

CAUSES AND CONSEQUENCES OF VARIABLE EXTRAFLORAL NECTAR SECRETION IN  
QUAKING ASPEN (*POPULUS TREMULOIDES* MICHX.)


By


Jonathon R. Newman

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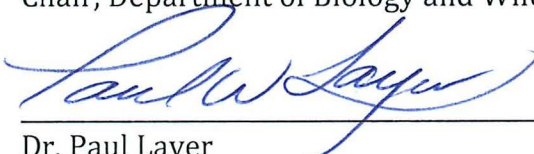
  
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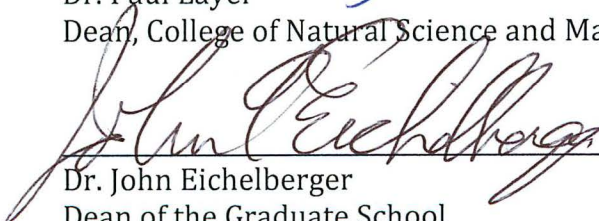
  
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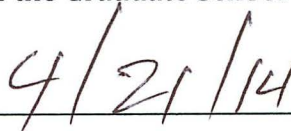
  
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CAUSES AND CONSEQUENCES OF VARIABLE EXTRAFLOURAL NECTAR  
SECRETIONS IN QUAKING ASPEN (*POPULUS TREMULOIDES* MICHX.)

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By

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## Abstract

Extrafloral (EF) nectaries mediate a defensive mutualism in many plant populations, wherein plants attract predatory arthropods by providing nectar rewards. The primary objectives of this study were to identify abiotic and biotic factors that may affect secretion by EF nectaries in quaking aspen (*Populus tremuloides* Michx.) and to determine how variation in secretion rate affects attractiveness of *P. tremuloides* ramets to predatory arthropods such as ants. I investigated the effects of water stress, defoliation, and genotype on extrafloral sugar secretions in *P. tremuloides* and tested how variations in EF sugar quantity affect ant visitation to *P. tremuloides* ramets in interior Alaska. Additionally, I analyzed *P. tremuloides* sugar composition from three genotypes. Extrafloral sugar secretions were inducible by defoliation, and the induction response was not inhibited by water stress. Irrespective of defoliation, water stress had a variable effect on sugar secretion rates between genotypes, with one out of four genotypes exhibiting a reduction in secretion rate in response to low water availability. Genotypes differed in secretion rates overall, which could potentially influence defensive levels among clonal stands. Ant visitation to ramets with experimentally increased sugar availability was increased for one of three genotypes in early summer, though in mid-summer ants did not respond to nectar supplementation. There was no effect of nectar reduction on ant visitation in either early or mid-summer trials. Genotypes attracted different average numbers of ants, which may have been a result of intrinsic variation in volatile organic compound emission, EF

nectar secretion rates, or nectar composition. Analysis of EF sugar secretions of *P. tremuloides* using nuclear magnetic resonance spectroscopy revealed that EF nectar tends to be dominated by sucrose over glucose and fructose. This composition may increase attractiveness to mutualistic ant species, which tend to favor sucrose dominated nectar blends. This study expands our knowledge of the sources of variation in EF nectar secretion and their impact in a widespread, ecologically important tree species.

## Table of Contents

Page

<b>Signature page</b> .....	<b>i</b>
<b>Title page</b> .....	<b>iii</b>
<b>Abstract</b> .....	<b>v</b>
<b>Table of Contents</b> .....	<b>vii</b>
<b>List of figures</b> .....	<b>ix</b>
<b>List of tables</b> .....	<b>xi</b>
<b>Acknowledgments</b> .....	<b>xii</b>
<b>Introduction</b> .....	<b>1</b>
<b>References</b> .....	<b>3</b>
<b>Chapter 1: The influence of water availability and defoliation on extrafloral nectar secretion in quaking aspen (<i>Populus tremuloides</i> Michx.)</b> .....	<b>7</b>
<b>1.1 Abstract</b> .....	<b>7</b>
<b>1.2 Introduction</b> .....	<b>8</b>
<b>1.3 Methods</b> .....	<b>11</b>
1.3.1 Study species.....	11
1.3.2 Ramet propagation.....	11
1.3.3 Experimental design.....	13
1.3.4 Nectar sampling.....	14
1.3.5 Sugar analysis.....	15
1.3.6 Statistical analysis .....	16
<b>1.4 Results</b> .....	<b>17</b>
<b>1.5 Discussion</b> .....	<b>19</b>
<b>1.6 References</b> .....	<b>25</b>
<b>Chapter 2: The impact of extrafloral nectar availability on ant visitation to boreal <i>Populus tremuloides</i> (Michx.)</b> .....	<b>41</b>
<b>2.1 Abstract</b> .....	<b>41</b>
<b>2.2 Introduction</b> .....	<b>42</b>
<b>2.3 Methods</b> .....	<b>43</b>
2.3.1 Propagation .....	43
2.3.2 Experimental setup .....	45
2.3.3 Statistical Analysis.....	48
<b>2.4 Results</b> .....	<b>49</b>
<b>2.5 Discussion</b> .....	<b>50</b>

2.6 References .....	57
<b>Chapter 3: Sugar composition of extrafloral nectar of <i>Populus tremuloides</i> ...</b>	<b>65</b>
3.1 Abstract .....	65
3.2 Introduction.....	66
3.3 Methods.....	67
3.3.1 Statistical analysis .....	68
3.4 Results/Discussion .....	69
3.5 References .....	71
<b>Conclusion .....</b>	<b>75</b>

<b>List of Figures</b>	<b>Page</b>
<b>Figure 1.1</b> EF sugar production by defoliated (white bars) and undefoliated (black bars) <i>Populus tremuloides</i> ramets at 2, 4, and 6 days after defoliation. ....	37
<b>Figure 1.2</b> Effect of water availability on EF nectar production in different <i>Populus tremuloides</i> genotypes (denoted by different symbols).....	38
<b>Figure 1.3</b> EF sugar secretion among <i>Populus tremuloides</i> genotypes at 2, 4, and 6 days after defoliation.....	39
<b>Figure 2.1</b> Setup of experimental <i>P. tremuloides</i> ramets around a central ant colony. ....	62
<b>Figure 2.2</b> Mean number of ants on ramets with reduced, unmanipulated, and supplemented nectar by <i>P. tremuloides</i> genotype. ....	63
<b>Figure 2.3</b> Mean number of ants on ramets of different <i>P. tremuloides</i> genotypes. ....	64
<b>Figure 3.1</b> NMR spectrum of extrafloral sugars present in nectar of <i>P. tremuloides</i> . ....	73
<b>Figure 3.2</b> Relative sugar abundance in extrafloral nectar of three <i>P. tremuloides</i> genotypes.....	74





<b>List of Tables</b>	<b>Page</b>
<b>Table 1.1</b> Results of repeated measures ANOVA comparing effects of defoliation, water availability, and genotype on mean EF sugar secretion rates in <i>Populus tremuloides</i> .....	35
<b>Table 1.2</b> Locations of <i>Populus tremuloides</i> genotypes, collected from naturally occurring stands in interior Alaska.....	36

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## Introduction

Plants employ a wide variety of defenses to reduce herbivory, ranging from direct chemical or physical defenses to indirect biotic defenses. Direct defenses are chemicals or structures that injure herbivores or deter them from feeding on plant tissues. Indirect defenses involve the attraction of predatory or parasitic arthropods which reduce herbivore damage by consuming or molesting the herbivore (Rudgers *et al.* 2003). Indirect defenses are traits that attract predators, and can be predator rewards such as food bodies, extrafloral (EF) nectar secretions, or domatia (structural components which house predators), as well as chemical attractants such as volatile organic compounds (VOCs) (reviewed by Heil 2008). EF nectaries are nectar-secreting glands located on non-flowering structures; typically stems, leaves, and bracts, and are found in over 100 plant families (Keeler 2008). In many plant populations, EF nectaries have been shown to reduce herbivory by attracting predators and parasitoids of herbivores (Bentley 1977; Rosumek *et al.* 2009). The primary nutritional component of EF nectar is carbohydrate, chiefly in the form of fructose, glucose, and sucrose (Wäckers 2005).

For many plant species, plasticity exists in levels of defense expression. Plant defense theory predicts that there are trade-offs between the expression of defensive traits and growth and reproduction, and that the relative costs and benefits of a defense can change based on a variety of biotic and abiotic factors such as inherent growth rate, nutrient availability and tissue susceptibility (reviewed by Stamp 2003). Due in part to these variable costs and benefits, plants may increase

investment in defenses during periods of increased vulnerability to herbivore feeding or decrease investment during severe environmental stress, such as drought (Herms and Mattson 1992). Increased expression of plant defenses in response to herbivory (induction) is mediated by the octadecanoid pathway, a signaling cascade involving synthesis of jasmonic acid, which ultimately triggers transcription factors for defense-related genes (reviewed in Lambers *et al.*, 2008).

Plasticity in defense expression may affect subsequent herbivory, impacting overall plant fitness. In the case of indirect defenses, variable investment in rewards may affect the strength of the interaction between plants and defending mutualists. Experimental induction of EF nectar secretions using jasmonic acid can increase plant fitness (Heil 2004), but this method for testing the defensive benefit of nectar induction does not control for induction of other defenses, including VOCs involved in attracting predatory mutualists (discussed in Kost and Heil 2005). Supplementing EF nectar with synthetic nectar can increase ant visitation to plants with EF nectaries (Kost and Heil 2005). Conversely, experimental reduction in EF nectar quantity led to reduced ant visitation in several plant populations (e.g., Apple and Feener 2001; Rudgers and Strauss 2004; Chamberlain and Holland 2008).

*Populus tremuloides* is the most widespread native tree species in North America (Barnes and Han 1993). It is an ecologically important tree species, serving as a major source of browse (e.g., Beck *et al.* 2006) and supporting high plant and animal biodiversity (e.g., Chong *et al.* 2001). In recent years, *P. tremuloides* has undergone large scale mortality and dieback in many regions of the United States

and Canada, which has been largely attributed to drought stress (Michaelian *et al.* 2011; Huang and Anderegg 2012). In interior Alaska, *P. tremuloides* has sustained high levels of insect herbivory over the past decade, largely due to recent population irruptions of *Phyllocnistis populliella* (aspen leaf miner) and *Choristoneura conflictana* (large aspen tortrix) (Wagner and Doak 2013; U.S. Forest Service, 2014). In addition to direct effects on survival, these stresses may affect expression of defenses such as EF nectar, which may impact attraction of predatory mutualists to *P. tremuloides* ramets.

