.000	0.000	0.065	0.020	0.066	0.020	0.002
Leaf out	Inner bark	0.025	0.008	0.059	0.013	0.085	0.011	0.468
Leaf out	Root collar	0.003	0.004	0.055	0.019	0.058	0.019	0.061
Leaf out	Twigs	0.026	0.019	0.051	0.016	0.077	0.026	0.545
Shoot expansion	Branches	0.001	0.000	0.040	0.010	0.041	0.010	0.023
Shoot expansion	Coarse roots	0.080	0.043	0.071	0.007	0.150	0.041	1.156
Shoot expansion	Fine roots	0.003	0.004	0.059	0.022	0.062	0.021	0.070
Shoot expansion	Leaves	0.012	0.011	0.085	0.010	0.097	0.019	0.131
Shoot expansion	Inner bark	0.003	0.004	0.048	0.008	0.050	0.009	0.054
Shoot expansion	Root collar	0.011	0.009	0.049	0.005	0.059	0.005	0.242
Shoot expansion	Twigs	0.001	0.001	0.061	0.012	0.062	0.012	0.008
Shoot expansion	Xylem	0.001	0.001	0.011	0.005	0.012	0.006	0.068
Bud set	Branches	0.021	0.017	0.047	0.009	0.069	0.014	0.507
Bud set	Coarse roots	0.046	0.038	0.075	0.017	0.121	0.042	0.638
Bud set	Fine roots	0.009	0.008	0.047	0.014	0.056	0.021	0.180
Bud set	Leaves	0.037	0.032	0.114	0.012	0.151	0.039	0.313
Bud set	Inner bark	0.015	0.007	0.046	0.006	0.061	0.010	0.329
Bud set	Root collar	0.004	0.003	0.064	0.011	0.067	0.010	0.065
Bud set	Twigs	0.017	0.014	0.072	0.009	0.089	0.017	0.236
Leaf fall	Branches	0.064	0.018	0.035	0.011	0.100	0.018	1.943
Leaf fall	Coarse roots	0.156	0.035	0.058	0.006	0.215	0.035	2.711
Leaf fall	Fine roots	0.044	0.021	0.048	0.012	0.092	0.031	0.906
Leaf fall	Leaves	0.010	0.011	0.105	0.018	0.114	0.029	0.084
Leaf fall	Inner bark	0.043	0.013	0.062	0.014	0.105	0.019	0.730
Leaf fall	Root collar	0.039	0.011	0.052	0.011	0.091	0.015	0.777
Leaf fall	Twigs	0.035	0.022	0.052	0.007	0.088	0.020	0.702
Leaf fall	Xylem	0.021	0.005	0.018	0.002	0.039	0.004	1.203

Table 2.S8. NSC pools (kg; ± SE) for a media-sized tree across tissues and collection times. (median DBH=10.61 cm, corresponding to Ht=10.3 m and whole-tree biomass= 43.75 kg ± 4.7 SE). Standard error around means incorporate error from both allometric and NSC concentration models.

	Dormancy	Sap flow	Leaf out	Shoot expansion	Bud set	Leaf fall
Leaves						
Starch	0 (0.001)	0.032 (0.014)	0.097 (0.027)	0.025 (0.013)	NA	NA
Sugar	0.213 (0.029)	0.286 (0.044)	0.377 (0.054)	0.348 (0.048)	NA	NA
Total NSC	0.213 (0.031)	0.322 (0.051)	0.495 (0.071)	0.374 (0.056)	NA	NA
Twigs						
Starch	0.005 (0.001)	0 (0)	0.004 (0.001)	0.008 (0.002)	0.001 (0.001)	0.002 (0.001)
Sugar	0.012 (0.002)	0.016 (0.002)	0.018 (0.003)	0.013 (0.002)	0.02 (0.003)	0.019 (0.003)
Total NSC	0.019 (0.003)	0.016 (0.003)	0.022 (0.004)	0.022 (0.003)	0.022 (0.003)	0.021 (0.004)
Branches						
Starch	0.257 (0.048)	0.006 (0.008)	0.128 (0.041)	0.427 (0.081)	0.032 (0.018)	0.135 (0.047)
Sugar	0.258 (0.04)	0.278 (0.051)	0.322 (0.066)	0.241 (0.043)	0.453 (0.065)	0.396 (0.06)
Total NSC	0.526(0.257)	0.282 (0.006)	0.467 (0.128)	0.673 (0.427)	0.488 (0.032)	0.539 (0.135)
Inner bark						
Starch	0.094 (0.023)	0.007 (0.006)	0.053 (0.018)	0.162 (0.03)	0.002 (0.003)	0.019 (0.013)
Sugar	0.223 (0.032)	0.181 (0.025)	0.175 (0.035)	0.234 (0.031)	0.47 (0.061)	0.451 (0.061)
Total NSC	0.32 (0.046)	0.191 (0.028)	0.231 (0.041)	0.398 (0.055)	0.478 (0.069)	0.472 (0.07)
Xylem						
Starch	NA	0.013 (0.017)	NA	0.406 (0.13)	NA	NA
Sugar	NA	0.186 (0.059)	NA	0.331 (0.073)	NA	NA
Total NSC	NA	0.203 (0.076)	NA	0.743 (0.166)	NA	NA
Root collar						
Starch	0.001 (0)	0.003 (0.001)	0.001 (0.001)	0.011 (0.002)	0 (0)	0 (0)
Sugar	0.016 (0.002)	0.014 (0.002)	0.019 (0.003)	0.015 (0.002)	0.02 (0.003)	0.022 (0.003)
Total NSC	0.016 (0.003)	0.018 (0.003)	0.02 (0.004)	0.026 (0.004)	0.02 (0.003)	0.022 (0.004)

Table 2.S8 continued

	Dormancy	Sap flow	Leaf out	Shoot expansion	Bud set	Leaf fall
Coarse roots						
Starch	0.85 (0.125)	0.693 (0.124)	0.375 (0.092)	1.443 (0.203)	0.615 (0.144)	0.906 (0.183)
Sugar	0.653 (0.087)	0.671 (0.096)	0.699 (0.105)	0.55 (0.091)	1.1 (0.151)	0.879 (0.128)
Total NSC	1.55 (0.186)	1.398 (0.194)	1.112 (0.164)	1.997 (0.25)	1.835 (0.243)	1.808 (0.25)
Fine roots						
Starch	0.009 (0.002)	0.001 (0.001)	0.005 (0.003)	0.028 (0.008)	0.017 (0.005)	0.007 (0.004)
Sugar	0.034 (0.005)	0.04 (0.006)	0.031 (0.006)	0.032 (0.005)	0.054 (0.008)	0.039 (0.006)
Total NSC	0.044 (0.007)	0.042 (0.008)	0.037 (0.008)	0.06 (0.01)	0.075 (0.012)	0.049 (0.009)

CHAPTER 3. INTER- AND INTRA-SPECIFIC COMPETITION MODERATES BELOWGROUND NONSTRUCTURAL CARBON STORAGE IN AMERICAN CHESTNUT *(CASTANEA DENTATA)*

3.1 Abstract

Competition affects the productivity and survival of individual plants, and the cumulative balance of competitive interactions between individuals determines species abundance and ecosystem structure. While nonstructural carbohydrates (NSC) storage is critical for temperate deciduous trees, how competition affects trees' internal balance of growth vs NSC accumulation is not well understood. We studied the effect of intra- and inter-specific competition on American chestnut (*Castanea dentata* (Marsh.) Borkh.) biomass accumulation (DBH) and NSC reserves using mixtures of tree species with strong functional differences. Coarse root total NSC pools, the largest and most dynamic pools in American chestnut, responded to density and species composition through their combined effects on NSC concentrations and tree biomass. There was a strong density × composition interaction: while NSC pools were largest in plots with the lowest planting density where chestnut was planted with black cherry (*Prunus serotina*) or in a three-species mixture with black cherry and northern red oak (*Quercus rubra*), chestnut NSC pools were reduced when planted in monoculture or in a two-species mixture with northern red oak. Thus, neighbor identity was more important than species richness for NSC pools in our system. Inter- vs. intra-specific interactions had no effect on biomass accumulation or NSC pools at our mid-density plots, suggesting need for further study.

3.2 Introduction

Inter- and intra-specific interactions affect the productivity and survival of individual plants, and the cumulative balance of these interactions determines species abundance and ecosystem structure (Carrick and Forsythe 2020, and references therein). Forest productivity (biomass accumulation) is thought to be positively related to increased diversity (Zhang et al. 2012; Liang et al. 2016), presumably because competition between individuals growing in mixed-species stands is less intense than competition among individuals growing among conspecifics (Forrester and Bauhus 2016). However, while productivity is often associated with biomass accumulation it

often does not separate between the structural and reserve mass as part of a plant's total fixed carbon. Non-structural carbohydrates (NSCs) are critical for stable plant functioning, but it is unclear how trees balance growth (increased resource capture) and storage (resilience), and what factors control these processes (Hoch et al. 2003; Wiley and Helliker 2012; Sala et al. 2012; Palacio et al. 2014). Inter- and intra-specific interactions among individual plants may play a role in this balance, as they are known to influence patterns of resource acquisition and mass allocation in individuals through competition and/or facilitation (Zapater et al. 2011; Poorter et al. 2012; Forrester and Bauhus 2016).

Nonstructural carbohydrates (NSCs; soluble sugars and starch) can accumulate as reserves in plant tissues when photosynthesis outpaces immediate demands for carbon (Kozlowski 1992). Stored NSCs are therefore an indirect but useful measure of a plant's ability to respond to and recover from damaging agents and environmental change (Chapin et al. 1990; Canham et al. 1999; Landhäusser and Lieffers 2002; Dietze et al. 2014; Deslauriers et al. 2015). While NSCs readily support routine functions like respiration, the biosynthesis of secondary compounds, and growth processes, stored NSCs (often stored as starch) also provide support for repair and regrowth from injuries after disturbance and for osmotic adjustments for cold and drought tolerance (Payton and Brasch 1978; Chapin et al. 1990; Kozlowski 1992). There has been a substantial effort to relate diversity to the resistance, the ability to function normally during a disturbance, or resilience, the ability to recover normal function after the disturbance has occurred (Lake 2013), of forests to biotic and abiotic stress (Ruiz-Benito et al. 2017; Grossman et al. 2018). NSC play a central, but understudied, role in these hypothesized relationships by fueling processes linked to resistance (e.g. increased sugar concentrations to avoid damage from freezing) and to resilience (e.g. resprouting after topkill by fire) (Kozlowski 1992; Piper and Paula 2020).

Neighboring plants compete for a common pool of above- and below-ground resources by adjusting biomass allocation and organ morphology (Poorter et al. 2012). Plants generally direct their growth towards organs that capture the most limiting resource; for example, plants facing nitrogen or water limitation increase biomass allocation to roots (Kozlowski et al. 1991). Competition intensity increases with forest stand density, although it can be ameliorated through complementary species mixtures (Forrester and Bauhus 2016). The responses described above assume that species directly compete for these resources at the same time and space. However, depending on the life history traits of species in a mixture, individual plants and species can vary

in their requirements and quantities for resources at specific times, and they meet their needs by adopting different resource acquisition strategies (O'Brien et al. 2017). For instance, competition for light limits photosynthesis rates in suppressed individuals (Kozlowski et al. 1991). But a structural partitioning of the canopy results in increased light interception and a greater source of photosynthate at the stand level (Pretzsch 2014; Tatsumi 2020). In another study, European beech (*Fagus sylvatica* L.) deepened and intensified its root system in mixture with Norway spruce (*Picea abies* L.) to better exploit soil water resources (Schume et al. 2004), illustrating plastic biomass allocation in response to different competition regimes (del Río et al. 2019). Complementary species mixtures exploit species-specific differences to reduce competition for resources and promote overyielding, whereby mixed stands have higher structural biomass production than monocultures (Tilman et al. 2001; Forrester and Bauhus 2016).

While there is a substantial body of literature on the effect of species mixtures on the productivity and allocation of structural mass, research explicitly linking inter- and intra-specific interactions to nonstructural carbohydrate (NSC) dynamics, particularly for large trees, is scarce. Previous studies of these processes have mostly focused on herbaceous plants (Lacey et al. 1994; García-Cervigón et al. 2013) or tree seedlings in pot experiments (Guo et al. 2016, 2020; Wu et al. 2020). Guo et al. (2020) found that intra-specific competition modulated NSC storage in *Cunninghamia lanceolata* seedlings, where it increased root starch concentrations under drought, while starch concentrations were dramatically lowered when faced with added competition (Guo et al. 2020). Wu et al. (2020) found a positive relationship between competition (vs. single-grown seedlings) and starch concentrations in two *Larix* species, but there was no statistical difference in NSC dynamics between the seedlings grown in inter- vs. intra-specific competition; however both species had similar life history strategies and nearly indistinguishable morphology. We hypothesize that studying mixtures of species with strong functional differences (*sensu* Díaz and Cabido 2001) might be a more appropriate approach for evaluating the potential effects of interactions on productivity and NSC reserve storage.