This thesis explores the causes and consequences of variation in EF nectar secretion in *P. tremuloides*. The study detailed in the first chapter examines the effects of water stress, defoliation, and genotype on EF sugar secretion rates. The second chapter explores how variations in sugar secretion affect the strength of the interaction between *P. tremuloides* and a local ant species, *Formica fusca*. Finally, in the third chapter, I describe the sugar components of *P. tremuloides* EF nectar, analyzed using nuclear magnetic resonance spectroscopy.

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## **Chapter 1: The influence of water availability and defoliation on extrafloral nectar secretion in quaking aspen (*Populus tremuloides* Michx.)<sup>1</sup>**

### **1.1 Abstract**

In recent years, water stress has led to widespread growth declines and dieback of several North American tree species. In addition to its direct effects on tree physiology, water stress may compromise anti-herbivore defenses. We tested whether extrafloral (EF) sugar secretion rate in *Populus tremuloides* Michx. (quaking aspen) increases in response to defoliation, and whether water stress impairs constitutive and inducible EF sugar secretion. We subjected *P. tremuloides* ramets of four genotypes to water restriction and defoliation in a factorial design, and measured EF sugar secretion rates 2, 4 and 6 days after defoliation. The sugar secretion rate of defoliated ramets was significantly higher than that of undefoliated ramets 6 days post-defoliation. Low water availability reduced the sugar secretion rate of one of the four aspen genotypes, but did not prevent induction. *Populus tremuloides* genotypes secreted EF sugar at different average rates, suggesting genetic variation for this trait. The results indicate that EF nectar secretion is inducible in *P. tremuloides*, which could increase the effectiveness of indirect defense following herbivory. Genotypic differences in the response of *P. tremuloides*

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<sup>1</sup> Newman JR, and Wagner D (2013) The influence of water availability and defoliation on extrafloral nectar secretion in quaking aspen (*Populus tremuloides* Michx). *Botany* 91(11): 761-767. (With slight modifications)

to water stress suggest that some clonal stands may be at a disadvantage when faced with compound stresses of drought and herbivory.

## **1.2 Introduction**

Increased frequency of drought associated with climate change has led to dieback and mortality of several tree species in western North America (van Mantgem *et al.* 2009; Allen *et al.* 2010). For instance, *Populus tremuloides* Michx. (quaking aspen) has suffered widespread dieback in several regions of North America, attributed largely to drought stress (Michaelian *et al.* 2011; Huang and Anderegg 2012). In general, drought may directly impact tree performance through hydraulic failure or carbon starvation (McDowell *et al.* 2008). These physiological responses may also lead to greater susceptibility to herbivores due to compromised anti-herbivore defenses (Breshears *et al.* 2009). The interaction between drought and herbivory are complex, but overall water-stressed plants tend to sustain higher foliar herbivory than controls, which may be a result of increased nutritional quality in foliar tissues or compromised defenses of drought-stressed plants (Jactel *et al.* 2012). A better understanding of how water stress impacts plant defenses is critical to our ability to predict the impact of future drought stress on plant populations.

The growth-differentiation balance hypothesis predicts that investment in carbon-based defenses will decrease in response to severe water stress (Herms and Mattson 1992). Prolonged water stress increases stomatal closure, reducing photosynthetic capacity and carbon assimilation, which is predicted to reduce the

available pool of carbon for allocation to carbon-based defenses (McDowell 2011). One such defensive trait that might be compromised by drought is extrafloral (EF) nectar, a secretion composed chiefly of carbohydrates that mediates indirect defense in some plant species (Koptur 2005). Additionally, during times of low water availability, water resources may be diverted away from defenses that require aqueous transport, such as EF nectar. In addition to impairing defenses overall, low water availability might inhibit the damage-induction response, preventing plants from responding to herbivory with increased levels of defense (e.g., Olson *et al.* 2009). Several studies have investigated the effects of water stress on damage-induction of chemical defense (Lombardero *et al.* 2000; Hale *et al.* 2005; Olson *et al.* 2009; Halpern *et al.* 2010; and Gutbrodt *et al.* 2011), yielding mixed results; the effect of water stress on damage-induction of EF nectar secretion is currently unknown.

Extrafloral nectar is predicted to be inducible in populations subject to variable levels of herbivory, especially those with high growth demands (Karban 2011). There are two ways in which EF nectar can be induced: plants can express more nectaries on new tissues and, on shorter time scales, increase nectar concentration or secretion rates from existing nectaries. Increases in EF nectary expression following damage have been documented for several plant species (Ness 2003; Mondor *et al.* 2006; Wooley *et al.* 2007; Pulice and Packer 2008). Additionally, increases in EF nectar secretion rate have been reported following

mechanical damage, herbivory, or application of jasmonic acid or volatile organic compounds (e.g. Heil 2004; Choh and Takabayashi 2006; Holland *et al.* 2009).

*Populus tremuloides* (quaking aspen) bears EF nectaries on a subset of its leaves. EF nectaries appear to function as an indirect defense against herbivory for *P. tremuloides*, as ramets with a greater proportion of nectary-bearing leaves suffer less damage from the leaf miner *Phyllocnistis populiella* Cham. (Mortensen *et al.* 2011; Mortensen *et al.* 2013). Nectary expression in *P. tremuloides* is highly variable. The frequency with which *P. tremuloides* expresses EF nectaries is heritable (Wooley *et al.* 2007), but within ramets, expression varies with ramet ontogeny and leaf position along the shoot (Doak *et al.* 2007). The proportion of *P. tremuloides* leaves expressing EF nectaries has been shown to increase the year after heavy defoliation (Wooley *et al.* 2007); however, this delayed induction response cannot contribute to defense until new leaves develop. An increase in the quality or quantity of EF nectar produced by existing nectaries might allow a ramet to recruit predators on a shorter time scale, providing a more rapid defensive response. Here we test the hypotheses that EF nectar secretion rate is inducible by defoliation in *Populus tremuloides*, and that water stress reduces the secretion rate of EF nectar and prevents induction of EF nectar.

## 1.3 Methods

### 1.3.1 Study species

*Populus tremuloides* Michx. (Salicaceae) has the widest distribution of any native tree species in North America, and is the second most widely distributed tree species in the world (Barnes and Han 1993). *Populus tremuloides* forest stands support high plant and animal biodiversity (Turchi *et al.* 1995; Stohlgren *et al.* 1997; Stohlgren *et al.* 1999; Chong *et al.* 2001), and *P. tremuloides* tissue serves as an important source of browse for valued wildlife species (Oldemeyer 1997; Beck *et al.* 2006). In interior Alaska, *P. tremuloides* is an early successional species commonly found on ridgetops and south-facing slopes, sometimes intermixed with white spruce (*Picea glauca* (Moench) Voss), balsam poplar (*Populus balsamifera* L.) and Alaskan birch (*Betula neoalaskana* Sarg.). In addition to sexual reproduction, *P. tremuloides* propagates via suckering, forming clonal stands. The average area of monoclinal stands in the Pacific west of North America has been estimated to be 0.32 to 0.46 ha (Hipkins and Kitzmiller 2004; De Woody *et al.* 2009). *Populus tremuloides* expresses nectaries on a subset of its leaves, located at the junction of the leaf and the petiole. The number of EF nectaries per leaf varies, with most leaves expressing zero or two nectaries.

### 1.3.2 Ramet propagation

Replicate ramets from *P. tremuloides* genets were propagated from root stock in the Institute of Arctic Biology Research Greenhouse, located on the University of

Alaska Fairbanks campus. We collected a single sample of root tissue from each of four putative *P. tremuloides* genets located a minimum of 2.1 km apart in the Tanana Valley in interior Alaska (Supplementary Table S1). In the greenhouse, we propagated *P. tremuloides* using a protocol by Barry and Sachs (1968). Beginning in July 2010, we buried each root sample singly into vermiculite, and harvested the adventitious shoots that emerged when they reached 5-15 cm in height. Before planting, we dipped the bases of each shoot into an aqueous solution containing 1000 ppm indole-3-butyric acid, a rooting hormone. Shoots were then transferred to a vermiculite medium in individual pots and placed under intermittent mist until they had formed roots. We transferred rooted shoots (hereafter “ramets”) into 2L pots containing a mixture of vermiculite, perlite, and coconut coir (1:1:1 by volume). In December 2010, we placed all ramets into a growth chamber and forced them into dormancy by ramping down the temperature and hours of light over 7 days. In May 2011, we removed ramets from the growth chamber and placed them into the greenhouse under high pressure sodium lights with a 21:3 L:D light cycle to break dormancy. This overwintering process resulted in ramets with multiple shoots, rather than the single central stem of a seedling. Pots were placed in random order approximately 0.75 m apart on the greenhouse bench. Prior to the experiment, ramets were given aqueous fertilizer following every watering (17:5:7 N:P:K), approximately every 3 d.

### 1.3.3 Experimental design

We randomly assigned two ramets from each of the four clones to each of two water and two defoliation treatments in a factorial design (n = 32 experimental ramets). Because our goal was to measure the effect of water availability on defensive responses, we began the water treatments 11 d before ramets were defoliated. We applied two watering treatments: water stressed and well-watered (control). Ramets in the water stress treatment were watered to saturation only when soil moisture reached 8-10% water by volume, the moisture range at which *P. tremuloides* leaves began to wilt in the potting media (established through independent tests), on average every 2 d. Well-watered ramets were watered to saturation more often, whenever soil reached 20% water by volume. We measured soil moisture every 3 h between 9:00 and 21:00 using an SM-200 Moisture Sensor and an HH2 Moisture Meter (Delta-T Services). We recorded number of water applications as a measure of the rate at which the soil dried. Aqueous fertilization was discontinued once water treatments were applied, and plants were provided with 1.5 g/L time-release Osmocote fertilizer (14:14:14 N:P:K).

To assess the effects of simulated herbivory on EF nectar secretions, we mechanically defoliated ramets using scissors. Eleven days after initiating the water treatments, we removed a total of 40% of the area of each leaf for ramets in the defoliation treatment. Leaf area was removed from plants in two successive bouts spaced 12h apart. During each bout we removed approximately 20% of total,



original leaf tissue from the distal end of each leaf. Because EF nectaries occur near the petiole, removal of leaf tissue did not affect the number of EF nectaries present.

#### **1.3.4 Nectar sampling**

To determine the rate of nectar secretion, we repeatedly measured the total sugar content of nectar secretions on a subset of leaves. All sampled leaves were “preformed”, expanded from primordia in buds produced prior to dormancy. We randomly selected four shoots within each ramet, and tagged the two most proximal nectary-bearing leaves on each shoot ( $n = 8$  leaves per ramet). The number of nectaries per sampled leaf varied between one and five, with the majority of leaves (87%) expressing two nectaries. To control the duration of nectar accumulation, we rinsed EF nectaries with reverse-osmosis water 2 d prior to the first nectar collection. Nectar sampling was conducted 2 d before, and 2, 4, and 6 d after defoliation.

We sampled nectar on small filter paper wicks, following a protocol modified from McKenna and Thompson (1988). When more than one EF nectary occurred on a leaf, they were clustered too close together to sample individually, and we therefore collected nectar from all EF nectaries on a leaf at each sampling. On each sampled leaf, we injected 2  $\mu$ L of deionized water onto the site of the EF nectaries to dissolve nectar solids, and then collected the solution onto one or more small wicks cut from Whatman No.1 chromatography paper. We then repeated the injection and collection to remove any remaining nectar. In an independent test, we established

that collecting nectar twice in this fashion removed  $98.7 \pm 1.1\%$  ( $n = 12$ ) of secreted sugar from the nectary surface. After collection, we immediately placed wicks from a single leaf into a boil-proof microcentrifuge tube and placed the tubes on ice for transport. In the laboratory, we opened the tubes and placed them into a drying oven at  $60\text{ }^{\circ}\text{C}$  for 24 h, then transferred them to a desiccation cabinet for 48 h. Following drying, samples were stored at  $-80\text{ }^{\circ}\text{C}$  until they were analyzed for sugar content.

### **1.3.5 Sugar analysis**

In order to measure sugar content of the accumulated nectar, we used the anthrone spectrophotometry method, which determines total, combined content of glucose, sucrose, and fructose (Umbreit and Burris 1972, McKenna and Thompson 1988). To reconstitute nectar samples, we added 1 mL nanopure water to each microcentrifuge tube, then placed the tubes into a boiling water bath for 5 min. For each sample, we combined an aliquot of 0.5 mL of reconstituted nectar solution with 1 mL of anthrone reagent (0.1 g anthrone powder into 50 mL concentrated  $\text{H}_2\text{SO}_4$ ), placed the mixture into a boiling water bath for 10 minutes, then cooled samples on ice. Absorbance of the sample was read at 620 nm on a Beckman DU 640B spectrophotometer (Beckman Coulter, Brea, California, USA). We inferred the concentration of sugars from a best-fit curve created using sugar standards of equal parts fructose, glucose, and sucrose ranging in concentration between 0 and  $100\text{ }\mu\text{g}\cdot\text{mL}^{-1}$ . We also ran 15 blank samples containing clean, unused wicks. The wicks

produced a small, but measurable, absorbance. We subtracted the mean value for blanks from the measured sugar concentration of each nectar sample, after adjusting for the number of wicks on which nectar was collected. This yielded the calculated sugar concentration for nectar samples, which was averaged across all leaves within each ramet for each collection day. Values are reported as mean mass of sugar secreted per leaf per day.

### **1.3.6 Statistical analysis**

We assessed the main effects of defoliation, water availability, and genotype on *P. tremuloides* EF sugar secretion rate using a mixed-model, repeated-measures ANOVA. The main effects were crossed in a full factorial design with the repeated factor, date of collection (hereafter “time”). The repeated subject, the *P. tremuloides* ramet, was treated as a random variable and nested within the main effects. Sugar secretion over the 48 hours pre-defoliation was included in the model as a covariate to account for variation among leaves in the number of EF nectaries. The dependent variable, daily sugar secretion averaged by ramet, was square-root transformed to meet test assumptions. Means were compared following ANOVA using Tukey’s HSD tests.

A potential criticism of this experimental design is that defoliation and water treatments could interact through their effects on transpiration. Specifically, the reduction in leaf area in defoliated ramets could decrease transpiration and water use, slowing the rate of soil drying for defoliated relative to undefoliated plants

(Georgiadis *et al.* 1989), potentially leading us to underestimate the effect of water stress on defoliated plants. The number of watering events after defoliation provides a measure of the rate of soil drying. Therefore we examined whether the number of watering events after defoliation was related to the fixed effect of defoliation while controlling for the random effect of number of shoots per plant (a proxy for ramet size).

All analyses were conducted using JMP 9 software (SAS Institute, Cary, NC). Denominator degrees of freedom for *F*-tests were calculated using the Kenward-Rogers method (Kenward and Roger 1997).

#### **1.4 Results**

Defoliation induced a greater average rate of extrafloral sugar secretion by preformed leaves of *P. tremuloides* plants. The effect of defoliation on nectar secretion increased over time (Table 1.1). There was no significant difference in the mean secretion rates of defoliated and undefoliated ramets two and four days post-defoliation, but by day six the secretion rate of defoliated ramets was 23.2% higher than that of undefoliated ramets (Fig. 1.1). EF sugar secretion increased over time for undefoliated ramets as well as defoliated ramets, though to a lesser extent. This led to a significant overall effect of time on sugar secretion rate (Table 1.1), with ramets secreting, on average, 14.4% more sugar on day 4 than on day 2, and 23.9% more sugar on day 6 than on day 4.

Water stress reduced average, overall sugar secretion rate in a genotype-specific manner. Whereas three of the four *P. tremuloides* genotypes used in the study did not alter mean EF sugar secretion in response to water availability, one genotype reduced sugar secretion rates by an average of 28% when subjected to water stress; within this genotype, water stress affected defoliated and undefoliated ramets similarly (Fig. 1.2, Table 1.1). Overall, water treatment did not interact significantly with the combined effects of defoliation and time, indicating that water availability did not affect nectar induction (Table 1.1).

*Populus tremuloides* genotypes varied significantly in overall mean rate of EF sugar secretion (Table 1.1); the average sugar secretion rate of the most productive clone (clone WD,  $55.2 \mu\text{g}\cdot\text{leaf}^{-1}\cdot\text{d}^{-1} \pm 1.8 \text{ SE}$ ) was 18.7% higher than that of the least productive clone (clone NT,  $46.5 \mu\text{g}\cdot\text{leaf}^{-1}\cdot\text{d}^{-1} \pm 1.7 \text{ SE}$ ). Genotypes also varied in the change in rate of sugar secretion over time (Table 1.1): there was a significant overall increase in sugar secretion rate over time for three of four genotypes in the study (Fig. 1.3).

Average post-defoliation EF sugar secretion rates were positively associated with pre-defoliation rates (Table 1.1), indicating that differences among ramets in secretion rate were somewhat conserved over time, independent of genotype and treatment. These intrinsic differences among ramets in secretion rate may be related to EF nectary expression, because average secretion rate by a ramet prior to defoliation was positively related to the average number of EF nectaries per sampled leaf ( $\beta = 69.5$ ,  $R^2 = 0.21$ ,  $F_{1,30} = 8.16$ ,  $P = 0.0077$ ). However, differences in

EF nectary expression had little effect on the experimental results. There were no significant differences in the average number of EF nectaries per sampled leaf between defoliation treatments ( $F_{1,30} = 0.11, P = 0.75$ ), between water treatments ( $F_{1,30} = 0.16, P = 0.70$ ), or among genotypes ( $F_{3,28} = 0.53, P = 0.67$ ).

There was no evidence that defoliation slowed the rate of soil dry-down relative to undefoliated ramets. The number of watering events experienced by experimental ramets did not vary between defoliation treatments ( $F_{1,13} = 1.809, P = 0.20$ ).

## 1.5 Discussion

Recent declines in survival and growth rates of boreal forest *P. tremuloides* appear to be associated with both drought and insect attack (e.g. Soja *et al.* 2007; Welp *et al.* 2007; Hogg *et al.* 2008; Wagner and Doak 2013), and these environmental pressures are likely to continue in the future. Temperatures in interior Alaska increased approximately 1.4 °C between 1906 and 2006, with no significant change in precipitation between 1916 and 2006 (Hinzman *et al.* 2005; Wendler and Shulski 2009). Continued warming and a longer growing season may outweigh the modest increases in precipitation that are predicted for interior Alaska in the future (Rupp and Springsteen 2009), potentially leading to drier soils and an increased incidence of water stress for *P. tremuloides*. Climate change may also increase herbivore pressure imposed on *P. tremuloides* and other boreal forest tree species by insect populations (Volney and Fleming 2000; Soja *et al.* 2007).

Our results show that the effect of water availability on EF sugar secretion depended on genotype. Water stress caused a reduction in the average sugar secretion rate of the WD genotype, while the remaining three genotypes maintained secretion rates similar to well-watered plants. Ramets of the WD genotype also had the highest average secretion rate of the genotypes, suggesting there may be a trade-off between drought resistance and nectar secretion rate. While experimental evidence supports the hypothesis of a trade-off between drought resistance and defense (Haugen *et al.* 2008), a test involving greater numbers of genotypes will be necessary to test this hypothesis for *P. tremuloides*. More generally, our results indicate that drought stress may reduce nectar secretion rates and defense for some clonal *P. tremuloides* stands. To our knowledge, only one other published study has addressed how water stress affects EF nectar secretion. In that study, water stress reduced the average EF secretion rate of the southeast Asian shrub *Mallotus japonicus* (Yamawo *et al.* 2012).

Although water limitation reduced the average overall sugar secretion rate of one genotype, there was no evidence that water limitation prevented the induction response to leaf damage. This contradicts the growth-differentiation balance hypothesis, which predicts that induction of defenses should be inhibited in water-stressed plants (Herms and Mattson 1992). It is possible that the water restriction treatment was not strong enough or imposed for sufficient length to inhibit induction of EF nectar. However, if this were the case, we would not expect ramets of any genotype to show a response to the water reduction treatment. Given that

ramets of the WD genotype secreted EF sugar at a lower mean rate when water was restricted than abundant, it is unlikely that the lack of inhibition of induction was due to an insufficient water stress treatment. The results of studies testing the effects of water inhibition on defenses are mixed. While several studies report no effect of water availability on damage-induction of defenses (Hale *et al.* 2005; Gutbrodt *et al.* 2011), others report inhibition of defense induction in response to water stress (Lombardero *et al.* 2000; Olson *et al.* 2009; Halpern *et al.* 2010). Responses to water stress may vary among species or defense type; more research is required to understand the reasons for these differing results.

The strength of defensive mutualisms, such as those mediated by EF nectar, can vary based on the quantity and quality of rewards provided by the plant (Kessler and Heil 2011). If predatory arthropods respond to increases in nutritional rewards with increased visitation or intensity of protective behaviors, as has been reported for several other plant species (e.g. Heil *et al.* 2001; Ness 2003; Kost and Heil 2005), increases in sugar secretion could increase the protective benefit of EF nectaries for *P. tremuloides*. In our study, mechanical defoliation in *P. tremuloides* induced higher EF sugar secretion within 6 days of damage. There is no evidence to date that increases in nectar availability increase protective benefits for *P. tremuloides*, but blocking EF nectaries does reduce ant visitation (Mortensen *et al.* 2013).