We address this knowledge gap by comparing belowground NSC reserves in American chestnut (*Castanea dentata* (Marsh.) Borkh.) growing under different inter- and intra-specific competition regimes. Earlier work in this species has shown that chestnut has a propensity to store starch preferentially in its root system (Chapter 2), which is most likely an adaptation to its ability to vigorously resprout after above-ground disturbance (Wang et al. 2013; Belair et al. 2018). In

this species, we explored the relationships between the type of interaction (inter- or intra-specific), tree productivity, and NSC dynamics in a plantation experiment that manipulated planting density and species composition. We hypothesized that NSC concentrations, particularly starch, would increase with competition intensity, because of increased stress, resulting in reduced allocation to growth and a preferential allocation to reserves as suggested by Wiley and Helliker (2012). We further hypothesize that this relationship will be modulated by the species composition (chestnut monoculture or mixed species) of the stand due to expected niche differentiation between species in our mixed stands (see Methods for species descriptions). Conversely, we anticipated that NSC pools may be larger in plots with higher species richness and lower planting density as they are associated with greater productivity due to mild competition effects (Forrester and Bauhus 2016). We expected strong interaction effects between species composition and planting density for models predicting NSC concentrations and pool sizes in accordance with the stress gradient hypothesis, which suggests that inter-specific niche dimensionality becomes more important in resource-limited environments (Bertness and Callaway 1994; Harpole et al. 2016).

3.3   Methods

Site Description

The study was carried out in a mature plantation experiment located at Martell Forest, a research site owned and operated by Purdue University in West Lafayette, Indiana (40º26'42"N, 87º01'47"W). The experimental plantation is situated on a moderately well drained, productive site formerly used as an agricultural field. The main soil type is comprised of Rockfield silt loam (USDA NRCS 2017). Mean annual temperature in West Lafayette, IN is 11.4 C and mean annual precipitation is 996 mm (National Climatic Data Center 2019).

American chestnut (*Castanea dentata*), northern red oak (*Quercus rubra*) and black cherry (*Prunus serotina*) were planted as monocultures or mixtures at three different densities in three replicate blocks (Fig. 3.S1). The four different species mixtures are: 100% American chestnut (C), 50% cherry and 50% chestnut (BC), 50% oak and 50% chestnut (NC), one-third of each species (CNB). The spacings used in this study are 1 m (10000 stems ha^{-1}), 2 m (2500 stems ha^{-1}), or 3 m (1111 stems ha^{-1}) between trees. All species × spacing treatments consist of 30 trees (6 trees by 5 trees square). A buffer of a single tree row around the perimeter of each treatment was planted to minimize potential edge effects. For plots with two or more species, each species was planted

alternately in each row in a checkerboard pattern. The experiment was planted in the spring of 2007 and occupies roughly 2.4 ha. The plots have since reached canopy closure. Diameter at breast height (DBH) has been measured annually since 2017, and tree heights were measured in 2020 using a Vertex hypsometer (Haglöf, Avesta, Sweden). We excluded plots with > 20% mortality (6/36 plots) because density is a treatment in our study (see Table 3.S1 for plot characteristics). Therefore, we sampled chestnut trees in 30 plots (Table 3.S1). More detail on the experimental design and original study can be found in Gauthier et al. (2013).

Study species

The three temperate hardwood species included in our experiment have varied patterns of resource acquisition and use. Black cherry grows rapidly in the seedling, sapling and pole stages, reacts poorly to shading and competition and has a shallow root system (Marquis 1990). Northern red oak has intermediate shade tolerance and a plastic growth form in response to light and space: forest-grown trees are characteristically tall and straight while open-grown trees have short boles and spreading crowns (Sander 1990). Juvenile red oak have a single deep taproot that eventually becomes overshadowed by 5-10 large, deep woody lateral roots as the tree matures (Lyford 1980). American chestnut is highly competitive and fast-growing during early growth (Wang et al. 2013). Belair et al. (2018) found that juvenile chestnut (5-7 years) had a lower root mass fraction than northern red oak. Information on chestnut's rooting habit is conflicting and dated: Buttrick and Holmes (1913) described the system as shallow, while Zon (1904) and Ashe (1911) wrote that chestnut had a deep taproot and wide-spreading woody laterals similar to oak.

Tissue sample collection

In an earlier seasonal study (Chapter 2), we found that starch and total NSC pools were largest and most dynamic in the coarse roots. Coarse root starch and total NSC pools reached a minimum during shoot expansion and a maximum during leaf fall in roots; we therefore sampled belowground tissues at these two phenological stages for this study with the expectation that the most dramatic differences in NSC reserves would be found in root tissues. Samples were collected between 2019-2020, commencing with leaf fall (Oct. 2019), then shoot expansion (June 2020). Tissue samples from the root collar, coarse roots (2-15 mm diameter), and fine roots (<2 mm) were collected from two trees per plot. We obtained root samples by digging half a meter from the base of the tree and following roots back to the base. Tissue samples were promptly frozen at -4 ^{o} C until further processing.

NSC concentrations have been reported to vary with respect to root diameter (Wargo 1976; Kozlowski 1992; Barbaroux et al. 2003). We therefore tested for any differences in the diameter of our coarse root samples by fitting a generalized linear mixed-effect model with a Gamma distribution and a log link, collection, species composition, and density as fixed effects, and block as a random effect. Block and density were significant predictors of coarse root sample diameter (Table 3.S2). However, differences between competition treatments were minimal (Fig. 3.S3). Furthermore, coarse root diameter did not significantly affect starch, sugar, or total NSC concentrations at the range of diameters included in this study ($P>0.51$; Table 3.S3).

Quantification of soluble sugars and starch

Tissue samples were ground in a ball mill (Retsch MM-400, Haan, Germany) and shipped to the University of Alberta for analysis. Water-soluble sugars were extracted from the tissue sample and starch content was determined in the residue, following the procedures and protocols described in detail in Landhäusser et al. (2018). Briefly, 30 mg of dried ground tissue sample was extracted with 80% hot ethanol. The ethanol extract was analyzed for soluble sugar concentration using phenol–sulfuric acid against a GFG (1:1:1, glucose, fructose, galactose) standard, with 2% phenol and absorbance was read at 490 nm. Interference was corrected by running a parallel sugar assay without phenol. The sample residue was analyzed for starch concentration by enzymatic digestion with a mixture of α-amylase and amyloglucosidase for 20 h, followed by the colorimetric measurement of glucose hydrolysate with a peroxidase–glucose oxidase/odianisidine reagent. Absorbance was read at 525 nm after the addition of 75% H_2SO_4. A synthetic standard sample (Landhäusser et al. 2018) was analyzed in parallel with the tissue samples as a quality check.

Statistical analyses

A visual analysis of the coarse root, fine root, and root collar NSC concentration data (Fig. 3.S2) confirmed that coarse roots contained the highest concentrations and the greatest seasonal fluctuation in total NSC. Additionally, fluctuation in total NSC was driven primarily by starch concentrations (Fig. 3.S2). These findings, which support our data from Chapter 2, and the fact that the coarse roots are the largest component of the root system by mass, indicate that the effects of competition on NSC reserves are best captured by focusing on total NSC concentrations and pools in coarse roots. Our subsequent analyses were therefore restricted to total NSC concentrations and pools in coarse roots.

All statistical analyses were performed in R (R Core Team 2017). We fitted generalized linear mixed models (GLMMs) to assess the effects of competition on diameter at breast height (DBH; Table 3.S4), maximum NSC concentration (i.e. NSC at leaf fall, Table 3.S5), and minimum NSC concentration (NSC at shoot expansion, Table 3.S5). All models were fit in the *lme4* package (Bates et al. 2015) with a Gamma distribution and a log link function. Planting density, species composition, and a density × composition interaction were included as fixed effects, and block as a random effect. Tukey contrasts were implemented in the *emmeans* package (Lenth 2020) to evaluate differences between pairs of treatment means (α=0.05, Fig. 3.1). We adjusted *P* values for multiple comparisons using the false discovery rate (FDR) method (Benjamini and Hochberg 1995; Jafari and Ansari-Pour 2019).

NSC concentration models were then used to predict maximum and minimum coarse root NSC concentrations for each chestnut tree in our experiment (469 trees). Coarse root NSC pool sizes were calculated by multiplying concentration estimates and biomass estimates generated via allometric modeling, as outlined in Chapter 2 and fully described in the Appendix A. As we were only interested in coarse root pool sizes, a simplified allometric model (coarse root biomass ~ DBH + Ht) was used for this experiment (Table 3.S6). Generalized linear mixed models with a Gamma distribution and log link were built to analyze the relationship between maximum or minimum coarse root NSC pool size and our competition treatments. Planting density, species composition, and a density × composition interaction were included as fixed effects, and block was a random effect (Table 3.S7). We also tested whether ΔNSC pools (max NSC – min NSC) differed based on competition treatments using the same fixed and random effects described for max. and min. NSC pools (Table 3.S7). ΔNSC represents the contribution of NSCs to growth, respiration, storage, and other functions over a particular time (Hoch et al. 2003). Tukey contrasts (α=0.05) with an FDR correction were implemented in the *emmeans* package to evaluate differences between pairs of treatment means (Fig. 3.S4).

3.4   Results

Chestnut tree size across competition treatments

The average DBH of individual chestnut trees was significantly reduced by increasing planting density (*P*<0.001), but this reduction in DBH also depended on the species composition in the plot (composition × density interaction *P*<0.001, Table 3.S4). While the average DBH of

chestnuts varied with composition at a density of 1111 and 10000 stems ha^{-1}, no differences in DBH were observed between composition treatments at 2500 stems ha^{-1} (Fig. 3.1). Specifically, in our low-density treatment (1111 stems ha^{-1}), chestnuts were significantly larger when growing with all three species together compared to growing in monoculture or in two-species mixtures (Fig 3.1). At a high density (10000 stems ha^{-1}), compared to chestnut monocultures, chestnut DBH was larger in mixtures that contain black cherry (CB and CNB treatments) and were smaller when growing alongside northern red oak (CN treatment; Fig. 3.1). Species richness (1, 2, or 3 species) had less of an effect on chestnut DBH than species composition. Within a given planting density, the only significant difference was increased chestnut DBH in the three-species mixtures compared to monocultures and 2 species mixtures (Fig. 3.S6).
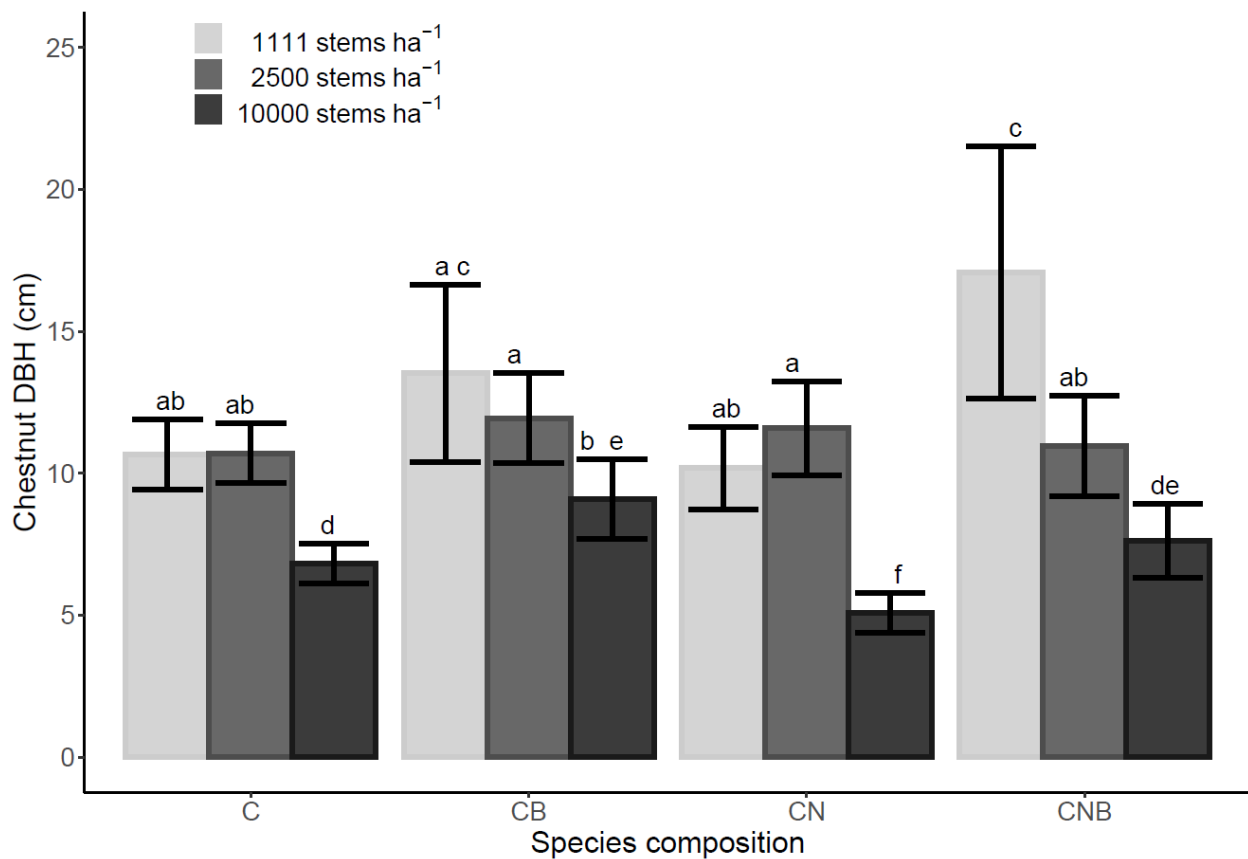
Figure 3.1. Diameter at breast height (DBH, in cm) of American chestnut (*Castanea dentata*) trees across competition treatments. Chestnut was planted in monocultures (C plots), with black cherry (*Prunus serotina;* CB plots), with northern red oak (*Quercus rubra;* CN plots), or in three-species mixtures (CNB plots). Error bars are 95% confidence intervals. Different letters represent differences in treatment means at α=0.05.