In addition to the induction response, we observed a general increase in EF sugar secretion in both defoliated and undefoliated ramets during the course of the



study. It is possible that nectar sampling itself increases the rate of sugar secretion by EF nectaries in *P. tremuloides*. Previous work on other plant species has shown EF nectar to be inducible in response to manual nectar removal (Heil *et al.* 2000) as well as ant visitation (Bixenmann *et al.* 2011). A second possibility is that undefoliated ramets in our study increased sugar secretion in response to volatile organic compounds (VOCs) released by defoliated neighbors. A recent study by Li *et al.* (2012) found increased EF sugar secretion in undamaged hybrid *Populus tremula* x *P. tremuloides* ramets exposed to volatiles from damaged conspecifics. An increase in EF nectar sugar secretion by undamaged ramets in response to VOC signals from damaged neighbors would tend to diminish differences between treatments groups. Therefore differences between defoliated and control treatments might occur earlier and at higher magnitude than suggested by the comparison of means in Fig. 1.

The EF induction response to damage was delayed by 4 to 6 days post-damage. One drawback of induced defenses is the associated lag time between defoliation and the activation of a defense (Heil and Baldwin 2002). While the true lag time of this induction response may be shorter than the observed, any lag represents a period of vulnerability. This is likely more pronounced for indirect than direct defenses, as there is yet an additional lag time between activation of the defense and the discovery by and recruitment of predatory defenders. Induction of EF nectar triggered by damage or application of jasmonic acid has been observed in other plant species after 8 h (Holland *et al.* 2009), 24 h (Koptur 1989; Heil *et al.*

2001; Wäckers and Bezemer 2003; Heil 2004), 36h (Ness 2003) and 48 h (Wäckers and Wunderlin 1999; Wäckers *et al.* 2001; Choh and Takabayashi 2006) following treatments. Plants with inducible nectar rewards, such as juvenile *P. tremuloides* ramets, may reduce the problem of lagged induction by maintaining lower constitutive levels of sugar secretion when undamaged, presumably sustaining baseline visitation by predatory arthropods and allowing rapid detection of increases in sugar secretion, as suggested by Wäckers *et al.* (2001).

Plants may respond differently to mechanical damage and true herbivory due to herbivore-derived elicitors including the timing, continuity, and mechanism of tissue removal, as well as compounds present in saliva (Lehtila and Boalt 2004). In cotton, EF nectar is induced to the same degree by both mechanical damage and herbivore feeding (Wäckers and Wunderlin 1999). In *P. tremuloides*, several defensive compounds are inducible by both mechanical damage and herbivory, including phenolic glycosides (Clausen *et al.* 1989; Young *et al.* 2010), trypsin inhibiting compounds (Haruta *et al.* 2001a), and polyphenol oxidase (Haruta *et al.* 2001b), though the magnitude of induction has not been compared between damage types. Our results differ from those of a recent study by Li *et al.* (2012), who found that EF sugar secretion in a *Populus tremula* x *P. tremuloides* hybrid decreased, rather than increased, following feeding by *Epirrita autumnata* (Li *et al.* 2012). Hypotheses to account for this decrease include damage to EF nectaries, depression of photosynthesis due to critical levels of leaf area removal, and the ability of *E.*

*autumnata* to suppress EF nectar induction (Li *et al.* 2012; Heil 2012). Suppression of plant defenses has been reported for several herbivore species (Alba *et al.* 2011).

We have provided the first evidence that defoliation in *P. tremuloides* leads to a higher rate of EF sugar secretion. In addition, we report significant variation among *P. tremuloides* genotypes in both overall EF sugar secretion rates and response to water stress. Genotypic variation in the expression of EF nectaries on leaves has been reported previously (Wooley *et al.* 2007). Taken together, these results suggest that there may be considerable variability in the costs and benefits of EF nectar as a defense among natural clonal stands of *P. tremuloides*, especially those subject to drought. Additional studies in other parts of *P. tremuloides*'s range are needed to clarify the function and responsiveness of EF nectaries in natural populations of this widespread and ecologically important tree species.

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**Table 1.1** Results of repeated measures ANOVA comparing effects of defoliation, water availability, and genotype on mean EF sugar secretion rates in *Populus tremuloides*.

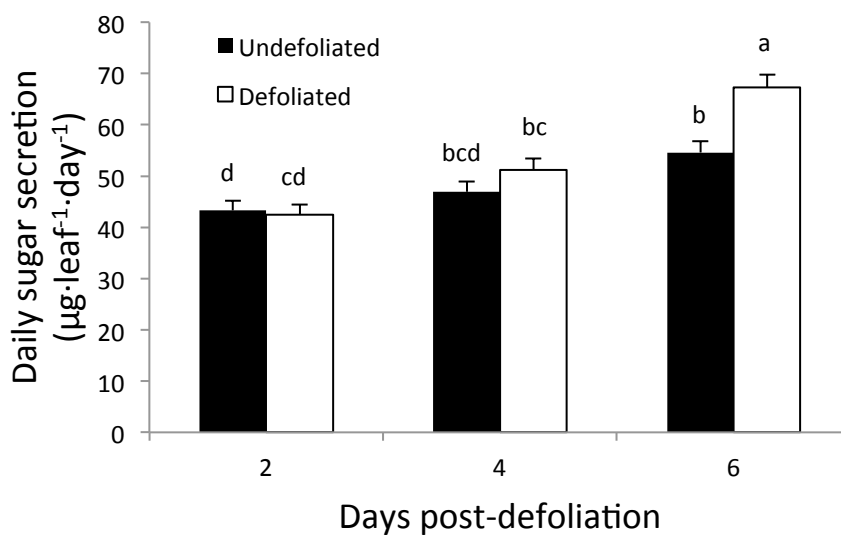
Source of Variation	DF		<i>F</i> -value	<i>P</i> -value
	DF	Denominator		
Water	1	15	2.974	0.1052
Defoliation	1	15	5.4354	<b>0.0341</b>
Genotype	3	15	4.4708	<b>0.0197</b>
Time	2	32	48.3628	<b>&lt;.0001</b>
Water x Defoliation	1	15	0.1153	0.7389
Water x Genotype	3	15	5.2883	<b>0.0109</b>
Water x Time	2	32	1.7379	0.1921
Defoliation x Genotype	3	15	0.6022	0.6235
Defoliation x Time	2	32	5.8109	<b>0.007</b>
Genotype x Time	6	32	4.6552	<b>0.0017</b>
Water x Defoliation x Genotype	3	15	2.6548	0.0863
Water x Defoliation x Time	2	32	0.236	0.7912
Water x Genotype x Time	6	32	1.0999	0.3839
Defoliation x Genotype x Time	6	32	1.1644	0.3495
Water x Defoliation x Genotype x Time	6	32	1.2064	0.3285
Pre-defoliation sugar secretion rate	1	15	429.8918	<b>&lt;.0001</b>

**Table 1.2** Locations of *Populus tremuloides* genotypes, collected from naturally occurring stands in interior Alaska.<sup>2</sup>

Genotype	Latitude	Longitude
BL	64.847	-147.945
NT	64.837	-147.881
PH	64.605	-149.090
WD	64.862	-147.901

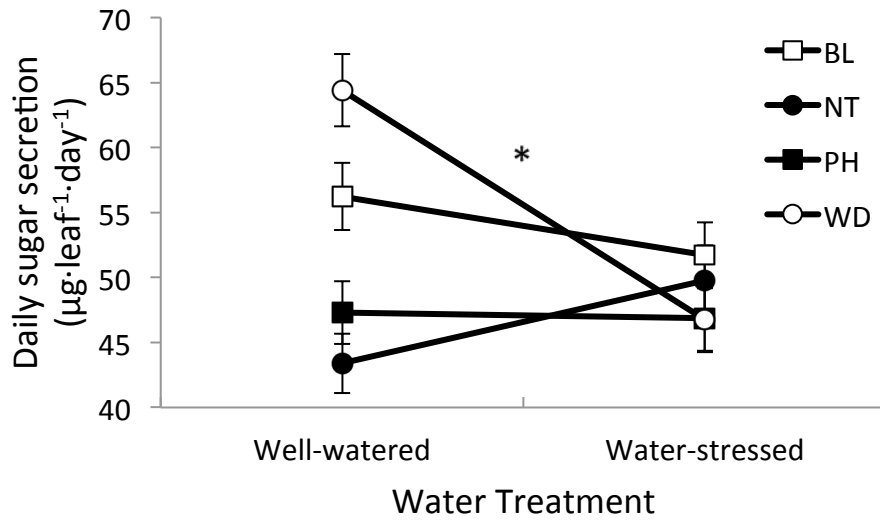
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<sup>2</sup> This table was included as electronic supplementary material in the manuscript published in *Botany*.

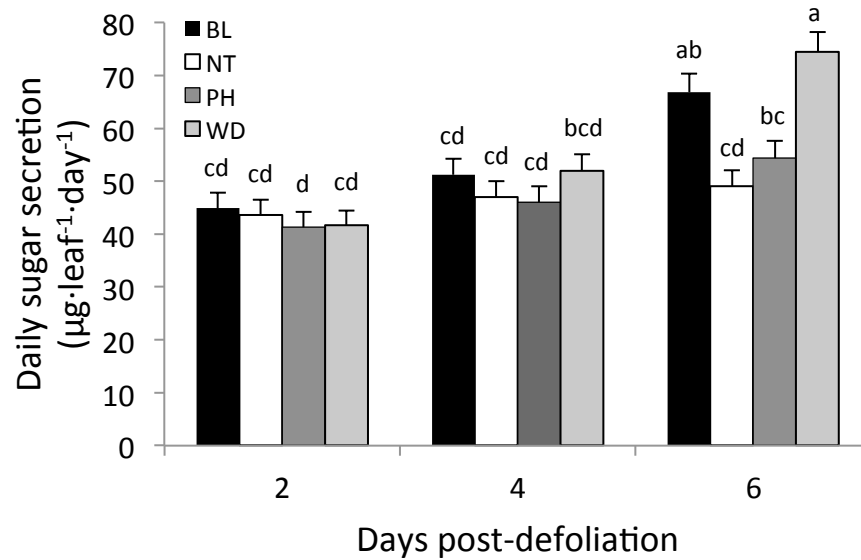


**Figure 1.1** EF sugar production by defoliated (white bars) and undefoliated (black bars) *Populus tremuloides* ramets at 2, 4, and 6 days after defoliation. Values shown are back-transformed LS means  $\pm$  SE,  $n = 16$  per group. Different letters denote significantly different mean sugar secretion (Tukey HSD,  $\alpha = 0.05$ ).





**Figure 1.2** Effect of water availability on EF nectar production in different *Populus tremuloides* genotypes (denoted by different symbols). Values shown are back-transformed LS means  $\pm$  SE,  $n = 4$  per group. Asterisk denotes significantly different mean sugar secretion by the WD genotype (open circle symbols) under the two watering regimes (Tukey HSD,  $\alpha = 0.05$ ).



**Figure 1.3** EF sugar secretion among *Populus tremuloides* genotypes at 2, 4, and 6 days after defoliation. Values shown are back-transformed LS means  $\pm$  SE. Different letters denote significantly different mean sugar secretion (Tukey HSD,  $\alpha = 0.05$ )



## **Chapter 2: The impact of extrafloral nectar availability on ant visitation to boreal *Populus tremuloides* (Michx.)<sup>3</sup>**

### **2.1 Abstract**

*Populus tremuloides* Michx. increases extrafloral (EF) nectar secretion rates in response to defoliation, and some genotypes decrease EF secretion rates in response to drought. Though blocking of EF nectar has been shown to reduce ant visitation to *P. tremuloides*, currently little is known about how variation in nectar availability typical of responses to drought or defoliation affect recruitment of ants to *P. tremuloides*. We tested whether variation in EF nectar availability affects visitation by *Formica fusca* ants to *P. tremuloides* ramets. We conducted a field experiment in which ramets of three genotypes were subjected to reduced, unmanipulated (control), or supplemented nectar availability. We measured ant visitation by conducting ant censuses three times per day. Censuses were conducted across two trials, in early and mid-summer, for a total of 30 days. Our results suggest that the behavior of *F. fusca* in this area is relatively insensitive to variation in nectar availability on *P. tremuloides*. In the early summer trial, mean ant visitation to ramets with supplemented nectar was significantly higher than to ramets with unmanipulated or reduced nectar within one *P. tremuloides* genotype. In the mid-summer trial, nectar availability had no effect on ant visitation, but mean ant visitation varied significantly among *P. tremuloides* genotypes. Increased nectar

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<sup>3</sup> Newman JR, Wagner D, and Doak P (2014) The impact of extrafloral nectar availability on ant visitation to boreal *Populus tremuloides* (Michx.). In preparation for submission to *Arthropod-Plant Interactions*.

availability may benefit *P. tremuloides* through improved ant recruitment, but this response depends on time of year and *P. tremuloides* genotype. Additionally, our study demonstrates that some *P. tremuloides* genotypes are more attractive to ants than others, which may result from genotypic differences in nectar secretion rates, nectar composition or volatile organic compound emissions.

## 2.2 Introduction

Extrafloral (EF) nectar mediates defensive mutualisms in many plant populations (Koptur 2005). Meta-analyses have supported that, in general, ant presence on plants expressing domatia, food bodies, or EF nectaries benefits those plants by reducing herbivory (Chamberlain and Holland 2009; Rosumek *et al.* 2009; Trager *et al.* 2010). The strength of defensive mutualisms, such as those mediated by EF nectar, may vary based on the quantity and quality of rewards provided by the plant. For example, increases and decreases in nectar availability can influence ant visitation to plants with EF nectaries (e.g., Rudgers and Strauss 2004; Kost and Heil 2005; Chamberlain and Holland 2008; Mortensen *et al.* 2013), and increases in EF nectar secretion can increase plant fitness (Heil 2004).

The rate of secretion from EF nectaries can vary in response to biotic and abiotic cues. Defoliation, application of jasmonic acid, and exposure to volatile organic compounds from damaged neighbors have been shown to increase the rate of EF nectar secretion across a broad range of EF nectary-bearing plant species (reviewed by Heil 2011). Induction of nectar secretion in response to herbivory

cues may reduce the cost of maintaining a constitutive indirect defense (Karban 2011). Competition, simulated as a decrease in the red:far red ratio of incident light, has been shown to decrease investment in EF nectar (Radhika *et al.* 2010). Additionally, drought stress may reduce EF nectar secretion rate (Yamawo *et al.* 2012; Newman and Wagner 2013).

Extrafloral nectaries appear to function as an indirect defense against herbivory for *P. tremuloides*, as ramets with higher EF nectary expression suffer less damage from the leaf miner *Phyllocnistis populiella* Cham. (Mortensen *et al.* 2011). However, little is known about how putative mutualists such as ants respond to variation in nectar secretion by *P. tremuloides*. The rate of EF sugar secretion in *P. tremuloides* is inducible in response to defoliation and inhibited by water stress, though this response varies among genotypes (Newman and Wagner 2013). Complete blockage of EF nectar on *P. tremuloides* reduces ant visitation to ramets (Mortensen *et al.* 2013), but how ants respond to more subtle changes characteristic of natural variation in EF nectar secretion is unknown. Here, we experimentally test the hypothesis that greater or lesser EF nectar availability correspondingly increases or decreases ant visitation to *P. tremuloides*.

## **2.3 Methods**

### **2.3.1 Propagation**

Individual aspen ramets of 3 genotypes were propagated for use in this experiment, using two similar propagation techniques. A single root from *P.*

*tremuloides* genets TT and TW was collected from spatially distinct naturally occurring stands in the Tanana Valley of interior Alaska in fall 2007. Roots were overwintered in natural soil in a refrigerator. In spring 2008, roots were cut into short (~15cm) segments, and placed into 2L pots containing a growth medium of 1:1:1 vermiculite:perlite:coconut coir. Ramets of these two genotypes were maintained in an outdoor garden until the summer of 2011. Ramets of the other genotype used in this study, BR, were propagated using a technique modified from Barry and Sachs (1968). In July 2010, we collected a single root from a naturally occurring *P. tremuloides* stand, and placed the root into vermiculite. Adventitious shoots were continually harvested when they reached 5-15 cm in height. We dipped the bases of each shoot into an aqueous solution of 1000 ppm indole-3-butyric acid, a rooting hormone, before placing the shoots (hereafter ramets) into a vermiculite medium under intermittent mist until they had formed roots. We transferred rooted ramets into 2L pots containing the same growth medium as the other two genotypes. Ramets of the BR genotype were overwintered in the outdoor garden with the other two genotypes. All ramets were fertilized following each watering with an aqueous fertilizer solution (17:5:7 N:P:K); 24 h prior to application of treatments, all ramets were fertilized using 3.0g/L time-release Osmocote fertilizer (14:14:14 N:P:K).

### 2.3.2 Experimental setup

The experiment took place in a naturally occurring *P. tremuloides* stand within a 0.14 ha plot on the University of Alaska Fairbanks campus (center of plot: 64.857225°, -147.859760°). We selected 17-18 juvenile potted *P. tremuloides* ramets from each of the three genotypes (n = 52). Ramets were randomly assigned to one of three treatments: reduced nectar, supplemented nectar, or unmanipulated nectar (control). We located six *Formica fusca* colonies and used 5 minute observation periods to identify the location of the most frequently used entrance to each colony. Colonies were located a minimum of 10m from each other. Around each focal ant colony, we arranged in a circle one *P. tremuloides* ramet from each combination of genotype and treatment (n = 9 ramets). Potted ramets were placed into holes in the soil with the lip of the pot at surface level, equidistant from each other and 1 m from the colony entrance as depicted in Fig 2.1. The order of the genotype x treatment combination was constant, although the array was rotated randomly at each site. Each day we visited all ramets at three times (excluding rainy days or times), 1200, 1500, and 1800, to count all ants present on each ramet. These censuses were conducted on colonies in the same order each day, though the direction by which the observer approached the array and the first ramet surveyed varied across censuses. During the experiment, ramets were watered as necessary, approximately every other day.

Ramets in the supplemented nectar treatment received repeated injections of an artificial nectar solution. The artificial nectar solution contained 95 g·L<sup>-1</sup> sugar



made up of equal parts fructose, glucose, and sucrose. The quantity of sugar in artificial nectar was based on the mean plus two standard deviations of daily sugar secretion by *P. tremuloides* leaves bearing EF nectaries, determined from a preliminary study (n = 229 leaves from 35 ramets; Newman and Wagner, unpublished data). Though the sugar composition of EF nectar was unknown at the time of this experiment, the composition of the artificial nectar we chose was similar to that later reported for a hybrid poplar (Li *et al.* 2012). We applied 2 $\mu$ L of the artificial nectar solution to the site of EF nectaries on the two most proximal leaf positions of the lowest six shoots (12 supplemented leaves per ramet). This selection of leaf locations for supplementation was intended to maximize the discovery of the extra sugar by ants. The artificial nectar solution was re-injected onto EF nectaries every 3 days between 0700 and 0730 for the duration of the experiment, resulting in an average increase in sugar availability of 63.3 $\mu$ g sugar per leaf per day, and a total increase of 759.6 $\mu$ g sugar per ramet per day. This constitutes a 113% increase in sugar availability on leaves with supplemented nectar, and a 10% increase in total sugar secretion for ramets of this size prior to the formation of neofoliated leaves (Newman and Wagner, unpublished data). For comparison, experimental defoliation-induction of EF nectar in *P. tremuloides* resulted in a 23% increase in sugar secretion per EF nectary bearing leaf (Newman and Wagner, 2013). It is unknown at this time if this induction response is systemic; however, it is possible that the total EF nectar availability per ramet may correspondingly increase 23% in response to defoliation. Ramets in the reduced

nectar treatment received a drop of fabric glue (Aleene's OK to Wash-It; Duncan Enterprises, Fresno, CA) on top of the EF nectaries at the two most proximal leaf positions bearing EF nectaries on all shoots. To control for any unintended effects of glue on ant behavior, ramets in supplemented and unmanipulated nectar treatments received a drop of glue next to the EF nectaries on the two most proximal EF nectary-bearing leaf positions on all shoots. We allowed the glue to dry for 24 h before placing ramets at the study site. Based on the total number of nectary-bearing leaves on experimental ramets, this treatment corresponded to an average 52.6% reduction in total nectar sugar availability per ramet. Water restriction does not affect EF sugar secretion in *P. tremuloides* genotypes equally; while some genotypes do not alter sugar secretion in response to drought, drought susceptible genotypes can experience as much as a 28% reduction in sugar secretion rate (Newman and Wagner 2013).