Coarse root NSC concentrations in response to treatments

Coarse root NSC concentrations were on average of 11.5% ($\pm$1.1% SE) higher during the leaf fall than during the shoot expansion period (Fig. 3.2). Models testing for the relationship between maximum or minimum coarse root NSC concentrations and our competition treatments were not significant overall ($P>0.25$). However, we did find a significant main effect of species composition on minimum NSC concentrations (Table 3.S5), which was driven by lower minimum NSC concentrations for chestnuts in CN plots relative to C or CB plots ($P<0.05$; Fig. 3.2). No significant differences were found between treatments when we analyzed NSC concentrations using species richness instead of composition (Fig. 3.S7).
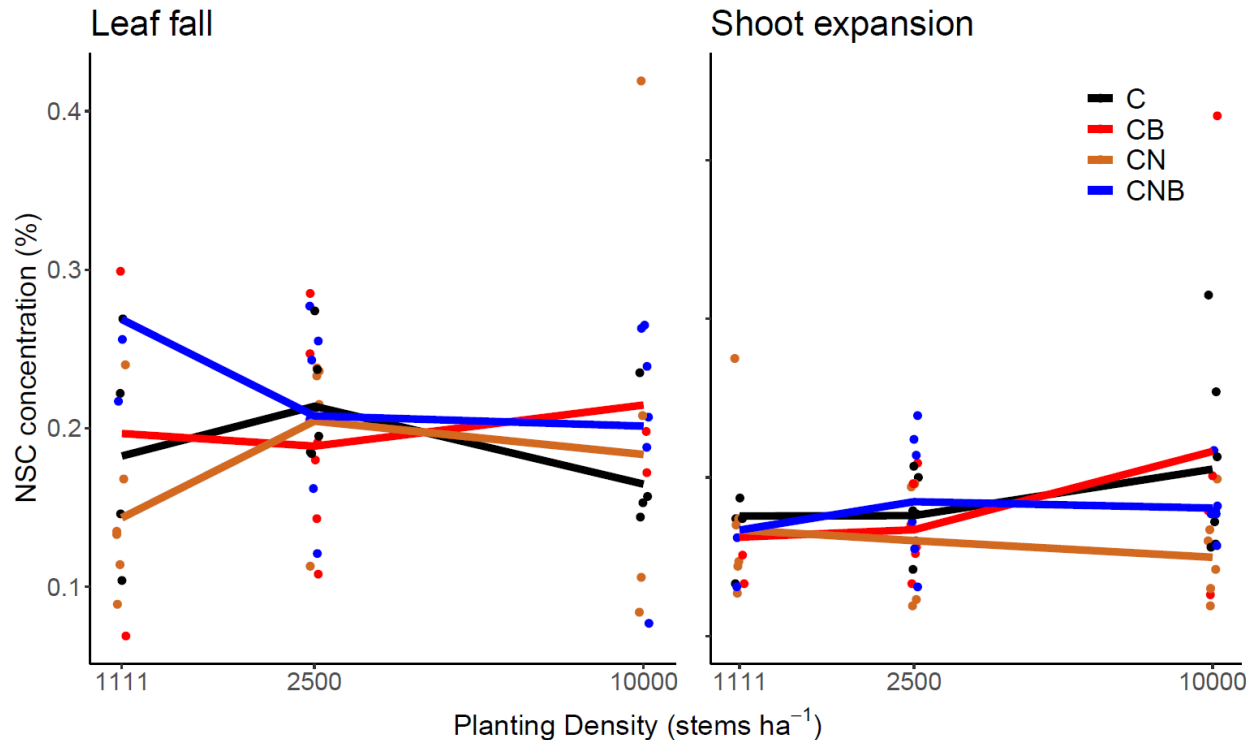
Figure 3.2. Coarse root NSC concentrations in American chestnut *(Castanea dentata)* across competition treatments during shoot expansion. Species composition treatments were: chestnut was planted in monoculture (C), chestnut with black cherry *(Prunus serotina;* CB plots), or with northern red oak *(Quercus rubra;* CN plots), or with all three species together (CNB plots). Lines connect treatment means; the only contrasts significant at α=0.05 were at 10000 stems ha^{-1} during shoot expansion, when NSC concentrations in C and CB were higher than in CN.

Effects of competition on coarse root NSC pools

Maximum, minimum, and ΔNSC coarse root pools responded in a similar fashion to our competition treatments. Planting density affected max. and min. NSC pools in coarse roots at every species composition: the smallest max., min, and ΔNSC pools were found at the 10000 stems ha^{-1} density, except for chestnuts in CB, which had similar pool sizes in the mid- and high-density plots (α=0.05). In all three of our analyses, NSC pools generally had a negative relationship with density (Fig. 3.3). However, there was a strong density × composition interaction in all models (*P*<0.001; Table 3.S7), and the response of chestnut coarse root NSC pools to reductions in planting density followed two distinct patterns according to species composition. Chestnuts in the CB and CNB treatments showed continued reductions in NSC pool size with increasing density (Figs 3.3 and S4). At high density, chestnuts in CB had larger minimum NSC pools than chestnuts in CNB (*P* < 0.05), but chestnuts in CNB plots at low density had the largest pools overall. Surprisingly, chestnuts in C and CN plots had larger coarse root max NSC pools when density increased from 1111 to 2500 stems ha^{-1}, before a reduction in pool size was observed between 2500 and 10000 stems ha^{-1}. The same pattern was observed for ΔNSC pools in C and CN and for minimum CN pools (Fig. 3.S4). Composition had no effect on chestnut coarse root max., min., or ΔNSC pools at the mid-density plots. The proportional change in ΔNSC (ΔNSC /max NSC) was high but relatively constant across treatments (Fig. 3.S5). Chestnuts in the high-density CN plots had the largest proportional ΔNSC (90.4%), and high-density chestnut monocultures had the smallest proportional ΔNSC (85.6%).

The different responses of chestnut in CB vs CN plots effectively cancelled each other out when analyzing with species richness (i.e. one, two, or three-species mixtures) instead of composition (Figs 3.S8 & 3.S9). At each planting density, NSC pools were the generally same in two-species mixtures as in chestnut monocultures at each planting density (Fig. 3.S8). The only exceptions occurred at our high-density plots, where chestnut monocultures had smaller maximum coarse root NSC pools (*P*=0.03) and ΔNSC (*P*=0.03) than two-species mixtures. Across all species richness treatments, chestnut maximum, minimum, and ΔNSC pools were smallest in high-density plots (Fig. 3.S9). At a given species richness level, minimum NSC pools were similar between mid-density and low-density plots except in the three-species mixtures, where low density plots had the largest NSC pools. As observed for the C and CN plots above, the one and two-species mixtures had smaller maximum and ΔNSC pools at low density than at high density.
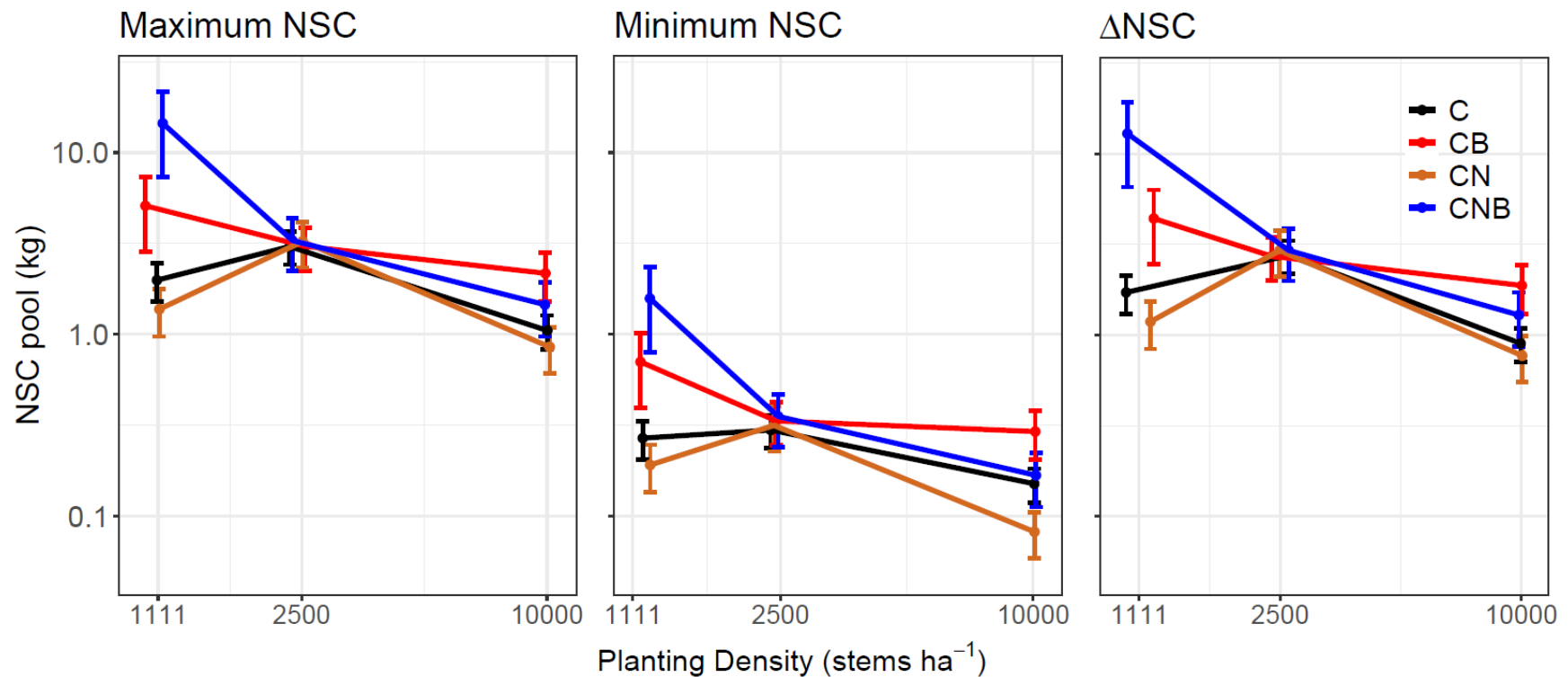
Figure 3.3. Coarse root NSC (starch + sugar) pools in American chestnut (*Castanea dentata*). Chestnut was planted in monoculture (C plots), with black cherry (*Prunus serotina;* CB plots), with northern red oak (*Quercus rubra;* CN plots), or with all three species together (CNB plots). Error bars are 95% confidence intervals around treatment means.

3.5 Discussion

Size differences across species composition and density treatments

Chestnut trees were generally smaller in monocultures than in mixtures, although this pattern was modulated by neighbor identity: chestnuts in CN plots at the lowest and highest planting densities had smaller DBH than chestnuts in monocultures. Positive effects of species mixing on chestnut biomass accumulation were expected because American chestnut, northern red oak, and black cherry have distinct life history strategies and biomass allocation patterns, as described above in Methods. These differences are expected to reduce competition intensity through resource partitioning (Forrester and Bauhus 2016; Barry et al. 2019). Furthermore, facilitation effects were predicted to be highest at 10000 stems ha^{-1} in accordance with the stress-gradient hypothesis (Bertness and Callaway 1994). For example, Harpole et al. (2016), showed a strong benefit for resource partitioning under limited resources: they found that increasing resource abundance (e.g. more space to harvest water, nutrients, and light) reduced niche dimensionality (the number of non-overlapping ecological niches), reduced diversity, and increased productivity (Harpole et al. 2016). Our results did not fit neatly with the predictions we made based the stress gradient hypothesis. The effects of species mixing on chestnut DBH were most pronounced in both our high-density and low-density plots (Fig. 3.1), the high and low extremes of our proposed stress gradient.

Some researchers have advocated for refining predictions generated by the stress gradient hypothesis based on these species-specific differences. Following the species classification scheme of Grime (1977), Maestre et al. (2009) illustrated that the balance of competitive/ complementary interactions changes along stress gradients when highly competitive species (characterized by rapid growth and poor reaction to competition) are paired with stress-tolerant species (characterized by slower growth rates and plastic growth forms). Following this scheme, black cherry would be characterized as a competitor, while northern red oak is stress-tolerant (see Methods for species descriptions). American chestnut is highly competitive and fast-growing during early stages (Jacobs and Severeid 2004) but also stores a substantial share of its carbon as NSCs, presumably as a long-term survival strategy (Ch. 2; Wang et al. 2013; Belair et al. 2018). Therefore, chestnut may reside in a grey area between competitive and stress-tolerant classifications. Pairing two stress-tolerant species produces negative effects in both high-stress and low-stress environments, but generally neutral effects in moderate environments, due to the

amelioration of stress in moderate environments but drawn-out competition for scarce resources in high-stress environments (Maestre et al. 2009). This theory may explain the reduction in chestnut DBH at high densities but neutral effects at mid- or low-density plots (Fig. 3.1).

Inter- and intra-specific competition moderates chestnut coarse root NSC reserves

NSC pools integrate the effects of competition on structural biomass and on NSC concentrations. We expected that these effects would have the opposite effect on growth vs. concentrations: low-density plots containing species mixtures were predicted to have the greatest chestnut productivity and the lowest NSC concentrations. As discussed previously, these expectations were largely met for productivity (DBH). However, chestnut coarse root max. and min. NSC concentrations were generally not affected by planting density or competition. The sole exception to this pattern was the lower minimum NSC concentrations of chestnut in high-density CN plots relative to chestnuts in high-density C or CB plots ($P<0.05$). Our concentrations results are surprising because chestnut productivity appears to be most constrained in the high-density CN plots, and trees under increasing abiotic stress are expected to allocate a higher proportion of fixed carbon to NSCs (Wiley and Helliker 2012). However, analyzing DBH only provides information about aboveground productivity, and our samples are from the belowground tissues. It is possible that chestnut allocates a higher proportion of its NSCs to root growth when planted near northern red oak to counter red oak's large and laterally spreading root system (Lyford 1980).