We conducted two trials of the experiment. The first trial (hereafter the early-summer trial) lasted for 18 days beginning June 18, for a total of 50 censuses per ramet. The second trial (hereafter the mid-summer trial) lasted for 12 days beginning July 14, for a total of 33 censuses per ramet. Between trials, ramets were removed from the experimental plots and returned to the greenhouse garden. During the period between trials, ramets were fertilized with aqueous fertilizer and all experimental ramets were checked to ensure that glue treatments had remained intact. Prior to the start of the mid-summer trial, we constructed "bridges" from within 5cm of the central colony entrance to the rim of the pot of each ramet in an

attempt to facilitate ant visitation to plants. Bridges were made from naturally occurring dead twigs from the surrounding area. Experimental ramets were replaced at the experimental plots at the same ant colony and the same position around the ant colony as in the early summer trial.

Aphids (Aphididae) were abundant on leaves and stems of experimental *P. tremuloides* ramets during the study. Phloem-feeders such as aphids excrete sugar-rich honeydew during feeding, which could potentially diminish differences in available carbohydrate sources between experimental ramets. Therefore, during each census and after ants were counted on a ramet, we manually removed aphids from the ramet by gently wiping them off the leaf. To control for possible negative effects of handling, we also handled leaves on ramets with no aphids present.

### **2.3.3 Statistical Analysis**

We tested the effects of nectar manipulation, ramet genotype, and the interaction between the two on ant abundance per ramet. We used the GENMOD procedure in SAS (SAS Institute, Cary, NY) to fit the model to repeated measures of ant counts using generalized estimating equations (SAS/STAT 9.2 User's Guide, 2008), assuming a Poisson distribution. Ant colony was included as a blocking variable. Individual ramet was included as the repeated subject. Means were compared using Tukey's HSD tests. Early- and mid-summer trial data were analyzed separately.

Because genotypes may vary in their tendency to express EF nectaries on leaves (Wooley *et al.* 2007) and this could impact the total nectar availability of a ramet, we additionally tested for systematic differences in the number of leaves bearing EF nectaries, counted prior to the experiment, between experimental genotypes. We conducted a one-way ANOVA in JMP (SAS Institute, Cary, NY), with total number of EF nectary-bearing leaves per ramet as the dependent variable and genotype as the independent variable.

## 2.4 Results

In the early-summer trial, the main effects of genotype and nectar manipulation were not statistically significant (genotype:  $\chi^2 = 3.61$ ,  $df = 2$ ,  $P = 0.164$ ; nectar manipulation:  $\chi^2 = 3.12$ ,  $df = 2$ ,  $P = 0.210$ ), but ant response to nectar manipulation differed among genotypes ( $\chi^2 = 10.80$ ,  $df = 4$ ,  $P = 0.029$ ). Within the TT genotype, ramets with supplemented nectar experienced significantly higher ant visitation than either the ramets with unmanipulated or reduced nectar (Fig. 2.2). TT ramets with supplemented nectar were visited by 58 and 80% more ants per census than ramets with unmanipulated and reduced nectar, respectively. There was no significant difference between the reduced and unmanipulated nectar treatments for this genotype (Fig. 2.2). Within each of the other two genotypes, there were no significant differences between nectar treatment groups (Tukey HSD,  $P > 0.05$ ).

Ant visitation rates were marginally reduced (7.8%) overall during the mid-summer trial compared to during the early-summer trial. In the mid-summer trial, ants did not respond significantly to nectar manipulation ( $\chi^2 = 3.96$ ,  $df = 2$ ,  $P = 0.138$ ), and there was no significant interaction between genotype and nectar manipulation ( $\chi^2 = 5.70$ ,  $df = 4$ ,  $P = 0.222$ ). However ants visited *P. tremuloides* genotypes at different frequencies ( $\chi^2 = 6.96$ ,  $df = 2$ ,  $P = 0.031$ ). Ramets of the TT genotype were visited by 40% more ants per census than ramets of the TW genotype (Fig. 2.3) while the other between genotype comparisons were not significant.

Because rain may have removed supplemented nectar from experimental ramets, we repeated the above analyses after excluding data from censuses that following a rainy period but prior to reapplication of supplemented nectar. The results of these analyses did not differ qualitatively from those obtained from analyses of the complete datasets.

The genotypes used in this study did not differ significantly in the mean number of EF nectaries per ramet ( $F_{2,49} = 0.6361$ ,  $P = 0.53$ ).

## 2.5 Discussion

The level of EF nectar manipulation applied in this study had little overall effect on ant visitation to *P. tremuloides*. In contrast to our results, the experimental removal or blocking of EF nectar has been shown to significantly reduce ant visitation (e.g.; Apple and Feener 2001; Rudgers and Strauss 2004; Chamberlain and

Holland 2008; Mortensen *et al.*, 2013). However, these studies all attempted to block or remove 100% of EF nectar resources. In our study, ramets with reduced nectar had secretions blocked on only a subset of nectaries, corresponding to a 52.6% reduction in total ramet EF sugar. Water stress has a variable effect on sugar secretion between genotypes, and drought-susceptible clones may experience as much as 28% reduction in EF sugar secretion (Newman and Wagner, 2013). Therefore, the reduction in nectar availability in our study was a more realistic representation of decreases in secretion rate typical of responses to water stress in *P. tremuloides* than blocking of all nectaries, but was nearly double the reduction in sugar secretion experienced by water stressed ramets. Moreover, the supplemented nectar treatment corresponded to a 10% increase in total sugar availability, though the perceived increase by ants may have been higher due to the placement of artificial nectar on more proximal leaf positions. Comparatively, defoliation-induction of EF nectar results in a 23% increase in sugar secretion (Newman and Wagner, 2013), though the total ramet increase in sugar secretion due to defoliation may be lower if induction is not systemic. The more subtle and realistic level of nectar manipulation achieved in our study may lead to less pronounced behavioral responses by foraging ants.

A potential criticism of this study is that the composition of the artificial nectar solution used differs somewhat from natural secretions by *P. tremuloides* ramets. The sugar constituents of EF nectar in *P. tremuloides* are sucrose, glucose and fructose; however, the artificial nectar solution used contained equal

contributions of these three sugars, whereas our analysis of the composition of EF nectar revealed that it contains  $48.1 \pm 3.9\%$  sucrose, with a 95% confidence interval that does not overlap the 33% sucrose used in our artificial nectar (see Chapter 3). Many ant species prefer sucrose over other sugars found in nectar (Blüthgen and Fiedler 2004). Perhaps more importantly, many ant species prefer nectar solutions containing amino acids over sugar solutions lacking amino acids (e.g., Blüthgen and Fiedler 2004), though it is noteworthy that a congener to our focal ant species, *Formica perpilosa*, does not exhibit preference for nectar with amino acids over nectar with sugar alone (Wagner and Kay 2002). At this time, there is no evidence that *P. tremuloides* EF nectar contains amino acids. However, numerous studies report small concentrations of amino acids in EF nectar secretions (e.g., Baker *et al.* 1978; Koptur 1994; Blüthgen *et al.* 2004). The differences between secreted EF nectar and the artificial solution used in this study may therefore contribute to the generalized lack of attraction to ramets in the supplemented nectar treatment.

The many sources of sugars available in a *P. tremuloides* stand may dull ant responses to within-ramet variation in nectar secretion. *P. tremuloides* tends to grow in high density stands, as was the case at our study site, which increases the availability of EF nectar to ants foraging in the area. In such a setting with abundant nectar resources, relatively subtle changes in secretion rate may have little impact on ant behavior. Additionally, aphid colonies such as we observed are common on *P. tremuloides* in the study area, and their honeydew secretion potentially reduces the importance of EF sugar resources to foraging ants (Katayama and Suzuki 2003).

Hemipteran phloem feeders such as aphids excrete sugar-rich honeydew as a product of feeding, which often serves as an attractant for ant protectors (Styrsky and Eubanks 2007). During the mid-summer trial of our study, we recorded that 7% of total ants were tending aphids at the moment the census was taken (Newman, personal observation). Despite low frequency of observed aphid-tending, it is possible that aphid honeydew attracted ants to all ramets, regardless of treatment, thereby blunting the effect of treatment on ant visitation.

The number of ants observed visiting *P. tremuloides* in interior Alaska is relatively low compared to the number reported visiting EF nectary-bearing plants in other study systems. For example, studies involving similarly sized plants in southern United States and Central America have reported mean values of ~9 ants per plant (Wagner and Kurina 1997) and 5 – 16 ants per plant (de la Fuente and Marquis 1999). By contrast, the mean ant visitation was 1.1 ants per census in our study and even lower in a previous study conducted in interior Alaska (Mortensen *et al.* 2013). Generally, ant abundance and diversity tend to decline with increasing latitude (reviewed in Rico-Gray and Oliveira 2007), which may account for low observed visitation rates to ramets in interior Alaska. Low visitation rates may limit our ability to discriminate changes in visitation in response to changes in nectar availability, but may also limit the effectiveness of ants as protectors.

The observed patterns of ant visitation differed between early and mid-summer trials. In the early summer trial, we found greater ant visitation to ramets with supplemented nectar than ramets with either unmanipulated or reduced



nectar for one of the three *P. tremuloides* genotypes; there was no trend in the predicted direction for either of the other two genotypes. In the mid-summer trial, we observed no effect of treatment for any genotype, although genotypes did differ overall in visitation by ants. Though little is known of the phenology of *Formica fusca* colonies in interior Alaska, differences in ant responses to treatment between early- and mid-summer trials may reflect temporal shifts in colony foraging requirements. In early summer, ant colonies include actively-feeding larvae. In mid-July, when the mid-summer trial was conducted, *F. fusca* colonies typically contain pupae (Wagner, unpublished data), which would tend to decrease the colony-wide demand for food. Larvae consume primarily protein, while workers consume primarily carbohydrates (reviewed in Blüthgen and Feldhaar 2010), but the presence of larvae in the nest may increase colony carbohydrate requirement indirectly by increasing the energetic demands of foraging. Experimental addition of ant larvae increased total colony intake of carbohydrates by *Rhytidoponera* sp. (Dussutour and Simpson 2009), likely in order to provide fuel for the additional foraging effort needed to sustain larvae. Moreover, Dussutour and Simpson (2008) showed that when there are larvae in the nest, colonies exhibit enhanced discrimination among sucrose solutions of varying concentrations, regulating their consumption of carbohydrates to more accurately meet colony demands, which is consistent with the greater discrimination among nectar treatments we detected early in the summer.