When integrated, the divergent effects on chestnut DBH and coarse root NSC concentrations produced NSC pools that differed dramatically based on an interaction between neighbor identity and planting density (Fig. 3.3). While the NSC pools of chestnuts growing in all species composition treatments increased when planting density was reduced from 10000 stems ha^{-1} to 2500 stems ha^{-1}, responses diverged when density was further reduced to 1111 stems ha^{-1}. Chestnuts in the CB and CNB plots appeared to take advantage of the extra resources (e.g. more space to forage for light and nutrients) in low-density plots by increasing NSC pools. In contrast, chestnuts in C and CN plots had smaller NSC pools at 1111 stems ha^{-1} than at 2000 stems ha^{-1}.

Diverging responses after 2500 stems ha^{-1} may be accounted for by the refined stress gradient hypothesis (Maestre et al. 2009) outlined in the previous section. In this framework, chestnut-chestnut and chestnut-red oak interactions produce similar outcomes (Fig. 3.3), suggesting that both species are of the same competition type (stress tolerant). Competitive black cherry reduces net competition intensity when in mixture with chestnut. But the disappearance of

species-specific effects on NSC pools at 2500 stems ha^{-1} is puzzling: if species mixtures are predicted to be generally complementary at moderate stress (Maestre et al. 2009), then we would expect mixtures to have higher BA and NSC pools than the C monoculture at 2500 stems ha^{-1}. It is possible that our 13-year-old stands are self-thinning at the 10000 stems ha^{-1} density, but have not yet started thinning at 2500 stems ha^{-1} density; this would presumably increase the competition intensity and thereby highlight the effects of niche partitioning on biomass at high-density plots. However, a time lag in thinning would not explain the dramatic differentiation of responses at 1111 stems ha^{-1}.

Delta NSC pools

ΔNSC pools largely reflected differences in maximum NSC pool sizes, with the largest NSC pools also having the largest ΔNSC. However, small differences in proportional ΔNSC may reveal differences in seasonal NSC utilization among competition treatments. Similarly to the results of Hoch et al. (2003), we found that the trees with the lowest NSC concentrations (CN at 10000 stems ha^{-1}) had higher relative ΔNSC (Fig. 3.S5). ΔNSC could represent the necessity of using limited NSC stores to compete for light, water, and other resources. However, chestnuts growing in CNB had > 2% higher proportional ΔNSC than any other low-density treatments, and average proportional ΔNSC was highest in the mid-density plots. These results, paired with data showing that CNB chestnuts at low density were the most productive, CN chestnuts at high density were the least productive, and that there were no composition differences in productivity at mid-density plots (Fig. 3.1), suggest that a high proportional ΔNSC may be attributed to NSCs supporting many distinct functions. Higher proportional ΔNSC values indicate a smaller allocation of NSC reserves to long-term storage, and potentially indicate a greater necessity of using limited NSC stores to compete for light, water and other resources in certain treatments. Chestnuts with the highest proportional ΔNSC may be expending the most carbohydrate to fuel growth, respiration, symbioses, or other functions at differing priorities; i.e., an aboveground growth-forward strategy for stored carbohydrates at low density, and preferential use of NSCs for respiration and (perhaps) belowground growth at high-density CN plots. Given our experimental design, we cannot be certain that a ca. 5% difference in storage would be biologically meaningful. However, recent work in *Populus tremuloides* has shown that root NSC mass (and starch particularly) is linearly related to sprout mass, and that nearly all starch reserves may be remobilized to support resprouting

production (ca. 0.4% left at sprout death; Wiley et al. 2019). Therefore, small differences in NSC storage could affect chestnut resilience to damage and to stressful conditions.

Taken together, the high proportional ΔNSC found in this study (85.6-90.4%) corresponds to minimum coarse root NSC reserves of just 9.6-14.4% of maximum reserves. This fluctuation was much higher than Hoch et al (2003) found in a study of aboveground organs of ten different species (branches: mean minimum = 55% of maximum; stem wood: 67% of max.), which led those authors to conclude that temperate deciduous trees were not carbon-limited. Numerous differences exist between the two studies, including tree age (100-year-old stand vs our 14-year old chestnuts) and the specific organs sampled. Our study focused on coarse roots as a key indicator for NSC status in our trees (Ch. 2), and in other species such as aspen (Landhäusser and Lieffers 2012). Beyond the contribution of NSCs to annual phenological events observed in Chapter 2, coarse root NSC reserves are an important indicator for overall NSC status of trees because they represent the most distant NSC sink from the canopy, and they provide essential functions like defense and assimilation of water and nutrients (directly or through symbiotic partners). The large fluctuation in chestnut reserves observed across all treatments of this study indicate that chestnut productivity may be limited by NSC supply at our site.

3.6   Conclusions

We studied the effect of intra- and inter-specific competition on American chestnut biomass accumulation (DBH) and NSC reserves using mixtures of species with strong functional differences. The relationship between planting density and chestnut DBH and the relationship between planting density and NSC reserves were both modulated by species composition at high- and low-density plots. While treatment effects on chestnut DBH were similar to the effects on NSC pool sizes, they were not identical: chestnut DBH was not significantly different between the mid- and low-density plots in all composition treatments except CNB, but differences between maximum, minimum, and ΔNSC pools at mid- and low-density plots were generally significant and depended on species composition (Fig. 3.1). Therefore, neighbor identity was more important than species richness for NSC pools in our system. Finally, understanding species interactions in moderately stressful environments (e.g. mid-density plots in our study) is a fruitful area for further study. While we propose an explanation for the lack of composition effects at mid-density plots

in line with the refined stress gradient hypothesis, most studies invoking this hypothesis were conducted at the treeline or in other harsh habitats.

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3.8   Supplementary Materials for Chapter 3

Figure 3.S1. American chestnut (*Castanea dentata*) was planted in monoculture (C plots) or in mixture with black cherry (*Prunus serotina;* CB plots), northern red oak (*Quercus rubra;* CN plots), or with all three species together (CNB plots). Planting density varied from 10000 stems ha^{-1} (1 m between adjacent trees), 2500 stems ha^{-1} (2 m), and 1111 stems ha^{-1} (3 m). Biomass data used to build the allometric model is from excavating trees in the 'unrelated chestnuts' stand. On the right, a satellite photo of the experiment.
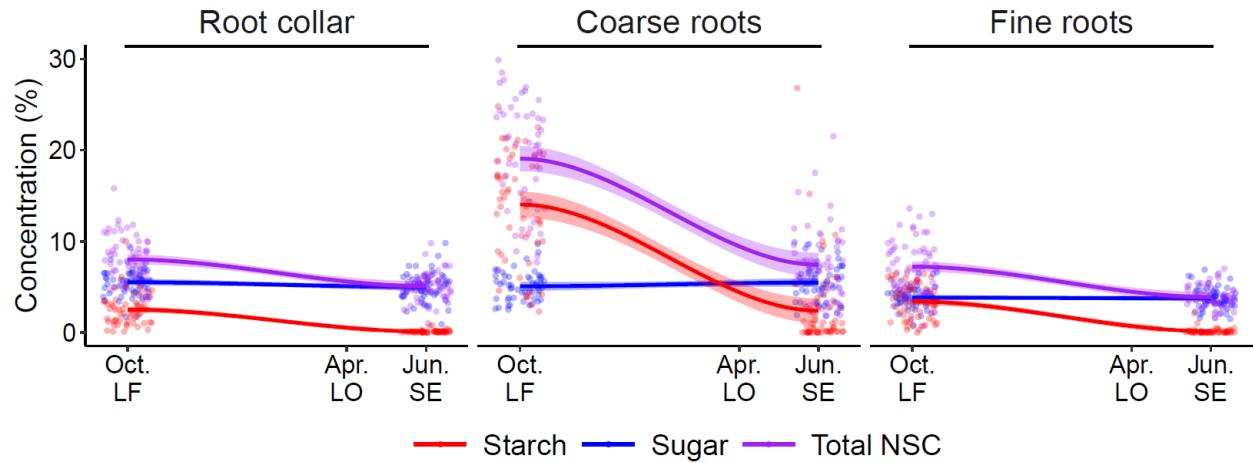
Figure 3.S2. NSC concentrations in American chestnut *(Castanea dentata)* across competition treatments during leaf fall (LF, October 2019) and shoot expansion (SE, June 2020). Shaded bands represent 95% confidence intervals around the fitted curve.
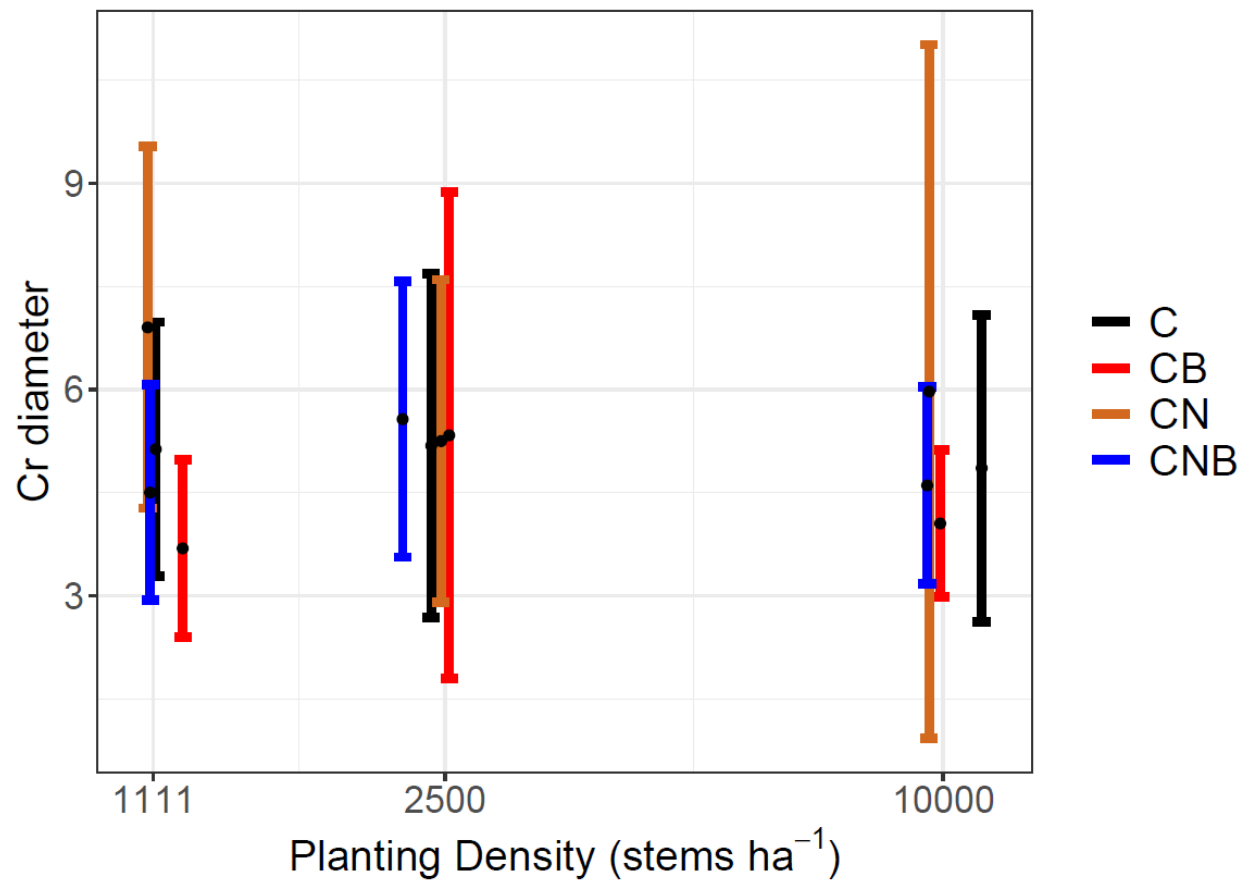
Figure 3.S3. Variation in coarse root diameter across competition treatments. Collection time did not have a significant effect on coarse root diameter at α=0.10. Data shown are means ±SD.
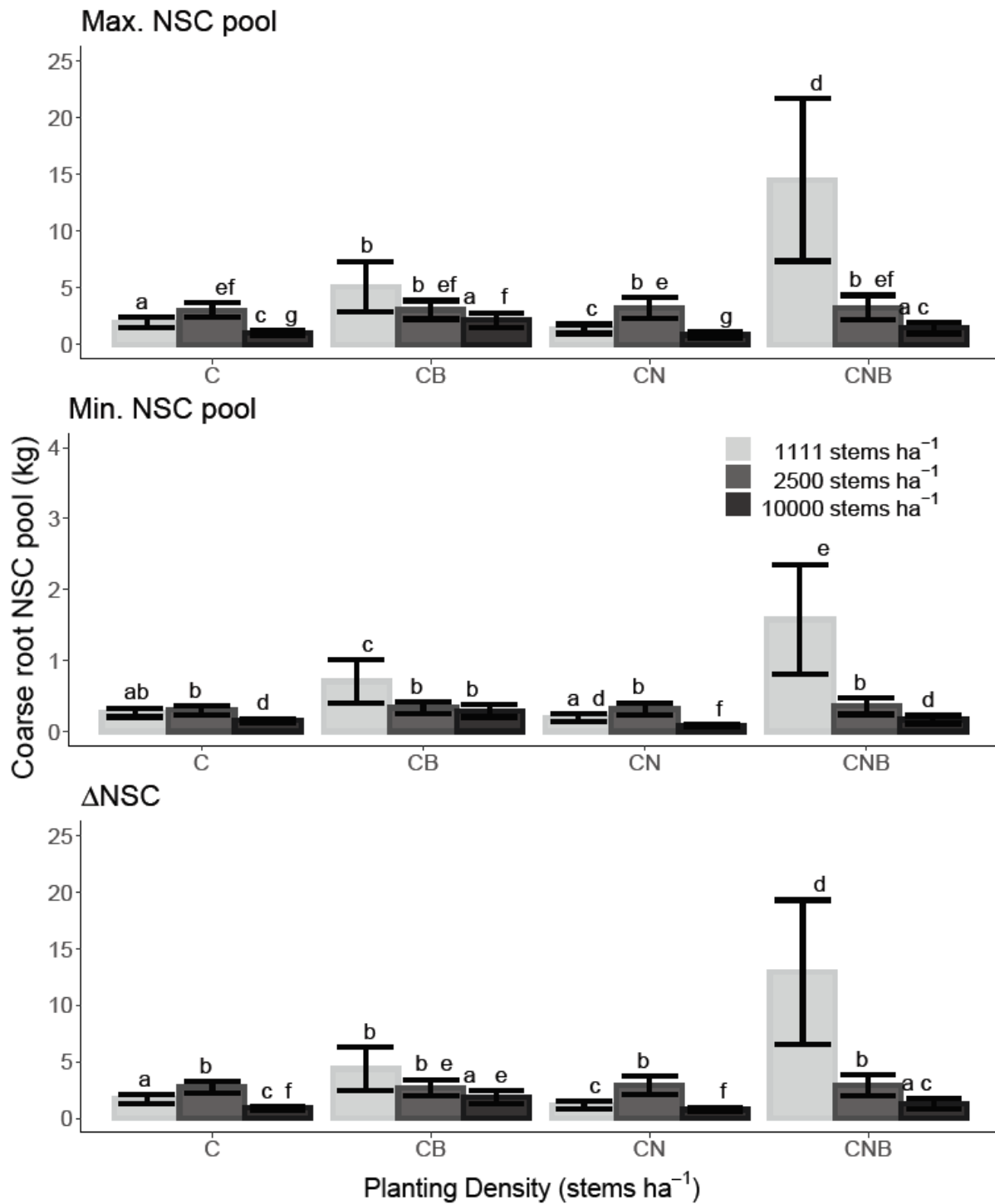
Figure 3.S4. Maximum, minimum, and ΔNSC coarse root pools in American chestnut (*Castanea dentata*). Chestnut was planted in monoculture (C plots), with black cherry (*Prunus serotina;* CB plots), with northern red oak (*Quercus rubra;* CN plots), or with all three species together (CNB plots). Error bars are 95% confidence intervals around treatment means, and different letters denote significant differences between pairs of means (α=0.05). Please note that the scale for the minimum pool figure was changed for ease of interpretation.
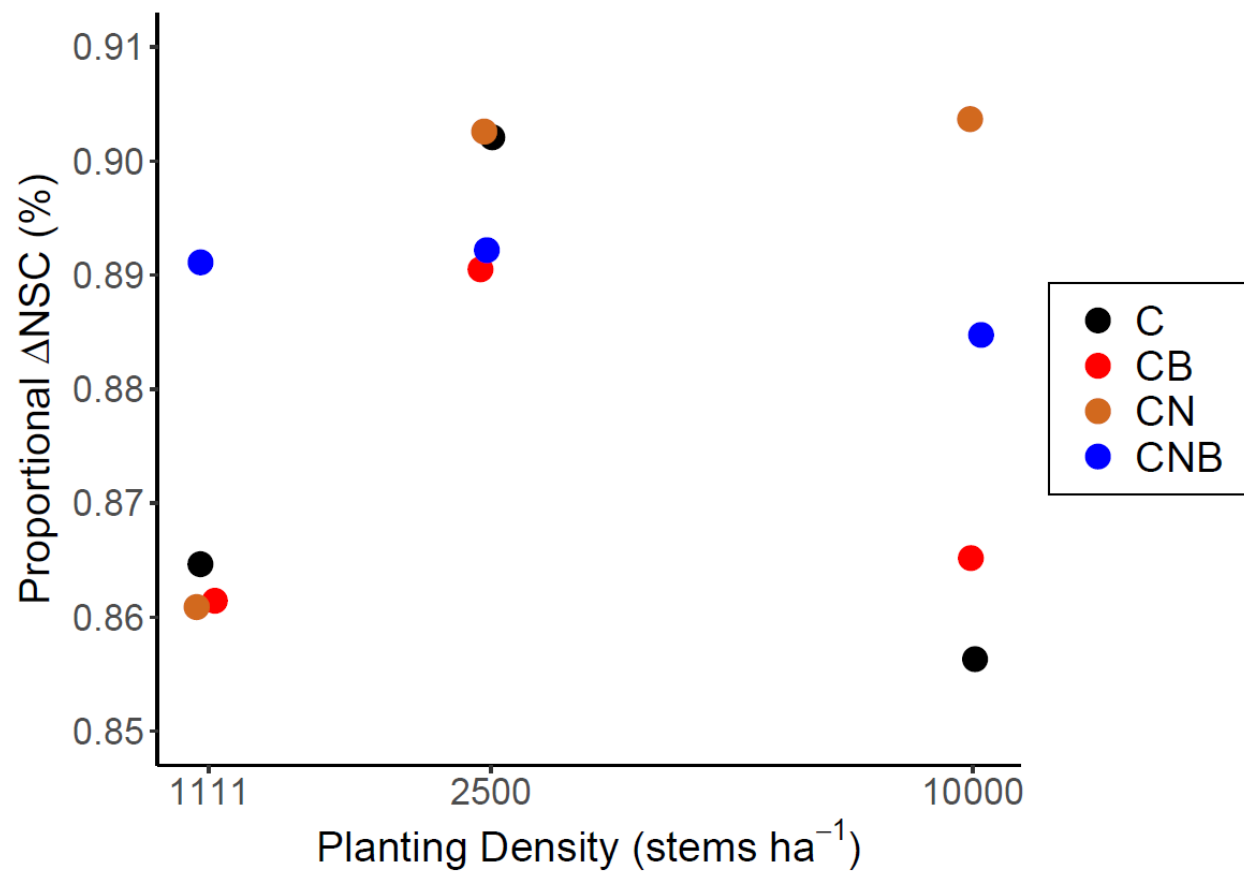
Figure 3.S5. Proportional change in ΔNSC pools, calculated as ΔNSC/ max.NSC.
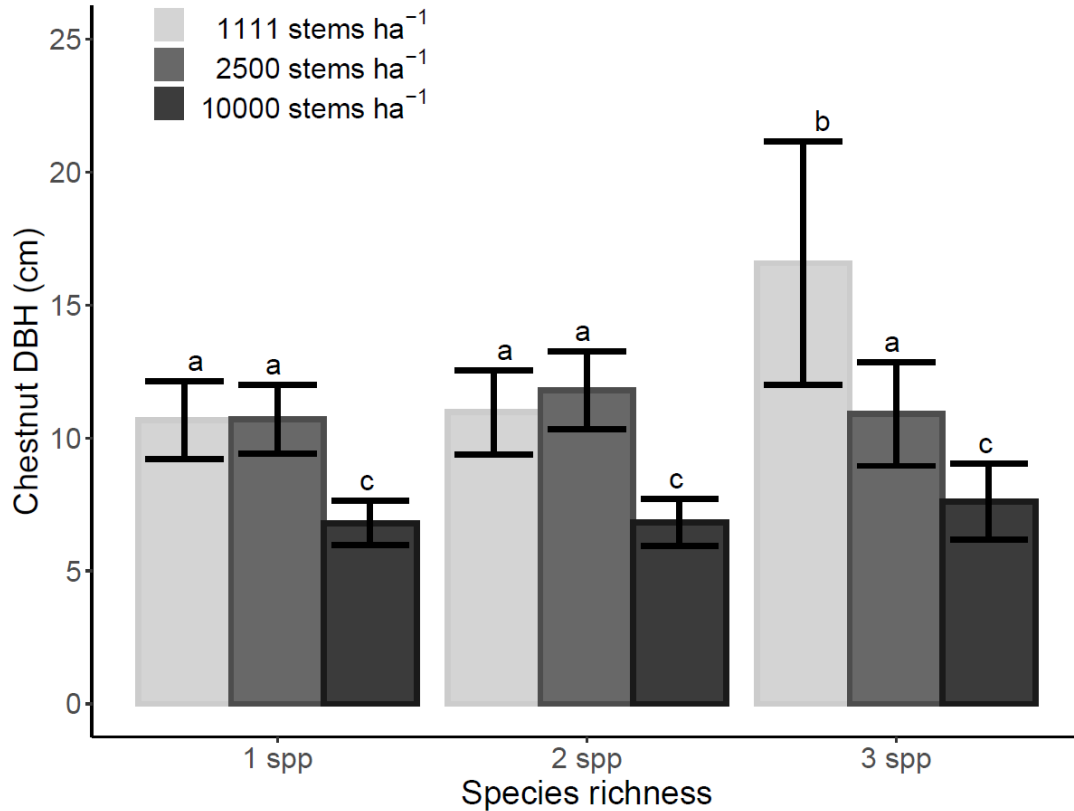
Figure 3.S6. Diameter at breast height (DBH, in cm) of American chestnut (*Castanea dentata*) trees across competition treatments. Chestnut was planted in monocultures (1 spp), with *Prunus serotina* or *Quercus rubra* (2 spp), or in three-species mixtures (3 spp). Error bars are 95% confidence intervals. Different letters represent differences in treatment means at α=0.05.
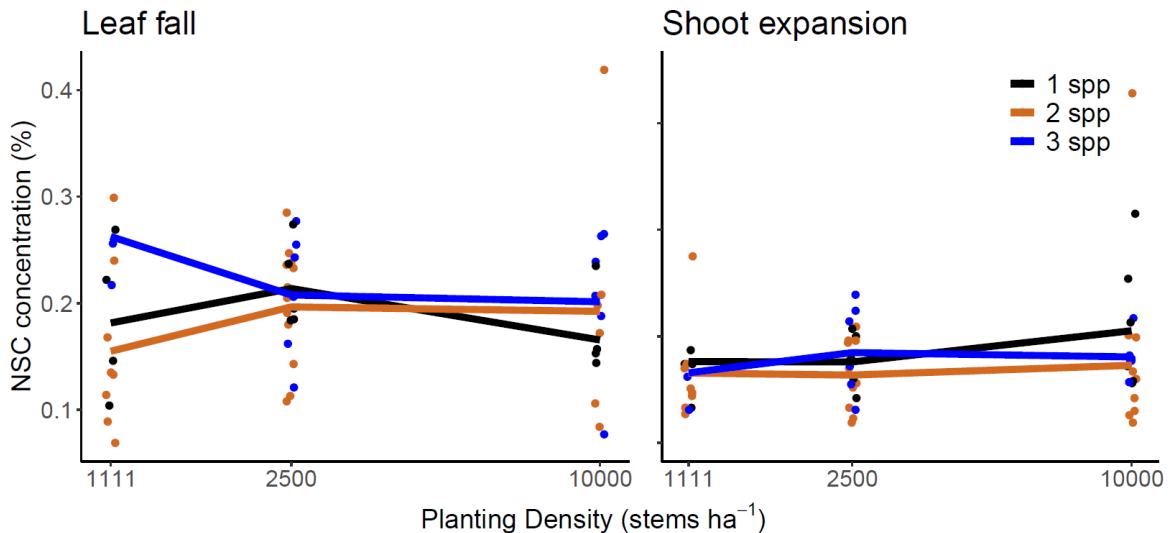
Figure 3.S7. Coarse root NSC concentrations in American chestnut *(Castanea dentata)* across competition treatments during shoot expansion. Chestnut was planted in monocultures (1 spp), with *Prunus serotina* or *Quercus rubra* (2 spp), or in three-species mixtures (3 spp).
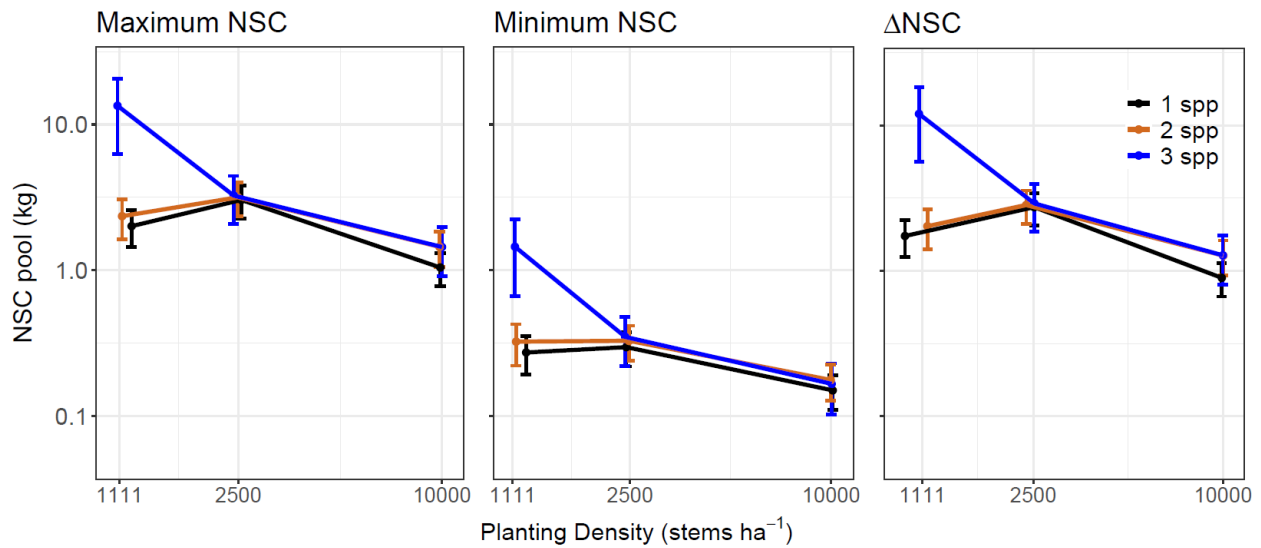
Figure 3.S8. Coarse root NSC (starch + sugar) pools in American chestnut (*Castanea dentata*). Chestnut was planted in monocultures (1 spp), with *Prunus serotina* or *Quercus rubra* (2 spp), or in three-species mixtures (3 spp). Error bars are 95% confidence intervals around treatment means.
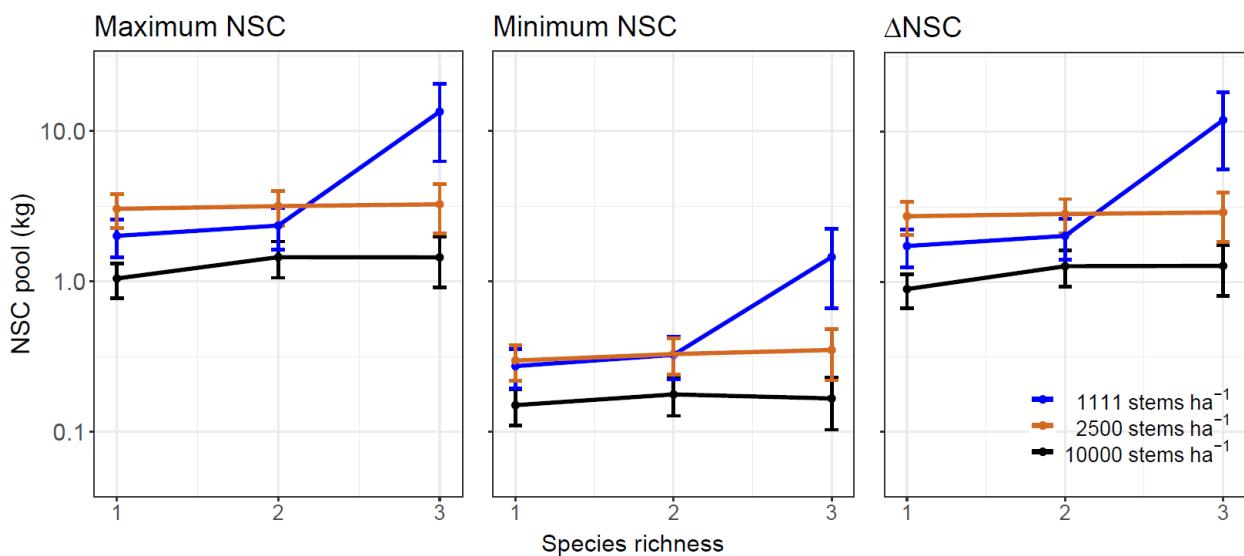
Figure 3.S9. Coarse root NSC (starch + sugar) pools in American chestnut (*Castanea dentata*). Chestnut was planted in monocultures (1 spp), with *Prunus serotina* or *Quercus rubra* (2 spp), or in three-species mixtures (3 spp). Error bars are 95% confidence intervals around treatment means.