Differences in the responses of ants to nectar manipulation across early- and mid-summer trials may also be related to a dilution in the treatment effect caused by the production of new EF nectary-bearing leaves throughout the summer. Neoformed leaves of *P. tremuloides*, produced by actively-growing plants during the summer, express EF nectaries much more consistently than the early, preformed leaves that arise from the overwintered buds, with over 80% of neoformed leaves expressing nectaries (Doak et al. 2007). As shoots elongated with neoformed leaves, leaves with supplemental nectar constituted a diminishing proportion of total nectar-producing leaves available, and ramets in the reduced nectar treatment possessed a lower proportion of blocked EF nectaries.

Across trials, our results indicate that the genetic identity of *P. tremuloides* plants affects the recruitment of ant visitors. In the mid-summer trial ant counts differed significantly between ramets of the TT and TW genotypes, and in the early summer trial the effect of treatment was dependent on genotype. Although the cause of genotype-related differences in discrimination among treatments by ants is not clear, average differences in visitation to different *P. tremuloides* clones could be attributable to differences in EF nectar quantity or quality, or non-nectar plant traits that affect attractiveness to ants such as foliar volatile organic compound (VOC) emissions. The mean number of preformed leaves with EF nectaries per ramet did not vary by genotype in our study. However, previous work has shown that the rate of EF sugar secretion per leaf can vary significantly among clones (Newman and Wagner 2013). Nectar quality may also impact attraction of ants to ramets, as

nectar blends with higher sucrose or amino acid concentrations are generally preferred by foraging ants (González-Teuber and Heil, 2009). Our nectar blend contained equal parts sucrose, fructose, and glucose, while later investigations of EF sugar content have found that EF nectar of *P. tremuloides* contains predominantly sucrose over fructose and glucose (Chapter 3). Volatile organic compounds emitted by plants have been shown to attract predatory insects (reviewed by Heil 2008), and several VOCs have been shown to vary among genotypes in *Populus* hybrids (Eller et al. 2012). Therefore, variation in VOC signals among *P. tremuloides* genotypes might also contribute to differences in ant visitation to plants, although more work will be needed to test this hypothesis.

This study provides evidence that increases in EF sugar availability by *P. tremuloides* correspond to only subtle increases in ant visitation that are dependent on *P. tremuloides* genotype and time of year. Additionally, our results indicate that ants can distinguish between *P. tremuloides* genotypes, and prefer some genotypes over others. Reasons behind these genetic differences in attractiveness remain unclear, but future work should focus on the effects of VOC cues as well as EF nectar quality or secretion rate as a possible source of variation in patterns of ant visitation among genotypes.

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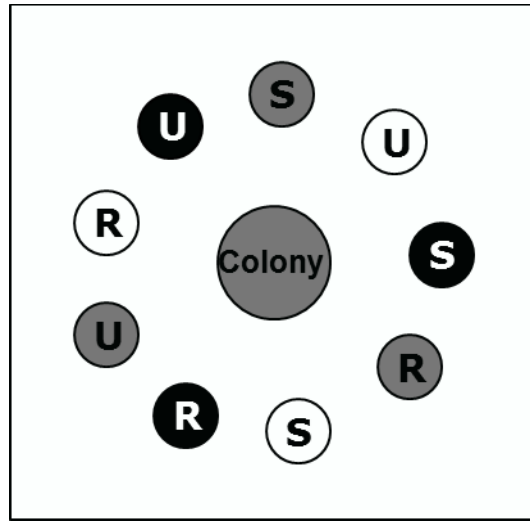
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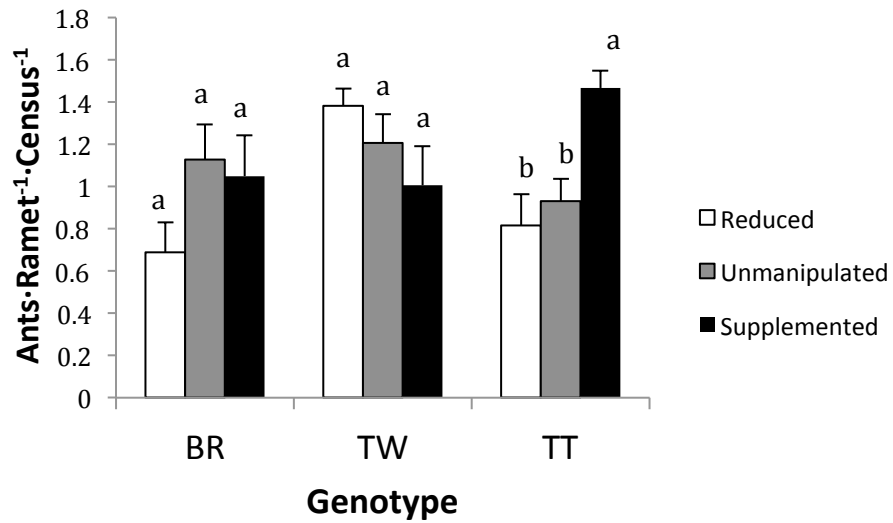
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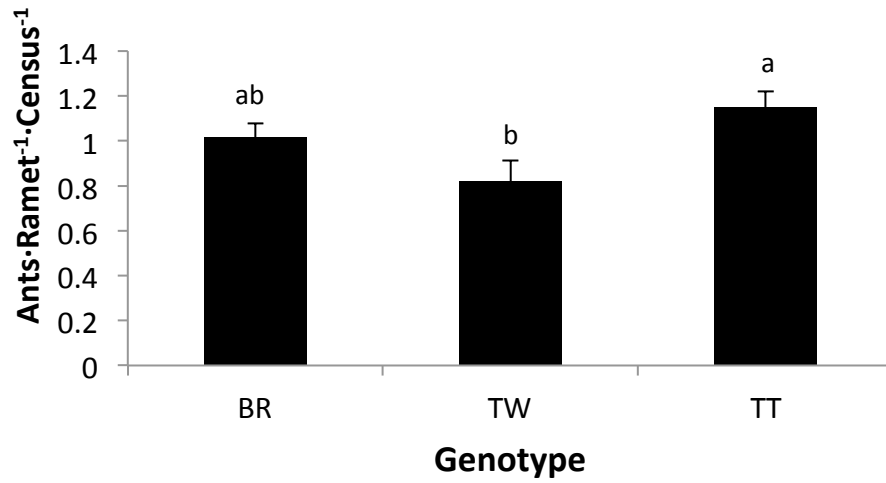




**Figure 2.1** Setup of experimental *P. tremuloides* ramets around a central ant colony. R, S, and U represent reduced, unmanipulated, and supplemented nectar treatments, respectively. Circle shading denotes distinct genotypes.



**Figure 2.2** Mean number of ants on ramets with reduced, unmanipulated, and supplemented nectar by *P. tremuloides* genotype. Values shown are LS means  $\pm$  SE. Different letters denote significantly different means within genotypes (Tukey HSD,  $\alpha = 0.05$ ).



**Figure 2.3** Mean number of ants on ramets of different *P. tremuloides* genotypes.

Values shown are LS means  $\pm$  SE. Different letters denote significantly different means (Tukey HSD,  $\alpha = 0.05$ ).

## Chapter 3: Sugar composition in extrafloral nectar of *Populus tremuloides*<sup>4</sup>

### 3.1 Abstract

Extrafloral (EF) nectar mediates defensive mutualisms in which plants provide food rewards for predatory mutualists, which in turn provide protection to the plant. EF nectar composition can impact the quality of nectar to predatory arthropods, affecting the strength of defense. We examined the sugar content of EF nectar of three genotypes of quaking aspen (*Populus tremuloides* Michx.) using nuclear magnetic resonance (NMR) spectroscopy. Overall, EF nectaries secreted  $25.6 \pm 2.8$  (48.1%),  $16.3 \pm 1.5$  (31.5%), and  $10.6 \pm 1.3$  (20.3%)  $\mu\text{g} \cdot \text{leaf}^{-1} \cdot \text{day}^{-1}$  of sucrose, glucose, and fructose, respectively. For two of the genotypes, sucrose dominated the nectar blend, while nectar from a third genotype contained approximately equal proportions of sucrose, glucose, and fructose. Despite qualitative differences, there was no significant difference in sucrose content between genotypes. Though our results provide no evidence of a genetic component in EF nectar composition, the sample size was modest and future work on EF nectar should incorporate a larger number of genotypes.

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<sup>4</sup> Newman JR, Wagner D, and Green T (2014) Sugar content of extrafloral secretions by *Populus tremuloides*. In preparation for submission, journal to be determined.

### 3.2 Introduction

The primary components of extrafloral (EF) nectar are carbohydrates, typically dominated by sucrose, fructose and glucose, although small amounts of other sugars and amino acids may be present (Heil, 2011). Carbohydrates in extrafloral nectar are a source of energy for foragers and may attract predatory arthropods that act as defending mutualists (Koptur 2005). Experimental evidence suggests that access to a high-sugar food source such as EF nectar and hemipteran-produced honeydew creates a desire for nitrogen in ants, enhancing predatory behavior (Ness *et al.* 2009; Pringle *et al.* 2011). The composition of sugars in nectar may further affect the attractiveness of EF nectar to predatory arthropods, as sucrose-dominated blends tend to be favored by ants over hexose-dominated (fructose, glucose) blends (reviewed in González-Teuber and Heil 2009a); the sugar preference of non-ant mutualists (e.g., parasitoid wasps) are unknown at this time. Knowledge of the composition of EF nectar secretions is therefore pertinent to understanding the strength of interactions between plants and EF nectary-visiting arthropods. Little is known about the composition of EF nectar secretions in *Populus tremuloides* Michx., though the sugar components of EF nectar secretions of a hybrid poplar have been described (Li *et al.*, 2012). In this study, we describe the carbohydrate components of the EF nectar secretions of *P. tremuloides*, determined through nuclear magnetic resonance (NMR) spectroscopy.

### 3.3 Methods

*Populus tremuloides* ramets from three distinct genotypes were propagated as described previously (see Chapter 1, Methods). In March 2012, we placed four overwintering ramets from each genotype (n = 12 ramets) into the Institute of Arctic Biology Research Greenhouse to break dormancy. In early April 2012, we tagged 10 leaves with visible EF nectar secretions on each of the ramets. To allow nectar to accumulate for a known period of time, the EF nectaries of all marked leaves were rinsed thoroughly with reverse-osmosis water 48 hours prior to nectar collection. Nectar sampling using a microcapillary tube was deemed inappropriate due to the small volume and high viscosity of the nectar and the tendency of this method to underestimate volume and yield inaccurate composition results (Marrant *et al.*, 2009). Therefore, in order to collect a more complete sample of EF nectar, we sampled using a washing technique (Li *et al.* 2012). Approximately 2 days following rinsing of nectaries, to collect nectar, we deposited 4  $\mu$ L of nanopure water onto the nectary surface, then recollected and transferred all resulting solution to a microcentrifuge tube using a micropipette. This was repeated two additional times to maximize the collection of sugars on the nectary surface. The nectar from 10 leaves per ramet was combined in a single microcentrifuge tube, which was then placed on ice for transport. This process yielded a total of 12 samples, one per ramet. In the laboratory, tubes were opened and placed into a drying oven at 60°C for 24 h, then placed into a desiccation cabinet for 48h to remove all water from the sample.