Table 3.S1. Growth and mortality characteristics for the 30 plots retained for this study. Plots were composed of American chestnut (*Castanea dentata*) monocultures (C), 50% chestnut- 50% black cherry (*Prunus serotina*) mixtures (CB), 50% chestnut - 50% northern red oak (*Quercus rubra*) mixtures (CN), or 33% of each species planted together (CNB). Each plot was planted with 30 trees. Plots with greater than 20% mortality (shaded grey) were excluded from the study, leaving 30 plots.

Plot #	Block	Density (stems ha^{-1})	Species composition	Plot-wide mortality	Chestnut mortality	Chestnut mean DBH (cm; ±SD)	Chestnut mean Ht (m; ±SD)	Chestnut mean dry whole-tree biomass (kg; ±SD)
1	1	1111	C	>20%	----	----	----	----
2	1	1111	CB	----	----	----	----	----
3	1	1111	CN	13.33%	3/15	8 (2.81)	7.54 (1.73)	7.54 (1.73)
4	1	1111	CNB	>20%	----	----	----	----
5	1	2500	C	13.33%	4/30	10.62 (3.26)	11.97 (1.73)	11.97 (1.73)
6	1	2500	CB	10.00%	0/15	11.38 (2.62)	11.6 (1.61)	11.6 (1.61)
7	1	2500	CN	13.33%	3/15	10.79 (3.18)	11.32 (2.12)	11.32 (2.12)
8	1	2500	CNB	10.00%	1/10	14.73 (1.99)	12.8 (0.64)	12.8 (0.64)
9	1	10000	C	13.33%	4/30	6.67 (1.88)	9.33 (1.6)	9.33 (1.6)
10	1	10000	CB	16.67%	0/15	8.85 (3.79)	10.29 (2.77)	10.29 (2.77)
11	1	10000	CN	16.67%	5/15	7.42 (3.21)	10.14 (3.37)	10.14 (3.37)
12	1	10000	CNB	16.67%	0/10	6.07 (3.12)	8.21 (3.07)	8.21 (3.07)
13	2	1111	C	4.33%	1/30	10.72 (3.63)	8.93 (1.33)	8.93 (1.33)
14	2	1111	CB	10.00%	2/15	13.89 (4.77)	10.38 (0.94)	10.38 (0.94)
15	2	1111	CN	10.00%	0/15	11.88 (3.51)	10.15 (2.27)	10.15 (2.27)
16	2	1111	CNB	0.00%	0/10	17.55 (5.01)	12.09 (1.46)	12.09 (1.46)
17	2	2500	C	4.33%	1/30	11.19 (3.71)	10.97 (1.51)	10.97 (1.51)

18	2	2500	CB	16.67%	3/15	13.08 (2.77)	11.34 (1.05)	11.34 (1.05)
19	2	2500	CN	16.67%	4/15	12.02 (2.89)	11.79 (1.22)	11.79 (1.22)
20	2	2500	CNB	6.67%	0/10	10.66 (3.1)	9.9 (1.6)	9.9 (1.6)
21	2	10000	C	6.67%	2/30	7.57 (2.45)	11.9 (2.88)	11.9 (2.88)
22	2	10000	CB	6.67%	0/15	9.59 (2.48)	11.57 (0.94)	11.57 (0.94)
23	2	10000	CN	4.33%	0/15	4.0 (3)	6.16 (4.09)	6.16 (4.09)
24	2	10000	CNB	16.67%	1/10	9.94 (2.36)	11.66 (1.11)	11.66 (1.11)
25	3	1111	C	13.33%	4/30	10.61 (4.73)	8.46 (1.7)	8.46 (1.7)
26	3	1111	CB	>20%	——	——	——	——
27	3	1111	CN	13.33%	3/15	10.51 (2.81)	8.69 (1.04)	8.69 (1.04)
28	3	1111	CNB	>20%	——	——	——	——
29	3	2500	C	4.33%	1/30	10.34 (2.97)	9.88 (1.85)	9.88 (1.85)
30	3	2500	CB	13.33%	3/15	11.53 (2.78)	10.64 (1.4)	10.64 (1.4)
31	3	2500	CN	4.33%	1/15	11.77 (3.02)	10.7 (1.48)	10.7 (1.48)
32	3	2500	CNB	16.67%	1/10	7.74 (3.85)	8.54 (2.4)	8.54 (2.4)
33	3	10000	C	13.33%	4/30	6.27 (2.34)	9.74 (2.84)	9.74 (2.84)
34	3	10000	CB	>20%	——	——	——	——
35	3	10000	CN	10.00%	1/15	4.59 (3.73)	6.55 (4.14)	6.55 (4.14)
36	3	10000	CNB	10.00%	2/10	7.4 (1.67)	10.67 (1.19)	10.67 (1.19)

Table 3.S2. Testing for a relationship between coarse root (cr) sample diameter, collection period, and our study variables (species composition × density). 174 coarse root samples ranging from 2-14 mm diameter were collected for this study.

	Chisq.	Df	*P* value	Pseudo R²
cr.diameter ~ collection + spp.comp + density + (1|block)	34.17	12	0.349	0.075
Collection	4.431	2	0.109	—
Spp.comp	2.640	3	0.021	—
Density	**7.454**	**2**	**0.021**	—
Random: Block	**228.88**	**1**	**< 0.001**	—

Table 3.S3. Results of generalized linear mixed models testing for the effects of coarse root (cr) diameter on a) starch, b) sugar and c) total NSC concentrations.

	Num df	Den df	a) starch		b) sugar		c) total NSC	
			Chisq	*P*	Chisq	*P*	Chisq	*P*
cr diameter	1	103	0.339	0.561	0.424	0.515	0.238	0.627

Table 3.S4. The effects of species composition and planting density on DBH (cm) of individual American chestnut trees.

	Chisq	Df	*P* value	Pseudo R²
DBH ~ Spp.comp × density + (1|block)	162.1	11	< 0.001	0.361
Spp.comp	35.656	3	< 0.001	—
Density	58.604	2	< 0.001	—
Spp.comp × density	25.250	6	< 0.001	—
Random: block	1354.456	1	< 0.001	—

Table 3.S5. Results of models relating maximum or minimum chestnut NSC (starch + sugar) concentrations to species composition (spp.comp) and planting density.

	Chisq	Df	P value	Pseudo R^2
Max. NSC % ~ **Spp.comp × density + (1|block)**	8.682	11	0.651	0.148
Spp.comp	1.032	3	0.794	—
Density	1.330	2	0.504	—
Spp.comp × density	4.436	6	0.619	—
Random: block	73.838	1	< 0.001	—
Min. NSC % ~ **Spp.comp × density + (1|block)**	13.567	11	0.258	0.130
Spp.comp	**9.120**	**3**	**0.028**	—
Density	1.861	2	0.394	—
Spp.comp × density	5.515	6	0.480	—
Random: block	67.219	1	< 0.001	—

Table 3.S6. Allometric model for coarse root (CR) biomass, based on data from excavating eight chestnut trees of a similar size distribution to our experimental trees. The model was fit as a GLM with a Gamma distribution and log link.

	Chisq	Df	P value	Pseudo R^2
CR.biomass ~ DBH + Height	-3.049	2	< 0.001	94.83
DBH	47.337	1	< 0.001	—
Height	7.108	1	0.048	—

Table 3.S7. NSC pool models relating planting density and species composition to coarse root total NSC pools in American chestnut. Max. coarse root NSC pools occur at leaf fall (LF, Oct.) and min. NSC pools occur at shoot expansion (SE, June). ΔNSC was NSC_{max} - NSC_{min}.

	Chisq	Df	P value	Pseudo R^2
Max. NSC pool **~ Spp.comp × density + (1|block)**	223.73	11	<0.001	0.339
Spp.comp	28.289	3	<0.001	—
Density	76.308	2	<0.001	—
Spp.comp × density	67.093	6	<0.001	—
Random: block	0.203	1	0.652	—
Min. NSC pool **~ Spp.comp × density + (1|block)**	191.94	11	<0.001	0.305
Spp.comp	43.883	3	<0.001	—
Density	34.653	2	<0.001	—
Spp.comp × density	58.607	6	<0.001	—
Random: block	307.599	1	<0.001	—
ΔNSC pool **~ Spp.comp × density + (1|block)**	229.78	11	<0.001	0.346
Spp.comp	27.397	3	<0.001	—
Density	83.691	2	<0.001	—
Spp.comp × density	69.038	6	<0.001	—
Random: block	0.974	1	0.324	—

CHAPTER 4.   CONCLUSIONS

4.1   Research objectives

The purpose of this research was twofold: first, I sought to contribute to American chestnut (*Castanea dentata* (Marsh.) Borkh.) restoration by establishing baseline NSC dynamics for mature chestnut trees. The second objective was to advance our understanding of nonstructural carbohydrates (NSCs) in terms of carbon allocation to reserves vs its use in structural growth or metabolism. This was accomplished by comparing NSC reserves in chestnut trees growing under varying competition regimes in a hardwood plantation experiment. NSCs play a critical role throughout the lifespan of trees by fueling routine cellular processes under varying environmental conditions and by facilitating resistance and resilience to stressors (Kozlowski et al. 1991). As long-lived, sessile organisms, trees must be able to withstand biotic stressors and environmental variability to survive (Petit and Hampe 2006). And in the 21st century, the combined stressors of climate change and the spread of exotic pests requires both active forest restoration and targeted forest management for increased resistance and resilience (Lake 2013; Nunez-Mir et al. 2015; FAO and UNEP 2020). We studied seasonal NSC dynamics and responses to inter- and intra-specific competition in American chestnut, a species of high restoration interest due to its functional elimination by the chestnut blight (*Cryphonectria parasitica*). Therefore, my thesis contributes to the restoration of a threatened tree species and to our understanding of the regulation of NSCs, which form the basis for resistance and resilience for temperate forest trees.

In Experiment I (Chapter 2), whole-tree NSC storage dynamics were tracked through one full seasonal cycle. Our specific objectives for the first experiment were to: I) track seasonal variation in NSC concentrations (i.e. sugars and starch) in all major organs, and II) compare organ-specific pool sizes with one another to infer the relative importance of different organs for NSC storage and remobilization. The effect of competition on belowground NSC reserves was tested in Experiment II (Chapter 3), where our objective was to explore relationships between the type of interaction (intra- or inter-specific), tree productivity, and NSC dynamics in a plantation experiment that manipulated planting density and species composition. In the Appendix, I present original biomass data and allometric modelling that enabled us to quantify and compare NSC pools (NSC concentration x biomass) of important storage organs in our trees.

4.2 Results synopsis

Experiment 1: Root system dominates carbohydrate reserve pools in American chestnut (*Castanea dentata*): implications for ecology and management

Three plots, each containing 30 pure American chestnut trees, were planted in 2007 at Martell Forest, a research site owned by Purdue University in West Lafayette, Indiana. Two different chestnut trees were sampled over the course of one year at different phenological stages. Organ-specific NSC concentration data was paired with biomass estimates from a bespoke allometric model to generate NSC pool sizes. Organ-level NSC concentrations and pools generally peaked at leaf fall (October) and were lowest during shoot expansion (June), although interactions between organ and collection period drove seasonal variation in pool sizes. Coarse root NSC reserves were replenished later in the growing season than aboveground organs. Coarse root reserves were also larger and more dynamic than in previous studies with other temperate deciduous trees, and they were the primary supplier of NSCs to support spring leaf-out.

Experiment 2: Inter- and intra-specific competition moderates belowground nonstructural carbon storage in American chestnut (*Castanea dentata*)

American chestnut, northern red oak, and black cherry were planted as monocultures or mixtures at three different densities in three replicate blocks at Martell Forest. We studied the effect of intra- and inter-specific competition on chestnut biomass accumulation (DBH) and NSC reserves. Coarse root total NSC pools, the largest and most dynamic pools in American chestnut (Ch 2) were evaluated across competition treatments. Coarse root NSC pools responded to density and species composition through their combined effects on NSC concentrations and tree biomass. Across composition treatments, chestnut NSC pools were smallest in high-density plots. There was a strong density × composition interaction: NSC pools were largest in plots with the lowest planting density where chestnut was planted with black cherry or in a three-species mixture with black cherry and northern red oak, but chestnut NSC pools were smaller when planted in monoculture or in a two-species mixture with northern red oak. Thus, neighbor identity was more important than species richness for NSC pools in our system. Inter- vs. intra-specific interactions had no effect on biomass accumulation or NSC pools at our mid-density plots.

4.3 Management implications

Our findings have specific relevance for forest managers seeking to restore American chestnut to the landscape, and they carry implications for forest resistance and resilience to challenges like drought, flooding, and pest outbreaks.

In the first experiment, we found that chestnut has a strong tendency to store NSCs belowground and that a large proportion of root NSCs are regularly mobilized, presumably to fuel growth and metabolism. Given the importance of the root system for NSC storage and mobilization, promoting robust root development will enhance outplanting success for chestnut. Vigorous root development can be encouraged at many points during the production of planting stock, starting with selecting genotypes or rootstocks with a large root-shoot ratio. In the nursery, careful root undercutting of bareroot seedlings or investing in a containerized system may be used to stimulate root development an improve outplanting success (Davis and Jacobs 2005; Landis 2008). Interplanting chestnut with other species may also promote belowground allocation of structural biomass and NSCs (del Río et al. 2019). Additionally, large and dynamic belowground pools in chestnut may reflect preferential allocation of carbohydrates to the roots as a secure storage site, similar to fire-adapted oak species. In fact, Native Americans promoted chestnut and other mast-bearing species at the expense of mesic species (e.g. beech and sugar maple) through clearing and burning forest areas in the pre-blight era (Black et al. 2006; Tulowiecki and Larsen 2015). Disturbance-based management may therefore be key to restoring (blight-resistant) chestnut to the landscape. (Belair et al. 2018). Additionally, large and accessible NSC reserves in the coarse roots may have prevented the total extinction of chestnut in forests of the eastern USA by fueling resprouting despite the endemic chestnut blight. It is possible that the blight acted as a strong selective force; strong belowground allocation may have been selected for as resprouting chestnut are effectively the only survivors of the blight.

In the second experiment, a strong density $\times$ composition interaction meant that the relationship of NSC pool size to density depended on neighbor identity (chestnut, northern red oak, and/or black cherry). Additionally, although the largest pools were found in the high-diversity plots, neighbor identity was a more important determinant for chestnut NSC pools than species richness. Given the importance of NSC pools for resprouting (Wiley et al. 2019), defense against biotic damaging agents (Goodsman et al. 2013), and surviving drought (Adams et al. 2013; Sevanto et al. 2014), chestnuts planted near black cherry are likely better primed for resistance and

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resilience to stressors. Therefore, if chestnut success (i.e. high rates of productivity and survival) is the primary goal, then care should be taken when selecting species to include in mixtures. Chestnut had larger DBH and root NSC pools when it was planted with black cherry, a 'competitor' species according to Grime's classification scheme (Grime 1977). American chestnut is highly competitive and fast-growing during early stages (Jacobs and Severeid 2004) but also stores a substantial share of its carbon as NSCs, presumably as a long-term survival strategy (Ch. 2; Wang et al. 2013; Belair et al. 2018). Therefore, chestnut may reside in a grey area between competitive and stress-tolerant classifications. Pairing chestnut with black cherry or other competitor species like tulip poplar (*Liriodendron tulipifera*) or black walnut (*Juglans nigra*) may foster success for chestnut at the detriment of the competitor. Conversely, pairing chestnut with stress-tolerant species like northern red oak or itself (i.e. chestnut monocultures) is likely to reduce the productivity and NSC reserves of individual chestnut trees. The planting site should be considered when choosing competitor species to interplant with chestnut, as inter- and intra-specific relationships are predicted to change along abiotic stress and resource availability gradients (Maestre et al. 2009).

The second experiment also tested whether there was a trade-off between NSC storage and growth. We did not find evidence of this trade-off: NSC concentrations were relatively unchanged between our competition treatments, while biomass increased when planting density was decreased and in plots containing black cherry. In other words, NSC pools increased in a parallel fashion with biomass, and competition treatments that increased biomass also increased NSC pool size. The apparent absence of a tradeoff between growth and NSC reserves makes management for both attributes more straightforward because increasing productivity also increases potential resiliency (NSC reserves) in chestnut.

4.4   Future directions

Future studies could investigate inter- and intra-specific interactions in moderate-stress environments, test the tradeoff between growth and NSCs in other species and settings, and refine the allometric model based on competitive effects (e.g. planting density and neighbor identity). First, the negligible effects of species composition at mid-density plots in (our moderate-stress treatment) are puzzling. While the refined stress-gradient hypothesis predicts roughly equal performance of competitors and stress tolerators in moderate-stress environments (Maestre et al.

2009), most studies invoking this hypothesis were conducted at the treeline or in other harsh habitats. More data on the changing nature of inter- and intra-specific interactions along stress gradients would clarify this pattern. Fortunately, a growing number of tree diversity experiments are coming online (Grossman et al. 2018), which could be utilized to test this question. These sites could also be used to test for a tradeoff between growth and NSC reserves in species besides American chestnut. Additionally, studying NSC dynamics in the other species in our experiment, northern red oak and black cherry, would shed light on the growth-NSC reserves tradeoff for a stress-tolerator (oak) and a competitor (cherry). Collecting data on oak and cherry in our experiment would also enhance our interpretation of chestnut NSC dynamics by providing other species for comparison.

Finally, modifying the allometric model used to scale NSC concentrations up to pool sizes would improve the accuracy of this work. Our allometric model was built using data from chestnuts planted in monoculture with 2.25 m between stems. However, structural biomass allocation changes in response to competition. For example, plants responded to increasing density by decreasing the total mass and the fraction of leaf and root mass, and by increasing the stem mass fraction in a meta-analyses using 18 studies (Poorter et al. 2012), although the root mass fraction may increase with density if nutrients are limiting (Berendse and Möller 2009). Given that the response of allocation to competition may be situation-specific, and that many of these studies were done on herbaceous plants or tree seedings, it would be useful to have a flexible allometric model that can be tuned to the specific competition regime. Excavating a small number trees in each competition experiment would be an enormous task, but data from a controlled experiment would be extremely valuable for researchers seeking to predict allometric relationships in response to competition in mature trees. Other avenues of modifying the allometric equation include comparing leaf litter collections across treatments, soil coring or trench excavation to estimate coarse and fine root biomass or using root ingrowth cores to estimate fine root productivity.

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APPENDIX A. ALLOMETRIC MODELLING

A.1. Project summary

Quantifying nonstructural carbohydrate (NSC) reserves involves taking tissue samples from major tree organs, each representing storage pools with different biomass, sugar and starch concentrations and NSC residence times. Laboratory analysis of tissue samples provides data on sugar and starch concentrations (Landhäusser et al. 2018). However, it is not possible to compare organ-specific NSC pools (organ biomass x NSC concentration) without knowing the biomass of each organ (Kozlowski 1992). There are no whole-tree allometric models available for American chestnut (Chojnacky et al. 2014). While Jacobs et al. (2009) modelled chestnut above-ground biomass in a mixed-hardwood plantations, they did not include below-ground biomass, a potentially important NSC reservoir (Loescher et al. 1990). Therefore, we excavated eight American chestnut trees and measured above- and below-ground biomass in great detail. We then fit an allometric model that predicts the dry biomass of American chestnut organs based on DBH and height**.**

A.2. Methods

Site description

This study was carried out at Martell Forest, a research forest owned and operated by Purdue University in West Lafayette, Indiana (40º26'42"N, 87º01'47"W). The trees selected for excavation and intensive biomass measurement were pure American chestnuts planted in 2008, immediately adjacent to the competition trial established by Gauthier et al. (2013) in 2007 ('unrelated chestnuts, Fig. 3.S1). The this stand of chestnuts was planted with 2.25 m between trees. The site is moderately well drained and was formerly used as an agricultural field. The main soil type is comprised of Rockfield silt loam, although the western edge of the experiment is characterized as 'somewhat poorly drained', with soil belonging to the Starks-Fincastle complex (USDA NRCS 2017). Mean annual temperature in West Lafayette, IN is 11.4º C and mean annual precipitation is 996 mm (data from 1981-2018, National Climatic Data Center 2019).

Data collection

Biomass measurements were made over six weeks, from late June through early August 2019. This sampling time was selected as the period of maximum leaf area for our trees. Eight trees were selected with a range of DBH measurements that was representative of the range of tree sizes in our adjacent competition trial. A single DBH measurement for multi-stemmed trees was calculated as the square root of the sum of each stem squared:

$$DBH_{total} = \sqrt{DBH_1^2 + DBH_2^2 \dots DBH_n^2}$$

Aboveground biomass measurements were taken following the guidelines of Picard et al. (2012). Trees were felled onto a tarp using a chainsaw. Biomass components were separated as follows: leaves, reproductive organs, branches (current-year, canopy, scaffold), and bole. Dry-weight biomass was determined following the 'rule of three' (Picard et al. 2012): total biomass of a given compartment was measured fresh in the field, an aliquot was also measured fresh the field, and that aliquot was measured again after it has been oven-dried to constant mass. The ratio of fresh:dry weight of the aliquot was then applied to the total fresh weight to give the dry weight for each component. The ratio of xylem vs phloem in the stem was determined in the lab by separating xylem and phloem tissue in 25 tree cookies sourced from the eight excavated trees. The diameter of tree cookies ranged from 2-18 cm.

Belowground biomass was excavated using a commercial pneumatic system (Air Spade® Series 2000, Concept Engineering Group, Inc., Verona, Pennsylvania) (Lavigne and Krasowski 2007). A pneumatic system has the advantage of dislodging soil around the root system without breaking even fine roots. We excavated roots to a maximum depth of c. 1 m. Not many roots grew deeper than 1 m; therefore, we did not correct for missing root mass. The excavated root system was partitioned into the stump, fine roots (<5 mm), roots 5-10 mm, roots 10-20 mm, roots 20-50 mm, and roots >50 mm. Dry-weight biomass of each compartment was determined following the 'rule of three' as above, with the addition of a rough washing step before oven drying in the lab.

Model fitting

Principal components analysis (PCA) was performed to assess relationships between predictor variables (DBH and height) and the biomass of each organ. Models were fit in the R statistical environment (R v.3.3.2) using generalized linear mixed models (GLMMs) from the *lme4*

package (Bates et al. 2015). A set of nested GLMMs encompassing a range of additive and multiplicative interactions between DBH, height, and biomass (both whole-tree and organ-specific) were created (Table A.1). Tree ID was included as a random effect, and we adapted the model optimizer using the *optimx* package (Nash et al. 2011). Models were evaluated based on AIC, log-likelihood, marginal R^2 (Nakagawa and Schielzeth 2013), and *P* value from likelihood ratio test. The best-performing model (lowest AIC, largest log-likelihood, highest marginal R^2) failed to converge with a maximum gradient of 0.784, perhaps due to over-fitting. Therefore, we selected a slightly simpler model, with height included as an additive fixed effect, as our final allometric model.