Each sample was dissolved in 600  $\mu\text{L}$  of  $\text{D}_2\text{O}$  and transferred to a 5mm NMR tube. A solution of 5mM trimethylsilyl propionate (TSP) in  $\text{D}_2\text{O}$  was used as an external reference for peak position and concentration.  $^1\text{H}$  NMR spectra, which display the resonance frequencies of hydrogen-1 nuclei, were recorded for each sample using a Bruker Ascend 600 NMR machine (600MHz). Baseline correction and integration of peaks were performed using Topspin 3.0 (Bruker, Billerica, Massachusetts, USA). Sugar peaks were identified by comparison to pure standards and a previously published source (Cazor et al., 2006). Because the molarity of TSP and the number of hydrogen-1 nuclei for TSP are known constants, the integral of the TSP peak was used for quantification of absolute concentrations of sugar components. Sugar content is reported on a per-leaf basis. From the absolute concentrations of sugars, we then calculated the percent contribution by mass of each sugar present to total sugar content.

### **3.3.1 Statistical analysis**

To determine whether the composition of nectar varied between genotypes, we conducted a one-way ANOVA with genotype as the independent variable and percent sucrose as the dependent variable. To assess the probability of detecting a significant difference among genotypes with such a small sample size, we conducted a power test. All analyses were conducted using JMP 9 software (SAS Institute, Cary, NC).

### 3.4 Results/Discussion

Sugar analysis of *P. tremuloides* EF nectar revealed the presence of sucrose, glucose, and fructose, with no additional carbohydrate compounds detected in any of the samples (example  $^1\text{H}$  NMR spectrum of EF nectar shown in Fig 3.1). Overall, *P. tremuloides* EF nectar contained  $25.6 \pm 2.8$  (48.1%),  $16.3 \pm 1.5$  (31.5%), and  $10.6 \pm 1.3$  (20.3%)  $\mu\text{g} \cdot \text{leaf}^{-1} \cdot \text{day}^{-1}$  of sucrose, glucose, and fructose, respectively. The percent composition of sucrose, glucose, and fructose produced by the three *P. tremuloides* genotypes are shown in Fig 3.2. Ramets of the PH and WD genotypes secreted EF nectar dominated by sucrose, whereas ramets of the NT genotype secreted EF nectar with approximately equal sucrose and glucose content. Across genotypes, EF nectar contained a lower proportion of fructose than either glucose or sucrose.

Despite qualitative differences, the percent sucrose did not vary significantly between genotypes ( $F_{2,2} = 3.17$ ,  $P = 0.09$ ). A power test revealed that an ANOVA given our sample size, variance, and effect size has a 24% chance of detecting a significant difference between genotypes at a significance level of 0.05. Due to the low replication within genotypes, our statistical analysis has low power and is thus inconclusive with regard to variation in composition among genotypes.

Our analysis suggests that in the sampled genets, *P. tremuloides* EF nectar is dominated by sucrose over fructose and glucose. These three sugars dominate the carbohydrate content of most EF nectars (Koptur, 2005). Much interspecific variation exists in relative composition of fructose, glucose, and sucrose, with some



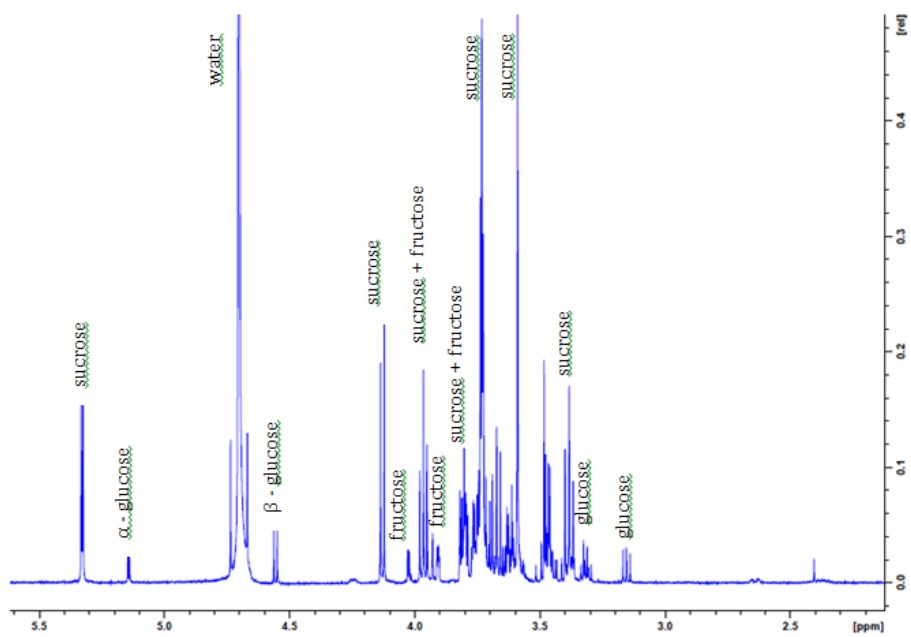
species secreting nectar containing equal proportions of the three, some containing predominantly sucrose, and some with little to no sucrose content (Blüthgen *et al.*, 2004). Analysis of extrafloral nectar in other plant populations has revealed that amino acids are frequently present in EF nectar in small quantities, and may be important attractants of mutualist ants (reviewed in González-Teuber and Heil, 2009b). At this time, there is no evidence that EF nectar of *P. tremuloides* contains amino acids.

The sugar composition of EF nectar might affect the attractiveness of nectar to predatory mutualists such as ants. For example, many ant species prefer nectar blends containing higher proportion of sucrose (Blüthgen and Fiedler 2004). For ant species lacking invertase, the enzyme necessary for digesting sucrose, sugar preference likely does not follow this generalized trend. Obligate mutualist ant species, for example, tend to lack invertase, and prefer nectars lacking sucrose (González-Teuber and Heil 2009a). However, no obligate ant-plant interactions occur in interior Alaska, and it is unlikely that *P. tremuloides* engages in obligate ant-plant mutualisms in any part of its range. *Formica fusca*, which is commonly observed feeding at EF nectaries of *P. tremuloides* in interior Alaska, possesses the capacity to digest sucrose (Ayre, 1967). Future work should focus on sugar preference of ant visitors to *P. tremuloides*.

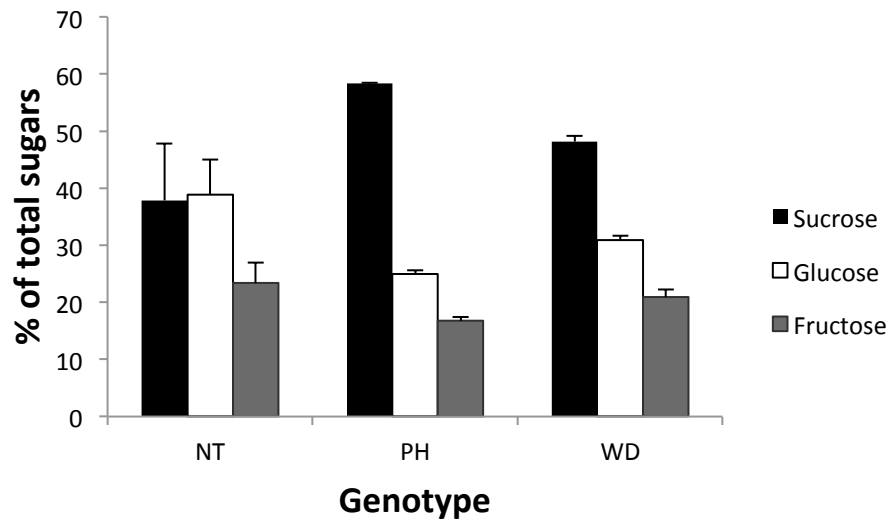
### 3.5 References

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**Figure 3.1** NMR spectrum of extrafloral sugars present in nectar of *P. tremuloides*.



**Figure 3.2** Relative sugar abundance in extrafloral nectar of three *P. tremuloides* genotypes. Values shown are mean  $\pm$  SE.

## Conclusion

This study revealed several potentially important sources of variation in extrafloral (EF) nectar secretion in *Populus tremuloides*. The greenhouse experiment described in Chapter 1 provides the first evidence for induction of EF sugar secretion in *P. tremuloides*. Defoliation increases EF sugar secretion, and induction is uninhibited by water restriction. The effect of water stress on EF sugar secretion varies by genotype, as water stress reduced sugar secretion rates for one of four genotypes. *P. tremuloides* genotypes also vary overall in sugar secretion rate. These results suggest that the benefit of EF nectar secretions may vary among clonal stands, particularly during times of drought. Carbohydrate components of EF nectar in *P. tremuloides* are sucrose, glucose, and fructose, with sucrose comprising the majority of EF sugars by weight. There is no evidence at this time that this composition varies with genotype, though future testing could include a more robust sample size.

The extent to which variation in sugar secretion rate affects defense depends on the response of predators. Ant predator response was predominantly insensitive to variation in sugar availability; however, this response varied during the growing season. In early summer, ant visitation to one of three genotypes increased in response to supplemented EF sugar and was unaffected by reduction in EF sugar availability. In mid-summer, ant attraction varied by ramet genotype but not by nectar availability. It is possible that the effects of variation in EF sugar availability

on ant visitation are higher at lower latitudes, where ant abundance and species richness are higher.

Further work should address effects of *P. tremuloides* genotype on indirect defense mediated by EF nectar. The genotype with the highest EF sugar secretion rate was the only genotype in our study to respond to water restriction with lowered secretion rates. This suggests the possibility of a trade-off between EF nectar secretion and traits that enhance drought tolerance, which may vary by genotype. Additionally, different genotypes varied significantly in ant visitation. Future work should investigate the possible causal linkage between natural variation in EF nectar secretion rates or VOC emission and ant visitation between genotypes. Finally, more work should be done to investigate genotypic variation in sugar and amino acid composition of EF nectar, which may impact the attractiveness of EF nectar of different *P. tremuloides* genotypes, and to different mutualists.