A.3. Results

We excavated chestnuts with a DBH ranging from 4.3-15.2 cm and height ranging from 5.95-10.0 m, which corresponded to whole-tree, dry-weight biomass of 5.92-99.16 kg (Fig. A.1). The first two principal components of our PCA analysis explained 94.6% of the variation in our biomass data and showed that DBH was more closely associated with the biomass of most organs than height (Fig. A.2). Our chosen model (biomass ~ organ $\times$ DBH + height + (1|tree)) had a marginal R^2 of 0.989, demonstrating that organ-specific and whole-tree biomass are strongly related to DBH and height (Table A.2). The error associated with our biomass estimates increased with DBH (Fig. A.1) as would be expected with an exponentially distributed response variable.

The strong interactions between DBH and organ type (Table A.2) indicate that the proportion of total biomass in each organ may change with tree size. We found a negative relationship between DBH and root-shoot ratio (RSR) ($F_{1,6}=3.941$; $R^2 = 0.396$; *P*=0.09; Fig. A.3), although our small sample size limits our ability to predict RSR with great precision. Our mean RSR was 32.4±2.9% SE. As DBH increased, we observed a reduction in the proportion of biomass in coarse roots, current-year twigs, and leaves, and we found an increase in the proportion of biomass in branches (Fig. A.4). The proportion of biomass in fine roots, root collar, xylem, and phloem did not vary appreciably with DBH.

A.4. Tables and Figures

Table A.1. Comparison of allometric models predicting whole-tree and organ biomass from DBH, height, and organ type. Tree ID was included as a random effect in all models (1|tree).

Model	Residual DF	AIC	log-likelihood	marginal R^2	*P* value
Tissue + DBH + (1|tree)	60	155.6	-65.8	0.977	< 0.001
Tissue × DBH + (1|tree)	52	145.6	-52.8	0.985	< 0.001
Tissue + height + (1|tree)	60	164.9	-70.4	0.955	< 0.001
Tissue × height + (1|tree)	52	156.5	-58.3	0.963	< 0.001
Tissue + DBH + height + (1|tree)	59	152.1	-63.0	0.982	< 0.001
Tissue × DBH + height + (1|tree)	**51**	**141.9**	**-50.0**	**0.989**	**< 0.001**
Tissue × DBH × height + (1|tree)*	34	136.7	-30.3	0.993	< 0.001

*model failed to converge with a maximum gradient of 0.784

Table A.2. Coefficients from allometric model. We used a Gamma distribution and log link. Tree ID was included as a random effect.

Model statement: biomass ~ organ × DBH + height + (1|tree)

| Parameter	Estimate	SE	t value	*P* value
(Intercept)	-2.293	0.378	-6.078	< 0.001
DBH	0.254	0.026	9.845	< 0.001
organ – coarse roots	1.225	0.321	3.818	< 0.001
organ – fine roots	-1.178	0.316	-3.727	< 0.001
organ – leaves	0.079	0.317	0.248	0.804
organ – inner bark	0.010	0.329	0.030	0.976
organ – root collar	-2.158	0.333	-6.473	< 0.001
organ – total biomass	2.456	0.323	7.615	< 0.001
organ – twigs	-1.730	0.308	-5.611	< 0.001
organ – xylem	1.634	0.329	4.960	< 0.001
Height	0.147	0.043	3.429	< 0.001
DBH × organ – coarse roots	-0.086	0.030	-2.882	0.004
DBH × organ – fine roots	-0.107	0.029	-3.664	< 0.001
DBH × organ – leaves	-0.076	0.031	-2.573	0.010
DBH × organ – Inner bark	-0.056	0.031	-1.841	0.066
DBH × organ – root collar	-0.094	0.030	-3.015	0.003
DBH × organ – total biomass	-0.057	0.029	-1.894	0.058
DBH × organ – twigs	-0.148	0.028	-5.201	< 0.001
DBH × organ – xylem	-0.059	0.031	-1.940	0.052
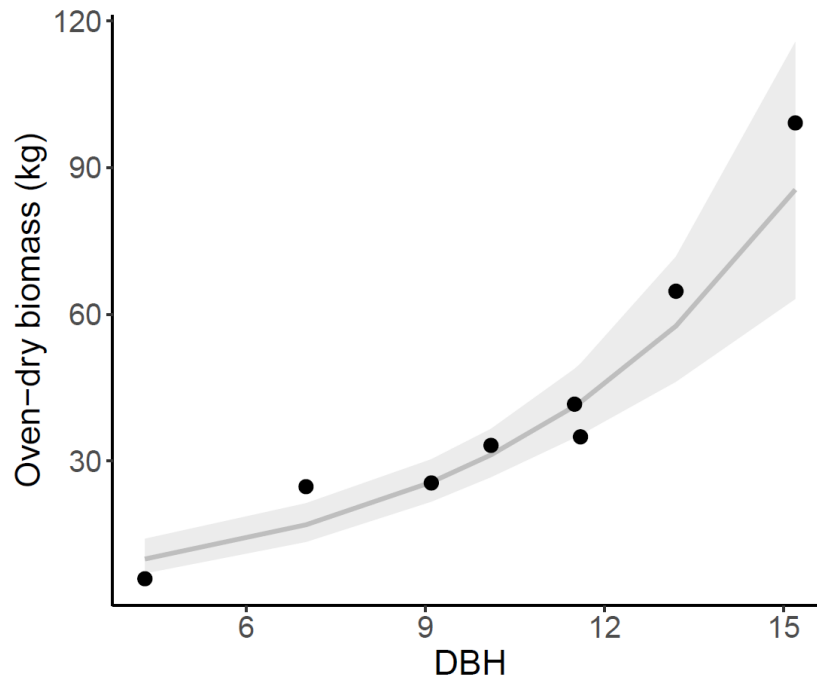
Figure A.1. Whole-tree biomass of the eight chestnuts excavated for this study. The grey line is predicted biomass from the allometric model, and the shaded area is our 95% CI. Height was fixed to the median height of excavated trees (8.7 m) for this figure to simplify interpretation.
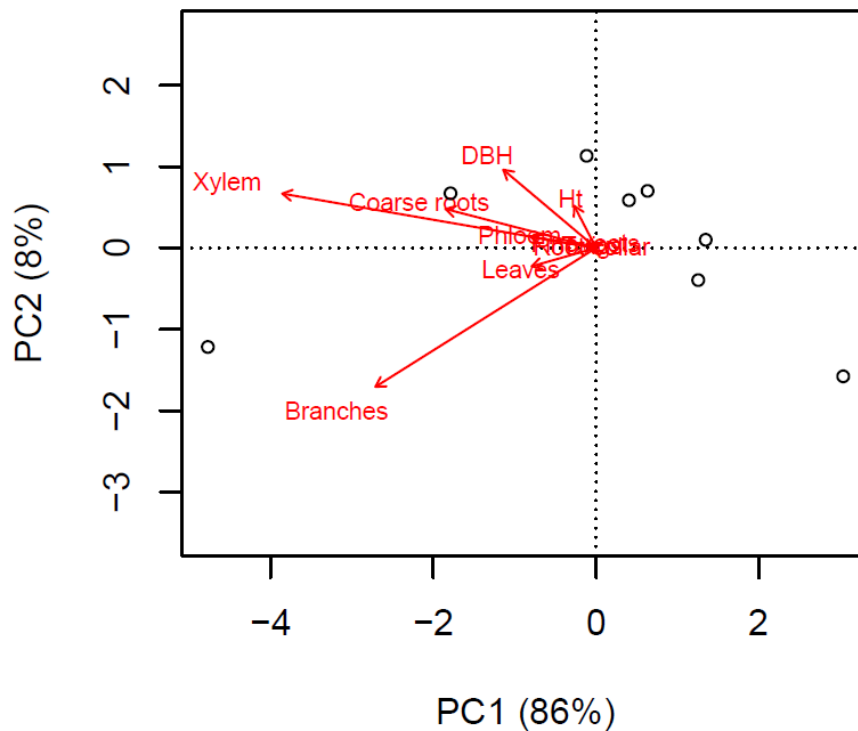
Figure A.2. Principle components analysis for DBH, height, and biomass data for each tree organ. The root collar, fine roots, and twigs are clustered around the center of the plot (0,0).
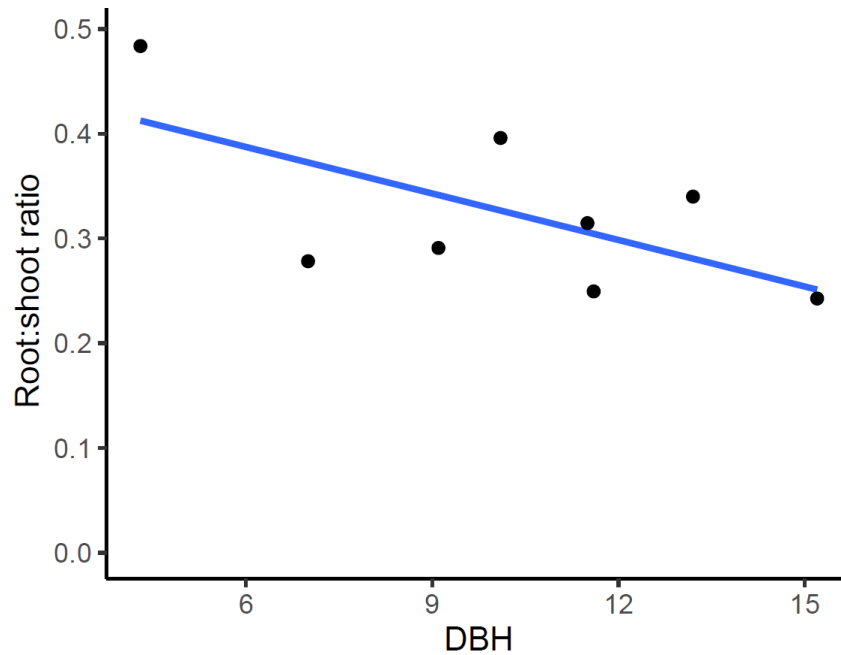
Figure A.3. Root:shoot ratio of the eight trees excavated for our allometric model.
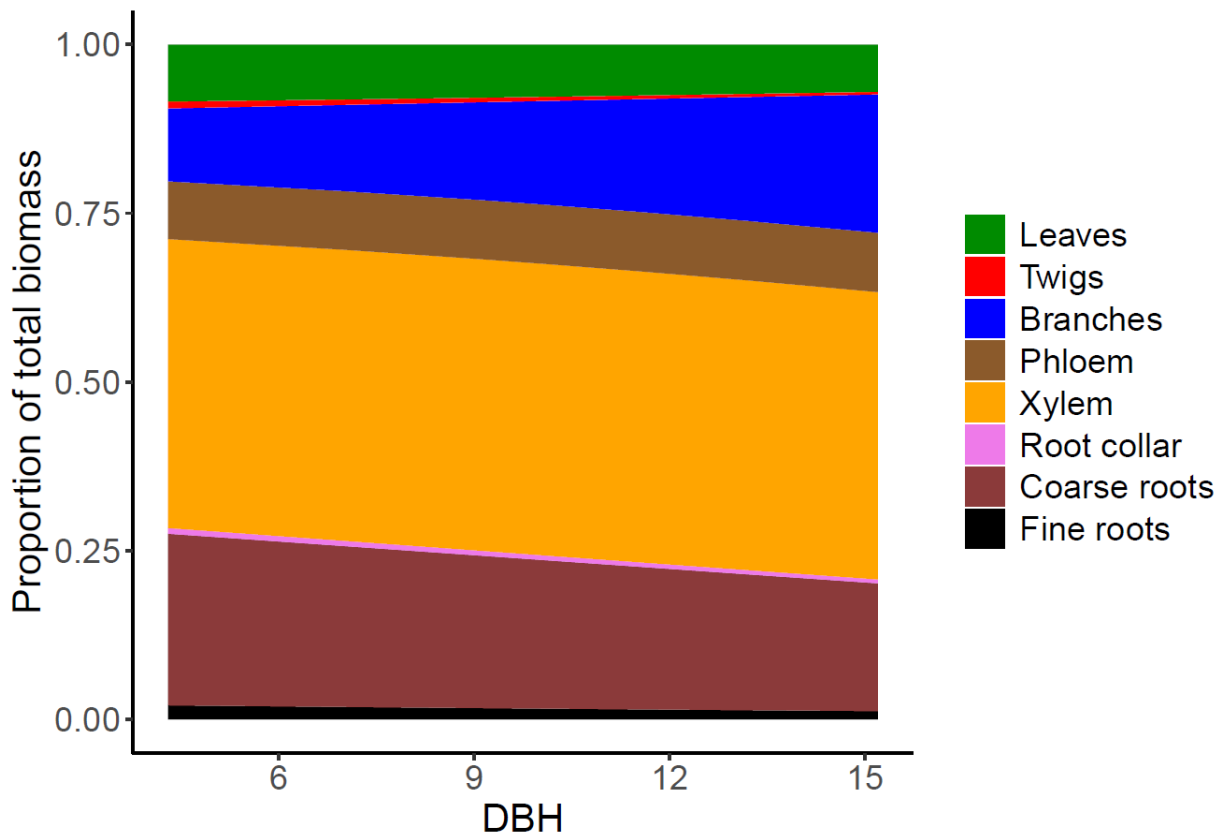
Figure A.4. Estimated mean biomass for each organ as a proportion of total biomass, based on our allometric model (n=8). Height was fixed to the median height of excavated trees (8.7 m) for this figure to simplify interpretation.

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